EFFECT OF ETHYLENE-MEDIATED PRIMING ON CHICKPEA SUBJECTED TO HEAT STRESS

by

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TABLE OF CONTENT

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACC	1-Aminocyclopropane-1-carboxylic acid
ETH	Ethephon
MDA	Malondialdehyde
GABA	Gamma-aminobutyric acid
ROS	Reactive Oxygen Species
TBARS	Thiobarbituric Acid Reactive Substances
MDA	Malondialdehyde
BHT	Butylated Hydroxytoluene
TBA	Thiobarbituric Acid
TCA	Trichloroacetic Acid
NADP	Nicotinamide Adenine Dinucleotide Phosphate
SSA	Succinic Semialdehyde
SOD	Superoxide Dismutase
CAT	Catalase
POD	Peroxidase
GPOX	Glutathione Peroxidase
ABA	Abscisic Acid
JA	Jasmonic Acid
MEL	Melatonin
AMP	Adenosine Monophosphate

ABSTRACT

Crop productivity is crucial to meet the food demand of the growing global population. Climate changes and other environmental factors are influencing the seasonal crops, leading to abiotic stress conditions and ultimately impacting crop production and yield. Thus, different studies are being done to improve crop resilience towards environmental stresses such as heat, drought, salinity, etc. This study focuses on an important legume chickpea which is a highly nutritious food. Being a winter legume, at high-temperature conditions chickpea plants experience abiotic stress resulting in reduced crop productivity and yield. To adapt to diverse environments plants have developed adaptative mechanisms, involving many hormones and biomolecules to enhance abiotic stress tolerance. Ethylene is a plant hormone that regulates plant growth and development while playing a crucial role in promoting abiotic stress tolerance in plants.

This study investigates the physiological parameters in ACC (1-aminocylopropane-1-carboxylic acid) and Ethephon-treated ICC-12726 and Genesis-836 chickpea plants. Both chickpea plants were treated with different ACC and ethephon concentrations and the optimal ethylene precursor and concentration were determined by analyzing physiological parameters such as germination percentage, shoot and root length, shoot, and root biomass. The effect of ethylene pretreatment on the growth and heat tolerance of chickpea plants was further investigated through these physiological parameters in combination with key biochemical signatures. The oxidative stress level of chickpea plants under stressed and control conditions was also quantified through TBARS (Thiobarbituric Acid Reactive Substance) assay, a proxy for lipid peroxidation which measures MDA (malondialdehyde) equivalents. The GABA (Gamma-Aminobutyric Acid) level of each plant subjected to heat and control conditions was quantified using GABase. Finally, the starch content was measured by using a starch assay.

Both chickpea seeds were pretreated with 0 µM, 10 µM, 50 µM, and 100 µM ACC concentrations and results showed that ACC did not have a significant impact on seed germination and root length in both ICC-12726 and Genesis-826 chickpea plants compared to the controls. In contrast, both chickpeas were pretreated 0 µM, 5 µM, 50 µM, 500 µM, and 1 mM ethephon concentrations and ethephon-pretreated plants exhibited more significant changes in shoot and root length, fresh root, and shoot biomass compared to control plants. According to the results, both ICC-12726 and

Genesis-836 chickpea plants showed reduced shoot and root length as ethephon concentration increased. This study has addressed the biochemical responses of chickpea plants subjected to heat stress along with ethephon pretreatment and foliar treatment. Moreover, this study examined the biochemical responses of chickpea plants subjected to heat stress, along with ethephon pretreatment and foliar treatment., overall 5µM ethephon-treated plants under heat stress conditions exhibited higher significance in MDA values compared to those under control conditions. However, ethephon pretreatment did not perform significant changes in MDA levels in both chickpea varieties. Overall, the ethephon pretreated both chickpea plants did not show significant changes in GABA level and starch content. In contrast, foliar-treated plants exhibited significant changes in MDA level, and GABA levels compared to ethephon pretreatment. In conclusion, these findings will help to elucidate the role of ethylene in stress signaling pathway in chickpea plants subjected to heat stress and ultimately these findings and strategies can be utilized to enhance abiotic stress resilience and productivity of chickpea plants subjected to heat stress

DECLARATION

I certify that this thesis: done not incorporate without acknowledgment my material previously submitted for a degree or diploma in any university and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University and to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due to reference in made in the text.

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CHAPTER 1

INTRODUCTION

1.1 Background

Crop productivity is crucial to meet the food demand of the growing global population which is estimated to be nearly 10 billion by 2050. Thus it has been estimated that food production should be increased by 70% to fulfill the global food demand (Bahar et al., 2020). Urbanization leads to limited arable lands and high crop productivity contributes to global food demand without relying on additional lands. Moreover, higher crop productivity helps to decrease food shortage by ensuring food security. Climate changes and other environmental factors are influencing the seasonal crops, leading to abiotic stress conditions and ultimately impacting crop production and yield. Thus, different studies are being done to improve crop resilience towards environmental stresses such as heat, drought, salinity, etc.(Li et al., 2024). However, it is important to use biotechnology applications to develop climate-resilient crops to gain more crop productivity and ultimately succeed in feeding the global population**.**

1.2 Abiotic stress and its effect on plant growth.

Plants require several essential components to grow including energy, water, carbon, and mineral nutrients. Abiotic stress describes as the nonliving factors challenging environmental conditions that can significantly hinder plant metabolism, growth patterns and development, yield, and altered expression of inherited genes even in optimal conditions. Plants can experience abiotic stress from various factors such as extreme temperatures, salinity, drought, and mineral toxicity. The plant response to stress conditions depends on tissue or organs affected by stress and it is important to identify that the magnitude and duration of stress can significantly impact the complexity of an individual's response (Dinneny et al., 2008). The various factors that influence the plant tissues interrupt their normal metabolism plants experience stress, but they have a remarkable ability to adapt and develop new metabolic reactions that help them resist harsh environmental conditions (Takahashi et al., 2004). These stress response reactions play a critical role in regulating and sustaining the plant's overall health and well-being, enabling it to cope with

a variety of environmental factors, including extreme temperatures, drought, salinity, etc. Over time, plants have evolved a range of advanced mechanisms to cope with stress, including changes in gene expression, biochemical pathways, and physiological processes, all of which work together to help the plant survive and thrive in challenging conditions.

Abiotic stress has been found to affect the internal metabolism of plants, which can ultimately lead to decreased productivity. The negative impacts of abiotic stress factors are well-documented and can have a significant impact on plant growth and development (Hasanuzzaman et al., 2020). Temperature plays a crucial role in the growth and development of plants. Each plant species has a specific temperature range which it can thrive and maintain its sustainability patterns. This temperature range varies depending on the plant's genetic makeup and environmental factors. When the temperature falls outside the optimal range, it can have negative effects on the plant's growth and development, leading to stunted growth, reduced yields, or even death (Kosová et al., 2018). Therefore, it is crucial to maintain the appropriate temperature range for each plant to ensure its overall health and sustainability. Plants possess a multiple array of responses to osmotic stress, from the molecular to the whole plant level. These responses may lead to the inhibition of shoot and root growth, modifications in ion transport (including uptake, extrusion, and compartmentalization of ions), and changes in metabolic pathways (such as carbon metabolism and synthesis of compatible solutes). While some of these reactions are caused by primary osmotic stress signals, others may result from secondary stressors or signals induced by the primary signals(Kosová et al., 2018). These secondary signals can include phytohormones (such as ABA and ethylene), ROS, and intracellular second messengers (such as phospholipids). Some of these secondary signals may have far-reaching effects beyond the primary stress sites, such as the roots. For instance, root-produced ABA can travel through transpiration flow to modulate stomatal aperture in leaves during drought.

Soil salinity is a prevalent stress condition that significantly impacts plant growth and development. Commonly found in the natural environment, saline and alkaline soils coexist, forming saline-alkaline soils (Rengasamy, 2010). Unfortunately, most plants struggle to flourish in such environments, and even moderate salt concentrations (100mM) can lead to a sharp reduction in crop yield (Cheeseman, 2015). When the salt content in the soil exceeds a particular threshold, it creates a higher water potential within the plant than the soil, hindering water

absorption from the external environment. The inadequate water content in the seed impedes germination and growth. Moreover, saline soil has high metal ion concentrations, which can have a toxic effect on plants, further impeding their growth(Zhang et al., 2011).

1.2.1 Abiotic stress and effect at the cellular level

Abiotic stresses such as cold, heat, drought, and salt can be reasons for common cell disturbance while membrane damage, oxygen-reactive species generation and damage, and protein denaturation are considered secondary stresses (He et al., 2018). The response of plants to unfavorable conditions is determined by a complex network of cellular, physiological and morphological defenses. These defense mechanisms are organized as a complex regulatory network, that involves various signaling molecules including stress-related hormones (ethylene-ETH, abscisic acid), reactive oxygen species (ROS), calcium ions (Ca2+), hydrogen sulfide (H2S), nitric oxide (NO), polyamines and phytohormones (Krasensky & Jonak, 2012; Ku et al., 2018).

1.2.2 Abiotic stresses signal integration and adaptive responses

Plants naturally experience various stress signals via the primary receptors that are found in the plasma membrane. These sensors are usually activated, which leads to a blockade of ions across the membrane that results in an entry of Ca^{2+} into the cytosol (Fu et al., 2020). The energy balance of the photosystem and electron transport chain in mitochondria are disrupted because of physical reactions to abiotic stress and ultimately leads to ROS production. The cytosol contains only the amount of Ca^{2+} which is equal to the cascade abiotic stress signaling, through the vacuolar antiport transporters. Other secondary messengers such as cyclic AMP (adenosine monophosphate) trigger these signals. These secondary messengers are produced because of the activation of adenylate cyclase as a response to Ca2+ and ROS. These signals induce the activation of protein kinases such as Ca^{2+} dependent kinases (CDKs) and signaling influx of MAP kinases induces biosynthesis of hormones such as abscisic acid. Ultimately, stress transcription factors are activated by hormone signaling. These hormonal signals contribute to the regulation of many genes that are responsible for transcription.(Akpinar et al., 2012; Fraga et al., 2021; Gill et al., 2016).

Abiotic stresses cause different imbalances in cellular functions, which are mostly characterized as an increase in Ca^{2+} influx into the cells. The various ionic channels related to calcium ion accumulation keeping the level of calcium ion upon stimulation with stress but also react to the secondary stimulus from the unfavorable conditions regularly faced by them (Liu et al., 2018). Thus, cytosol Ca^{2+} influx is caused by abiotic stresses such as osmolarity, salinity and temperature that the plants are exposed to. Salt stress is recognized by plasma membrane, microdomains in plasma membrane rich in phosporylcerimide and enhance sensitivity towards salinity stress due to MOCA1. MOCA1 has been identified as a glucuronosyltransferase which adds glucuronic acid residue at inositol phosphorylceramide (Rennie et al., 2014). This generates a negative ionic pole for binding extracellular Na+ and the interactions between acidic ceramides and Na+ induces depolarization of cell surface. This triggers the opening of ANN1/4 ionic channels, which enhance Ca2+ influx. For homeostasis, downstream system activates SOS proteins(salt overly sensitive) and urges a negative feedback on ANN1/4 transporters by avoiding calcium spikes formation in cytosol (Ma et al., 2019).

Heat stress is also recognized by the plasma membrane as a secondary messenger, interconnected with Ca2+. Temperature alters flexibility and mobility of biomolecules and this is the case with fluidity changes reflected in the lipid bilayer, recognized by associated proteins like CNGG channels (Cui et al., 2020). Moreover, temperature leads to denaturation and eventually works as temperature sensors. Thus, misfolding proteins are identified by heat shock proteins (HSPs) that bind heat stress-responsive transcriptional factors (HSFs) in normal temperatures. When the HSPs detached from HSFs, stress-responsive genes become activated, and triggering downstream signals (Scharf et al., 2012). It is interesting to note that the promoter region of HSFs is in special heterochromatin, where nucleosomes are abundant in the H2A.Z histone variant. This variant resist for DNA unwrapping, but high-temperature conditions cause H2A.Z to dissociate from DNA. ultimately this enhances the accessibility of transcriptional factors to promoter regions of HSF genes, effectively controlling heat stress (McAinsh & Pittman, 2009).

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1.2.3 Role of Reactive Oxygen Species in stress signaling

The ROS ions are synthesized from atmospheric oxygen due to unstable unpaired electrons which have the potential to reactive with biological molecules. However, mechanisms of ROS in plants are not entirely recognized, the role of ROS in cellular mechanisms is identified such as abiotic stress responses, tolerance, and adaptation mechanisms. During stress conditions, ROS is produced in two main pathways (Choudhury et al., 2017): (1) the signaling pathway, in which ROS are produced as a response to the abiotic stress signal transduction pathway, and (2) the metabolic pathway, where ROS are generated as a result of metabolic imbalances. Thus, RO is capable of producing radicals that can lead to damage to cellular membranes and oxidation of both proteins and DNA, and this, in turn, can even accelerate oxidative destruction of cells(Choudhury et al., 2017).

Plants synthesize many proteins that are involved in ROS detoxification such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPOX) to prevent ROS damage. These components, which act as a modulator, are able to affect on plant stress responses by interfering with the interactions of hormonal signaling, metabolic, and developmental signaling (Choudhury et al., 2017). ROS act as secondary messengers in the signal transduction process while generating responses to stress conditions. Often Ca2+ influx or phosphorylation of downstream causes ROS production target by stress-responsive kinases. The ROS will transfer the message through all the cell components that occur as a result of the redox status. Such changes may cause protein function alteration, for example, regulation of transcription factor binding modulates the transcription of stress-responsive genes. Furthermore, they effectively act through the alteration of the oxidation state of intracellular regulatory enzymes and manipulate their metabolic function several times in different ways (You & Chan, 2015).

1.3 Chickpeas as an important legume for the world population

This study focuses on an important legume chickpea which is a highly nutritious food, rich in protein, beta-carotene, and essential minerals such as phosphorus, calcium, magnesium, iron, and zinc. They play a vital role as a source of both protein and starch in nations with significant vegetarian populations. On a global scale, over 2 billion individuals lack essential nutrients, specifically anemia, zinc, and vitamin A (Ritchie & Roser, 2024). The need arose for research and development efforts to unveil novel crop varieties exhibiting enhanced yield potential, wherein nutritional interventions are regarded as a mitigating factor. Chickpea is a rich source of protein, Fe, and Zn, leading to consumption as a part of a balanced diet that can alleviate malnourishment. Moreover, the chickpea plant has depicted several beneficial agronomic benefits such as adaptation to low nutrients in soils, readily grow on stored soil moisture, tolerance of dry periods, and efficient nitrogen extraction from soil at 80% of the total requirement (Sharma et al., 2013).

Chickpea is considered a winter season legume while performing unusual among other cool season legumes, in that it is tolerant to warmer climates. Chickpea plants are adaptative to cultivation in low moisture environment conditions and possesses some heat tolerance (Wery et al., 1993). Abiotic stresses such as heat and drought cause over 70% of global yield losses and many studies have been conducted to reveal new cultivars that could improve yield based on heat tolerance

(Kumar et al., 2013). However, heat stress is a major factor that significantly hinders chickpea production by causing adverse effects on seed germination, seedling establishment, and overall crop yield. To adapt diverse environments plants have developed adaptative mechanisms, involving many hormones and biomolecules to enhance abiotic stress tolerance such as ethylene, Gamma-Aminobutyric acid (GABA), abscisic acid (ABA) salicylic acid, jasmonic acid (JA), melatonin (MEL), cytokinin, etc.

1.4 Role of Ethylene in plant's physiological development and stress tolerance

Ethylene plays a crucial role in promoting abiotic stress tolerance in plants (Gamalero & Glick, 2012). Although ethylene is synthesized in plants under normal and abiotic stress conditions, ethylene plays a significant role in abiotic stresses such as high temperature and drought, high salinity, iron deficiency, flooding conditions, etc. Some studies have explored that the biosynthesis of ethylene is comparatively more effective under salt stress. In addition, it has been observed that ethylene-insensitive mutant plants of *Arabidopsis thaliana* are not tolerant to heat stress (Bharadwaj et al., 2022). Ethylene regulates enzymatic and non-enzymatic antioxidants against oxidative stress induced by abiotic stress (Husain et al., 2020). Also, ethylene interacts with other hormones and secondary metabolites such as flavonoids, phenolic compounds, alkaloids, phenolic compounds, amino acids and their derivatives, glucosinolates, etc. to coordinate and enhance stress tolerance. Ethylene modulates ion homeostasis under salt stress conditions. Not only that ethylene helps to maintain photosynthetic efficiency under abiotic stress by regulating chloroplast function and antioxidant metabolism (Fatma et al., 2022). Abiotic stress conditions cause reduction of seed germination resulting in declining seed quality, gemination potential, and seed vigor. In order to obtain sustainable crop yield, it is crucial to improve seed germination under abiotic stress (Wang et al., 2024). Seed germination is sensitive towards hormones while ethylene promotes seed germination. Seed priming techniques improve seed germination, where seeds are treated with chemical compounds to increase seed germination and seedling vigor under abiotic stress conditions. Ethylene plays an important role in plant development including seed germination and seedling growth. Thus ethylene-mediated seed priming is suggested as the best seed priming technique to enhance seed and survival rate under abiotic stress conditions (Wang et al., 2020)

1.4.1 Mechanism and regulation of ethylene biosynthesis

The metabolic pathway of ethylene production is one of the most widely studied and understood aspects of the plant hormone (Kende, 2003). This marked a significant advancement in the synthesis of ethylene by defining S-adenosylmethionine (S-AdoMet) and ACC as the ethylene precursors. S-AdoMet is formed from the methionine used in the reaction by SAM synthetase and the reaction costs one ATP per molecule of S-AdoMet produced. S–AdoMet is brought into a methylated acceptor molecule, where it transfers a methyl group to a variety of cellular molecules such as nucleic acids, proteins, and lipids (Yang & Hoffman, 2003). On the same note, we learn that S-AdoMat is involved in the synthesis of polyamines and that is through the Spermidine/Spermine biosynthesis pathway. Ethylene is closely related to ACC and is the second constituent but the immediate predecessor of ethylene. The S-AdoMet-dependent pathways are generally acknowledged to be constrained at the conversion of S-AdoMet to ACC by ACC synthase under most circumstances. This has been confirmed by the high levels of ethylene production that were obtained from growing explants in the presence of AVG. MTA is formed along with ACC during ACC synthesis through the action of ACC enzyme. Recycling of the MTA back to methionine helps to preserve the methylthio group and there is capacity to maintain a steady pool of methionine in the cell in case ethylene is synthesized at a higher rate. The reduction of ACC pool by conversion of ACC to its malonylated form, that is malonyl-ACC (MACC), minimizes the ethylene synthesis. ACC oxidase plays the role of participating in the last step of the synthesis of ethylene with the help of ACC as a substrate and results in the production of carbon dioxide and cyanide. Hatching indicates speculative relationships to regulation by transcription factors of both ACC synthase and ACC oxidase. Reversible phosphorylation of ACC synthase is assumed and its dephosphorylation can be mediated by some phosphatase while the phosphorylation may be done by some kinases which are assumed to be controlled by stresses. While both native and phosphorylated form (ACC synthase-Pi) of ACC synthase operate, the native ACC synthase might not be stable or active in vivo. Depending on the present status of the inhibitor it is bound to the carboxyl end of ACC synthase but when the enzyme is phosphorylated in that area, the inhibitor may get detached from the enzyme (Wang et al., 2002).

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1.4.2 Effect of Ethylene on Seed Germination

Abiotic stress conditions affect plant growth and development and it is evident that plant growth, development and senescence are mediated by hormones(Qin & Wang, 2020). These major three events are widely controlled by ethylene and thud ethylene(Eth) is considered a multifunctional phytohormone due to its diverse role in plant biological processes(Iqbal et al., 2017). The ethylene signal is detected by the endoplasmic reticulum to initiate a signaling cascade that stimulates the various development processes of transcriptional regulation in the nucleus. Exogenous ethylene can induce or impede plant growth and senescence, based on cell type, plant species, level of ethylene, and application time(Dubois et al., 2018). Ethylene leads to induction or inhibition of senescence depending on its optimal concentrations. Although Eth emissions cannot be well regulated the gaseous nature of Eth is the cause that limits its uses in investigational and applied contexts. Nonetheless, ethephon [(2-chloro-ethyl)-phosphonic acid], which is an Eth-emitting compound possessing a wide spectrum of applications, is used comprehensively in agriculture as a replacement for Eth (Hu et al., 2017). Ethylene upgrades seed germination and apical hook development. Further ethylene is responsible for hypocotyl elongation in light while inhibiting in dark. Further through interactions with auxin, ethylene enhances primary root and lateral root development. However low level of ethylene induces auxin biosynthesis and promotes lateral root initiation, while a higher level of ethylene inhibits growth of the lateral root.

Seed germination is considered the critical stage in plant growth as affects the final yield of the plant. Many plant hormones are responsible for seed dormancy and germination(Ibrahim, 2016). several studies have reported that seed dormancy is initiated by ABA while gibberellins are responsible for breaking seed dormancy and inducing seed germination. Moreover, cytokinins, auxins, Jasmonates, and brassinosteroids perform significant contributions toward seed germination(Miransari & Smith, 2014). However, However, there is still not enough evidence provided about crosstalk with ethylene (In Arabidopsis, Eth moves inversely to ABA while it interacts positively with GAs in Arabidopsis at the stage of seed germination. Thus , with respect

to ET, these hormones have interactive effects, with both ABA and GA regulating germination and seed dormancy through their biosynthesis and signaling (Arc et al., 2013).

