"Investigating effect of ethylene pre-treatment on seed to enhance heat stress tolerance in wheat".

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Contents

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1. Abstract:

Extreme high temperatures have become more common due to global warming posing considerable difficulties to plant growth and agricultural productivity. High temperature is one of the most damaging abiotic stresses to crop growth and production, requiring appropriate mitigating techniques. This study looks at how ethylene pre-treatment affects growth and heat stress responses in two wheat cultivars (Scepter and E60) using ethephon for seed priming. We investigated biomass accumulation, oxidative stress markers (malondialdehyde (MDA) levels) and metabolic changes (gamma-aminobutyric acid (GABA) and starch content) under both optimum (17°C) and heat stress conditions (27°C for up to 144 hours). Ethephon pre-treatment with 5µM significantly increased biomass accumulation in both wheat varieties. Furthermore, short-term heat stress enhanced antioxidant defences in ethylene pre-treated plants as demonstrated by lower MDA level indicators of oxidative stress. However, prolonged heat stress (144 hours) increased MDA accumulation indicating that ethylene protective effects may be restricted during a lengthy duration of stress. Furthermore, ethylene priming was found to increase GABA concentration during heat stress, demonstrating its involvement in stress tolerance mechanisms. Ethephon pre-treatment also helped to stabilise starch levels during heat stress indicating increased metabolic activity. These findings emphasise the complexities of ethylene-mediated responses in wheat which are significantly impacted by genotype. This work highlights the efficacy of ethylene-based seed priming as a feasible approach to enhance heat stress tolerance in wheat. It promotes cultivar-specific optimisation strategies to improve the effectiveness of ethylene treatments. This study offers significant insights into wheat's physiological and biochemical reactions to heat stress, facilitating future studies focused on establishing sustainable agricultural methods in the context of climate change problems.

2. Introduction

The growing global population and drastic climate changes are significantly affecting global food security. Among the various challenges, drought and heat stresses are major limiting factors to crop production. Wheat (Triticum aestivum L.) is the staple food for approximately 35% of the world's population and provides a significant source of carbohydrates, protein, fats, zinc, and other essential minerals. Wheat fulfils roughly 25% of global calorie needs and 22% of protein requirements. (Bhandari et al., 2024)

Wheat is grown worldwide throughout the winter. One of the major wheat-producing regions South Asia experiences terminal heat stress during the reproductive and ripening stages of wheat growth. The optimum temperature ranges for wheat are 16–22 °C, 12–22 °C, and 21–25 °C for growth, anthesis, and ripening. (Djanaguiraman et al., 2020) However, temperatures in South Asia frequently surpass these ideal ranges, especially during important reproductive stages, resulting in heat stress. For this study, temperatures between 27 and 30°C are symptomatic of heat stress. Previous studies reveal that high temperatures have a significant impact on wheat at several developmental stages. Temperatures above 20 °C during the terminal spikelet stage delay spikelet initiation and lower spikelet numbers, and temperatures above 31 °C around anthesis reduce grain set via decreasing pollen viability. Temperatures above 35.4 °C during grain filling might reduce grain weight and protein quality, resulting in a considerable loss in production.(Jacott & Boden, 2020). This moderate heat stress range is relevant to many wheat-growing countries such as India, China, Nepal, and Türkiye and provides a useful framework for investigating wheat's physiological and biochemical responses to increased temperatures.

2.1. Effect of Heat Stress on the plant:

Heat stress can affect several stages of wheat development, including germination, vegetative growth, and reproductive stages, resulting in reduced photosynthesis, decreased plant height, lower grain yield, and poor grain quality.

→ **Germination Stage:** The germination stage is an important phase in a plant's life cycle and exposure to high temperatures such as 45 degrees Celsius can lead to serious effects. The embryo within the seed is Highly fragile and prolonged exposure to intense heat can cause embryo death. It prevents seeds from germinating and growing as seedlings. Heat stress during germination can inhibit the formation of

the radicle (embryonic root) and plumule (embryonic shoot) resulting in shorter roots and shoots in established seedlings. These seedlings frequently show lesser dry mass accumulation indicating slower growth and development. (Cheng et al., 2010).

→ **Reduced Photosynthesis:** High temperatures impact photosynthesis. Heat stress damages chloroplasts and deactivates enzymes such as Rubisco, which is necessary for photosynthesis. High temperatures also damage thylakoid membranes, which are responsible for photosynthesis. This damage breaks down chlorophyll and affects the plant's ability to transport electrons. As a result, plants struggle to assimilate carbon dioxide (CO2) slowing down photosynthesis and lowering overall plant productivity. (Buckley, 2017)

→ **Cellular and Membrane Damage:** Heat stress increases the synthesis of reactive oxygen species (ROS). It is responsible for cellular component damage and affecting membrane stability. This increases cell membrane permeability and disrupts cellular processes like lipid peroxidation, protein breakdown and enzyme inactivation. (Wada et al., 2020) (Cavanagh et al., 2022)

→ **Morphological Changes:** High temperatures cause changes in plant structure, such as stem elongation and leaf expansion, but they also limit green leaf area and several productive tillers. These morphological changes impact a plant's overall growth and yield potential because they limit photosynthetic surface area and resource allocation. (Djanaguiraman et al., 2010)

→ **Leaf Senescence:** Higher temperatures accelerate the ageing process of leaves known as senescence. This affects transpiration, leaf water content, and stomatal cell conductivity, lowering photosynthetic activity. Premature leaf senescence can decrease grain-filling time, resulting in smaller, lighter grains and thus poorer yields. (Sharma et al., 2015)

→ **Flower and Grain Development:** High temperatures at the post-anthesis stage (after bursting) harm wheat reproductive growth. Temperatures higher than 26°C can drastically hamper grain quality and yield. Heat stress during this period can lead to pollen sterility and reduced pollen viability, disruption of pollen germination and pollen tube growth, ovule abortion and poor fertilisation, and decreased seed set and grain number per spike These consequences eventually lead to reduced grain yield because fewer grains are produced and developed. (Prasad et al., 2011)

→ **Starch and Protein Content:** Heat stress during grain development affects the starch and protein composition of the grains. High temperatures tend to reduce starch accumulation while increasing protein concentration.(Yu et al., 2018)

→ **Assimilate Translocation**: High temperatures inhibit the transport of assimilates (photosynthesis products) from leaves and stems to growing grains. This is attributed to decreased membrane integrity and disruption of phloem loading and unloading processes during heat stress conditions.(Sharma et al., 2015)

→ **Photorespiration:** Photorespiration is the process that competes with photosynthesis by consuming oxygen and releasing carbon dioxide. High temperatures accelerate photorespiration in wheat, particularly in flag leaves. It plays an important role in grain filling. (Keys et al., 1977)

→ **Oxidative damage:** Heat stress causes oxidative damage in plant cells as reactive oxygen species (ROS) accumulate. ROS such as superoxide ($\bullet O^{2-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and singlet oxygen are naturally produced by cellular metabolism, especially during stressful situations such as heat. (Kalaipandian et al., 2023) Plants exposed to high temperatures produce more ROS than the antioxidant defence system can scavenge. It causes oxidative stress. Excess ROS damage a wide range of biological components, including lipids, proteins, carbohydrates, and DNA. Oxidative damage to lipids impacts cell membrane stability. This causes leakage of cellular contents and finally cell death. Lipid peroxidation leads to the destruction of the plasma membrane, mitochondrial membrane, and chloroplast membrane. Protein oxidation can lead to loss of function and aggregation, affecting cellular activity. Carbohydrates oxidise and alter their structure and function. ROS trigger DNA damage, leading to mutations and genetic instability. (Hasanuzzaman et al., 2019) (Choudhary et al., 2020)