The treatments of ABA and GA during germination alter the regulation of the enzyme ACC oxidase (ACO) besides ACC synthase (ACS) and influence Eth synthesis. Further, in Eth biosynthesis mutant aco2, a deficiency in ACO2 causes altered ET production that also hinders ABA-mediated inhibition during endosperm rupture. Eth also affects the responsivity of plant during the regulation of germination esoctetoy 3 (eto3) and constitutively triple response 1 (ctr1) mutants exhibited reduced sensitivity to ABA while ethylene receptor 1 (etr1), ethylene-insensitive 2 (ein2) and ethylene insensitive 6 (ein6) showed hypersensitive to ABA (Linkies & Leubner-Metzger, 2012).

1.4.3 Interactions between ethylene and other hormones toward abiotic stress

Besides involvement in the regular development, Eth is also known to participate in the regulation of growth and development with respect to abiotic stress, which has only recently been focused on. ERFs are vital downstream transporters that localize stress-mediated growth regulatory pathways in Eth-affected plants. For example, transgenic tobacco with overexpression of an ERF protein exhibits enhanced seedling tolerance to drought, salt, and freezing stresses, which is related with reduced accumulation of ROS which imply that ERFs have a very vital role in Eth mediated abiotic stress tolerance (Wu et al., 2008). Furthermore, under salt stress conditions, there is the activation of EIN3/EIL1 that enhances ABS sensitivity and helps weather salt stress directly, enhancing ROS removal at the seedling level of Arabidopsis thaliana in the Petri dish. Additionally, using ACC, an ethylene-producing plant hormone that is synthesized through the conversion of glycine, reduces the effect of salt on seed germination in Arabidopsis. Further, salinity treatment affects the ETR1 gene expression decrease in Arabidopsis, and ETR1 or EIN4 gene function disruption enhances seed germination under salt stress (Wilson et al., 2014). It can be noted that gain of function mutants namely etr1-1, ein4-1, and etr2-1 do not respond to Eth and exhibit higher salt sensitivity than the wild-type. Also, the downregulation of EIN2 contributes to a reduced seed germination rate and enhanced mortality rate of seedlings under salt stress, pointing to a positive role of EIN2 in salt stress tolerance. Salinity treatment was also observed to cause

accumulation of EIN3 in Arabidopsis; however, overexpression of EIN3 helps the plant to acclimatize to salt stress (Peng et al., 2014).

The previous findings suggest that Eth production and signaling improved or hampered plant tolerance depending on a particular species' sensitivity to salt stress, as well as the condition analyzed (Dubois et al., 2018). However, the positive function of ET in making tolerance of plant to salt stress has been widely accepted. They are encoded by C-REPEAT BINDING FACTOR (CBF) genes that have the ability to bind CRT/dehydration-responsive element (DRE; G/ACCGAC) of cold-responsive (COR) genes. The CBF triple mutants with the knockout of CBF1, CBF2, and CBF3 genes exhibit a reduced germination frequency compared to wild-type seeds at 4°C on MS media. Additionally, 7 transcription factors that have been implicated in hormone (Eth, GAs, auxin and BRs, JAs mediated transcriptional regulation are connected nodes between CBF-regulated cold acclimation and other hormones namely ABA, SA, and ETH (Zhao et al., 2016).

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From this literature, it is evident that Eth plays a role both in initiation of development of lateral root from pericycle as well as outgrowth as well as elongation of this lateral root. Investigations in this publication showed that both Eth and NO work oppositely to regulate the formation of lateral roots during Se stress in Arabidopsis. First, Se concentration causes the suppression of primary/root growth and the promotion of lateral roots. It is well known that Eth has the ability to inhibit NO synthesis in plants and the pharmacological experiments and mutant analysis indicate that Se stress influences the emergence of lateral roots of Arabidopsis by inhibiting NO synthesis in plants. On the other hand, Eth triggers lateral root formation in legumes and similarly, ABA has a positive impact on lateral roots (Herrbach et al., 2017). Compared to this, wheat transcription factor TaWRKY51 raises lateral root formation by diminishing Eth biosynthesis and reducing the

expression of AUX1, an auxin transport gene, thus minimizing auxin transport outside the zone of differentiation of the primary root, which in turn increases auxin concentration in the same zone and suggests that the promotion of lateral root formation by TaWRKY51 uses the auxin signaling pathway as well, a detailed view of ARFs is presented pointing out its significant functions in plant growth and development regulated by auxin (Hu et al., 2018).

1.4.4 Mechanistic view of ethylene

S-AdoMet is the precursor for ethylene biosynthesis (Kende, 2003; Yang & Hoffman, 2003). Methionine is also a critical constituent of proteins and almost 80% of methionine is converted to S-AdoMet with the help of enzyme S-AdoMet synthetase (SAM synthetase, EC 2.5. 1. 6) with depleting ATP in the process (Ravanel et al., 1998). S-AdoMet is the main methyl donor in plants, in addition, S-AdoMet is used in polyamines and ethylene biosynthesis since it is the methyl donor in various biochemical pathways (Ravanel et al., 1998). Moreover, S-AdoMe and S-AdoMet are also involved in the methylation process of lipids, proteins, and nucleic acids. According to the Yang cycle, the first constitutively regulated step in ethylene biosynthesis is the conversion of S-AdoMet to ACC by ACC synthase (S-adenosyl-L-methionine methylthioadenosine-lyase, EC 4. 4. 14) The created ACC biosynthesise ethylene through a cascade, for which it has been named after Yang (Yang & Hoffman Another product formed by ACC in this reaction is 5'-methylthioadenosine that is later metabolised to methionine, and this occurs through a modified methionine (Bleecker & Kende, 2000). This salvage pathway is useful so that the methyl group from MAG can be used again in the synthesis of ethylene. Thus, the catalytic pyrolysis of methionine makes it possible to obtain ethylene in continuous process without the need for ever larger amounts of methionine. On the same note, the sulfur group of the methionine is also conserved The MCP-1 bifunctional protein lacks sequences. Lastly, ethylene is synthesised by converting ACC to ethylene through the action of ACC oxidase and CO2 while the toxic cyanide is converted to β-cyanoalanine by β-cyanoalanine synthase (β-CAS, E. C. 4. 4. 1. 9 because high rates of synthesis harm plants.

Ethylene synthesis is believed to be controlled most specifically at the final point within the biosynthesis and synthesis of this important chemical compound from S-AdoMet to ACC with the help of an enzyme called ACC synthase (Kende, 2003). These include: First, the generation of the

ACS genes is only elicited by the variety signals Secondly the active ACC synthase is trans isotropic is short supply, these observations all point towards the realization that the synthesis of ethylene is stringently regulated activity. The present study showing feedback inhibition of ethylene biosynthesis in *Carica papaya* fruits and other plant species under similar conditions are supported by several previous studies (Barry et al., 2000; Kende, 2003; Nakatsuka et al., 1998) . Surprisingly, several types of ACS appear to constitute among the special action's primary goals. For example Le-ACS2 and Le-ACS4 are auto induced whereas Le-ACS6 is auto repressed during fruit ripening of ethylene tomato in the process (Nakatsuka et al., 1998). Most previous studies on endogenous regulation of ACS have focused on which of the ACS genes are activated when exposed to specific internal signals or extracellular signals. The only proof that can be deduced from the above results is that ACS enzymes are in different spatial and temporal contexts and the activity of ACS enzymes is controlled by sundry internal and external signals.

1.4.5 Ethylene signaling

Ethylene is received and through the process of transduction, there are biological reactions that are initiated. However, following the triple response observed using dark grown Arabidopsis seedling mutants that slow down their response to ethylene has been identified. Ethylene signal transduction pathway can be illustrated on the basic level using molecular biology techniques such as Mutant analyses in combination with cloning and characterization of the corresponding disrupted genes. In this species there are five ethylene receptors; that include, ETR1, ETR2, EIN4, ERS1, and ERS 2 (Chang et al., 1993). ETR1 has three TM helices and histidine kinase domain while ERS1 has five leucine rich repeat domains, which are the characteristic features of these proteins (Wurgler-Murphy & Saito, 1997). These facts suggest that both the proteins work in dimeric forms. The proteins ETR2, EIN4, and ERS2 are classified as the four-pas family of proteins that contains degenerate histidine kinase domains within their sequence of amino acids. From the domain annotation, it can be noted that only ETR1, ETR2, and EIN4 possess receiver domains at the C-terminus of the proteins (Hall et al., 2000; Schaller et al., 1995). It has been observed that a copper transporter named as RAN1 is necessary in the transport of copper to the ethylene receptor. In case there is no ethylene signal produced the protein named as CTR1 kinase that is like Raf comes into action and the CTR1 may again put off the further ethylene signal active

pathway theoretically through MAP-kinase chain. The EIN2/TEX1/KEG complex may actively promote the degradation of CTR1, and when the degradation of CTR1 is completed, EIN2 becomes a positive signal for the ethylene signal transduction pathway (Schaller & Bleecker, 1995). EIN2 c protein contains the N-terminal subdomain, which can be described as a bacterial Nramp metal transporter protein, and the C-terminal hydrophilic extension (Hall et al., 2000). Downstream EIN2 proceeds as a signal to the nucleus to alter the members of the EIN3 family of transcription factors. This study has provided evidence demonstrating that EIN3 is capable of binding to the promoter of the ERF1 gene and modulating its expression by stimulating transcription and this normally occurs through the action of ethylene. ERF1 and other subfamily proteins of EREBPs can directly interact with GCC box of promoter regions in target genes and modulate the subsequent signals of ethylene (Fankhauser et al., 1999) .

Heat stress has the possibility to lead to either enhancement or inhibition of ethylene production influenced by the activation or inhibition of ACS activity. A study also indicated that exposed heat stress increased the production of ethylene in kiwifruit while, some plants like tomato (Lycopersicon esculentum), it was reported to decrease (Biggs et al., 1988). Heat stress excites the production of ROS, which in turn leads to oxidative stress. Specifically, it is suggested that the rise in ROS to a specific level is able to give a signal for synthesis of the ethylene. MARYLENE; Whereas H_2O_2 interacts synergistically with ethylene as a positive feedback mechanism whereby H_2O_2 increases ethylene synthesis and H_2O_2 is responsible for the process of leaf senescence and chlorophyll degradation under heat stress. Ethylene production is thought to be elevated during heat stress, which may help explain why heat stress has been linked to reduced pollen germination and subsequent growth (Valluru et al., 2017). On the other hand, the decrease in the biosynthesis of ethylene using AVG causes a decline in chlorophyll accumulation and affects the process by increasing electrolyte leakage in *Lolium perenne*. Furthermore, it was also found that heat stress causes changes in ethylene production and responsiveness in floral and fruit of pea plant. Higher CV of GA and lower retention of these fruits are apparent when ethylene biosynthesis gene expression is up-regulated under heat stress in pre-pollinated ovaries(Savada et al., 2017). Some papers demonstrated that ACS regulation is mainly concerned with ACS gene regulation during the different stimuli. Moreover, these enzymes are mostly localized in specific compartments and organelles, regulated by space and time and also regulated and controlled by different external and internal signals. At high temperatures whether at about 35 °C or even above, there are no increases

in the level of ethylene in creeping bent grass. Hence, this suggests that that heat tolerance or heat stress responses in stress ethylene production cross different species of plant and ethylene has a time and dose-dependent effect on the plants during heat stress (Poór et al., 2022).

1.5 Justification

A previous study, similar to my study, has demonstrated the role of ethylene in Arabidopsis seedling development which affects plant growth and stress tolerance. Ethylene-pretreated plants have exhibited significant changes in plant development such as root growth, tissue growth, etc., and shown more tolerance to high temperatures. Moreover, ethylene pretreatment increased carbon assimilation (23%), glucose (266%), sucrose (446%) and starch (87%) content and ultimately increased photosynthesis rate (Brenya, 2023).

Chickpea is considered as an economically important food legume and plays vital role vital role as a source of protein and starch in nations with significant vegetarian populations. Being a winter crop, chickpea production and crop yield are reduced under different abiotic stresses specifically in high-temperature stress. Therefore, a necessity has arisen to develop strategies for stress resilience in chickpea plants under abiotic stress conditions. This study aims to investigate the effects of ethylene pretreatment of chickpea seeds on the physiological, biochemical, and molecular biological responses of chickpea plants subjected to heat stress. Specifically, this research will analyze physiological and biochemical parameters such as photosynthetic efficiency, antioxidant enzyme activity, and GABA level. The objective is to elucidate if ethylene pre-treatment of seeds mediates improved stress tolerance via increased germination, early vigor, and growth in chickpea plants. The objectives of this study were

I. Determine the best ethylene precursor and optimize concentration.
II. Analyze the difference between foliar-treated plants and ethylene-priming plants.
III. Investigate the effect of ethylene and foliar treatment on physiological and biochemical responses subjected to heat stress.

Hypothesis

Ethylene (ACC and ethephon) treatment will enhance the abiotic stress of chickpea plants by resulting their physiological and biochemical responses. Ethylene and Foliar treated plants will exhibit higher GABA level, Lower MDA level and alternated starch content when compared to controls.

CHAPTER 2

METHODS

2.1 Determine the optimal concentration and best ethylene precursor for chickpea growth

Two different ethylene precursors were applied, and their effective concentrations were tested for the chickpea growth affecting factors, namely ACC (1- amino cyclopropane-1-carboxylic acid) (A3903, Sigma Aldrich) and ethephon (C0143, Sigma Aldrich)

2.1.1 ACC treatment

Forty seeds from the ICC-12726 variety and forty from the Genesis-836 variety were obtained as a gift from Dr. Crystal Sweetman. Seeds from each variety were divided into four groups with ten seeds per replicate for both varieties. Each replicate of the ten seeds was weighed to find out if all the replicates had similar weights before treatment. The seeds' surfaces were sterilized with 100% bleach solution (v/v, 6% sodium hypochlorite) for 5 minutes. Then the seeds were rinsed briefly with sterile water for one minute. Four petri dishes were prepared for each variety at 0 µM, 10 µM, 50 µM, and 100 µM ACC treatment. Each petri dish was accommodated with 10 seeds. The seeds were transferred onto filter paper and 10 ml of different concentrations of ACC solution dissolved in distilled water was added to petri plate. Each petri plate was wrapped with aluminum foil and incubated for 3 days at room temperature.

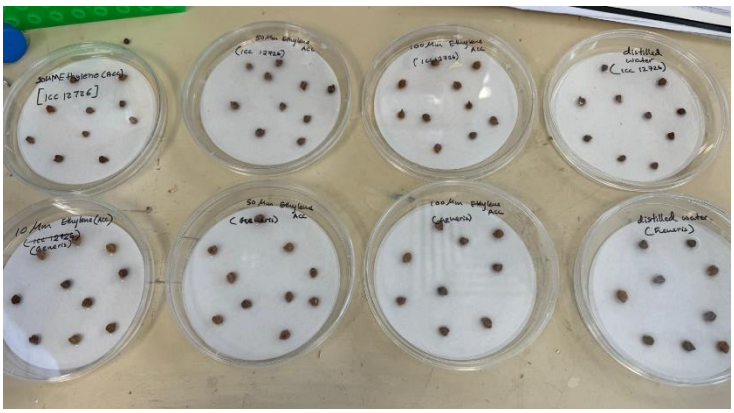

Figure 2.1: Ten chickpea seeds from each variety were accommodated in petri dishes and treated with 0 µM, 10 µM, 50 µM, 100 µM ACC solutions and placed for seed germination.

Preparation of ACC solution

Different concentrations of ACC solutions were prepared using 10 mM stock solution diluted with distilled water. Aliquoted each volume ACC derived from the stock solution into the 50 ml sterile falcon tubes and made up to the required 50 ml mark using distilled water.

$C_1V_1=C_2V_2$

Eg: 10000 µM × V_1= 10 µM × 50 ml

V_1= 0.05 ml

ACC concentration	0 µM	10 µM	50 µM	100 µM
Volume of ACC (ml)	0	0.05	0.25	0.50
Volume of Distilled water(ml)	50	49.95	49.75	49.5
Total Volume (ml)	50	50	50	50

After three days of incubation, seeds were scored for germination percentage and root and shoot length. Then seeds were transferred into a pot filled with bio-grow soil obtained from the University of Adelaide. Biogrow soil is referred to organic soil enriched with nutrients and other beneficial micro- organisms for healthy plant growth Pots were clearly labelled and kept in a greenhouse for growing. For two weeks plants were watered and growing was observed. After 2 weeks plants were carefully removed from the soil and root length, shoot length, fresh shoot biomass, and fresh root biomass were measured. Roots and shoots were placed in brown paper bags and kept in the oven at 60 ° C for three days to obtain dry weights (DW).

Figure 2.2: ACC- treated chickpea seeds were sown in pots filled with bio grow soil and kept for greenhouse for two weeks for growing.

2.1.2 Ethephon treatment (ETH)

Fifty seeds from the ICC-12726 variety and fifty from the Genesis-836 variety were obtained. Seeds from each variety were divided into five groups containing 10 seeds per replicate for both varieties. Each replicate of the ten seeds was weighed to find out if all the replicates had similar weights before the treatments. Five plastic containers were prepared for each variety for five different concentrations of ethephon treatments. 0 µM, 5 µM, 50 µM, 500 µM, 1 mM ethephon concentration series was prepared using ethephon powder dissolved in distilled water. Each container had 10 seeds from each variety. At the bottom of the container, two filter papers were placed with seeds moistened with 10 ml each of various concentrations of ethephon. Container lids were closed immediately after treatment and wrapped with aluminum foil for dark incubation. Then all the containers were kept at room temperature for four days for germination.

After four days of incubation, seeds were observed for germination percentage and scored for root and shoot length. This was followed by transplanting seeds to pots containing a brand of soil called bio grow. Pots were clearly labeled and kept in a growth chamber under a 12-hour photoperiod for

2 weeks. For two weeks plants were watered and growing was observed. After 2 weeks, plants were carefully removed from the soil and root length, shoot length, fresh shoot biomass, and fresh root biomass were measured. After obtaining fresh shoot and root biomass, roots and shoots were placed in brown paper bags and kept in the oven at 60 ° C for three days to obtain dry weights.

Figure 2.3: Ethephon- treated chickpea seeds were sown on pots filled with bio grow soil and placed in the growth chamber under 12-hour photoperiod for two weeks.

2.2 Full scale study

Based on data from the root and shoot biomass from the pilot study, 5 µM was determined as the optimum ethephon concentration for seed germination of two chickpea varieties. Four plastic containers were prepared with filter papers, at 0 µM and 5 µM ethephon concentrations for each variety. Then all together 8 containers were prepared for both varieties including two containers for each treatment. Twenty seeds were placed in each relevant container and the seeds were weighed prior to treatment. Two different 0 µM and 5 µM ethephon solution were prepared using ethephon powder dissolved in distilled water. Each container had 10 seeds from each variety. At the bottom of the container, two filter papers were placed with seed moistened with 20

ml from two concentrations of ethephon. Container and wrapped with aluminum foil for dark incubation. Then all the containers were kept at room temperature for five days for proper seed germination. After five days of incubation period, seeds were observed for germination percentage and measured root and shoot length. Then seeds were transferred into clearly labelled pots (2.8L) filled with biogrow soil and at greenhouse 3 under 17° C for 34 (5 weeks) days.

Figure 2.4: After incubation seeds were sown in 2.8L pots which filled with biogrow soil and kept at greenhouse 3 under 17° C until heat treatment.

Figure 2.5: Ethephon treated and untreated plants chickpea plants after four weeks of sowing; grown at 17 ° C

2.2.1 Foliar treatment

Four containers were prepared for both varieties while two for each. Two containers were prepared for ICC-12726 variety and 20 seeds were accommodated in each container. Another two containers were prepared for Genesis-836 variety and 25 seeds were accommodated in each container. After that the seeds were treated with distilled water and incubated for three days wrapped in aluminum foil. After incubation of three days, 12 pots were prepared and clearly labelled. A well germinated seed was selected and transferred into each pot. 12 whole pots were transferred into greenhouse allowing them to grow under 17 °C. On 31 days from seed potting, plants were exposed to foliar treatment with GABA spray for two days (second foliar treatment was done after two days of first foliar treatment). 5mM GABA solution was prepared for foliar treatment. The amount of 0.515g GABA powder was dissolved in 1000 ml distilled water and 0.3ml of silwet (0.03%) surfactant was added in to 5mM GABA solution to promote better adhesion and reduce surface tension on foliar surfaces. After the second day of foliar treatment, plants were transferred into a different bay in greenhouse for heat treatment at 27°C for 24 and 72 hours.

Figure 2. 6: Foliar treatment with GABA spray; 5mM GABA solution was spray in to each plant at three times for two days. All twelve controlled chickpea plants from both varieties were exposed to foliar treatment prior to heat treatment.

2.2.2 Sampling

After applying 24 hours of heat stress for ethephon-treated (0 µM and 5 µM) plants and foliar-treated plants, leaves samples were collected from each plant. The top two leaves were gently removed and placed in a falcon tube. Likewise, two leaves were collected from each and all plants and leaves were collected into falcon tubes separately. Falcon tubes were placed in the liquid nitrogen container immediately after sampling. Then all samples were placed in the -80 C freezer for biochemical analysis. Sampling was done at 4-week and 12-week sampling points.

2.2.3 Harvesting

At week 4 and week 12 after sampling, the plants were harvested. The entire plants including roots and shoots, were carefully removed from the pots, and the root-bound soil was washed with water and dried to remove access water. Once clean, the roots and shoots were carefully excised and root and shoot length were measured. Immediately following the length measurements, the root and shoot biomass was measured. The fresh sample weights were important for understanding the growth and development of the plant. After that, the shoots and roots were labeled and placed into separate bags. Then the labelled samples were placed in an oven for three days at 60° C to obtain dry weight. During the drying process, moisture was fully removed allowing for accurate measurement of the dry weight of both roots and shoots.

2.3 Biochemical analysis

Prior to conducting the biochemical assays, the frozen samples (72 samples) were ground using metal beads. After grinding, samples were weighed and aliquoted into the required amount for all biochemical analyses. During the weighing process samples were maintained under super cool condition using liquid nitrogen to prevent any unnecessary thawing. This ensured the integrity of the sample throughout this process.

2.3.1 TBAR assay

The TBAR assay (Thiobarbituric Acid Reactive Substances assay) is used to detect MDA (malondialdehyde). MDA is synthesized via autooxidation and enzymatic degradation of polyunsaturated fatty acids (lipid peroxidation). In TBAR assay, MDA which is contained in samples reacts with TBA under an acidic heat environment. The reactions between MDA and TBA yield a pinkish-red chromogen with maximum absorbance at 532nm(Ghani et al., 2017).

A TBAR assay was conducted on 72 samples collected at the week 4 and week 12 sampling points, resulting in a total of 144 samples.

Materials - 5% TCA, solution A, solution B, Malondialdehyde (MDA)

Solution preparation

250 ml 5% TCA

12.5g of TCA was dissolved in distilled water.

150 ml solution A

30g of TCA (20%), 0.015g of BHT (0.01%) and 0.75g of TBA (0.5%) were completely dissolved in distilled water using magnetic stirrer with heat function.

150ml solution B

30 g of TCA (20%) and 0.015 g of BHT (0.01%) were completely dissolved in distilled water.

0.1mM Malondialdehyde (MDA)

To prepare 100mM MDA solution, 0.0360g was dissolved in 5 ml. From this solution, 1 µL of the 100 mM MDA was taken and diluted with 999 µL of 5% TCA to create a 0.1 mM MDA solution.

Standard preparation

Standard concentration(nmoles)	Amount of MDA to add to TCA	Amount of 5% TCA to add
0	0	1000
1	10	990
3	30	970
4	40	960

Approximately 40g- 70g of homogenized tissue was weighed and aliquoted into 2.0 ml tubes. 72 homogenized tissue samples were prepared. 1 ml volume of 5%(w/v) TCA was added into each tube and vortexed for a few seconds. After that samples were centrifuged at max speed for 15 minutes. Then the 400µl of supernatant was transferred into two centrifuged tubes named tube ' a' and 'b'. 400µl of 'solution A' which contained 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid(TBA) was added to tube 'a'. 400 µl of solution B which contained 20%(w/v) TCA containing no 0.5% (w/v) thiobarbituric acid (TBA) was added to 'tube B'. Likewise, two tubes were prepared with two different solutions for each sample. Samples were heated at 96°C for 30 minutes. The lids of each tube were opened to avoid pressure during the heating process. Immediately after 30 minutes, samples were placed on ice for 5 minutes sometimes and centrifuged at 9500g for 10 minutes. After centrifugation 100 µl of sample was added to 96 well plates as duplicates. Readings were taken at 440nm, 532nm, and 600nm wavelengths using a CLARIOstar microplate reader machine(Hodges et al., 1999; Singh et al., 2012).

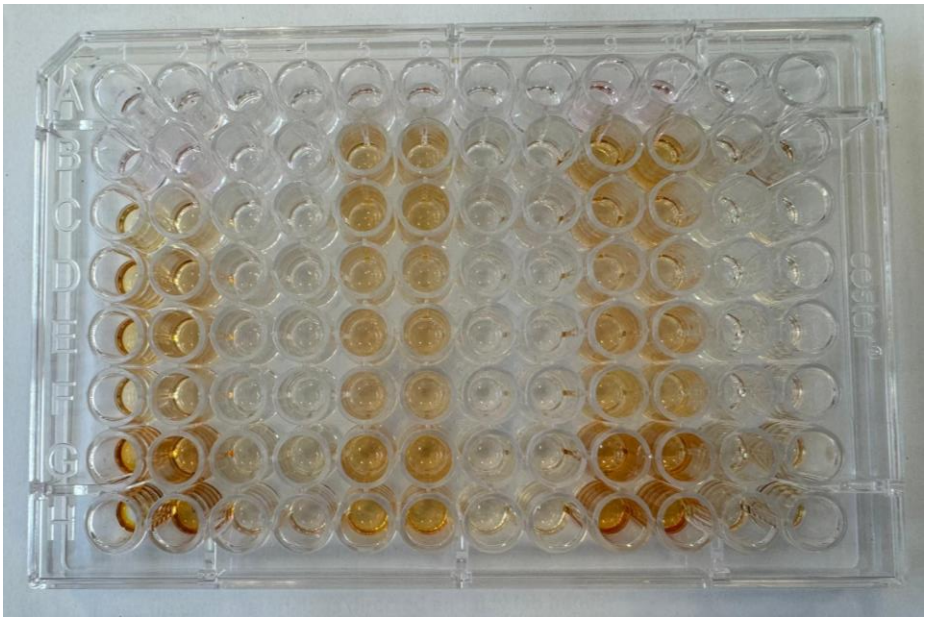

Figure 2.7: The 96 microplate well view just before taking absorbance: Yellow colored wells indicating samples with ‘solution a’ while wells with pale yellow or colorless indicated samples with ‘solution b’. Wells with a pink color were represented standards with ‘solution a’ and wells without any color change indicated standards with ‘solution b’

Calculation for MDA equivalent value.

1) $[(\text{Abs } 532_{+TBA}) - (\text{Abs } 600_{+TBA}) - (\text{Abs } 532_{-TBA} - \text{Abs}600_{-TBA})] = A$

2) $[(\text{Abs } 440_{+TBA} - \text{Abs } 600_{+TBA})\ 0.0571] = B$

3) MDA equivalents ($\text{nmol} \cdot \text{ml}^{-1}$) = $((A-B)/157\ 000)\ 10^6$

A Standard curve was plot for each plate using MDA equivalents values. The equation derived from the standard curve, along with the weight of each sample, was used to calculate the MDA level (nmol/g) in each sample.

2.3.2.GABA assay

GABase is the main enzyme used in GABA assay. Under optimal pH and temperature conditions, GABase enzyme catalyzes the GABA into succinic semialdehyde(SSA) with the presence of α-ketoglutarate as co-substrate.

GABA+α-ketoglutarate ⟶ SSA + Glutamate

In further reaction, SSA is oxidized into succinate while NADP+ is reduced into NADPH.

$SSA+NADP^{+} \longrightarrow Succinate + NADPH + H^{+}$

The amount of NADPH produced via enzymatic reaction is directly proportional to the GABA level in the sample and NADPH exhibits maximum absorbance at 340nm wavelength(Wu et al., 1981).

A TBAR assay was conducted on 72 samples collected at the week 4 and week 12 sampling points, resulting in a total of 144 samples.

Materials: Methanol 100%, 70 nM Lanthanum chloride, 1M potassium hydroxide, 9.0 pH 0.5M potassium pyrophosphate ,100 % 2-Mercaptoethanol, 20mM α-ketoglutarate, 10Mm Nicotinamide adenine dinucleotide phosphate (NADP), GABase 5U/ml.

Sample preparation

70mM Lanthanum chloride: 5.199 g of lanthanum chloride was completely dissolved in 200 ml distilled water.

1M KOH: 5.610g of KOH fully dissolved in 100 ml of distilled water.

9.0 pH 0.5M potassium pyrophosphate- 24.775g of potassium pyrophosphate was completely dissolved in 150 ml of distilled water and pH was adjusted to 9.0 using 10 M KOH.

20mM α-ketoglutarate: 0.672g of α-ketoglutarate was fully dissolved in 200 ml of distilled water.

GABA extraction

Approximately 50.0mg of frozen tissue powder was weighed and aliquoted in to 2.0 ml centrifuge tube. 400 µl of methanol was added in to each tissue powder sample and incubate at 25 °C for 10 minutes in a heating block. Then the samples were allowed to dry in the speed vacuum. Speed vacuum was run for 2 hours to dry the samples. After obtaining dry samples, pellet was resuspended in 500 µl of Lanthanum chloride and shaken at 100 rpm for 15 minutes. Then the samples were centrifuged at 12000 rpm for 5 mins. The 400 µl of supernatant was again transferred in to 2.0ml tubes which was containing 160 µl of 1M KOH. Then the tubes were inverted in order to mix the solutions and allowed them to shake at 100 rpm for 5 minutes. After shaking samples were centrifuged at 12000 rpm for 5 minutes. Then the supernatant was carefully pipetted out to new 2.0 ml tube and store at 80° C until analysis(Ramesh et al., 2015).

GABase enzyme assay

The volume of 45.2 µl of sample extract was transferred to 96 microwell plates as duplicates. After plating the samples, the master mixture was freshly prepared just before taking the readings. Below reagents were mixed in the following order. Special care was taken to minimize NADP exposure to light, as it is highly light-sensitive. BME, being highly toxic, was added last and mixed in the fume hood to ensure safety.

Reagent	Per sample (per well)	For 100 samples(for 100 wells)
1. 0.5M $K_4P_2O_7$	15 µl	1500 µl
2. 20mM α-ketoglutarate	25 µl	2500 µl
3. 10nM NADP	12.5 µl	1250 µl
4. 2-Mercaptoethanol(BME)	0.33 µl	33 µl

Then 52.8µl of master mix was added to each well. The pre reaction absorbance was measured before adding GABase at 340 nm for 3 cycles at 120 secs cycle time at 25 °C. After obtaining pre

absorbance reaction, 2 µl of GABase was added to each well and absorbance was measured at 340 nm for 30 cycles of 90 secs cycle time at 25 °C.

A standard curve was plotted for each plate using pre reaction absorbance values(A1) and absorbance values obtained after adding GABase enzyme(A2). The equation derived from the standard curve, along with the weight of each sample, was used to calculate the GABA level (µmoles) in each sample(Ramesh et al., 2015).

2.3.3 Starch assay

The starch assay is derived from the enzymatic hydrolysis method which quantifies the starch content in the sample. The starch molecules are hydrolyzed into glucose units by amylase and amyloglucosidase enzymes and the amount of glucose is quantified by using the colorimetric method. GOPOD reagent plays and key role in this enzymatic reaction. GOPOD contains two enzymes known as glucose oxidase and peroxidase. Glucose oxidase oxidizes free glucose into gluconic acid and hydrogen peroxide. Peroxidase enzyme reacts with hydrogen peroxide and chromogenic substance in GOPOD reagent to produce colored compound. The intensity of color directly proportional to the glucose content in the sample and absorbance is measured at 510 nm wavelength (GOPOD is very light-sensitive because of the chromogenic compound. Expose into light causes photodegradation and GOPOD is always stored under dark conditions) (McCleary, 2019)

$$\text{Glucose} + O_2 \xrightarrow{\textit{Glucose Oxidase}} \text{Oxidase Gluconic Acid} + H_2O_2$$

$$H_2O_2 + \text{Chromogen} \xrightarrow{\textit{Peroxidase}} \text{Colored Compound}$$

A starch assay was conducted on 72 samples collected at the week 4 and week 12 sampling points, resulting in a total of 144 samples.

Materials- 80% v/v ethanol, 1.7 M sodium hydroxide, pH 3.8 sodium acetate buffer, amylase enzyme, amyloglucosidase enzyme, GOPOD, glucose standard

Sample preparation

80% v/v ethanol- 80 ml of 100% ethanol was poured into a graduated cylinder and distilled water was added to bring the total volume up to 100ml

Chilled 1.7M sodium hydroxide- 6.8 g of sodium hydroxide was weighed and completely dissolved in 100ml of distilled water. Then a solution was placed in the refrigerator to obtain chilled sodium hydroxide.

4M sodium hydroxide- 8.0g of sodium hydroxide was completely dissolved in 50 ml of distilled water.

pH 3.8 sodium acetate buffer-17.4 ml of glacial acetic acid was added to 400 ml of distilled water and pH was adjusted to pH 3.8 using 4M sodium hydroxide. Then 0.444g of calcium chloride dihydrate was added and dissolved. The volume was adjusted to 600 ml with distilled water and stored at room temperature.

Glucose standard(1mg/ml)- 20 mg of glucose was weighed and dissolved in 20 ml of distilled water.

Starch Extraction:

Approximately 50mg of homogenized tissue was weighed and aliquoted into 2.0ml tube and stored at -80 °C. 150 µl of 80% v/v ethanol was added to each frozen sample and vortex for few seconds. Then refrigerated chilled 1.7M sodium hydroxide was added and vortex again for few seconds. After that samples were shaken for 15 minutes at 100 rpm. Samples were centrifuged at 10000g for 2 minutes to obtain supernatant.

Starch Assay:

A volume of 150 µl of supernatant was pipetted to two microcentrifuge tubes named as unknown sample extracts (UK) and unknown sample blank (UKB). Then 400 µl of pH 3.8 sodium acetate buffer was added to both UK and UKB samples. A volume of 5µl of amylase and 5 µl of amyloglucosidase were added to each UK sample while 10 µl of pH 3.8 sodium acetate buffer was added to each UKB sample. Then all the samples were vortex for 5 seconds and incubated at 50

°C for 30 min. After incubation samples were allowed to cool for minutes and inverted to mix well. 200 µl from each UK and UKB extract were pipetted into new microcentrifuge tubes. All the samples were centrifuged at 13 000 rpm for 5 min. After centrifugation volume of 100 µl from each sample was pipetted into new micro centrifuge tube. A 5µl from each sample (UK and UKB were pipetted into 96 micro wells in plate triplicates. Additionally, 5 µl of glucose standards and 5 µl of distilled water were pipetted into microwell plate as triplicates.

After pipetting all the samples and blanks into 96 well plates, 150 µl of GOPOD reagent was added to each well. Then the 96 microwell plate was covered with aluminum foil and incubated at 50° C for 20 minutes. After the incubation absorbances were measured at 510 nm using CLARIOstar microplate reader. Prior to measuring absorbance, the shake function on the microplate reader was used to mix for 5s after incubation(McCleary et al., 2019).

Starch % calculation

$$\textbf{Starch \%} = \frac{\Delta A \times F \times EV \times 1 \times 100 \times 162}{0.005 \times 1000 \times W \times 100}$$

ΔA = Absorbance of sample solution read against reagent blank, less the absorbance of the sample blank read against reagent blank

F = Sample extraction volume(0.71ml)

0.005 ml = Volume of sample analyzed

1/1000 = Conversion from µg to mg

100/ W = conversion to 100mg sample

162/180 = Factor to convert free glucose, as determined to anhydroglucose as occurs in starch (McCleary et al., 2019)

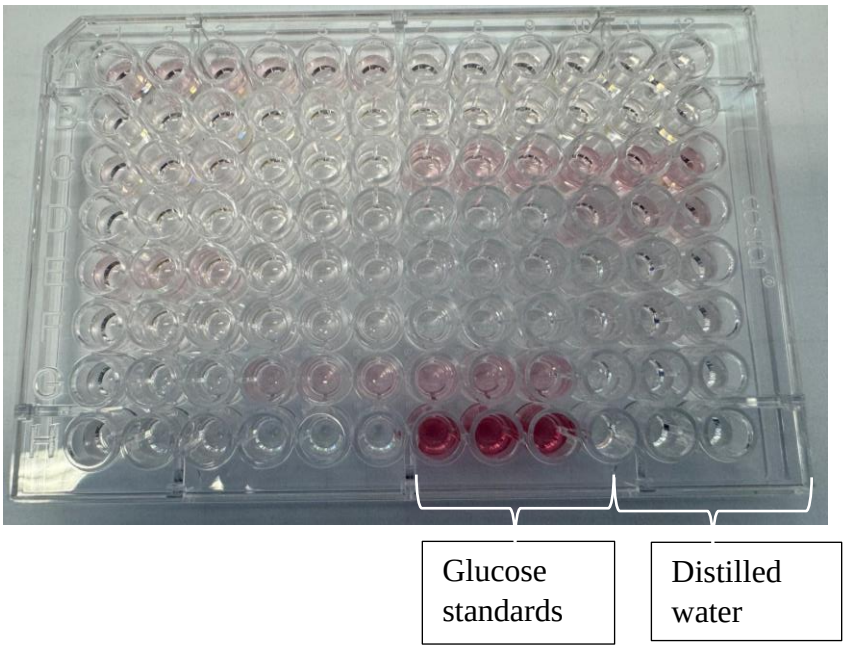

Figure 2.8: The 96 microplates well view after adding GOPOD and 20 minutes incubation at 50 °C. Most wells containing UK samples indicated light pink color. Most of UKB samples have shown no color change while some wells have shown pale pink. Dark pink color wells represented glucose standards.

CHAPTER 3

RESULTS

3.1 Determine the best ethylene precursor and concentration

Forty seeds from each ICC-12726 and Genesis-836 chickpea varieties were obtained and seeds were grouped into 8 groups comprising 10 seeds in each group. Then seeds were treated with ACC and ethephon which are ethylene precursors at different concentrations (0µM and 5 µM) to determine the best ethylene precursor and concentration.

3.1.1 Physiological parameters of ACC-treated ICC-12726 and Genesis-836 chickpea plants

The germination percentage for ACC-pretreated ICC-12726 chickpea plants ranged from 20%-70% while the germination percentage for ACC-pretreated Genesis-836 chickpea plants ranged from 40%-70% (Figure3.1). There was a significant difference between 0 µM and 100µM ACC treated ICC-12726 and Genesis-826 chickpea plants for germination percentage. The germination percentage of 100µM ACC treated Genesis-836 chickpea plants was 2.3 folds significantly higher than those treated with 50 µM. The 100µM ACC treated ICC-12726 chickpea plants was 3.5 folds significantly higher than those treated with 0 µM ACC (Figure 3.1). After 3 days of ACC pretreatment, root length was measured for both chickpea varieties. The root length of 100µM ACC-treated ICC-12726 chickpea plants was 3.4 folds significantly higher than 0µM ACC-treated chickpea plants (Figure 3.1). Considering the Genesis-836 plant,0µM ACC-treated chickpea plants have shown 1.3 folds significant in root length compared to 100µM ACC-treated chickpea plants.

The fresh shoot and root biomass of ACC pretreated chickpea plants was measured after two weeks of seed potting. The fresh shoot biomass of ICC-12726 plants ranged from 0.357 g- 0.164g, 0.108g-0.434g, 0.288g-0.432g, 0.373g-0.471g for 0µM, 10µM, 50µM,100µM ACC treated plants respectively (Figure3.1). In contrast, fresh shoot biomass for Genesis-836 plants ranged from 0.4450g-0.571g, 0.409g-0.637g, 0.142g-0.526g, 0.441g-0.595g for 0µM, 10µM, 50µM,100µM ACC treated plants respectively (Figure 3.1). Notably, only ACC-treated ICC-12726 chickpea plants exhibited significant differences for fresh shoot biomass. ICC-12726 plants treated with

100µM ACC have shown a 2.1-fold significant in fresh shoot biomass than those treated with 10µM ACC (Figure 3.1). The fresh root biomass of ICC-12726 plants ranged from 0.160g-0.194g, 0.073g-0.304g, 0.163g-0.321g, 0.290g-0.495g for 0µM, 10µM, 50µM,100µM ACC treatments respectively (Figure 3.1). In comparison fresh shoot biomass for Genesis-836 plants ranged from 0.523g-0.655g, 0.415g-0.680g, 0.147g-0.618g, 0.409g-0.713g for 0µM, 10µM, 50µM,100µM ACC treated plants respectively. Significantly for root biomass, there was no significant difference between ACC treatments for both varieties (Figure 3.1).

According to the results ACC pre-treated chickpea plants didn't show significant differences in physiological parameters such as germination percentage, root length, shoot and root biomass, etc. Thus, ICC-12726 and Genesis-836 chickpea seeds were again pretreated with different concentrations of ethephon solutions to determine the significance of physiological parameters.

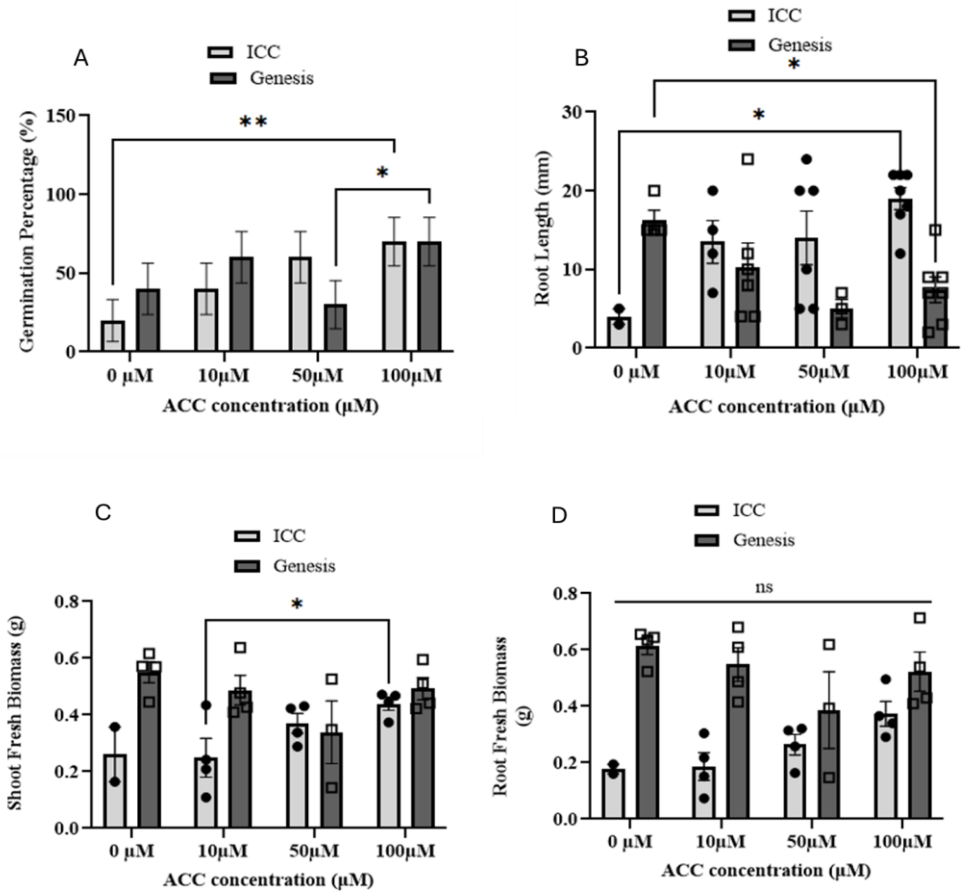

Figure 3.1. Physiological responses of ACC-treated ICC-12726 and Genesis-836 chickpea plants. A) The germination percentage of ACC-treated chickpea seeds was examined after 3 days of treatment. Ten seeds were treated with 0µM, 10µM, 50µM, and 100µM varying ethephon concentrations, and germination percentage was determined by analyzing the number of germinated seeds out of the total seeds. B) The root length of ACC-treated chickpea plants after 3 days of ACC treatment. C) The fresh shoot biomass of ACC pretreated ICC-12726 and Genesis-836 chickpea plants measured at harvesting, after 2 weeks of seed potting. D) The fresh root biomass of ACC pretreated chickpea plants was measured at a two-week harvesting point. Data in A-D represent the mean±SD ($n \leq 4$), the data were analyzed by two-way ANOVA, and the * indicates a significant difference ($p<0.05$) while ** indicates a significant difference ($p<0.005$)

3.1.2 Physiological parameters of ETH-treated ICC-12726 and Genesis-836 chickpea plants

The germination percentage of ethephon pretreated ICC-12726 and Genesis-836 chickpea seeds ranged from 80%-100% , 20%-40% respectively. Both varieties didn't show a significant difference in germination percentage with different ethephon concentrations. In comparison, ethephon-pretreated ICC-12726 chickpea seeds have shown higher germination percentage than

ethephon-pretreated Genesis-836 chickpea plants. According to the results, no germination was observed in Genesis-836 seeds which have been treated with distilled water while equal germination percentage was observed in 5µM, 50µM, and 1000µM ethephon pretreated seeds. For ICC-12726 chickpea seeds, 5µM and 500µM ethephon pretreated chickpea seeds were observed to have a higher germination percentage compared to other ethephon treatments (Figure 3.2).