2.2. Plant Responses to Heat Stress:

→ **Heat shock protein**:

Plants undergo a range of biochemical and physiological reactions in response to heat stress, which inhibits normal protein production.(ul Haq et al., 2019) Plants respond by quickly synthesising new proteins known as heat shock proteins. These proteins contribute to the maintenance of physiological balance within cells by assisting in the proper folding of newly synthesised polypeptides and preventing denaturing proteins

from irreversibly aggregating. HSPs are classified into five classes based on their molecular weight: HSP100, HSP90, HSP70, HSP60, and small heat shock proteins (sHSP). While all HSPs have been linked to heat tolerance sHSPs particularly those found in chloroplasts and mitochondria are thought to be more important for plant heat resistance. They preserve the heat-sensitive photosynthetic system II protein complex. Which ensures regular electron transport and ATP generation allowing plants to develop in hot weather. (Wada et al., 2020)

→ Wheat (Triticum aestivum) contains a small heat shock protein (sHSP) called TaHSP23.9 which is essential to the plant's reaction to heat stress. (J. Wang et al., 2020) TaHSP23.9 is strongly expressed in filling grains under normal conditions indicating that it plays a role in grain development. Its expression is elevated in response to heat stress demonstrating its critical role in wheat's heat stress response mechanisms. This increase shows that TaHSP23.9 functions as a molecular chaperone preventing protein denaturation and aggregation at high temperatures. The protein has an alpha-crystallin domain (ACD) and is found in the endoplasmic reticulum (ER), indicating an involvement in protein synthesis, folding, and transport within the cell. Transgenic Arabidopsis plants overexpressing TaHSP23.9 demonstrated improved heat stress tolerance as indicated by lower levels of malondialdehyde (MDA) and higher levels of total soluble proteins and proline. These findings highlight TaHSP23.9's potential as a molecular marker of heat tolerance in wheat breeding programmes. This sequence similar to OsHSP23.2 a heat tolerance marker in rice, contributes to its utility in detecting and selecting heat-tolerant wheat genotypes. (X. Chen et al., 2014) TaHSP23.9 expression levels are much greater in heat-tolerant wheat varieties than in heat-sensitive wheat varieties, indicating that it is a viable marker for breeding efforts targeted at boosting wheat's tolerance to heat stress. (J. Wang et al., 2020)TaHSP23.9 incorporation into wheat breeding procedures, whether by marker-assisted selection or transgenic approaches has the potential to improve wheat's capacity to endure high temperatures while maintaining yield and quality. TaHSP23.9 provides a road towards generating more robust and climate-resilient wheat varieties, which is critical for assuring agricultural productivity in the face of climate change problems.

→ **Enzymatic Antioxidants**: Antioxidants are molecules that prevent other molecules from oxidising, thereby protecting cells from damage caused by reactive oxygen

species (ROS). ROS, which includes superoxide anions (O2•-), hydrogen peroxide (H2O2), and hydroxyl radicals (OH•), are extremely reactive substances that can cause oxidative damage to proteins, lipids, and DNA in cells. Antioxidants neutralise these toxic ROS, protecting cellular health and reducing oxidative stress. Oxidative stress arises when the generation of ROS exceeds the cell's ability to detoxify these reactive intermediates or repair following damage. Various abiotic factors can cause oxidative stress in plants, including drought, salinity, and heat, which can inhibit growth, development, and production.

Wheat Antioxidant Defence System

Several studies on wheat have found that the activity of several antioxidant defence system enzymes alters in response to oxidative stress caused by environmental conditions. Under distinct conditions, wheat plants show changes in the activity of SOD, APX, CAT, GR, and POX, as well as changes in ROS levels. (Noctor et al., 2014) These studies demonstrate that ROS detoxification mechanisms in wheat are positively activated in response to stress.

- **Superoxide Dismutase (SOD):** The enzyme superoxide dismutase (SOD) plays a critical role in this defence system. SOD activity increases in response to oxidative stress turning superoxide radicals into hydrogen peroxide. Under drought and salinity stress increased SOD activity protects cellular components from oxidative damage and contributes to the plant's overall stress resistance. During heat stress enhanced SOD activity serves to maintain cellular homeostasis and protects the photosynthetic machinery photosystem II from oxidative damage. This protection is critical for maintaining photosynthetic efficiency in stressful settings. Molecular and biochemical studies have demonstrated that the expression of SOD genes such as Cu/Zn-SOD and Mn-SOD is elevated in wheat under diverse stress situations resulting in higher SOD enzyme activity. (Mohi-Ud-Din et al., 2021)
- **Catalase (CAT):** Catalase (CAT) is an important factor in response to abiotic stress including heat and drought. Studies have shown that heat stress causes a large increase in CAT activity in seedlings, which is strongly associated with other heat tolerance elements. (Mohi-Ud-Din et al., 2021) Similarly, drought circumstances enhance CAT activity to deal with the higher levels of hydrogen peroxide (H2O2) produced by stress-induced metabolic alterations. CAT is required for H2O2 detoxification especially in peroxisomes during

photorespiration prevent oxidative damage to cellular components such as proteins, lipids, and DNA. Different wheat genotypes have different amounts of CAT activity, with heat-tolerant cultivars frequently having more CAT activity than heat-sensitive ones. (Mohi-Ud-Din et al., 2021) CAT activity varies across tissues with leaves showing higher levels than roots under drought stress.

- **Peroxidase (POX):** Peroxidase (POX) is important in wheat's response to a variety of stress including drought, heat, salinity, and heavy metal stress. For example, short-term heat stress (45°C for 2 hours) has been demonstrated to boost POX activity in wheat seedlings. POX aids in the detoxification of hydrogen peroxide (H2O2) which is critical for maintaining cellular redox balance and preventing oxidative damage. POX also plays a role in lignin production and cell wall crosslinking, which improves stress tolerance by reinforcing cell walls.
- **Ascorbate Peroxidase (APX):** Ascorbate peroxidase (APX) is essential for scavenging hydrogen peroxide (H2O2). Reactive oxygen species (ROS) that can oxidise wheat lipids, proteins, and DNA. By converting H2O2 to water, APX helps to protect cells from oxidative damage. It is a crucial enzyme in the ascorbate-glutathione cycle, collaborating with MDHAR, DHAR, and GR to replenish ascorbate and maintain cellular redox equilibrium. APX is especially important in chloroplasts and mitochondria because it detoxifies H2O2 generated during photosynthesis and respiration, efficient photosynthesis under stressful situations. APX activity rises in response to abiotic conditions such as heat, drought, and salinity, reducing oxidative damage.

→ **Non-Enzymatic Antioxidants**

- **Ascorbic Acid (AA):** Ascorbic Acid (AA), often known as vitamin C, is a vital non-enzymatic antioxidant in wheat that plays an important role in stress response. AA directly scavenges reactive oxygen species (ROS) such as superoxide, singlet oxygen, and hydroxyl radicals, acting as the first line of defence against oxidative damage in stressed plants. It is a vital component of the ascorbate-glutathione cycle in which ascorbate peroxidase (APX) uses AA to convert hydrogen peroxide (H2O2) to water so maintaining cellular redox equilibrium. This activity is essential for maintaining the photosynthetic apparatus in leaves under high light or temperature stress. The increased level

of AA contributes to the protection of critical cellular components from oxidative damage and the maintenance of redox balance.