Root and shoot length and shoot and root biomass were measured after two weeks of seed potting. The shoot length of ethephon pretreated ICC-12726 chickpea seeds ranged from 8.5cm-18.0cm, 16.5cm-19.5cm, 15.0cm-19.5cm, 8.0cm-10.5cm, 7.5cm-9.5cm for 0µM, 5µM, 50µM, 500µM, 1000µM ethephon treatments respectively. For both ICC-12726 and Genesis-836 chickpea plants, 5µM, and 50µM, ethephon-pretreated chickpea plants have shown higher shoot length compared to other ethephon-pretreated plants. 1000µM ethephon pretreated ICC-12726 and Genesis-836 plants were observed to have significantly less shoot length compared to other treatments. ICC-12726 plants that were treated with 0µM, 5µM, and 50µM ethephon concentrations have shown significant differences ($p<0.0001$) towards 1000µM ethephon-treated plants. Moreover, for shoot length, 0µM, 500 µM ethephon-treated Genesis-836 chickpea plants have shown less significant differences to 1000µM ethephon-treated plants while 5µM, 50µM ethephon-treated plants were observed higher significant($p<0.0001$) to 1000µM ethephon treated plants (Figure 3.2).

The root length for ethephon pretreated ICC-12726 chickpea plants ranged from 14.0cm-22.0 cm, 19.0cm-21.5cm, 18.0 cm-23.0 cm, 12.0 cm-17.5cm, 8.0 cm-18.0 cm for 0µM, 5µM, 50µM, 500µM, 1000µM ethephon treatments respectively. In contrast, the root length for ethephon pretreated Genesis-836 chickpea plants ranged from 2.2 cm-11.5 cm, 15.0 cm-24.7 cm, 14.0cm-19.5cm, 7.0cm-17.0cm, 1.5 cm - 6.0 cm for 0µM, 5µM, 50µM, 500µM, 1000µM ethephon treatments respectively. Based on the results, both chickpea plants have shown higher root lengths at 5µM and 50µM ethephon treatment conditions. Notably, 50µM and 5µM ethephon-treated Genesis-836 chickpea plants have shown 12 folds and 16 folds significant than 1000µM ethephon-treated plants while other ethephon-treated plants did not show any significant difference in root length (Figure 3.2).

The fresh shoot biomass for ethephon-treated ICC-12726 plants ranged from 0.7560g-1.6900g, 1.271g -1.409 g, 1.134g-11.294g, 0.518 g-0.895 g, 0.439g- 0.613g for 0µM, 5µM, 50µM, 500µM, 1000µM. The fresh shoot biomass of ethephon-treated Genesis-836 plants ranged from 0.218g -

0.748g, 1.082g -1.917g, 0.843g-1.177g , 0.555g-0.549g , 0.279g- 0.389g for 0µM, 5µM, 50µM, 500µM, 1000µM. In comparison, 5µM and 50µM ethephon-treated ICC-12726 and Genesis-836 chickpea plants have shown higher fresh shoot biomass than other ethephon-treated plants. ICC-12726 plants treated with 0 µM, 5 µM, and 50 µM ethephon exhibited significant differences($p<0.0001$) compared to those treated with 1000 µM. Similarly, Genesis-836 plants treated with 5 µM and 50 µM ethephon showed significant differences($p<0.0001$) when compared to plants treated with 1000 µM (Figure3.2).

For ethephon treated ICC-12726 plants fresh root biomass ranged from 0.5259g-1.8859g, 1.026g-1.4115g, 1.027g-1.492g, 0.495g-1.098g, 0.363g-0.695 for 0µM, 5µM, 50µM, 500µM, 1000µM. The fresh root biomass of ethephon-treated Genesis-836 plants ranged from 0.172g-0.774g , 1.194g-2.507g , 0.734g -1.694g , 0.457g – 0.801g and 0.228g-0.1923g for 0µM, 5µM, 50µM, 500µM, 1000µM. Both ICC-12726 and Genesis-836 chickpea plants that have been treated with 5µM and 50µM ethephon concentrations have shown significant differences to chickpea plants treated with 1000µM ethephon concentration in fresh root biomass. ICC-12726 plants treated with 5 µM and 50 µM were 1.8 folds and 2.6 folds significantly higher than 1000 µM ACC treated plants Genesis-826 plants treated with 5 µM and 50 µM were 7.9 folds and 5 folds significantly higher than 1000 µM ACC treated plants (Figure 3.2).

Based on results obtained from ethephon treatments, both varieties have shown higher shoot and root length, shoot and root biomass toward 5µM and 50µM ethephon pretreatment while Both varieties exhibited diminished values towards 1000µM ethephon treatments for the aforementioned physiological parameters.

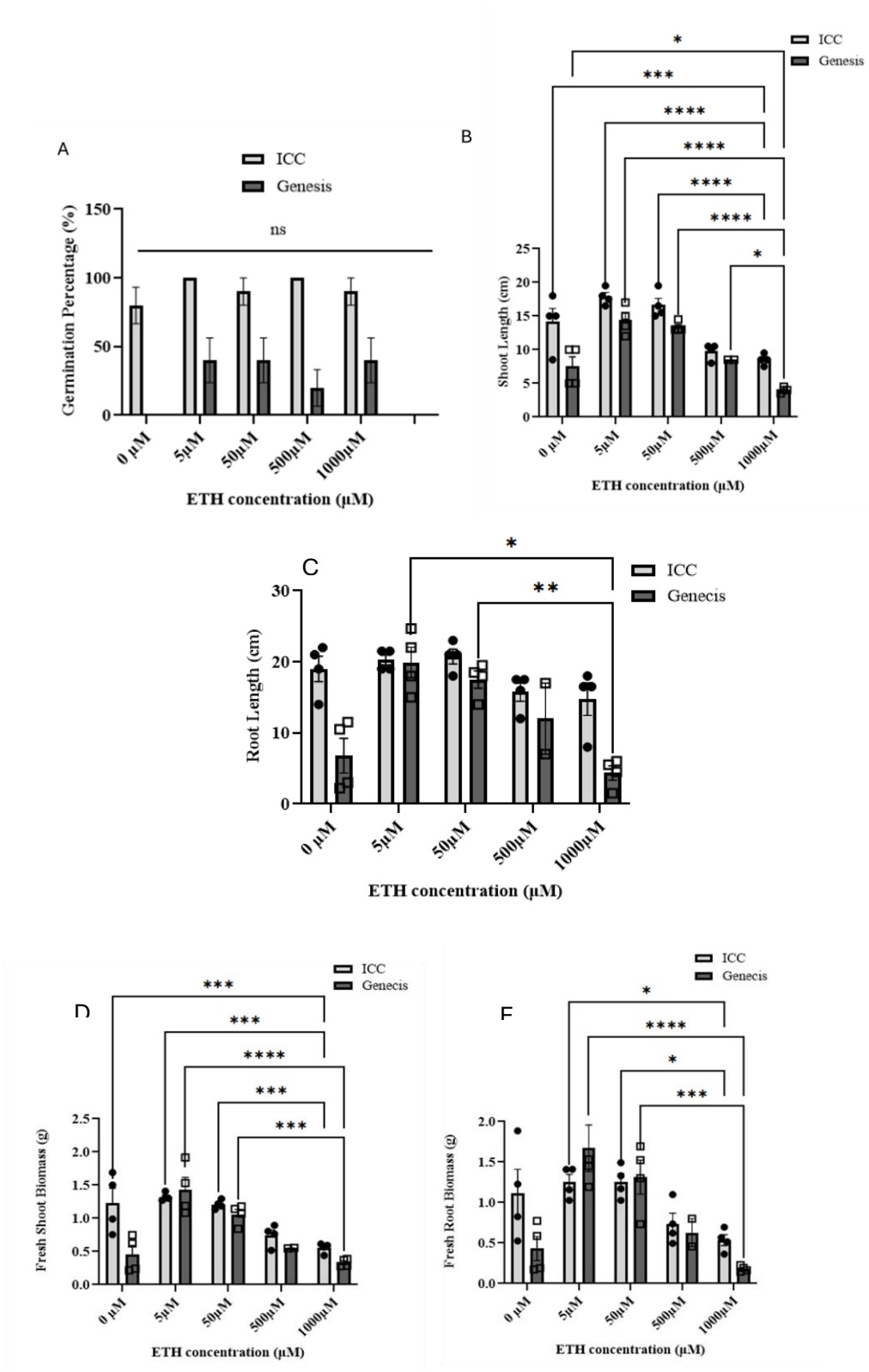

Figure.3.2. Physiological responses of ICC-12726 and Genesis-836 chickpea plants at 0µM, 5µM, 50µM, 500µM, and 1000µM ethephon concentration. A) The Germination percentage of ethephon pretreated

chickpea seeds was examined after 3 days of treatment. Four different ethephon concentrations, each containing 10 seeds were subjected to treatments, and the germination percentage was determined by analyzing the number of germinated seeds out of the total seeds. B) The shoot length of ethephon-treated chickpea plants was measured after 2 weeks of seed potting. harvesting of ethephon treatment. C)The root lengths of ethephon-treated chickpea plants were measured after 2 weeks of seed potting) The fresh shoot biomasses of ethephon pretreated ICC-12726 and Genesis-836 chickpea plants were measured at two two-week harvesting points. E) The fresh root biomasses of ethephon pretreated chickpea plants were measured after two weeks of seed planting. Data in A-E represent the mean±SD ($n \leq 4$), the data were analyzed by two-way ANOVA, and the * indicates a significant difference ($p<0.05$) while **** indicates a significant difference ($p<0.0001$)

3.2 Determination of MDA equivalent value of ICC-12726 and Genesis-836 chickpea plants under different treatments

Heat stress was applied for both varieties of chickpea plants at 4-weeks and 12-weeksafter germination. At 4-week sampling point heat stress (27°C) was applied for 24 hours and 72 hours and during 12-week sampling point heat stress (27°C) was applied for 72 hours and 144 hours (extreme condition). MDA levels are measured to examine reactive oxygen species (ROS), considered biomarkers for oxidative stress in chickpea plants subjected to heat stress. During the experiment, MDA equivalent levels of heat-treated and non-heat-treated chickpea plants which were treated with different ethephon concentrations and foliar treatments were measured. Plants were treated with 5mM GABA solution as foliar treatment. This assessed the oxidative stress of chickpea plants with different treatments under heat stress conditions.

3.2.1 Determination of MDA equivalents level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under control and heat stress conditions at 4-week sampling point

MDA level of foliar untreated ICC-12726 plants under control and heat stress conditions varied from 6.90nmol/g- 11.36nmol/g and 7.59nmol/g-12.41nmol/g respectively. In contrast, foliar-treated ICC-12726 plants under control and heat stress conditions varied from 5.11nmol/g-11.35 nmol/g and 5.11nmol/g-12.91 nmol/g respectively (Figure 3.3).

Considering MDA values for ethephon treated ICC-12726 chickpea plants, 5 μM ethephon plants under control and heat stress conditions varied from 5.87nmol/g- 6.00nmol/g and 13.84nmol/g-23.83nmol/g correspondingly. Notably, 5 μM ethephon-treated ICC-12726 plants were 2-fold

higher than 0 μM ethephon-treated plants under heat stress conditions. Moreover, 5 μM ethephon-treated ICC-12726 plants at heat stress condition were 5-fold higher than those treated with heat stress conditions (Figure 3.3).

Genesis-836 chickpea plants that have been treated with foliar treatment under heat stress and control conditions did not show a significant different towards Foliar untreated (control) plants under heat stress and control conditions. MDA level of control Genesis-836 plants under control and heat stress conditions varied from 6.88nmol/g- 9.52nmol/g and 7.78nmol/g-8.27nmol/g respectively. In comparison foliar-treated Genesis-836 plants under control and heat stress conditions ranged from 7.20nmo/g-16.27nmol/g and 9.52 nmol/g- 13.78nmol/g (Figure 3.3).

Ethephon-treated (5μM) Genesis-836 chickpea plants under both control and heat stress conditions exhibited less MDA values compared to ethephon-untreated plants under similar conditions. But there no significant difference was exhibited among these two groups. MDA values of 5μM ethephon treated Genesis-836 plants at control and heat stress conditions ranged from 7.01 nmol/g-8.39nmol/g and 7.00 nmol/g-8.17 nmol/g correspondingly (Figure 3.3).

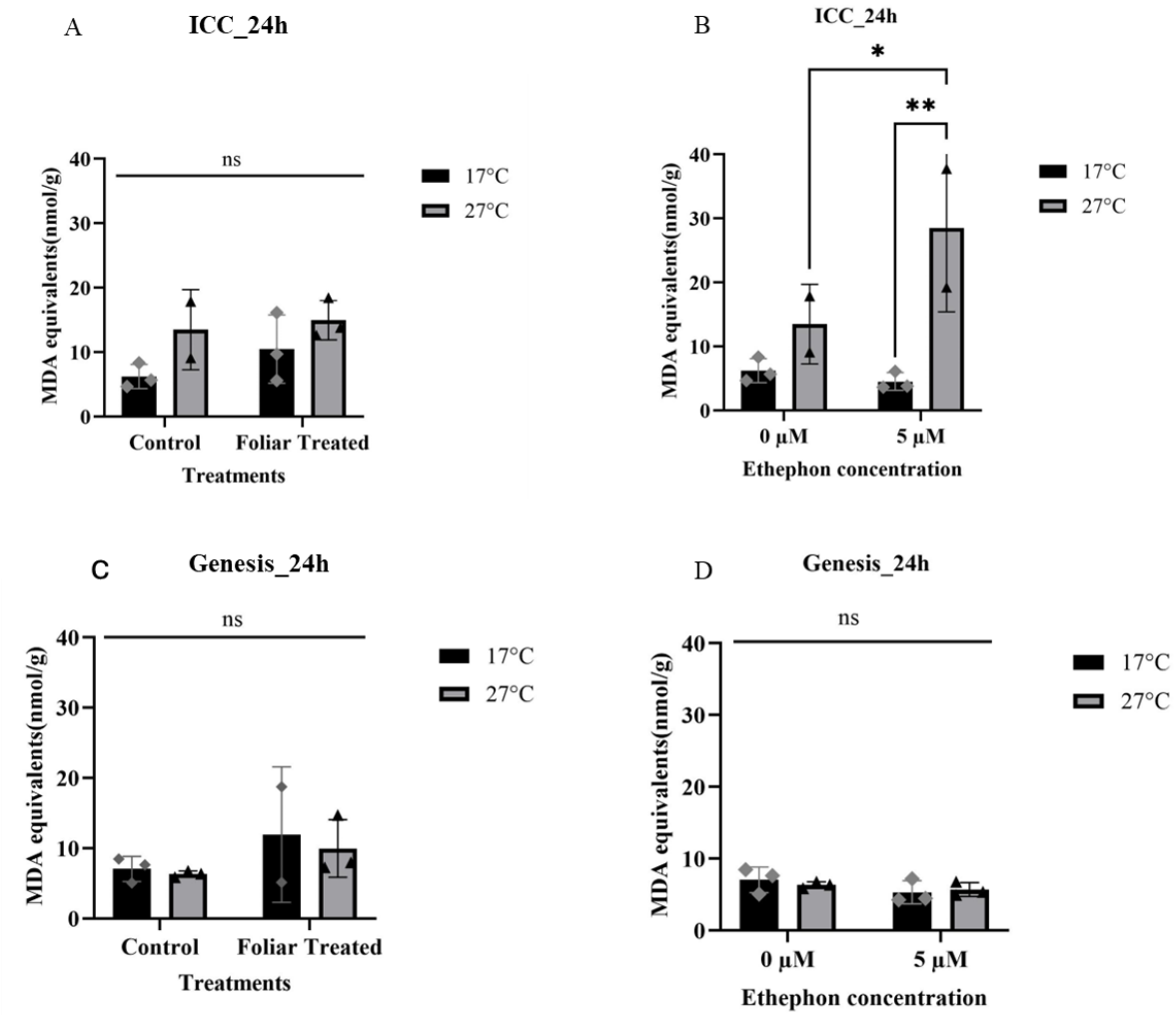

Figure 3.3. MDA equivalent level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions for the 24-hour time period at 4-week sampling point. The heat stress condition was 27∘C while the control condition was 17∘C.A) MDA equivalent level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) MDA equivalent levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) MDA equivalent level of foliar treated and foliar untreated Genesis-836 chickpea plants. D) MDA equivalent levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD (n≤3), two-way ANOVA analyzed the data, and the * indicates significant difference($p<0.01$) while ** indicate a significant difference($p<0.001$) . 'ns' indicates a non-significant difference.

MDA level of foliar untreated ICC-12726 plants under control and heat stress conditions varied from 11.80 nmol/g- 14.73 nmol/g and 20.95 nmol/g-24.08 nmol/g respectively. In contrast,

foliar-treated ICC-12726 plants under control and heat stress conditions varied from 12.15 nmol/g-10.43 nmol/g and 13.12 nmol/g- 27.73 nmol/g respectively. Notably, foliar untreated plants at heat stress 1.6 fold higher than foliar untreated plants at control conditions (Figure 3.4).

Considering MDA values for ethephon treated ICC-12726 chickpea plants, 5 μM ethephon-treated plants under control and heat stress conditions (72 hours) have shown higher MDA values than 0 μM ethephon treated plants at control and heat stress conditions. However, no significant difference can be found between these two groups. MDA levels of 5μM ethephon treat ICC-12726 plants at control and heat stress conditions varied from 13.34 nmol/g- 18.70 nmol/g and 18.16 nmol/g-64.04 nmol/g correspondingly (Figure 3.4).

Foliar-treated Genesis-836 chickpea plants at both control and stress conditions were 1.7 fold and 2 fold higher than foliar untreated plants at control and heat stress conditions. The MDA level of control Genesis-836 plants under control and heat stress conditions varied from 7.10 nmol/g-7.26nmol/g and 10.73nmol/g- -14.33 nmol/g respectively. In comparison foliar-treated Genesis-836 plants under control and heat stress conditions ranged from 12.23 nmol/g-14.79 nmol/g and 19.54 nmol/g- 21.19 nmol/g. Notably, both foliar untreated and foliar-treated Genesis-836 plants at heat stress conditions (17∘C) were 1.5-fold and 1.7-fold higher than those under control (17∘C) conditions (Figure 3.4).

Both 0μM and 5μM ethephon-treated Genesis-836 chickpea plants under heat stress conditions(27∘C) were 1.5 fold and 1.8 fold higher than those under control conditions. Considering the MDA level of ethephon-treated Genesis-836 plants, 5μM ethephon-treated Genesis-836 plants at control and heat stress conditions ranged from 5.90 nmol/g- 8.55 nmol/g and 14.33 nmol/g-15.72 nmol/g correspondingly (Figure 3.4).

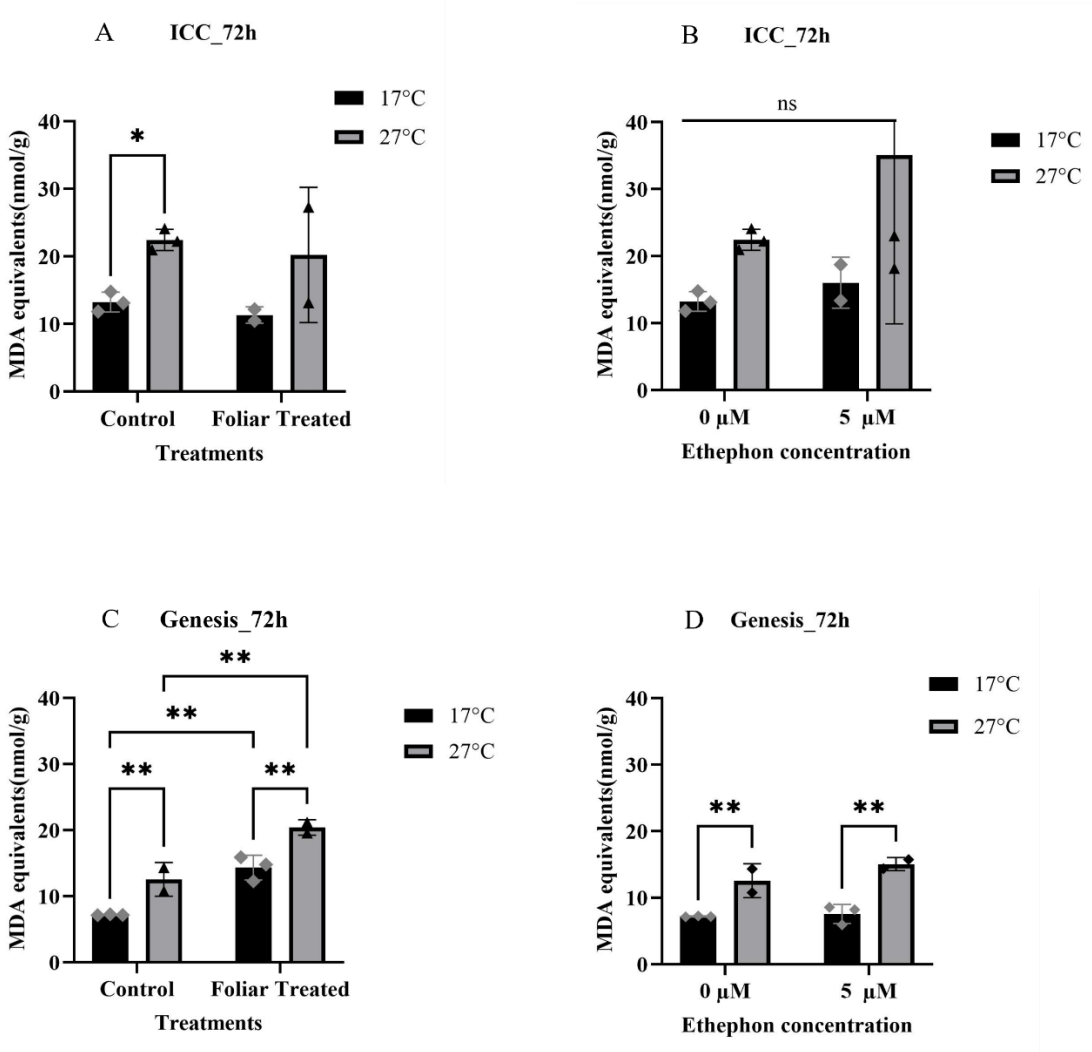

Figure.3.4 MDA equivalent level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions for 72 hour time period at 4-week sampling point. Heat stress condition was 27∘C while control condition was 17∘C.A) MDA equivalent level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) MDA equivalent level of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) MDA equivalent level of foliar treated and foliar untreated Genesis-836 chickpea plants.D) MDA equivalent levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD ($n \leq 3$), the data were analyzed by two-way ANOVA, and the * indicate significant difference($p<0.01$) while ** indicates a significant difference($p<0.001$) . 'ns' indicates a non-significant difference.

3.2.2. Determination of MDA equivalents level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under control and heat stress condition at 12-week sampling point

MDA level of foliar untreated (control) ICC-12726 plants under control and heat stress conditions varied from 15.65 nmol/g- 26.56 nmol/g and 10.45 nmol/g-27.39 nmol/g respectively. In contrast, foliar-treated ICC-12726 plants under control and heat stress conditions varied from 12.02 nmol/g- 17.52 nmol/g and 15.88 nmol/g- 25.12 nmol/g respectively. However, there was no significant difference between foliar untreated and foliar-treated groups under control and heat stress conditions. Considering MDA values for ethephon-treated ICC-12726 chickpea plants, 5 μM ethephon treated plants under control and heat stress conditions varied from 18.64 nmol/g-21.09 nmol/g and 15.01 nmol/g-36.71 nmol/g correspondingly (Figure 3.5).

Foliar untreated(control) Genesis-836 chickpea plants at both control and stress conditions were 2.8-fold and 1.7 fold higher than the foliar-treated plants at control and stress conditions. Notably, both foliar untreated and foliar treated plants at heat stress conditions (27∘C) were 1.6 folds and 2.6 folds higher than the plants under control temperature conditions(17∘C). The MDA level of foliar untreated Genesis-836 plants under control and heat stress conditions varied from 14.61 nmol/g-23.17 nmol/g and 21.27 nmol/g- 25.24 nmol/g respectively. In comparison, foliar-treated Genesis-836 plants under control and heat stress conditions ranged from 5.64 nmol/g-8.87 nmol/g and 14.92 nmol/g- 17.18 nmol/g (Figure 3.5).

Ethephon untreated Genesis-836 chickpea plants exhibited high MDA levels at heat stress conditions compared to control conditions while ethephon-treated Genesis-836 chickpea plants have shown less MDA levels at heat stress conditions compared to control conditions. However, there was no significant difference between the two groups for MDA level under control and heat stress conditions. MDA values of 5μM ethephon treated Genesis-836 plants at control and heat stress conditions ranged from 15.45nmol/g-26.57nmol/g and 9.10nmol/g - 30.27nmol/g correspondingly (Figure 3.5).

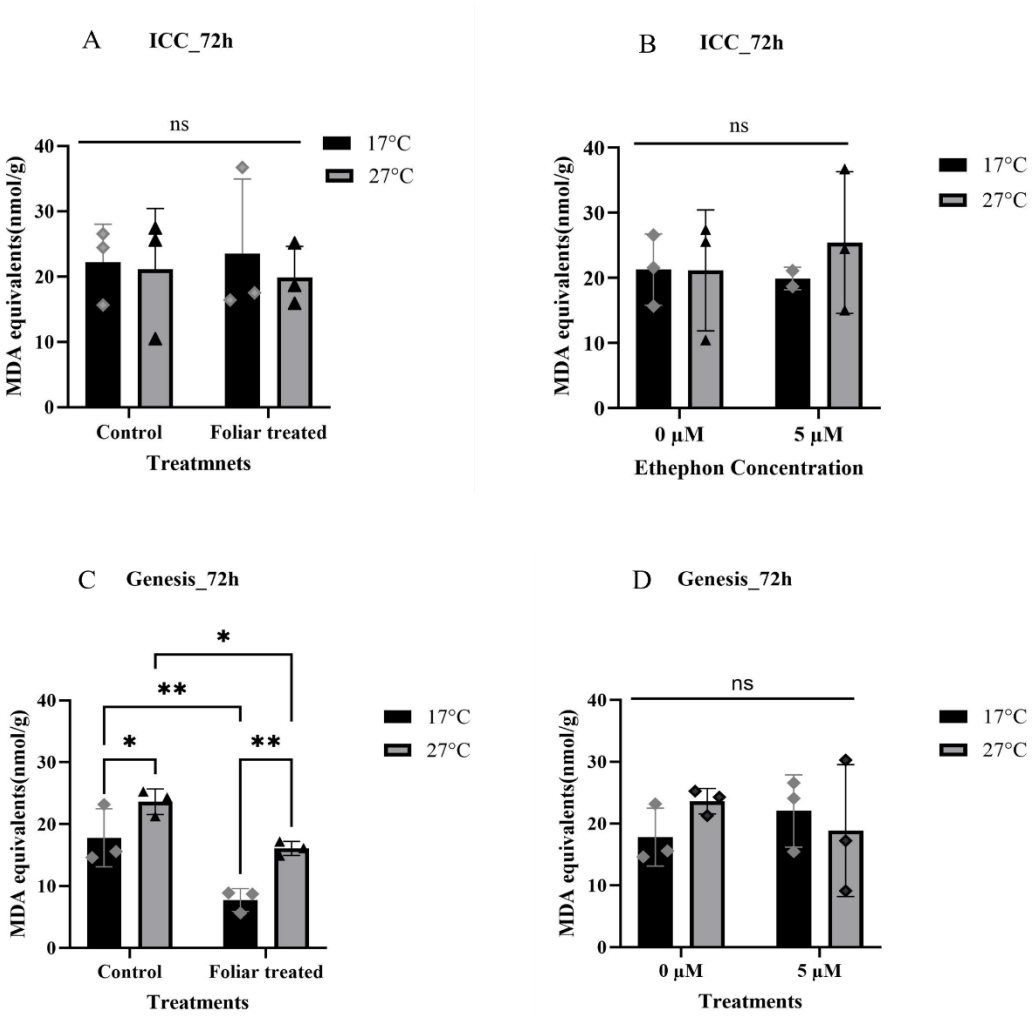

Figure 3.5. MDA equivalent level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions for 72-hour time period at 12-week sampling point. The heat stress condition was 27∘C while the control condition was 17∘C.A) MDA equivalent level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) MDA equivalent levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) MDA equivalent level of foliar treated and foliar untreated Genesis-836 chickpea plants.D) MDA equivalent levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD ($n \leq 3$), the data were analyzed by two-way ANOVA, and the * indicates significant difference ($p<0.01$) while ** indicates a significant difference($p<0.001$) . 'ns' indicates a non-significant difference.

Foliar untreated and foliar treated ICC-12726 under heat stress conditions were 3.2 folds and 5.6 folds higher than those under control conditions(17∘C). Considering the MDA level of ethephon-treated ICC-12726 plants, foliar untreated Genesis-836 plants at control and heat stress conditions ranged from 9.56 nmol/g- 16.44 mol/g and 30.735nmol/g- 44.97 nmol/g correspondingly. In contrast, foliar-treated ICC-12726 plants at control and heat stress conditions varied from 8.47 nmol/g- 15.69 nmol/g and 28.39 nmol/g-48.11 nmol/g (Figure 3.6).

Similarly, Foliar untreated and foliar-treated Genesis-826 plants under heat stress conditions(27∘C) were 2.4 folds and 2.7 folds higher than those under control conditions(17∘C). Foliar untreated Genesis-836 plants at control and heat stress conditions varied from 12.837nmol/g- 19.86 nmol/g and 20.37 nmol/g- 47.432 nmol/g while foliar-treated Genesis-836 plants at control and heat stress conditions varied from 11.13nmo l/g- 17.25 nmol/g and 26.12 nmol/g- 34.06 nmol/g correspondingly (Figure 3.6).

The 0μM and 5 μM ethephon-treated ICC-12726 plants under heat stress conditions were 3.2 folds and 2.5 folds higher than plants under control conditions. The 5 μM ethephon-treated ICC-12726 plants under control and heat stress conditions varied from 13.99nmol/g- 17.22 nmol/g and 30.51 nmol/g- 44.97 nmol/g correspondingly. Similarly, the 0μM and 5 μM ethephon-treated Genesis plants under heat stress conditions were 2.4 folds and 2.6 folds higher than plants under control conditions. The 5 μM ethephon-treated Genesis-836 plants under control and heat stress conditions varied from 10.77 nmol/g- 15.31 nmol/g and 38.64 nmol/g- 45.49 nmol/g correspondingly (Figure 3.6).

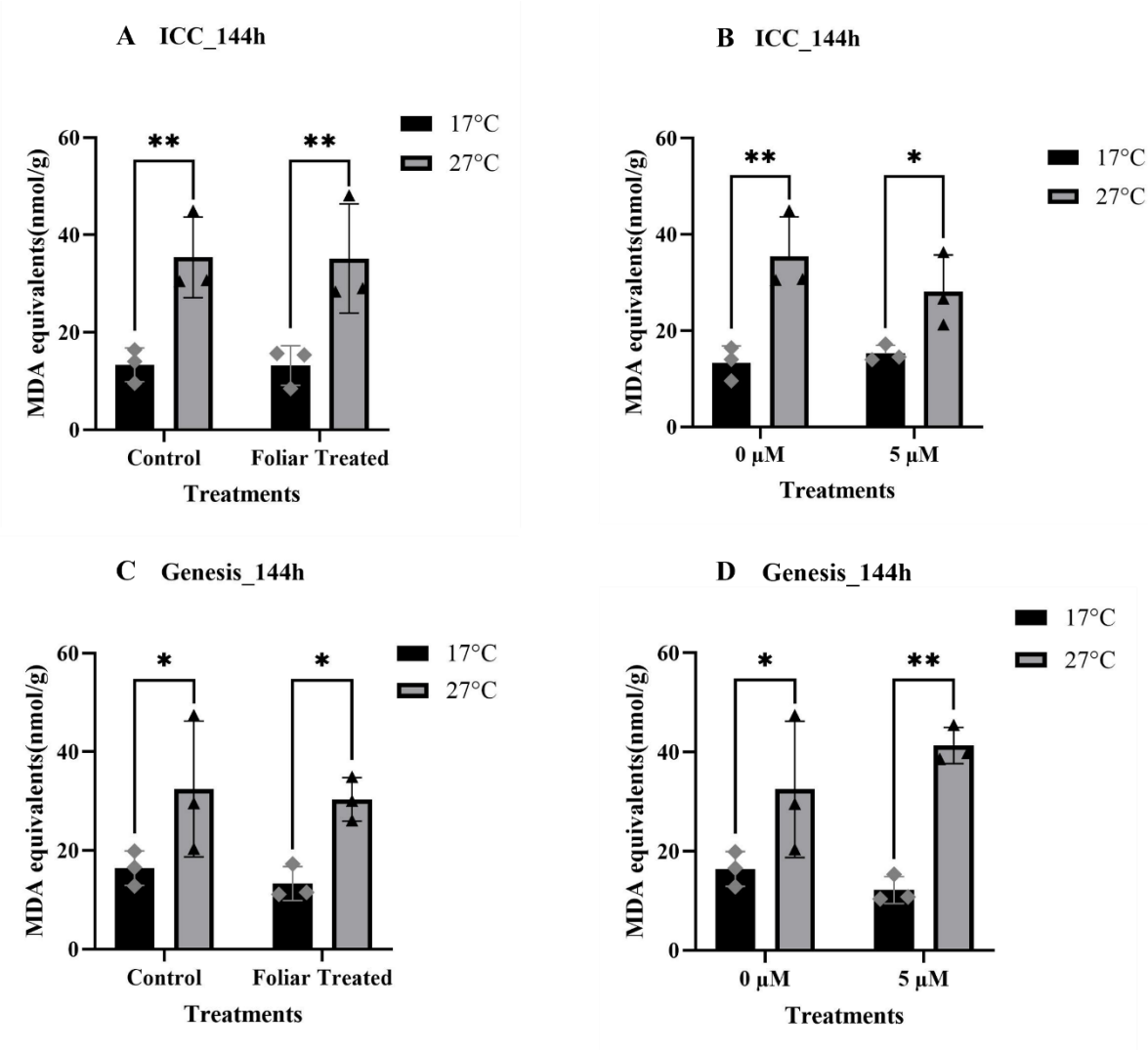

Figure 3.6 MDA equivalent level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions for 144-hour time period(extreme) at 12 week sampling point. The heat stress condition was 27∘C while control condition was 17∘C.A) MDA equivalent level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) MDA equivalent level of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) MDA equivalent level of foliar treated and foliar untreated Genesis-836 chickpea plants. D) MDA equivalent levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD (n≤3), the data were analyzed by two-way ANOVA, and the * indicate significant difference($p<0.01$) while ** indicates a significant difference($p<0.001$).

3.3 Determination of GABA level of foliar-treated and ethephon treated chickpea plants under control and heat stress conditions at 4-week and 12-week sampling points

GABA levels were measured for both ICC-12726 and Genesis-836 chickpea plants harvested at 4-week and 12-week sampling points. At 4 weeks, both chickpea varieties were applied with heat stress for 24 hours and 72 hours while at 12 weeks heat stress was applied for both chickpea plants for 72-hour and 144-hour time periods. GABA level was determined by using GABase enzyme assay GABA is a non-protein amino acid and interacts with ethylene under stress conditions to mitigate the stress responses. Therefore, the GABA level indicates the level of stress experienced by plants subjected to heat stress.

3.3.1 GABA level of foliar-treated and ethephon treated chickpea plants under control and stress conditions at weeks 4

According to the results foliar treated ICC-12726 plants at heat stress conditions were 1.7 folds higher than those under control conditions. Moreover, foliar untreated plants at control conditions were 2.8 folds higher than those at heat stress conditions. GABA level of foliar untreated ICC-12726 plants under control and heat stress conditions varied from 0.83µmoles/g-1.17µmoles/g and 0.21µmoles/g-0.34µmoles/g. GABA level of foliar-treated ICC-12726 plants control and heat stress conditions varied from 0.64µmoles/g-0.92µmoles/g and 0.54µmoles/g-1.67µmoles/g. 0µM ethephon-treated ICC-12726 plants were 3.5 folds higher than the 5µM ethephon-treated ICC-12726 plants at control temperature. Additionally, GABA levels of 0µM ethephon-treated ICC-12726 plants at control temperature conditions were 2.8 folds higher than those under heat stress temperature. 5µM ethephon-treated ICC-12726 plants under both control and heat stress temperatures varied from 0.16µmoles/g-0.23µmoles/g and 0.14µmoles/g-0.17µmoles/g respectively (Figure 3.7).

Considering Genesis-836 plants, foliar-treated plants at both control and stress temperatures exhibited higher GABA levels than foliar-untreated plants at control and stress conditions. Notably, there was no significant difference between control and foliar-treated plants under different heat treatment conditions. GABA level of foliar untreated Genesis-836 plants under control and heat stress conditions varied from 0.24µmoles/g- 0.68µmoles/g and 0.39µmoles/g-0.78µmoles/g while Foliar treated plants at control and heat stress conditions ranged from 0.13

moles/g-1.00 µmoles/g and 0.60 µmoles/g-0.62µmoles/g respectively. Genesis-836 plants that have been treated with 5µM exhibited higher GABA levels at both control and heat stress conditions than 0µM ethephon-treated plants under the same conditions. GABA level of 5µM ethephon treat plants under control and heat stress temperature ranged from 0.22µmoles/g-0.38µmoles/g and 0.11µmoles/g-0.37 µmoles/g (Figure 3.7).

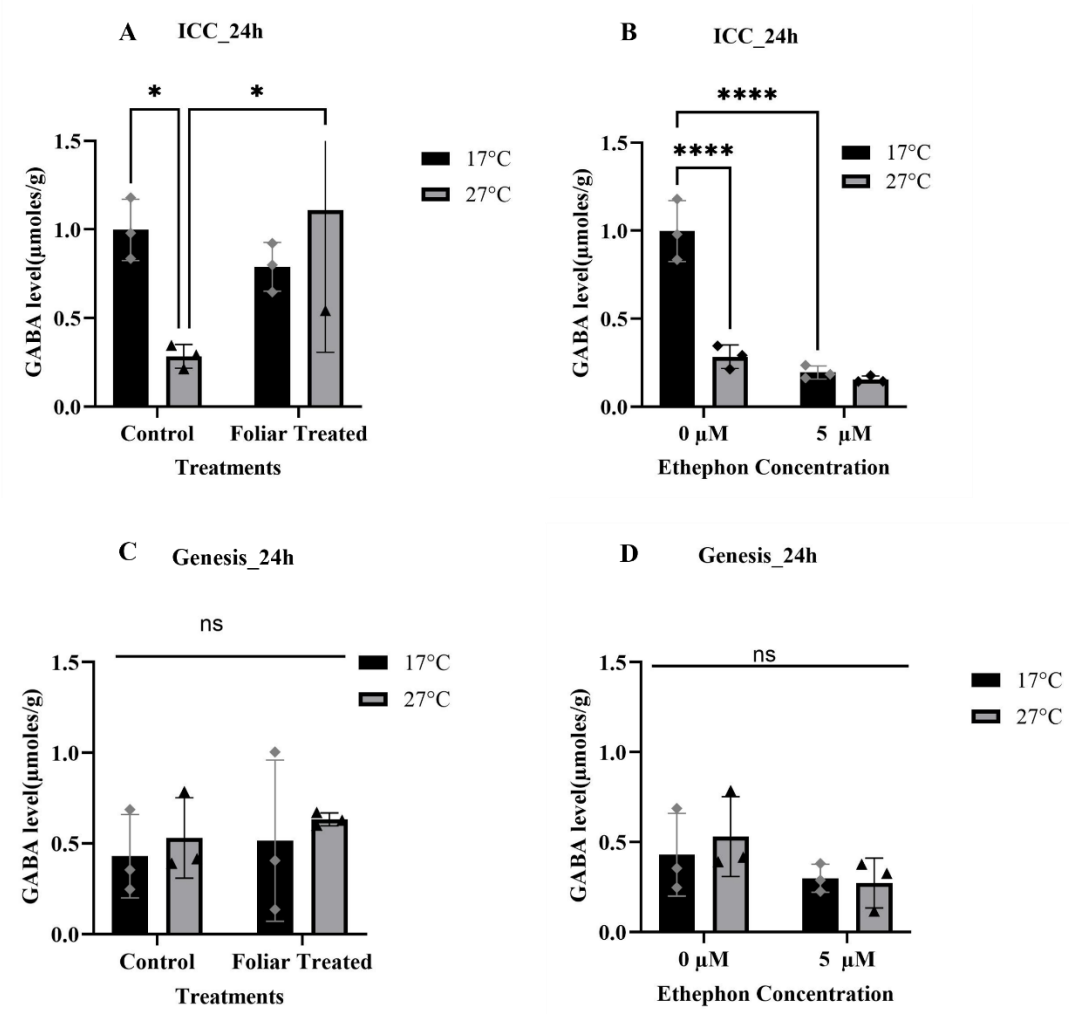

Figure 3.7 GABA level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions applied for 24 hours at 4 week sampling point. Heat stress condition was 27∘C while the control condition was 17∘C.A) GABA level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) GABA level of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) GABA level of foliar

treated and foliar untreated Genesis-836 chickpea plants. D) GABA levels of 0 μM and 5 μM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD (n≤3), two-way ANOVA analyzed the data, and the * indicates significant difference($p<0.05$) , ****indicates a significant difference($p<0.0001$) and 'ns' indicates non-significance.