- **Proline:** Proline amino acid essential functions as both an osmolyte and potent antioxidant. Proline is derived from glutamic acid and has a unique cyclic structure that allows it to accumulate significantly during stress conditions like drought, heat, and salinity. (Lim et al., 2015) This buildup occurs through increased synthesis or reduced breakdown, which improves the plant's ability to deal with environmental challenges. Proline is an important antioxidant because it neutralises reactive oxygen species (ROS) such as hydroxyl radicals and singlet oxygen, protecting cellular components from oxidative damage. Its function also includes inhibiting lipid peroxidation, which is critical for preserving membrane integrity during stress. Proline also functions as an osmolyte, assisting in osmotic adjustment to maintain cell turgor and water balance in wheat plants during water deprivation. (Hussain et al., 2019)
- **Reduced Glutathione (GSH):** Glutathione (GSH) tripeptide consisting of glutamic acid, cysteine, and glycine, is essential for stress responses and general physiology. (Noctor et al., 2002) GSH maintains cellular redox equilibrium by balancing oxidation and reduction activities. This activity is crucial for protecting cells from oxidative damage caused by reactive oxygen species (ROS) during stress. GSH plays a critical role in hydrogen peroxide (H2O2) detoxification via the ascorbate-glutathione cycle. Where it interacts with enzymes such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). (Noctor et al., 2002) This cycle not only detoxifies H2O2 but also regenerates important antioxidants such as ascorbate. GSH protects biological components like lipids, proteins, and DNA from oxidative damage by directly scavenging different ROS, including superoxide radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($OH^{\bullet}$).
- **Carotenoids:** Carotenoids are vital pigments present in plants particularly in protecting the photosynthetic mechanism from oxidative stress. They accomplish this mostly by quenching reactive oxygen species (ROS), especially singlet oxygen, transforming potentially damaging ROS into innocuous ones. This protective process maintains the integrity of chlorophyll molecules which is critical for photosynthetic efficiency, particularly in

> drought, heat, and salt. Plants control their carotenoid levels in response to various challenges, hence improving photosynthetic protection. Carotenoids play an important function in sustaining photosynthesis and boosting plant growth and production under stress by reducing ROS levels. Carotenoids operate as antioxidants during heat stress, preventing chlorophyll and other photosynthetic components from being damaged. Similarly, carotenoids protect cellular components from oxidative damage under drought and salinity stress, allowing cellular homeostasis to remain intact.

To enhance crop resilience in the context of climate change, it is essential to comprehend the molecular distinctions between heat and dehydration stress. Primarily, heat stress induces the activation of heat shock proteins (HSPs), which are essential for the stabilisation of proteins and the protection of cellular structures from denaturation. On the other hand, drought stress is primarily characterised by the regulation of osmotic adjustments and the induction of stomatal closure to reduce water loss through abscisic acid (ABA) signalling. Sato et al. underscore that these stresses trigger distinct gene expression profiles and signalling mechanisms, in addition to activating overlapping pathways, such as oxidative stress responses. For instance, the "dry air effect" is a phenomenon that occurs when reduced stomatal conductance, which is exacerbated by heat stress, elevates leaf temperatures in conjunction with drought. Heat stress can increase transpiration rates under normal conditions. In addition, metabolic pathways exhibit variations, with drought stress increasing the production of flavonoids for oxidative protection and heat stress increases the production of thermoprotective metabolites. These observations emphasise the intricate relationship between heat and drought stress, emphasising the necessity of molecular interventions and targeted breeding strategies that recognise and mitigate their individual and combined effects. (Sato et al., 2024)

2.3. Seed Priming:

In response to these challenges, developing strategies to enhance abiotic stress tolerance in wheat has become a crucial area of research within the field of agricultural biotechnology. One promising approach that has gained attention is the priming of seeds with ethylene involves treating wheat seeds with ethylene or ethylene-releasing compounds (like ethephon) before sowing to enhance germination, seedling Vigor, and stress tolerance. Recently seed priming with ethylene a gaseous plant hormone known for its role in regulating various

physiological processes, including stress responses has been reported (Zhang et al., 2021). Ethylene's role in plant growth and development has been extensively studied and its potential to improve abiotic stress tolerance is now attracting notable interest (Bharadwaj et al., 2022)

Brenya et al. (2023) discovered considerable benefits of ethylene priming in Arabidopsis, indicating its efficacy in improving stress tolerance. The plants that were primed with ethylene showed 100% survival rates when exposed to high temperatures (43°C) and saline stress. In contrast, the plants that were not treated experienced bleaching and eventually died. Ethylene-primed plants demonstrated significant morphological improvements under stress, including longer primary roots, enhanced lateral root development, and larger aerial tissues than controls. These structural enhancements show that ethylene has a role in increasing plant growth resistance even in stressful situations.(Brenya et al., 2023)

2.4. Ethylene:

Ethylene is a fundamental olefin gas and the first gaseous molecule demonstrated to act as a hormone. (Lin et al., 2009) It is biosynthesized by plants and is well-known for regulating numerous developmental processes, such as seed germination, fruit ripening, senescence, and abscission, as well as responses to various stresses, such as flooding, excessive salt, and soil compaction.

Biosynthesis Pathway of Ethylene:

The ethylene biosynthesis path in plants is a well-studied mechanism that involves several enzyme processes that transform the amino acid methionine into ethylene. Methionine is an amino acid that can be converted into ethylene in two phases. SAM synthetase initiates the synthesis process by forming the intermediate SAM from methionine in an ATP-dependent phase. The enzyme ACS first converts the substrate SAM to ACC and 5′-methylthioadenosine (MTA). **(Figure 1.1)** An adenosyl group is transferred from adenosine triphosphate (ATP) to methionine to generate SAM. Methionine is a sulphur-containing amino acid, and the enzyme SAM synthetase helps the adenosine bind to the sulphur in the methionine to generate SAM. In the following phase, ACS converts SAM into ACC, and methionine is generated via the Yang cycle. In this reaction, ACS not only produces ACC but also 5′-methylthioadenosine, which is converted to methionine via a modified methionine cycle and stores the methyl group for use in a subsequent round of ethylene synthesis. The enzyme SAM decarboxylase then catalyses the conversion of methionine to decarboxylated

SAM. In plants, the immediate ethylene precursor or ACC can travel acropetally in the xylem or basipetally in the phloem. A recent study found that the direct ethylene precursor ACC is carried via the xylem via the LYSINE HISTIDINE TRANSPORTER (LHT1) or conjugated into malonyl-ACC or jasmonyl-ACC both are delivered through the xylem. (Choi et al., 2019) The final stage in the ethylene biosynthesis from ACC is catalysed by ACO, which requires O2 and carbon dioxide (CO2) as co-substrates and necessary activators. The ACO oxidises ACC to ethylene and cyanoformic acid, which spontaneously decarboxylates into cyanide and CO2. Cyanide is detoxified by β-cyanoalanine synthase. Ethylene is oxidised to ethylene oxide (ethylene monooxygenase) and ethylene-glycol.