GABA levels of foliar-treated ICC-12726 plants at heat stress conditions were 2.3 folds higher than foliar untreated plants under heat stress conditions. Additionally, foliar untreated plants at control conditions were 1.7 folds higher than the plants at heat stress conditions. GABA level of foliar untreated ICC-12726 plants under control and heat stress conditions varied from 0.40μmoles/g-0.92μmoles/g and 0.11 μmoles/g-0.33μmoles/g. GABA level of foliar-treated ICC-12726 plants control and heat stress conditions varied from 0.22 μmoles/g-0.62 μmoles/g and 0.64 μmoles/g -0.99 μmoles/g (Figure 3.8).

There was no significant difference among 0μM and 5μM ethephon-treated ICC-12726 under both control and stress conditions. GABA level of 5μM ethephon treated ICC-12726 plants under both control and heat stress temperatures varied from 0.22μmoles/g-0.24μmoles/g and 0.23μmoles/g-1.73 μmoles/g respectively (Figure 3.8).

Genesis-836 plants, that have been treated with foliar spray under control and stress temperature exhibited higher GABA levels than foliar untreated plants at control and stress conditions. Notably, there was no significant difference among control and foliar treated plants under different heat treatment conditions. GABA level of foliar untreated Genesis-836 plants under control and heat stress conditions varied from 0.14μmoles/g-0.26μmoles/g and 0.19μmoles/g-0.24 μmoles/g while Foliar treated plants at control and heat stress conditions ranged from 0.21 moles/g- 0.56 μmoles/g and 0.31 μmoles/g-0.74 μmoles/g respectively. Genesis-836 plants that have been treated with 0 μM concentrated ethephon under heat stress conditions exhibited 2.3 folds higher compared to 5μM ethephon-treated plants at the same conditions. GABA level of 5μM ethephon treat plants under control and heat stress temperature ranged from 0.11μmoles/g-0.16 μmoles/g and 0.11μmoles/g-0.14 μmoles/g (Figure 3.8)

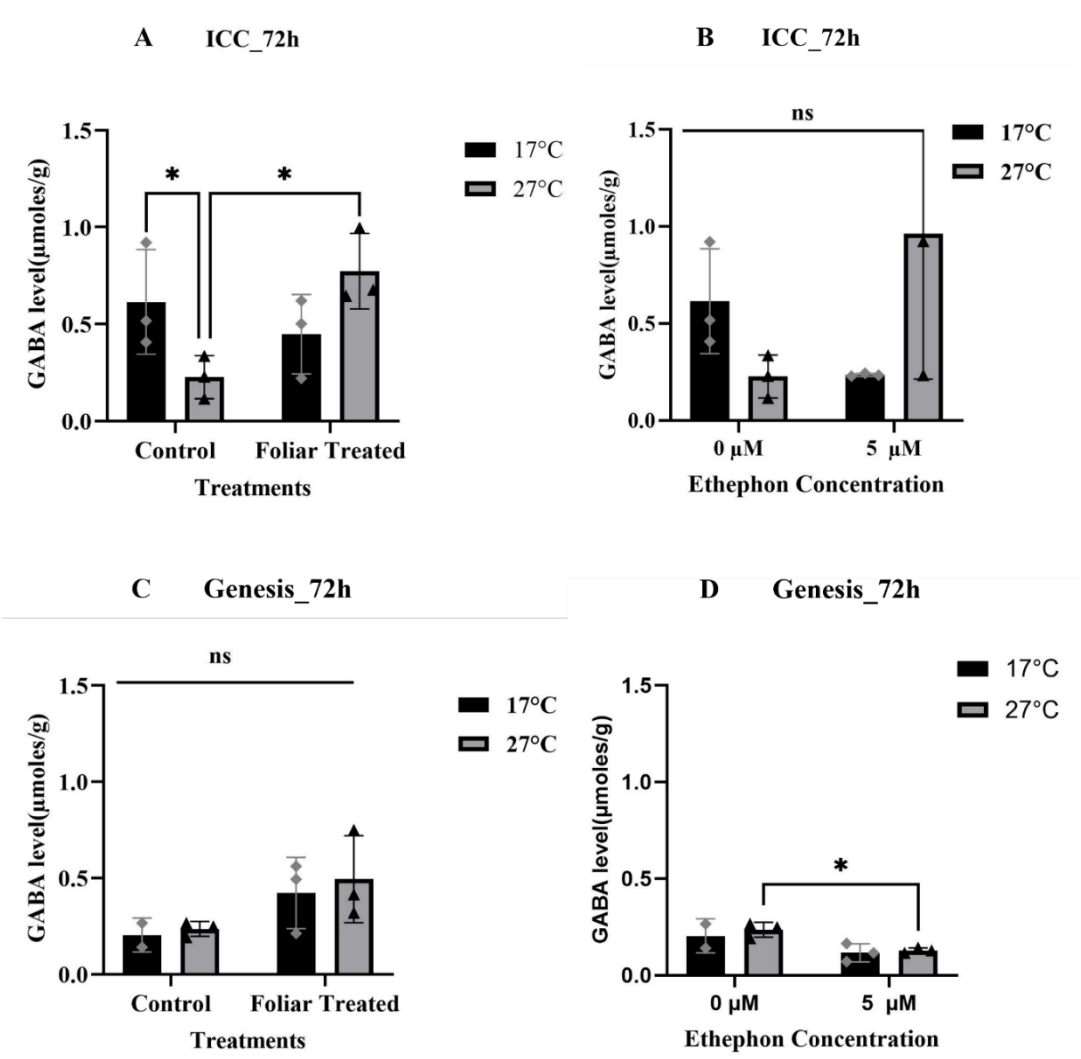

Figure 3.8 GABA level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions applied for 72 hours at 4 week sampling point. Heat stress condition was 27∘C while control condition was 17∘C.A) GABA level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) GABA levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) GABA level of foliar treated and foliar untreated Genesis-836 chickpea plants. D) GABA levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD (n≤3), the data were analyzed by two-way ANOVA, and the * indicate a significant difference($p<0.01$).

3.3.3 GABA level of foliar-treated and ethephon treated chickpea plants under control and stress conditions at 12 weeks

Foliar-treated ICC-12726 plants at both control and heat stress conditions exhibited higher GABA levels compared to foliar untreated plants under the same conditions. However, only the foliar-treated ICC-12726 plants at heat stress performed 5.5-fold significantly higher GABA levels compared to foliar untreated plants under the same conditions. GABA levels of foliar untreated plants under control and stress conditions Ranged from 0.20μmoles/g-0.93 μmoles/g and 0.12 μmoles/g- 0.37 μmoles/g. GABA level of foliar-treated ICC-12726 plants control and heat stress conditions varied from 1.32 μmoles/g-2.34μmoles/g and 0.30μmoles/g -3.31 μmoles/g (Figure 3.9).

There was no significant difference between 0μM and 5μM ethephon-treated ICC-12726 under both control and stress conditions. GABA level of 5μM ethephon treated ICC-12726 plants under both control and heat stress temperatures varied from 0.07μmoles/g-0.38μmoles/g and 0.37μmoles/g-1.25 μmoles/g respectively (Figure 3.9).

Foliar-treated Genesis-836 plants under both control and heat stress conditions exhibited 5.7 folds and 7.3 folds higher in GABA levels compared to foliar-untreated Genesis-836 plants under similar conditions. GABA level of foliar untreated Genesis-836 plants under control and heat stress conditions varied from 0.31μmoles/g-0.38μmoles/g and 0.18 μmoles/g- 0.24μmoles/g while Foliar treated plants at control and heat stress conditions ranged from 1.78moles/g- 2.37 μmoles/g and 0.26 μmoles/g-2.19 μmoles/g respectively. GABA level of 5μM ethephon treat plants under control and heat stress temperature ranged from 0.45μmoles/g-0.90 μmoles/g and 0.26- 2.19 μmoles/g (Figure 3.9).

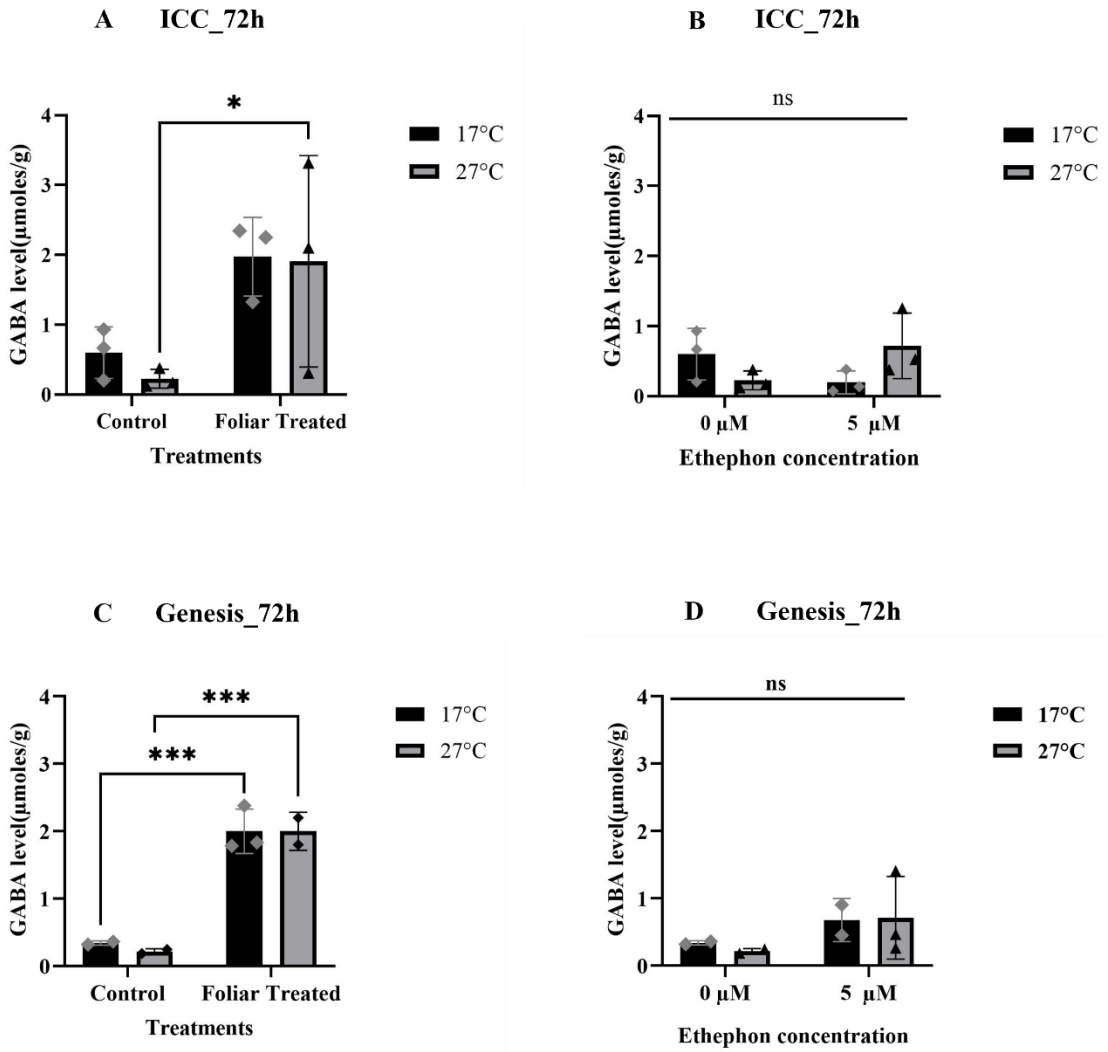

Figure.3.9 GABA level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions applied for 72 hours at 12 week sampling point . Heat stress condition was 27∘C while control condition was 17∘C.A) GABA level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) GABA levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) GABA level of foliar treated and foliar untreated Genesis-836 chickpea plants. D) GABA level of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD (n≤3), the data were analyzed by two way ANOVA, the * indicates a significant difference($p<0.05$), the *** indicates a significant difference($p<0.0001$), and 'ns' indicates nonsignificant.

According to the results, the GABA level of foliar-treated Genesis-836 plants under heat stress conditions was 2.1 folds higher than the foliar-treated plants under control conditions and foliar untreated plants under heat stress conditions. Also, GABA levels of foliar-treated Genesis plants under heat stress was 2.3 folds higher than foliar untreated plants under control conditions (Figure 3.10).

GABA level of foliar untreated ICC-12726 plants under control and stress conditions ranged from 0.03µmoles/g-0.56µmoles/g and 0.71 µmoles/g- 1.16 µmoles/g. GABA level of foliar-treated ICC-12726 plants control and heat stress conditions varied from 0.83 µmoles/g-1.51 µmoles/g and 1.63 µmoles/g -3.94 µmoles/g. In comparison, GABA levels of foliar untreated Genesis-836 plants under control and stress conditions ranged from 0.80µmoles/g- 1.51µmoles/g and 0.75 µmoles/g-1.25 µmoles/g. GABA level of foliar-treated Genesis-836 plants control and heat stress conditions varied from 0.90 µmoles/g- 1.77µmoles/g and 2.13 µmoles/g -2.88 µmoles/g (Figure 3.10)

The 0 µM ethephon-treated ICC-12726 plants under heat stress conditions exhibited 2 folds higher in GABA levels than those under control conditions. The GABA level of 5µM ethephon treated ICC-12726 plants under both control and heat stress temperatures varied from 0.62µmoles/g-0.86µmoles/g and 0.51µmoles/g-1.43 µmoles/g respectively. In contrast, the GABA level of 5µM ethephon-treated Genesis-836 plants under control and heat stress temperature ranged from 0.05µmoles/g-1.21µmoles/g and 0.36- 0.79 µmoles/g (Figure 3.10).

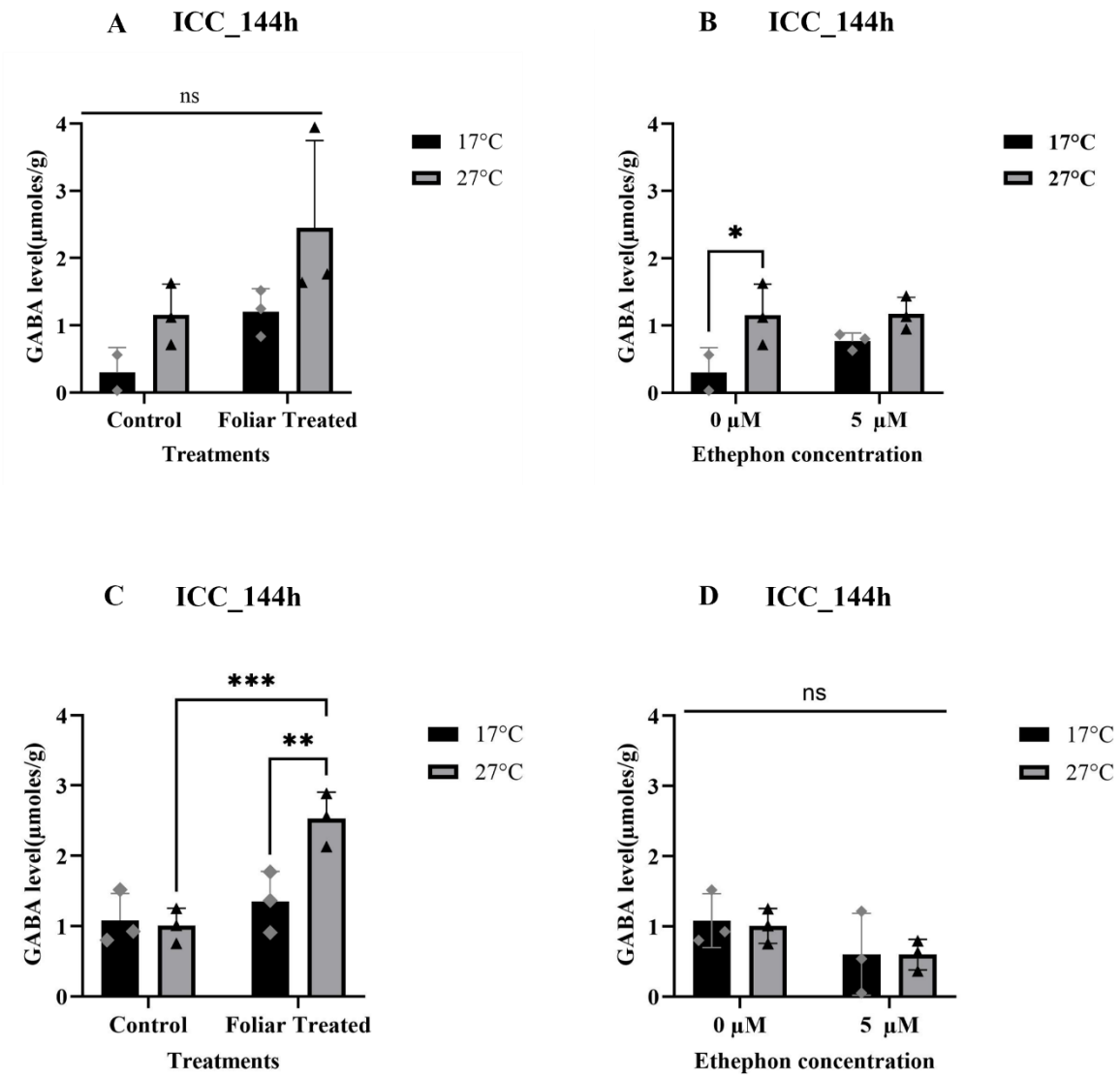

Figure 3.10. GABA level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions applied for 144 hours at 12-week sampling point. Heat stress condition was 27∘C while control condition was 17∘C.A) GABA level of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) GABA levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) GABA level of foliar treated and foliar untreated Genesis-836 chickpea plants. D) GABA levels of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD (n≤3), the data were analyzed by two-way ANOVA, the * indicates a significant difference($p<0.05$), the ***indicates a significant difference($p<0.0001$), and the 'ns' indicates non-significant.

3.4 Determination of the starch level of foliar-treated and ethephon-treated chickpea plants under control and heat stress conditions at a 12-week sampling point

According to the results, foliar-treated ICC-12726 at the control condition exhibited higher significance by 6-fold than foliar-treated plants under heat stress conditions, and no significance was found in foliar-treated Genesis-836 plants. Also, there was no significant difference found between ethephon untreated and ethephon-treated ICC-12726 and Genesis-836 plants under normal and heat stress conditions (Figure 3.11).

The starch content of foliar untreated ICC-12726 plants under control and stress conditions ranged from 0.71% -2.11% and 0.68%- 1.02%. The starch content of foliar-treated ICC-12726 plants control and heat stress conditions varied from 0.25%-5.77% and 0.21% -3.47%. In comparison, the starch content of foliar untreated Genesis-836 plants under control and stress conditions ranged from 0.84%- 2.82% and 1.17%-4.88%. The starch content of foliar-treated Genesis-836 plants control and heat stress conditions varied from 1.97%-2.82% and 1.03%-2.72% (Figure 3.11).

The 0 μM ethephon-treated ICC-12726 plants under heat stress conditions exhibited higher significant difference for starch content compared to the plants under control conditions. GABA level of 5μM ethephon-treated ICC-12726 plants under both control and heat stress temperatures varied from 0.11 %-2.27% and 0.26%-1.55% respectively. In contrast, the starch level of 5μM ethephon-treated Genesis-836 plants under control and heat stress temperature ranged from 1.37%-2.78% and 1.37%-5.04% (Figure 3.11).

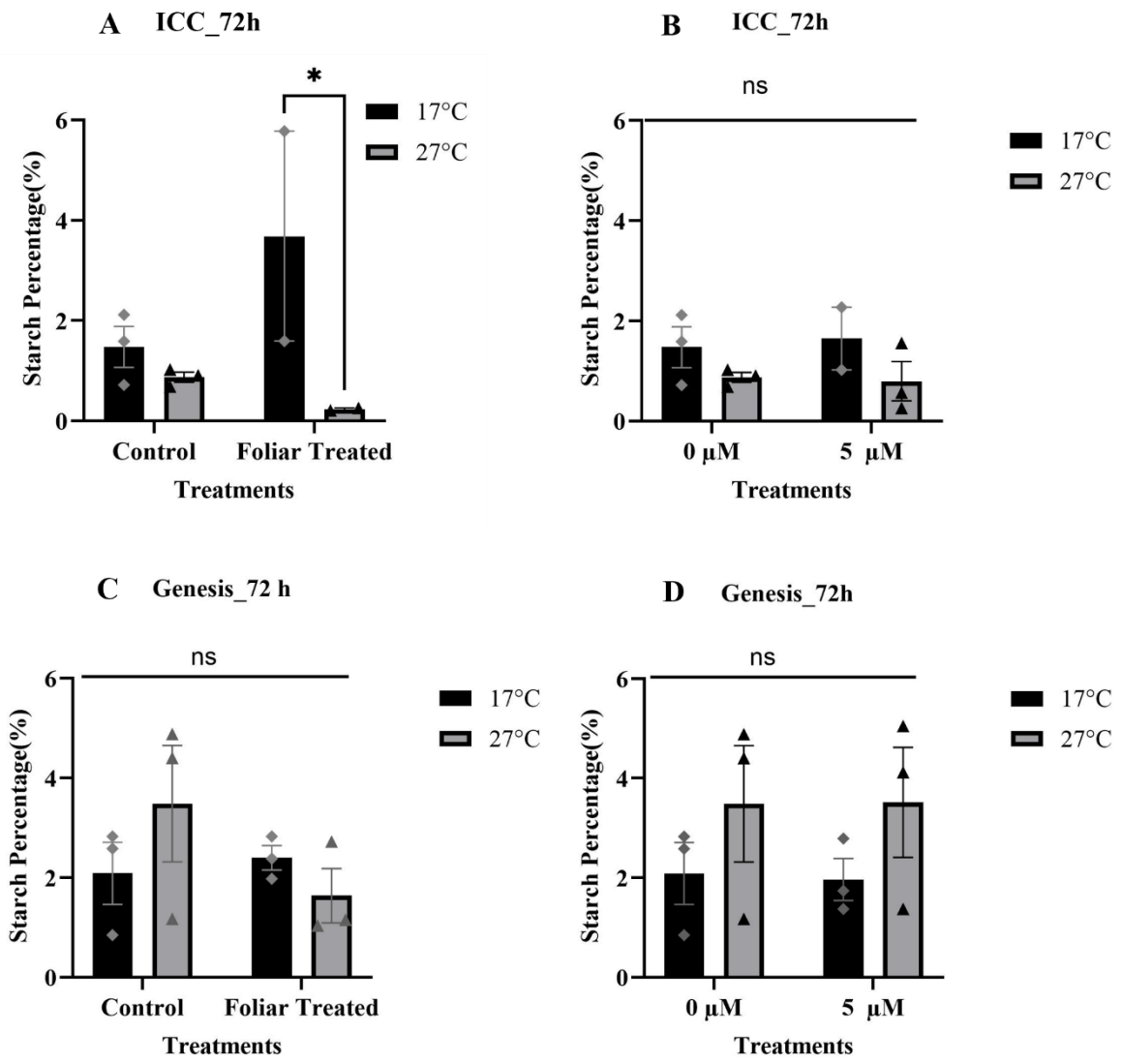

Figure 3.11 Heat stress condition was 27∘C while control condition was 17°C.Starch percentage was measured per 100mg. A) Starch content of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) Starch content of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) Starch content of foliar treated and foliar untreated Genesis-836 chickpea plants. D) Starch content of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD ($n \leq 3$), the data were analyzed by two-way ANOVA, the * indicates a significant difference($p<0.05$), and the 'ns' indicates non-significant.

According to the results, ICC-12726 chickpea plants did not exhibit significant difference in foliar treatment and ethephon treatment for starch content. In contrast, foliar untreated Genesis-836

plants under stress conditions (144 hours) have shown 8 folds higher in starch content compared to foliar untreated plants at normal conditions. Similarly, ethephon untreated plants under stress conditions have shown higher significant difference to ethephon untreated plants at normal conditions for starch content. (Figure 3.12).

The starch content of foliar untreated ICC-12726 plants under control and stress conditions ranged from 0.46%-1.72% and 1.38%-3.29%. The starch content of foliar-treated ICC-12726 plants control and heat stress conditions varied from 0.75%-1.53% and 1.70%-3.63%. In comparison, the starch content of foliar untreated Genesis-836 plants under control and stress conditions ranged from 0.34%-6.88% and 2.64%-2.98%. Starch content of foliar-treated Genesis-836 plants control and heat stress conditions varied from 0.63%-3.21% and 0.51%-2.40% (Figure 3.12).

Starch level of 5μM ethephon treated ICC-12726 plants under both control and heat stress temperatures varied from 0.76%-0.99% and 0.76% -0.95% respectively. In contrast, the 0 μM ethephon-treated ICC-12726 plants under heat stress conditions were 7.6 folds higher in starch content compared to those plants under control conditions. The starch level of 5μM ethephon-treated Genesis-836 plants under control and heat stress temperature ranged from 0.40%-1.72% and 1.83%-3.82 % (Figure 3.12).

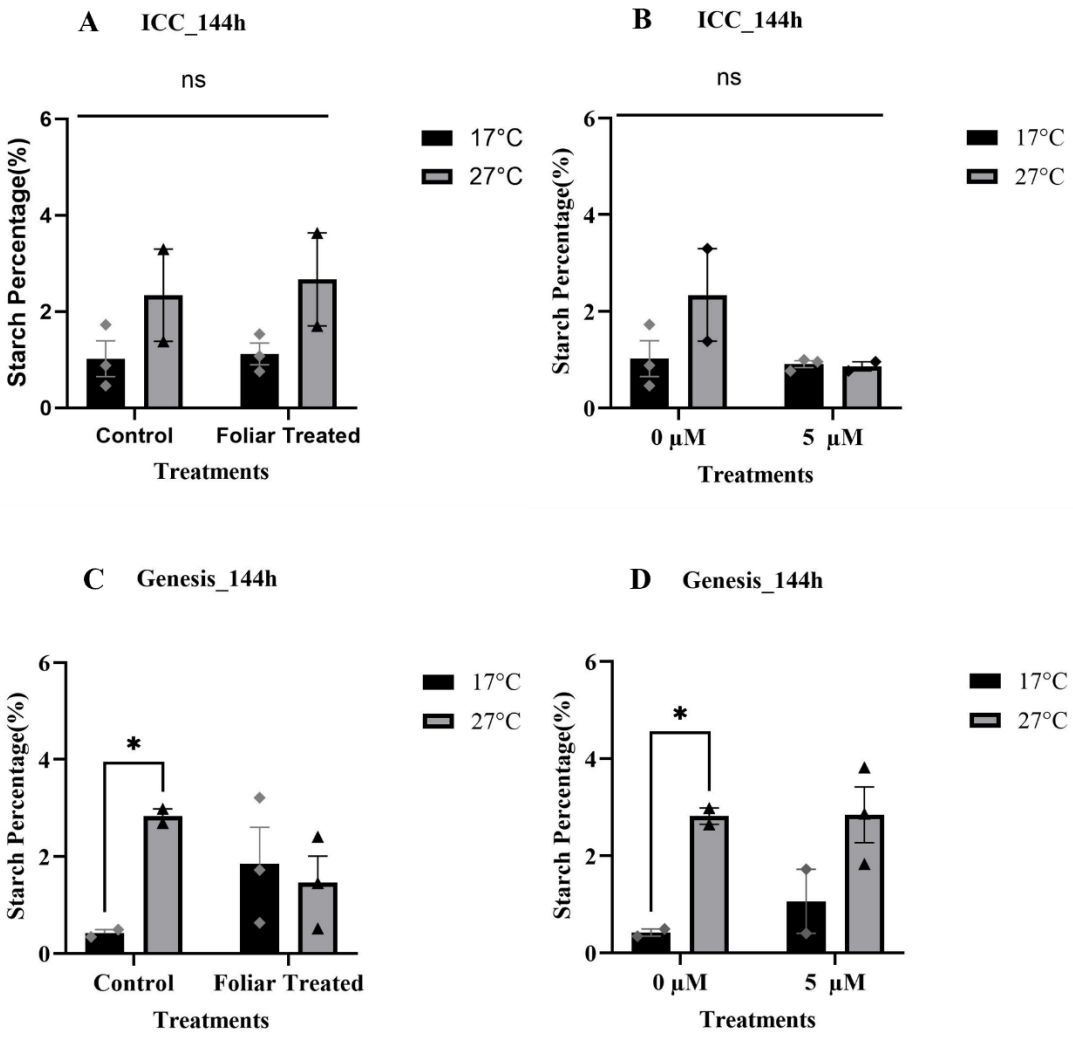

Figure 3.14. Starch level of ethephon and foliar treated ICC-12726 and Genesis-836 chickpea plants under heat stress and control conditions applied for 144 hours at 12-week sampling point. Heat stress condition was 27∘C while control condition was 17∘C.Starch percentage was measured per 100mg. A) Starch content of foliar treated and foliar untreated ICC-12726 chickpea plants under heat stress and normal conditions. B) Starch content of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. C) Starch content of foliar treated and foliar untreated Genesis-836 chickpea plants. D) Starch content of 0 µM and 5 µM ethephon-treated ICC-12726 plants were measured under heat stress and normal conditions. Data in A-D represent the mean±SD ($n \leq 3$), the data were analyzed by two-way ANOVA , the * indicates a significant difference($p<0.05$), and the 'ns' indicates non-significant.

CHAPTER 04
DISCUSSION

This study investigated physiological parameters in ACC and Ethephon-treated ICC-12726 and Genesis-836 chickpea plants, and the optimal ethylene precursor and concentration were determined by analyzing physiological parameters. The effect of ethylene pretreatment on the growth and heat tolerance of chickpea plants was further investigated through these physiological parameters in combination with key biochemical signatures. The oxidative stress level of chickpea plants under stressed and control conditions was also quantified through TBARS assay, a proxy for lipid peroxidation which measures MDA equivalents. Finally, the GABA level and starch content of each plant subjected to heat and control conditions were quantified by the use of GABase and starch assay.

During this study, both ICC-12726 and Genesis-836 chickpea plants were treated under normal (17°C) and heat stress (27°C) conditions to determine the MDA level, GABA level, and starch content under these conditions. According to the previous study, 15°C is considered the threshold temperature for the reproduction of chickpea plants while 21°C is considered the mean flowering temperature. However, above 32°C chickpeas face heat stress resulting in reduced seed size, yield etc. (Rani et al., 2020).

4.1 Determination of best ethylene precursor and ethylene concentration

According to the results, there was no significant difference in germination percentages between different concentrated ACC-treated seeds and seeds treated with distilled water. Considering germination percentage, ICC-12726 chickpea plants exhibited a gradual increase in germination percentage with the ACC concentration (Figure 3.1). Genesis-836 plants also exhibited an increase in germination percentage with the increase of ACC percentage except for a sudden drop in 50 µM ACC pretreated Genesis-836 plants (Figure 3.1). However, both plants performed a high germination percentage at 100 µM ACC concentration which means 100 µM would be the optimal concentration for seed germination. Based on the result, only 100 µM ACC-treated seeds exhibited higher significance different to distilled water pretreated seeds for root length. However, these results showed that ACC did not have a significant impact on seed

germination and root length. Considering fresh shoot and root biomass, different concentrated ACC-treated ICC-12726 and Genesis-836 chickpea plants did not show any significant difference towards ACC untreated plants (distilled water-treated plants). As ACC-treated seeds and plants did not exhibit significant physiological responses in germination percentage, root length, shoot, and root biomass, the plants were treated with ethephon to obtain more significant physiological responses (Figure 3.1). Previous studies done with alfalfa seedlings have found exogenous application of Ethephon produced plenty of ethylene more efficiently while exogenous application of ACC did not produce a sufficient amount of ethylene. Moreover, they measured ethylene content from alfalfa seedlings in the germination stage using gas chromatography and found that ethephon-treated seeds released a higher amount of ethylene while ACC did not release a sufficient amount of ethylene (Makleit et al., 2024)

In contrast, ethephon-pretreated plants exhibited more significant changes in shoot and root length, fresh root and shoot biomass. Considering germination percentage, almost all ICC-12726 seeds treated with ethephon have shown 100% germination percentage while Genesis-836 seed exhibited a germination percentage of less than 50%. Compared with ACC treatments, ethephon pre-treated seeds have shown a higher germination percentage (Figure 3.2). Ethephon pretreatment might trigger seed germination compared to the ACC pre-treatment and ICC-12726 chickpea seeds have shown more sensitivity towards ethephon treatment compared to Genesis-836 seeds. According to the results, both ICC-12726 and Genesis-836 chickpea plants showed reduced shoot and root length as ethephon concentration increased. At 5 µM and 50 µM, ethephon-treated plants have shown higher shoot and root lengths while 1000 µM ethephon-treated plants were observed with the least shoot and root lengths (Figure 3.2). Similar changes were observed considering fresh shoots and root biomass of ethephon-treated ICC-12726 and Genesis-836 plants. Higher fresh shoot and root biomass were observed at 5 µM and 50 µM ethephon concentrations, and at least shoot and root biomass were observed at 1000 µM ethephon treated plants (Figure 3.2). These results depicted that higher levels of ethephon concentration have inhibitory effects on root and shoot growth. Considering about controls, ethephon treated ICC-12726 plants have shown higher significance to ICC-12726 plants treated with 1000 µM ethephon in shoot length. Control plants did not show any significance to ethephon treated plants in root length. Fresh biomass control samples demonstrated higher significance to 1000 µM ethephon treated ICC-12726 plants. Moreover, control plants did not show any significance to ethephon treated plants in fresh root

biomass. Overall, ethephon-treated ICC-12726 and Genesis-836 836 chickpea plants exhibited a higher range of value for fresh shoots and root biomass compared to the ACC-treated chickpea plants. Therefore, ethephon has performed greater effectiveness towards shoot and root length and overall biomass of chickpea plants compared to the ACC.

Another study on ethephon seed priming of rice seedlings subjected to salt stress has shown that germination speed was 31.6% higher in ethephon primed seeds than no primed seeds under salt stress conditions. Also, ethephon-primed seeds increase root length by 1.1 % under salt stress compared to ethephon-unprimed plants. However, this study's results exhibited that the seed germination and growth of rice seedlings were natively affected by salinity while ethephon regulated it positively(Hussain et al., 2020). Moreover, a subsequent study done with artichoke seedlings exposed to heat stress examined the effect of ethylene concentrations on seed under normal (23℃) and heat stress conditions (30℃). Results have shown that ACC and ethephon treatment (ranging from 1-100 μM L^{-1}) increased early root growth including root hair density, root area, and lateral roots. Moreover, seedlings treated with 30 30 μM L^{-1} ethephon had increased primary root elongation which was inhibited at higher temperatures (Shinohara et al., 2017). Another study based on the application of ethylene as the exogenous source on rice under heat stress, have proven that the two rice varieties treated with 1.6mM increased plant height, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight. by 14.1%, 7.97%, 12.3%, 15.9%, and 16.1% in Taipei-309 and 10.0%, 6.07%, 10.5%, 13.5%, and 12.2%in Rasi, respectively, compared to the control (H. Gautam et al., 2022).

4.2 MDA level, GABA level, and starch content of ethephon pre-treated ICC-12726 and Genesis-836 chickpea plants

4.2.1 MDA level of ethephon pre-treated ICC-12726 and Genesis-836 chickpea plants

At the 4-week sampling point, 5μM ethephon-treated ICC-12726 plants under heat stress conditions treated for 24 hours, exhibited 5-fold significantly higher than 5 μM ethephon-treated plants under control conditions. Also, 5μM ethephon-treated plants were found 2 2-fold significantly higher than 0μM ethephon plants at heat stress conditions (Figure 3.3). A previous study was done to determine the ethylene-dependent regulation of antioxidant enzymes to mitigate heat stress on wheat plants. During the experiment plants were treated with 200 μl L^{-1} ethephon

under normal and stress conditions. Results have shown that heat stress significantly increased TBAR level by 169.1% compared to control plants and ethephon-treated plants have reduced heat stress-induced oxidative stress by 25.4% in TBARS (Sehar et al., 2023). Ethephon treatment might trigger ROS production under stress conditions which leads to a higher level of MDA, and this may explain the results in both scenarios. Considering 72-hour heat treatment, ethephon untreated and treated Genesis-836 plants at heat stress exhibited 1.5-fold and 1.8-fold higher significance in MDA values than plants under normal conditions respectively (Figure 3.4). Ethephon-treated and untreated plants that were experiencing stress conditions under high-temperature conditions **m**ight accelerate the ROS production in plants regardless of treatments.

Considering 72-hour heat treatment, MDA levels of ethephon-treated ICC-12726 and Genesis-836 chickpea plants, no significance was found in ethephon untreated and treated plants under both control and heat stress conditions treated for 72 hours (Figure 3.5). In previous studies, maize plants have been treated with ethylene under heat-stress conditions to determine MDA levels. during this experiment, plants' leaves were treated with ethylene solution and treated for heat stress for four hours. However, no significant was observed between control and treatments (Makleit et al., 2024) .Under the experimental conditions, both plants might not be sensitive to ethephon treatment and because of that, ethephon treatment might not be strong enough to reduce the oxidative stress in ICC-12726 and Genesis-836 chickpea plants under heat stress conditions.

According to 144 hours of heat treatment at 12 weeks, both ICC-12726 and Genesis-836 plants have exhibited similar changes in MDA level. Ethephon-treated and untreated ICC plants under heat stress conditions were 2.5 and 3.2 folds significantly higher than those under control conditions for MDA levels (Figure 3.6). Plants were treated with heat stress for 144 hours which means plants were experiencing extreme heat stress conditions. These extreme conditions might cause excessive production of ROS. High ROS levels cause oxidative damage in plants, resulting in increasing MDA levels. Another study based on ethephon pretreatment on rice seedlings under drought stress has shown that exposure to water shortage under drought conditions resulted in membrane injury which increased MDA levels in shoot and root by 60.9% and 44.0% respectively. However, ethephon seed priming have reduced MDA content by 16.3% and 20.0% in shoot and root(Zhang et al., 2024).

However, MDA levels of ethephon-treated plants were expected to be less than those of ethephon-untreated plants, there was no significant reduction of MDA levels in ethephon-treated plants compared to ethephon untreated plants under stress conditions. A previous research study related to rice cultivars has demonstrated that high-temperature stress significantly improved TBARS content by 100% in the Taipei cultivar and 140.0% in the Rasi cultivar. However exogenous application of 1.6mM ethephon treatment has significantly reduced TBARS content in both cultivars under heat stress(H. Gautam et al., 2022). According to the previous study, which has been done to determine the effects of bioregulators on chickpea plants subjected to heat tolerance. This has revealed that heat stress has induced lipid peroxidation in chickpea verities and the application of bioregulators like ABA. Salicylic acid decreased lipid peroxidation under high-temperature conditions (Kumar et al., 2020).

4.2.2 GABA level of ethephon pre-treated ICC-12726 and Genesis-836 chickpea plants

In 4-week and week 12 sampling points, some ethephon treated and ethephon untreated ICC-12726 and Genesis-836 plants didn't show any significant difference between control and heat stress conditions for 24 hours in terms of GABA level (Figure 3.7). Ethylene and GABA interact with two different signaling pathways. Ethylene is involved in the plant stress signaling pathway and ethylene is not directly involved in the GABA shunt pathway to control stress responses. Thus, ethylene treatment may not significantly increase GABA levels under control and stress conditions treated for 24 hours(Podlešáková et al., 2019). Moreover, in the 4-week sampling point, ethephon-untreated Genesis-836 plants were 2.3 folds significantly higher than ethephon treated plants under heat stress conditions treated for 72 hours (Figure 3.8). Ethylene is released by ethephon during metabolic pathways. In this case, ethylene might downregulate the GABA shunt pathway under heat stress conditions, resulting in a decreasing GABA level. However, previous studies have exhibited that GABA has been involved in stress tolerance mechanisms as a signaling molecule and endogenous GABA levels change towards stress conditions as a result of plants' defense mechanisms. In Arabidopsis plants, GABA accumulated via calcium-induced activation of glutamate carboxylase enzyme under heat stress conditions (Locy et al., 2000)

According to the results, ethephon untreated ICC-12726 plants under heat stress conditions exhibited 3.2 folds higher significance in GABA levels than plants treated with control conditions (Figure 3.10). Considering experiment conditions, plants were treated with heated stress conditions

for 144 hours which was extreme for plants. A previous study has shown that ethephon treatment increased endogenous GABA levels in aquatic plants (Lemna). According to the study results, *L. paucicostata* increased endogenous GABA levels with 0.5mM and 0.2mM ethephon concentrations(Kim et al., 2020).

4.2.3 Starch content of ethephon-treated ICC-12726 and Genesis-836 chickpea plants

Considering 144-hour heat treatment, ethephon untreated Genesis 836 plants under stress conditions have shown 7.6 folds higher significance to ethephon untreated plants at normal conditions for starch content (Figure 3.12). Previous studies based on two rice varieties (Taipei and Rasi) have shown that high-temperature conditions significantly reduced non-structural carbohydrates such as starch, and glucose by 35.8% in Taipei-309 and 40.4% in Rasi. In contrast, ethephon treatment significantly increased nonstructural carbohydrates by 6.67% in Taipei-309 and 4.17% in Rasi under high-temperature stress compared to the controls (Harsha Gautam et al., 2022)

4.3 MDA and GABA level and starch content of foliar-treated ICC-12726 and Genesis-836 chickpea plants

4.3.1 MDA level of foliar treated ICC-12726 and Genesis-836 chickpea plants

Considering heat treatments, in some cases, both ICC-12726 and Genesis-836 chickpea plants, did not exhibit a significant difference between control plants and foliar-treated plants under control (17°C) and heat stress (27°C) conditions (Figure 3.3). The short period of exposure to heat stress might be the one reason for this result. Chickpea plants were treated at 27°C under heat stress conditions and this heat stress treatment might not be strong enough to trigger oxidative responses in plants. Also, foliar treatment might not be strong enough to decrease oxidative stress in plants can be another reason. Some results were obtained from plants harvesting at a 4-week sampling point and might be earlier than their development stage. If foliar treatment is applied at an early stage of development, the expected outcomes may not be observed. A previous study demonstrated that the effects of bioregulators on chickpea plants subjected to heat tolerance. This has revealed that exogenous application of bioregulators like ABA, ethylene, and Salicylic acid decreased the lipid peroxidation of chickpea verities under high-temperature conditions(Kumar et al., 2020).

Considering about 72 hours of heat treatment, foliar untreated ICC-12726 plants under heat treatment were 1.6-fold significantly higher than those under control conditions for MDA levels (Figure 3.4). Foliar untreated ICC-12726 plants might be sensitive to heat stress conditions, which can lead to increased oxidative stress. Foliar-treated Genesis-836 chickpea plants at both control and heat stress conditions were 1.7-fold significantly higher in MDA level, than untreated chickpea plants at both conditions. Foliar treatment might trigger the ROS level, and the high ROS level ultimately increases the MDA level in plants (Figure 3.4). Moreover, foliar untreated and treated Genesis-836 plants at heat stress exhibited higher significance than plants under normal conditions. This can be the reason for increasing ROS levels due to heat stress. Foliar-treated and untreated plants that were experiencing stress conditions and high-temperature conditions might accelerate the ROS production in plants regardless of treatments. Another study has shown that plants treated with foliar spray containing growth regulators decreased lipid peroxidation and ultimately reduced the MDA level of rice plants. Also. This study has suggested that foliar application help to mitigate the negative effect of heat stress on plant physiology. (Pantoja-Benavides et al., 2021).