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Figure 1.1: Ethylene biosynthesis starting with methionine precursor converts into ACC through the Yang cycle. (Fatma et al., 2022)

Ethylene signalling pathway:

ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4) are ethylene receptors. Ethylene receptors are present on the membranes of the Golgi body and the endoplasmic reticulum. They play a negative role in ethylene signalling. When there is no ethylene, these receptors activate a protein called CONSTITUTIVE TRIPLE RESPONSE1 (CTR1), which is equivalent to the Raf protein kinase. (Ju et al., 2012) CTR1 attaches phosphate groups to EIN2's C-terminal end

(EIN2-CEND), preventing EIN2 from functioning. (Wen et al., 2012) This prevents ethylene-responsive genes from being activated.

When ethylene is present the receptors become inactive, which leads to inactivation of CTR1. EIN2 is to be dephosphorylated and cleaved, releasing EIN2-CEND into the cytosol and nucleus. EIN2-CEND in the cytosol inhibits the synthesis of EBF1 and EBF2 by binding to untranslated regions of their gene sequence and transporting them to specific areas of the cell known as processing bodies. EIN2-CEND in the nucleus aids in the activation of proteins known as EIN3 and EIL1. Which are critical for directing the activity of ethylene-responsive genes. These genes, including ETHYLENE RESPONSE FACTOR (ERF), regulate the plant's ethylene reactions.

EIN2-CEND also contributes to downstream signalling by controlling histone acetylation at H3K14 and H3K23. When ethylene is not present, EIN2 interacts with EIN2 nuclear-associated protein 1 (ENAP1) to keep the chromatin open. In the presence of ethylene, EIN2-CEND interacts with ENAP1, boosting histone acetylation at H3K14 and H3K23 and allowing EIN3 and EIL1 to regulate transcription rapidly. In plants, ethylene signalling comprises a complex network of interactions and changes that control gene expression and physiological responses.

2.5. Ethylene's role in the plant:

Table 1.1: Ethylene has several critical roles in regulating plant growth, development, and responses to environmental stress. Here are the key roles of ethylene in plants. (Van de Poel et al., 2015)

Cell Division	▪ Ethylene has numerous roles in plant development and stress responses. It works with auxins to drive cell division in the subepidermal layers, assisting in hook bending. Ethylene in the root system regulates cell development in the quiescent centre, influencing the growth of the root cap layers and the root apical meristem via pathways involving CTR1, EIN2, and ARR1. In leaf cells, ethylene responds to osmotic stress by creating a short pause in the cell cycle and affecting the MPK3/6 pathway. These different activities of ethylene show its significance in controlling plant growth and stress responses.

Cell Elongation	▪ Ethylene rapidly shifts CMTs in Arabidopsis roots and hypocotyls, inhibiting elongation and generating radial swelling. This repositioning of CMTs from a longitudinal to a transverse orientation limits cell elongation and encourages radial development.
Flower Development	▪ Ethylene controls flower senescence by regulating wilting, petal abscission, and the final death of floral components. It can also affect flower opening and petal expansion in certain plant species. ▪ In cucurbits, ethylene biosynthesis and signalling components are critical for sex expression and the formation of unisexual or bisexual flowers.
Root Hair Development	▪ Ethylene suppresses the growth of root hair. Elevated levels of ethylene reduce root hair growth, resulting in shorter and fewer root hairs. This process is mediated by the ethylene signalling system which includes receptors such as ETR1, ERS1, and EIN4, as well as downstream transcription factors like EIN3 and EIL1.
Chloroplast Development	▪ Ethylene has been shown to promote senescence. Chloroplasts experience structural changes and lose functionality during senescence. This process is accelerated by ethylene, which results in the breakdown of chlorophyll and other photosynthetic pigments. No-senescing leaves need basal levels of ethylene to maintain normal chlorophyll concentration.
Stomatal Regulation	▪ Ethylene influences the development of stomata during plant growth. Ethylene can control stomatal opening and closure in response to environmental stimuli. Ethylene induces stomatal closure by triggering H_2O_2 synthesis in guard cells. It triggers stomatal closure in stressful situations like drought and high light intensity. This reduces water loss through transpiration and keeps plants hydrated. Ethylene regulates stomatal function by interacting with other hormones like abscisic acid (ABA) and auxins.

Photosynthesis	Light Reaction of Photosynthesis ▪ Ethylene regulates energy-dependent non-photochemical quenching (NPQ) by suppressing the xanthophyll cycle. This reduces the conversion of violaxanthin to zeaxanthin. It results in increased ROS generation under high light situations. Dark Reaction of Photosynthesis ▪ Ethylene regulates the production and activity of Rubisco and other enzymes involved in carbon fixation. Elevated ethylene levels prevent photosynthesis by limiting Rubisco content and activity.
Fruit Ripening	▪ Ethylene, a crucial hormone in fruit ripening, initiates colour change, softening, and fragrance development in climacteric fruits.
Seed Germination	▪ Ethylene can either stimulate or inhibit seed germination, depending on the species and environmental conditions. It frequently interacts with other hormones such as gibberellins and abscisic acid.

Role of Ethylene on ROS: When plants are exposed to a variety of abiotic obstacles, they produce reactive oxygen species (ROS) which can damage biomolecules such as proteins, membranes, and DNA. Plants use antioxidative enzymes and antioxidants such as ascorbic acid, glutathione, and tocopherol, to neutralise the effect. Ethylene can have both beneficial and harmful impacts on plants under abiotic stress. Ethylene improves plant tolerance to salt stress by enhancing the expression of ROS scavengers. Salt stress increases the expression of ethylene-responsive genes in Arabidopsis, resulting in greater ROS scavenging and salt stress resistance. Mutants that were insensitive to ethylene displayed higher absorption of metals such as lead, as well as lower levels of glutathione (GSH) and altered antioxidant enzyme expression.(Peng et al., 2014)

Ethylene may increase oxidative stress by inhibiting the action of antioxidative enzymes and increasing ROS levels. Under salinity stress, ethylene signalling promotes ROS generation via modulating peroxidase (POD) and glutathione reductase activity in rice plants. Furthermore, several ethylene-responsive

transcription factors (ERFs) have been linked to ROS generation. ERF1 suppresses the expression of ROS-scavenging genes during drought and high salinity conditions.

2.6. Current research on ethylene pretreatment:

Ethylene affects the expression of genes involved in ROS metabolism and scavenging helps to reduce ROS (Reactive Oxygen Species) levels in plants under stress situations. The gene TERF1 has been identified as a crucial regulator in stress situations. (Li et al., 2009) The research demonstrates that ethylene signal transduction is mediated by a series of membrane receptors and downstream components such as CTR1, EIN2, EIN3, and ERF transcription factors. EIN3 and its homologs (EILs) attach directly to the promoters of ERF genes via elements, controlling their expression. It was discovered that EIL3 and EIL4 may bind to the promoter of the TERF1 gene, which is implicated in ethylene signalling and ROS response. When tobacco plants were overexpressed with TERF1, ROS accumulation decreased in response to ethylene exposure. This suggests that TERF1 may regulate ROS generation or scavenging in response to ethylene signalling.(Zhang et al., 2016)

ACC is an ethylene (ET) precursor, and its use has been demonstrated to improve plant heat stress responses. A recent study found that short-term heat stress initially reduces ET synthesis but eventually leads to a considerable increase. This increase in ET production is associated with the plant's tolerance to heat stress. Pre-treatment with ACC, which elevates ET levels in plants, has been demonstrated to boost heat stress tolerance in several plant species. Pretreatment with ACC enhanced survival rates and reduced lipid peroxidation in Arabidopsis seedlings under heat-stress conditions. In these grass species, ACC treatment boosted the activity of antioxidant enzymes while decreasing lipid peroxidation. Exogenous ACC treatment in rice seedlings increased heat stress tolerance by lowering lipid peroxidation and electrolyte leakage, maintaining chlorophyll content, and increasing antioxidant enzyme activity. Under heat stress conditions, ACC-treated rice seedlings expressed higher levels of heat shock factors, ET biosynthesis genes, and ET-signalling genes.(Wu & Yang, 2019)