In contrast, MDA levels of foliar untreated Genesis-836 chickpea plants under both control and heat stress were 2.8-fold and 1.7 fold significantly higher than foliar treated plants under both temperature conditions (Figure 3.5). The foliar treatment effectively worked for Genesis-836 plants at a 12-week sampling point. Foliar treatment compounds might induce antioxidants in Genesis-836 plants to mitigate oxidative stress and this ultimately led to a reduction of MDA levels. According to the previous study, based on GABA application on sunflowers has shown that MDA levels increased significantly under drought conditions (23.17 ± 0.15 nmol g−1 FW) and application with GABA decreased MDA value significantly to (67.90 ± 0.058 nmol g−1 FW)(Abdel Razik et al., 2021).

According to the results of heat stress treatment for 144 hours, both ICC-12726 and Genesis-836 plants have shown similar patterns of fluctuating MDA levels for foliar treatment and ethephon treatment (Figure 3.6). MDA levels of foliar treated and foliar untreated plants under heat stress conditions were significantly higher than those under control conditions. Plants were treated with heat stress for 144 hours which means plants were experiencing extreme heat stress conditions. These extreme conditions might cause excessive production of ROS. High ROS levels cause oxidative damage in plants, resulting in increasing MDA levels. Another study based on exogenous

GABA application (foliar treatment) on *P. vulgaris* showed that 0.5, 1, 2 mM GABA application reduced MDA content in drought-stress plants compared to controls (Abd El-Gawad et al., 2021).

4.3.2. GABA level of foliar-treated ICC-12726 and Genesis-836 chickpea plants

Considering about 4 and 12-week sampling point, higher significance was found in foliar-treated ICC-12726 plants than foliar untreated plants under heat stress in terms of GABA level. GABA compound was exogenously applied in foliar treatment, and GABA level might be naturally increased in foliar-treated plants compared to the foliar untreated plants. Also, ICC-12726 foliar untreated plants under normal conditions were significantly higher than those under heat-stress conditions (Figure 3.7). GABA is synthesized through the GABA shunt pathway and heat stress conditions might negatively regulate the GABA shunt pathway. In contrast, Foliar-treated Genesis-836 plants did not show any significance among foliar-treated and untreated groups under control and heat stress conditions. Genesis-836 plants might have produced exogenous GABA regardless of foliar treatments under heat stress and control conditions to control the stress response. Thus, in Genesis-836 plants, foliar treatment may not significantly contribute to increased GABA levels. Previous studies done on the application of GABA to improve heat stress of mung bean plants have revealed that exogenous application of GABA significantly increases the endogenous GABA concentrations by 6.5 fold in GABA-treated plants compared to GABA-untreated plants under heat stress(Priya et al., 2019)

According to 72-hour heat treatment, in comparison to 24-hour heat treatment, similar patterns were observed in GABA levels for ICC-12726 and Genesis-836 plants for foliar treatment under control and heat stress conditions for 72 hours (Figure 3.7 and Figure 3.8). During the first 24-hour treatment, the plant may increase GABA level up to saturation level. Therefore, extending heat treatment for 72 hours may not result in further increases in GABA levels.

In comparison, foliar-treated Genesis-836 chickpea plants under heat stress conditions treated for 144 hours exhibited higher significance in GABA levels compared to the foliar-treated Genesis-836 plants under normal conditions and foliar untreated plants under heat stress conditions (Figure 3.10). Heat stress might induce plants defense mechanism response to stress conditions producing molecules to mitigate the stress conditions, such as GABA molecules. This might be the reason

for high GABA levels in stress conditions. Moreover, applying exogenous GABA compounds might result in higher levels of GABA in foliar-treated plants. Another study based on heat priming of lentil seed and foliar treatment with GABA under heat stress has revealed that plants were treated with heat priming and GABA resulted in a significant increase in endogenous GABA levels in leaves. Moreover, the application of heat priming and GABA treatment considerably reduced the membrane damage compared to control plants while GABA treatment has contributed to enhancing the cellular oxidizing ability(Bhardwaj et al., 2021).

4.3.3. Starch content of foliar-treated ICC-12726 and Genesis-836 chickpea plants

According to the results, foliar-treated ICC-12726 at the control condition exhibited higher starch levels compared to foliar-treated plants under heat stress conditions (72 hours), and no significance was found between foliar untreated and foliar-treated Genesis-836 plants under control and heat stress conditions (Figure 3.11). Considering 144 hours of heat treatment, ICC-12726 chickpea plants did not exhibit significance between foliar-treated and untread plants under control and stress conditions for starch content. In contrast, foliar untreated Genesis-836 plants under stress conditions (144 hours) have shown higher significance to foliar untreated plants at normal conditions for starch content (Figure 3.12). Starch is considered the main energy source and the most important carbohydrate in chickpea plants. Plants release starch under stress conditions and accumulate under favorable conditions (Mahadevamma et al., 2004). A previous study demonstrated that chickpea plants treated with foliar treatment have shown a remarkable increase in starch content (+45.9%) (Fedeli., 2023).

Conclusion

In this study the changes in physiological parameters in ACC and Ethephon-treated ICC-12726 and Genesis-836 chickpea plants. Both chickpea plants were treated with different ACC and ethephon concentrations and the optimal ethylene precursor and concentration were determined by analyzing physiological parameters such as germination percentage, shoot and root length, shoot, and root biomass. The effect of ethylene pretreatment on the growth and heat tolerance of chickpea plants was further investigated through these physiological parameters in combination with key biochemical signatures. This study has addressed the biochemical responses of chickpea plants subjected to heat stress along with ethephon pretreatment and foliar treatment. Moreover, this study examined the biochemical responses of chickpea plants subjected to heat stress, along with ethephon pretreatment and foliar treatment. In conclusion, these findings will help to elucidate the role of ethylene in stress signaling pathway in chickpea plants subjected to heat stress and ultimately these findings and strategies can be utilized to enhance abiotic stress resilience and productivity of chickpea plants subjected to heat stress.

Limitations

Tissue samples, that were collected at a 4-week sampling point were not enough for starch analysis. Plants were not well grown and not able to collect a sufficient amount of plant tissues for more biochemical analysis.

During sample weighing and grinding some samples were exposed to liquid nitrogen and due to that samples were experienced with blasting.

During handling and grinding, samples should be kept in a super cool environment. Due to some handling errors, some samples become tough and destroyed.

In the starch assay, a low starch percentage was obtained due to less amount of plant tissue.

In the starch assay, some blank samples exhibited color change due to the possibility of hydrolyzed glucose by plant enzymatic reactions.

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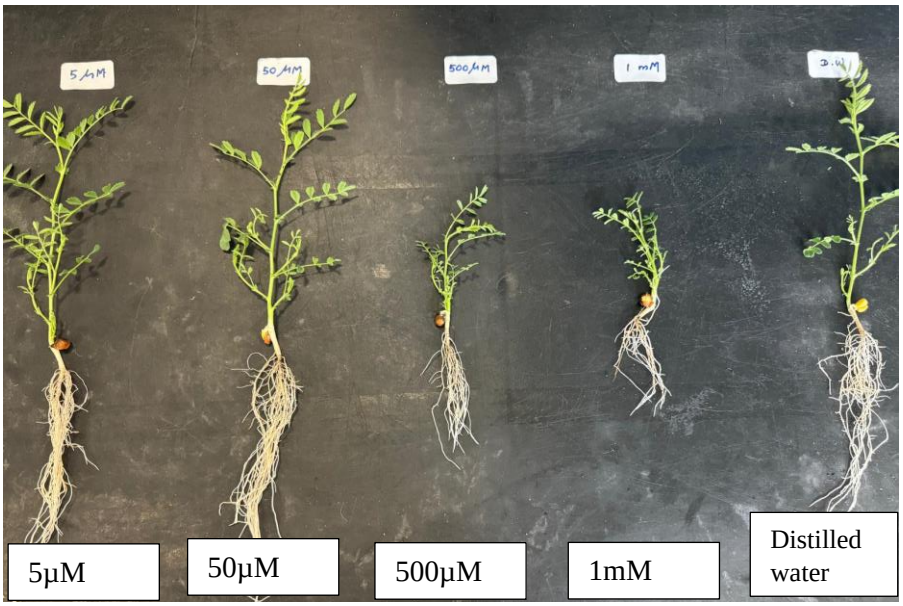
APPENDICES

Appendix 1: Ethephon treated ICC-12726 and Genesis-826 chickpea plants at 2 weeks harvesting.

5µM | 50µM | 500µM | 1mM

Appearnace of Ethephon treated ICC-12726 chickpea plants after two week of seed potting



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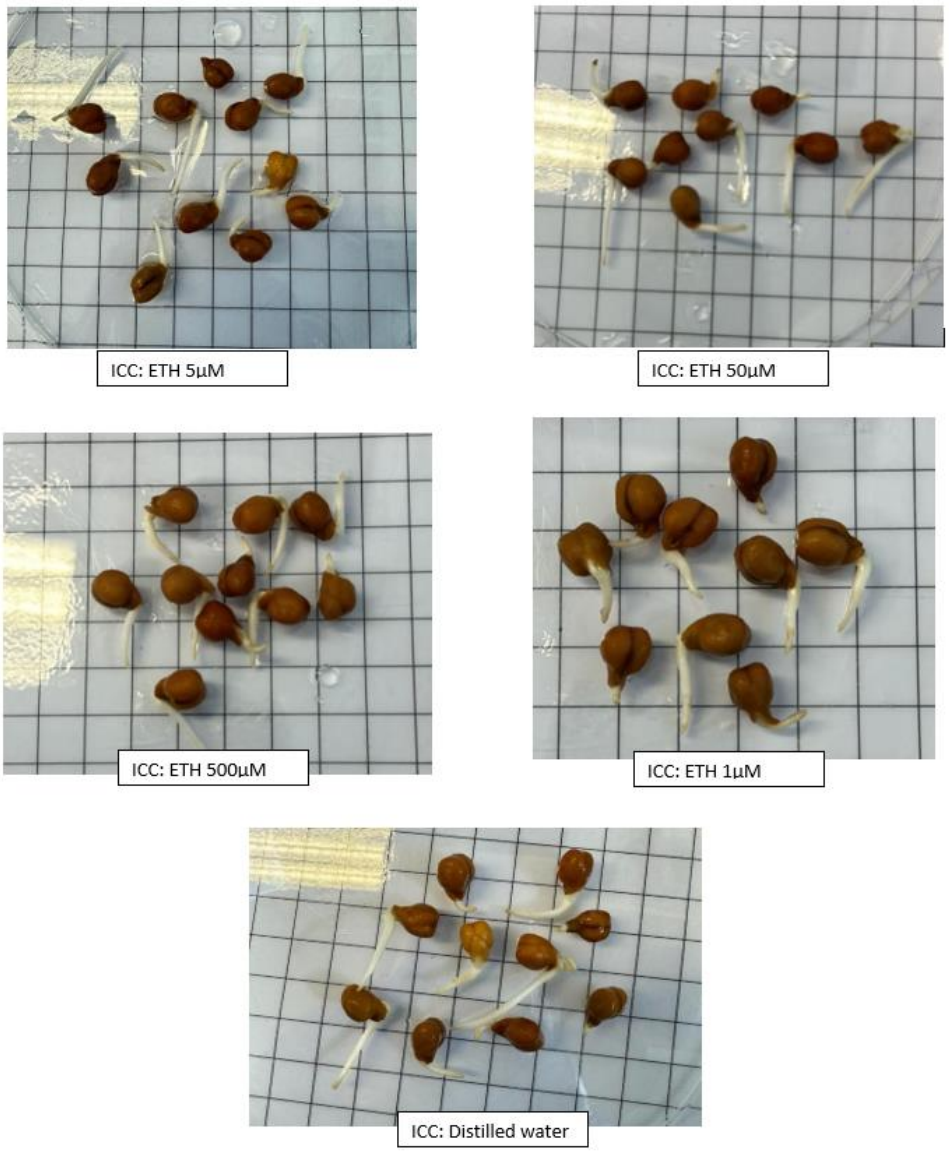
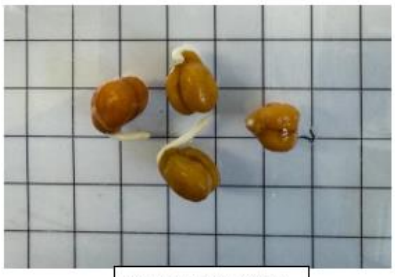
Appearnace of Ethephon treated Genesis-826 chickpea plants after two week of seed potting

Appendixes 2: Appearance of ICC-12726 and Genesis-826 chickpea seedlings after three days of ethephon treatments

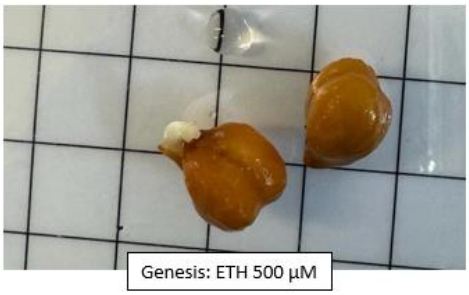


ICC seedlings treated with different concentrated ethephon after three days of treatment

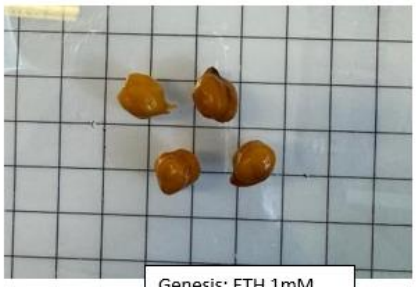
Genesis: ETH 5 µM



Genesis: ETH 50µM



Genesis: ETH 500 µM



Genesis: ETH 1mM

Genesis: Distilled water

Genesis seedlings treated with different concentrated ethephon after three days of treatment

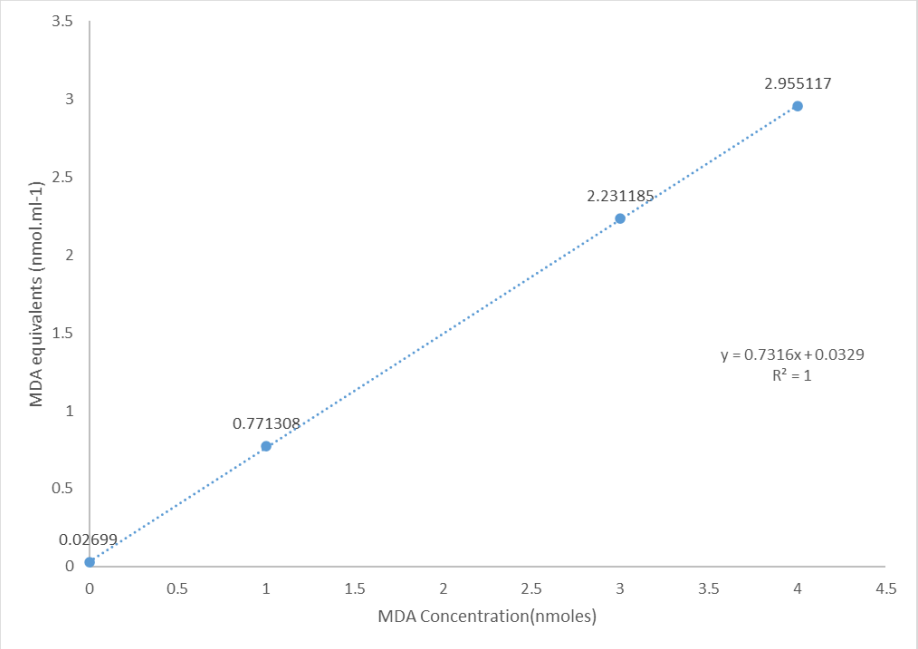
Appendix 3: Foliar treatment, Harvesting and sample storage

Foliar treatment with GABA spray; 5mM GABA solution was spray in to each plant at three times for two days. All twelve controlled chickpea plants from both varieties were exposed to foliar treatment prior to heat treatment.

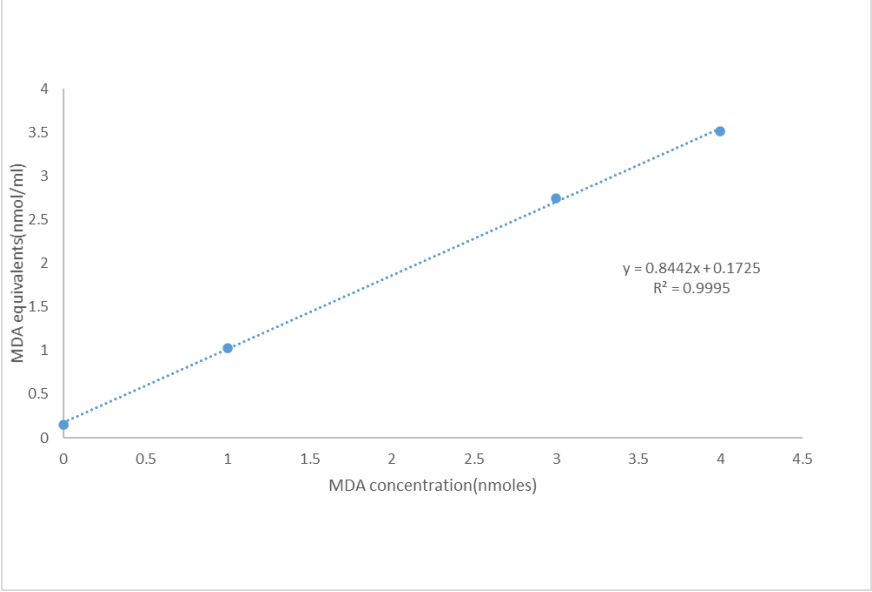
Top two leaves were gently excised from each chickpea plant after 24-hour heat treatment for biochemical analysis.

After excising of leaves, leaves were transferred in to falcon tube for storage for further analysis.

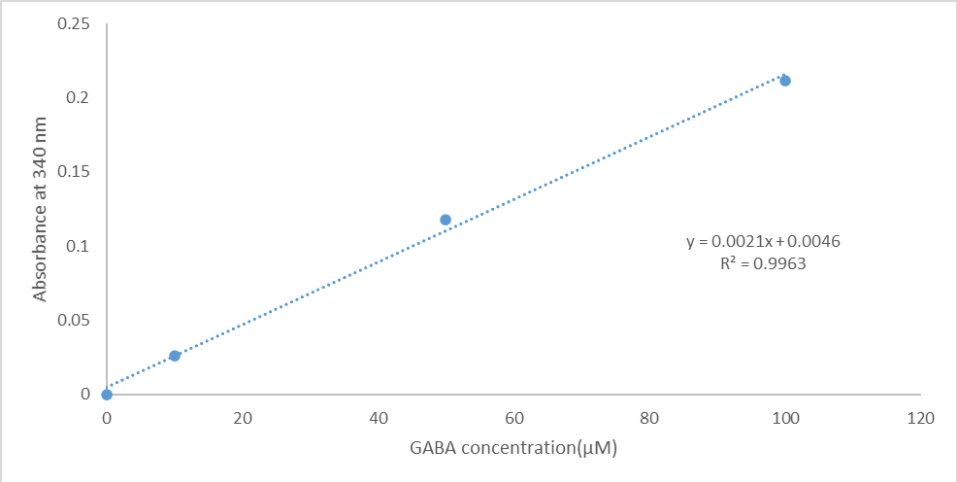
Falcon tubes contain leaf samples were placed in liquid nitrogen container immediate after sampling until storage.



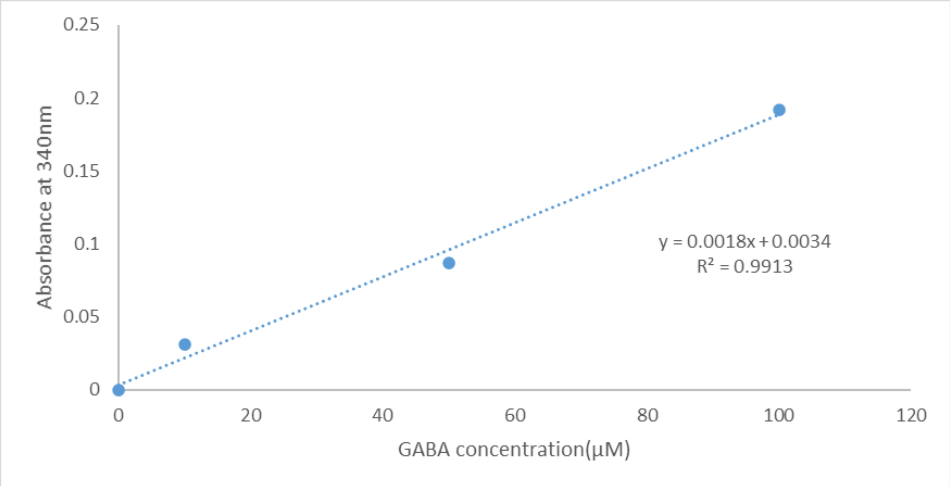
Appendix 4: The standard curve plotted with MDA equivalents(nmol.ml^{-1}) against MDA concentrations(nmoles). The standard curve equation was used to determine the MDA levels(nmol/g) in chickpea plants harvested at 4-week sampling point.



Appendix 5: The standard curve plotted with MDA equivalents(nmol.ml^{-1}) against MDA concentrations(nmoles). The standard curve equation was used to determine the MDA levels (nmol/g) in chickpea plants harvested at 12-week sampling point.



Appendix 6: The standard curve plotted with absorbance at 340nm against GABA concentrations. The standard curve equation was used to determine the GABA level(µmoles/g) in chickpea plants harvested at a 4-week sampling point.



Appendix7: The standard curve plotted with absorbance at 340nm against GABA concentrations. The standard curve equation was used to determine the GABA level(µmoles/g) in chickpea plants harvested at a 12-week sampling point.