Ethephon (2-chloroethylphosphonic acid; Ethrel) is a compound that leads plants to produce ethylene during metabolism. Since its release in the 1960s, ethephon has been widely used as a plant growth regulator. (Y. Chen et al., 2014) Ethephon is easily absorbed by photosynthetic plant tissues, thereby releasing ethylene directly into plant tissues. Ethephon is widely used across different crops to induce flowering, enhance fruit ripening, and facilitate abscission. A

recent study indicates that ethephon treatment helps mitigate glucose sensitivity. Which can inhibit photosynthetic activity under salt stress. By promoting ethylene production, ethephon improves chlorophyll content and net photosynthesis. This leads to increased leaf area and biomass even in stress conditions. plants treated with ethephon showed improvements in photosynthetic parameters, including stomatal conductance and intercellular CO_2 concentration, compared to control plants under salt stress conditions.(Sehar et al., 2021)

Plants treated with ethylene show considerable improvements in growth, photosynthetic efficiency, and stress tolerance. This treatment enhances vigorous growth in leaves and roots, increases chloroplast development, and boosts carbon fixation by 25%, resulting in increased amounts of essential carbohydrates such as glucose, sucrose, and starch.(Brenya et al., 2023) These alterations are connected to increased expression of photosynthesis-related genes such as RBCS1a and RBCS2b. Ethylene also enhances the plant's ability to manage abiotic stimuli such as heat and drought via metabolic priming, increasing energy availability for stress responses. Furthermore, ethylene's interaction with auxin and blue light photoreceptors influences growth independently of the typical auxin responses.(Brenya et al., 2023)

Ethylene pretreatment in plants has the potential to improve resilience to abiotic stresses. its efficacy varies depending on the type and severity of stress, as well as the treatment methods. It has been demonstrated that treating Arabidopsis seedlings with ethylene during their dark-grown stage promotes growth during the light phase and enhances abiotic stress tolerance. Experiments with young tomato plants exposed to salt, drought, and waterlogging stress gave mixed results: modest benefits during drought, harmful impacts during salinity, and no discernible influence during waterlogging. The research show that the timing, dose, and duration of ethylene pretreatment are all important for its effectiveness. 4-hour ethylene pretreatment may not be adequate to generate a strong stress resilience response, and longer pretreatment periods or alternative dosages may be required. Ethylene pretreatment causes stomatal closure to conserve water during a drought, but it may not affect photosynthesis or growth in high salinity or waterlogging conditions. (Hartman et al., 2020) Furthermore, ethylene-induced reactions like epinasty can reduce CO2 assimilation by diminishing light perception, demonstrating the complexities of ethylene's impact on plant stress tolerance.(Mohorović et al., 2023)

Cotton seed germination and growth are improved by ethylene pretreatment under salt stress, due to its ability to reduce oxidative damage and osmosis. High NaCl concentrations inhibit

seed germination by generating osmotic and ion toxicity, interfering with water intake, and causing metabolic abnormalities. (Li et al., 2023) However, ethylene generated by ethephon stimulates seed dormancy breakdown and germination by boosting α-amylase activity, which hydrolysis starch into soluble sugars to provide the necessary energy. Ethylene also increases the activity of defensive enzymes such as SOD and POD, lowering MDA levels and preserving cell membranes from lipid peroxidation. Furthermore, ethylene increases the production of proline and soluble carbohydrates, which improves osmotic control and stress tolerance. (Li et al., 2023)

Recent research on wheat under Salt stress causes plants to produce excess ethylene which inhibits photosynthesis. Sehar et all studies reveal that Ethephon treatment with glucose reduced ethylene production under salt stress. Which improved GSH production via the ascorbate-glutathione cycle. Ethylene enhanced stomatal conductance, allowing for higher CO2 input for photosynthesis, and stimulated the expression of psbA and psbB genes, (Zhang et al., 2017) which boosted PSII activity. The PsbA gene is encoded for the D1 protein which is a core component of psII. This protein is important for the oxygen-evolving complex where water is divided to release oxygen during photosynthesis. The PsbB gene encodes the psbB protein which helps to stabilize the psII complex and facilitate electron transfer. According to Sehar's findings, ethylene treatment helps plants maintain well-organized chloroplast structures and thylakoid systems when exposed to salt stress. This structural preservation improves photosynthetic efficiency allowing plants to endure and withstand adverse effects of salinity. (Sehar et al., 2021)

The regulation of genes involved in the metabolism of reactive oxygen species (ROS), antioxidant activity, and stress signalling pathways is a critical function of ethylene in the modulation of plant stress responses. ethylene signal transduction is mediated by key components, including CTR1, EIN2, EIN3, and ERF transcription factors. This process directly affects genes such as TERF1, which reduces ROS accumulation and improves stress tolerance. To enhance antioxidant enzyme activity, reduce lipid peroxidation, and maintain photosynthetic efficiency, treatments with ethylene precursors, such as ACC, or ethylene-releasing compounds, such as ethephon, have been demonstrated to improve heat stress tolerance. The precise mechanisms and optimal conditions for ethylene-based priming treatments, which vary depending on plant species, stress type, and treatment conditions, are, however, still complex. However, voids continue to exist in the comprehension of the impact of ethylene priming on the resilience of wheat to stress and early growth, despite recent

progress. This research aims to fill this void by proposing that ethylene priming optimises wheat germination and early development in response to heat stress, thereby enhancing tolerance through physiological and metabolic adaptations. The hypothesis is that “Ethylene priming enhances the germination rate and early growth of wheat leading to improved tolerance to heat.”

2.7. Aims of the study:

AIM:1: Determine the optimal concentration for wheat seed priming and evaluate the effect on seed germination rate, and early seedling growth parameters (root/shoot length, biomass).

AIM:2: To investigate the molecular and metabolic changes induced by ethylene priming in plants under heat stress, examine the alterations in reactive oxygen species (ROS) levels, gamma-aminobutyric acid (GABA) levels, photosynthetic parameters (chlorophyll content), and carbohydrate metabolism (starch).

3. Method and Material

3.1.1. Pilot Study: Seed Priming with 1-aminocyclopropane-1-carboxylic acid (ACC)

Two layers of filter paper were placed at the bottom of each plastic jar and soaked with 10 mL of the equivalent 1-aminocyclopropane-1-carboxylic acid (ACC) concentrations (10 µM, 50 µM, and 100 µM). Wheat seeds were placed in jars. Which has two layers of filter paper. The study examined ACC doses of 10µM, 50µM, and 100µM, with distilled water as the control. The jars were then coated in aluminium foil to avoid light exposure and left alone for 3 days to allow adequate seed priming. This study used two wheat genotypes: Beckom and E62. Each genotype and treatment group comprised ten replicates.

3.1.2. Pilot Study: Seed Priming with ethephon (ETH)

Wheat seeds were placed in the jar containing two layers of filter paper. Filter papers were soaked with 10 mL of the ethephon solution. Various concentrations of ethephon were evaluated specifically 5µM, 50 µM, 500 µM, and 1000 µM, with distilled water serving as the control. The jars were then wrapped in aluminium foil to prevent light exposure and left undisturbed for three days to facilitate effective seed priming. This study used two wheat genotypes Scepter and E62. Each genotype and treatment group consisted of ten replicates.

3.2. Seed priming:

Wheat genotypes Sceptre and E60 were chosen for this investigation because to their known variations in stress tolerance and reactivity to ETH and ACC treatments. Although Beckom was originally evaluated, early trials revealed no significant response to ACC priming, therefore it was discarded. Similarly, E62 was not chosen for a lack of seed availability, which restricted its participation in the experimental setting. Controls consisted of unprimed plants for growth analyses and distilled water for seed priming. The optimal priming conditions were established in pilot studies by evaluating germination rates, early growth parameters, and stress tolerance. Wheat seeds were placed in a jar containing two layers of filter paper soaked with 25 mL of 5µM ethephon solution. The jars were wrapped in aluminium foil to prevent light exposure and left undisturbed for three days to facilitate effective seed priming. This study used two wheat genotypes Scepter and E60. Each genotype and treatment group consisted of four replicates.

3.3. Heat Treatments:

After 5-week growth seedlings of two wheat genotypes (Scepter and E60) were transferred to separate growth rooms. The control group was kept at the optimal temperature of 17°C, while the heat stress group was placed in a growth chamber set to 27°C to simulate high-temperature stress. Both environments were maintained under a 12-hour photoperiod with relative humidity controlled between 50% and 60%. Each treatment consisted of four replicate plants per genotype.

3.4. Analysis of MDA Content:

Tissue samples homogenized in liquid nitrogen. 50–70mg of each was transferred into individual 1.5 mL microcentrifuge tubes. Each sample was then mixed with 1 mL of 5% TCA solution. Thoroughly vortexed to ensure complete homogenization. The tubes were centrifuged at maximum speed. After which 400µL of supernatant was aliquoted into two separate microcentrifuge tubes labelled **(A)** and **(B)**. Tube **(A)** received 400µL of **Solution A** which contained 20% TCA, 0.01% BHT, and 0.5% TBA. Tube **(B)** was supplemented with 400µL of **Solution B** consisting of 20% TCA and 0.01% BHT, with no TBA. Both tubes were heated at 96°C for 30 minutes with the lid slightly open to release pressure. After heating the samples were cooled on ice for 5 minutes. Followed by centrifugation at 9,500 g for 10 minutes. For each sample 100µL of supernatant from tubes (A) and (B) was transferred to individual wells of the 96-well plate. Absorbance was measured at 440 nm, 532 nm, and 600 nm using a spectrophotometer, and resulting values were used to determine TBARS content, which reflects lipid peroxidation levels in samples. Four biological replicates were included in each treatment group, with two technical replicates per sample to guarantee reproducibility.(Jakovljević et al., 2019; Singh et al., 2012).

To calculate MDA equivalents

1) [(Abs 532+TBA) - (Abs 600+TBA) - (Abs 532-TBA - Abs600-TBA)] = A

2) [(Abs 440+TBA - Abs 600+TBA) 0.0571] = B

3) MDA equivalents (nmol · ml-1) = ((A-B)/157 000) 106

3.5. Analysis of GABA concentration:

Frozen tissue powder (50-80 mg) was weighed and transferred to a 2.0 mL centrifuge tube. Add 400µL of methanol to the tube and incubate at 25°C for 10 minutes in a heating

block. Dry samples with SpeedVac to eliminate methanol resulting in the dry pellet. The pellet was resuspended in 500µL of 70 mM lanthanum chloride and shaken at 100 rpm for 15 minutes. After centrifugation at 12,000 rpm for 5 minutes supernatant (400µL) was transferred to a new tube containing 160 µL of 1 M KOH. The tube was mixed by inversion and shaken at 100 rpm for 5 minutes. The mixture was centrifuged again at 12,000 rpm for 5 minutes. The supernatant was then transferred to another 2.0 mL centrifuge tube and stored at -80 °C until further analysis. The master mix was prepared consisting of the following components: 0.5 M $K_4P_2O_7$ (pH 9.0) at 15µL per sample, 2-mercaptoethanol at 0.33µL per sample, 20 mM α-ketoglutarate at 25µL per sample, and 10 mM NADP at 12.5µL per sample. 45.2 µL of sample extract was transferred into each well of a 96-well microplate. Followed by the addition of 52.8 µL of the prepared master mix. Pre-reaction absorbance measurements were taken at 340 nm for three cycles of 120 seconds per cycle at a temperature of 25 °C. Following initial measurement 2µL of GABAase enzyme was added to each well. The absorbance at 340 nm was then measured for 30 cycles of 120 seconds per cycle to monitor the enzymatic reaction. Four biological replicates were included in each treatment group, with two technical replicates per sample to guarantee reproducibility. (Ramesh et al., 2015).

3.6. Analysis of starch content:

Tissue samples (50 mg) were placed in 2 mL microcentrifuge tubes and treated with 200µL of 80% (v/v) ethanol. Followed by vertexing. 200µL of refrigerated 1.7 M sodium hydroxide was added, and samples were vortexed again. Shaken at 100 RPM for 15 minutes into an incubator shaker before centrifugation at 10,000 rpm for 2 minutes. The supernatants were transferred into labelled tubes for unknown sample extracts (UK) and unknown sample blanks (UKB). 100µL of respective extract to each tube. 400µL of sodium acetate-calcium chloride buffer (pH 3.8) was added. 5µL of α-amylase and amyloglucosidase were transferred to the UK tube, while the UKB tube received an equivalent volume of buffer only. After vortex and 30-minute incubation at 50 °C samples were cooled. Transferred 200µL into new tubes which were then centrifuged at 13,000 rpm for 5 minutes. From each sample, 5µL was added to a well on a 96-well microplate along with glucose standard and distilled water in separate wells. Following the addition of 150 µL of GOPOD reagent to each well the microplate was incubated at 50 °C for 20 minutes. The contents were mixed using the shake function on the microplate reader for 5 min before measuring absorbance at 510 nm. Four

biological replicates were included in each treatment group, with two technical replicates per sample to guarantee reproducibility.(McCleary et al., 2019; McCleary et al., 2002)

3.7. Statistical analysis:

GraphPad Prism was used for all statistical analyses. Analysis of Variance (ANOVA) was used to determine statistical significance between sample groups. A p-value of Less than 0.05 was the threshold for statistical significance.

4. Result

4.1. Pilot: seed priming with ACC & ETH (Ethephon) seed priming

Applying 1-aminocyclopropane-1-carboxylic acid (ACC) as seed priming compound influenced biomass production in wheat cultivars (Beckom and E62). ACC pre-treated plants produced higher biomass compared to untreated plants after 2 weeks of growth. The application of 1-aminocyclopropane-1-carboxylic acid (ACC) as a seed priming agent affected biomass production in the wheat cultivars Beckom and E62. Plants pre-treated with ACC exhibited greater biomass than untreated plants following a growth period of two weeks. In the Beckom cultivar, pretreatment with 50 µM ACC led to optimal fresh weights for shoots and roots, achieving 0.5 g and 0.4 g, respectively. This corresponds to a 1.67-fold increase in shoot biomass and a 1.6-fold increase in root biomass relative to water-primed controls (Figures 4.1.1 a & b). Dry biomass patterns exhibited variation: the highest dry shoot weight (0.07 g) occurred with 50 µM ACC, representing a 1.75-fold increase, whereas the maximum dry root weight (0.05 g) was recorded with 100 µM ACC, indicating a 1.67-fold increase compared to controls **(Figures 4.1.1 c & d).** In the E62 cultivar, treatment with 100 µM ACC led to the maximum shoot fresh weight of 0.7 g, indicating a 2.33-fold increase, whereas the dry shoot weight reached 0.08 g, reflecting a 2-fold increase relative to water-primed controls **(Figures 4.1.1 a & c).** Root biomass exhibited a distinct response: the highest fresh root weight (0.5 g) occurred at 10 µM ACC, representing a 1.67-fold increase, while the maximum dry root weight (0.03 g) was also noted at this concentration, indicating a 1.5-fold increase compared to controls **(Figures 4.1.1 b & d)**. The findings indicate that the impact of ACC priming on biomass production is dependent on both genotype and concentration. Beckom demonstrated optimal growth at 50 µM ACC for both fresh and dry shoot weights, whereas E62 showed improved shoot biomass at 100 µM ACC, but enhanced root biomass at a lower concentration of 10 µM. The fold differences indicate that ACC may enhance biomass production

compared to water-primed controls, although optimal concentrations vary among genotypes and tissue types.

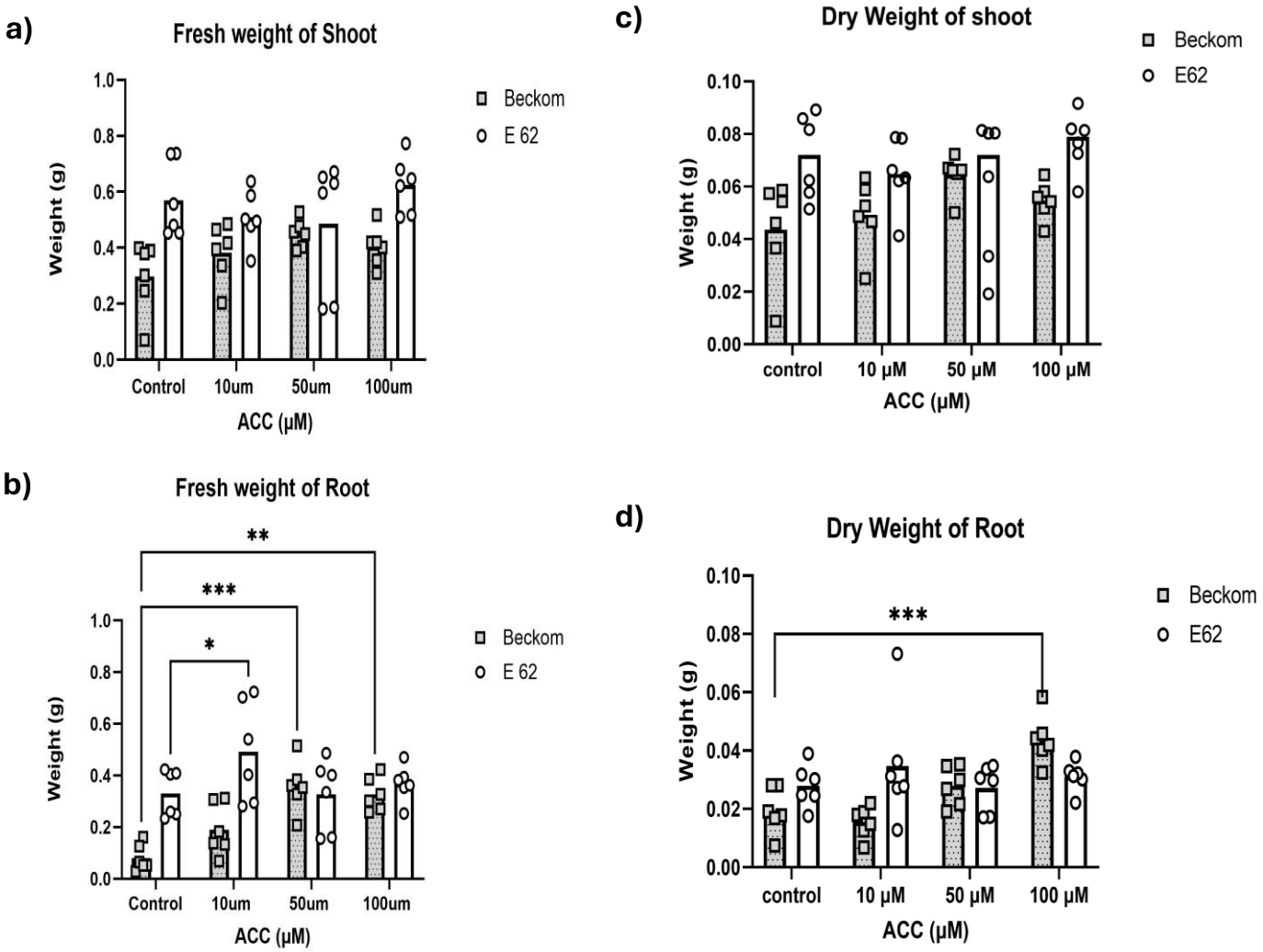


Figure 4.1.1.: Effect of 1-Aminocyclopropane-1-Carboxylic Acid (ACC) pre-treatment on fresh and dry weight of wheat plants. Fresh and dry weights of shoots and roots were measured in ACC pre-treated and untreated wheat seedlings of two cultivars (Beckom and E62) after 2 weeks of pot transfer. Plants were pre-treated with different concentrations of ACC (0, 10, 50, and 100 µM). (A) Fresh shoot weight. (B) Fresh root weights. (C) Dry shoot weight. (D) Dry root weights. Weights were reported in grams. Data represent means ± SE of six biological replicates (n = 6). (*) indicate significant differences between ethylene-treated and untreated plants (*$p<0.05$, ANOVA)

In the context of ACC seed priming, E62 exhibited a stable root length of 40 mm across all concentrations, which was marginally shorter than the control measurement of 45 mm, indicating a 0.89-fold reduction **(Figure 4.1.2.a).** In Beckom, root length exhibited variation in response to ACC concentration, with the shortest root length recorded at 100 µM ACC measuring 35 mm, representing a 0.81-fold decrease relative to the control value of 43 mm. At 10 µM and 50 µM ACC, root lengths measured 40 mm, indicating a 0.93-fold reduction relative to the control group. Beckom shoot growth was consistent at 8 mm across all ACC concentrations, representing a 0.8-fold reduction relative to the control (10 mm). Shoot length for E62 was approximately 10 mm in both ACC pre-treated and control seedlings, indicating no significant impact of priming on shoot growth (1.0-fold change) **(Figure 4.1.2.b).**

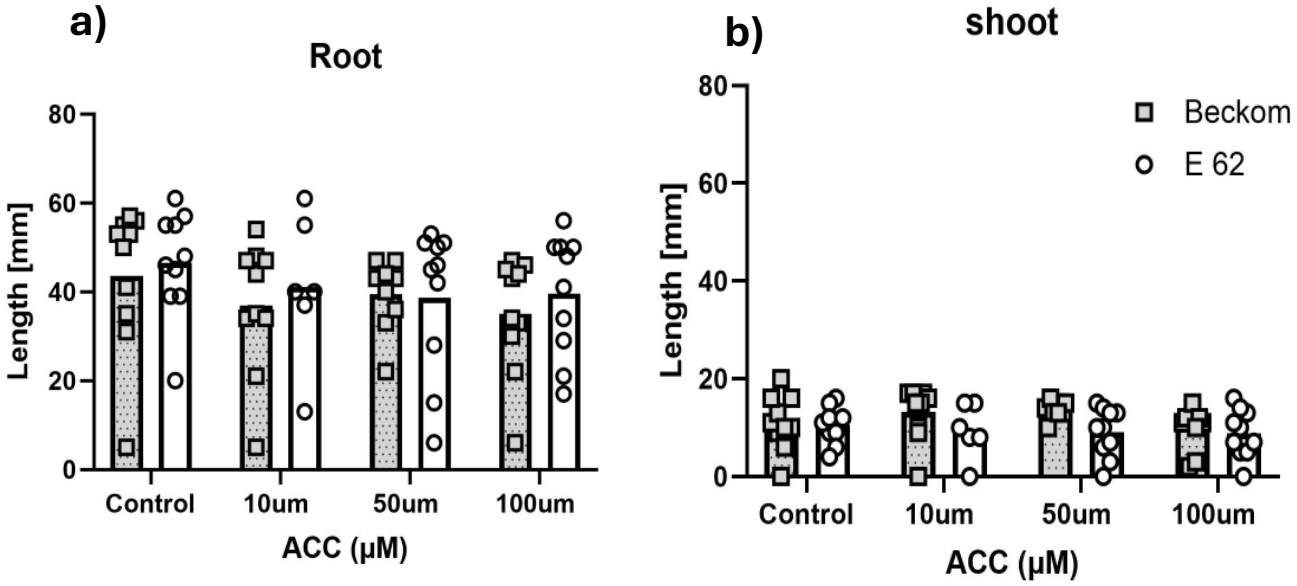


Figure 4.1.2.: Effect of 1-Aminocyclopropane-1-Carboxylic Acid (ACC) pre-treatment on root and shoot length of wheat seedling after 3 days of germination. Plants were treated with different concentrations of ACC (0, 10, 50, and 100 µM), (A) Root length of ACC treated. (B)Shoot length of ACC treated. Length was reported in mm. Data represent means ± SE of ten biological replicates (n = 10). (*) indicate significant differences between ethylene-treated and untreated plants (*$p<0.05$, ANOVA).

E62 demonstrated a concentration-dependent response in root development following ethephon pre-treatment. The control root length measured 50 mm, whereas treatment with 5 µM ethephon produced a root length of 40 mm, indicating a decrease of 0.8-fold. At a concentration of 50 µM ethephon, root length decreased to 30 mm, representing a 0.6-fold reduction. Higher concentrations of 500 µM and 1000 µM resulted in a significant decrease in root length to 10 mm, corresponding to a 0.2-fold reduction compared to the control **(Figure 4.1.3.d).** The control root length in Scepter measured 55 mm. Pre-treatment with 5 µM and 50 µM ethephon yielded root lengths of 50 mm, indicating a 0.91-fold reduction. Higher concentrations (500 µM and 1000 µM) significantly decreased root length to 10 mm, representing a 0.18-fold reduction relative to the control **(Figure 4.1.3.c).** Scepter exhibited comparable shoot lengths (18 mm) to both the control and the 5 µM ethephon pre-treatment (1.0-fold change). Nonetheless, shoot lengths were reduced to 10 mm (0.56-fold decrease)

with 500 µM ethephon and 10 mm (0.56-fold decrease) with 1000 µM ethephon **(Figure 4.1.3.a).** For E62, shoot growth measured 15 mm for the control group and 5 µM ethephon pre-treatment, indicating no significant difference (1.0-fold change). Nonetheless, shoot growth diminished to 8 mm (0.53-fold decrease) with 500 µM ethephon and to 5 mm (0.33-fold decrease) with 1000 µM ethephon **(Figure 4.1.3.b).**

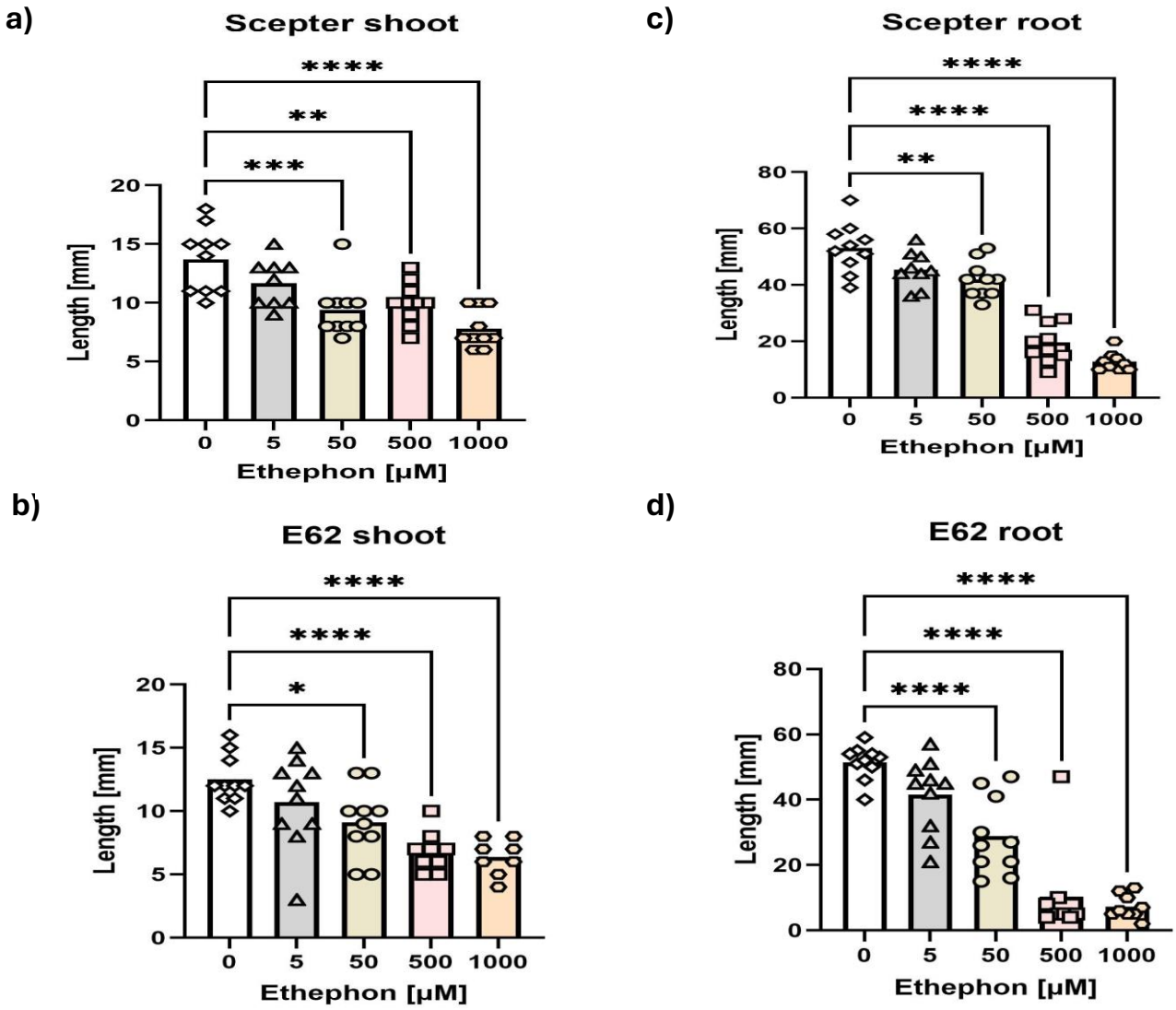


Figure 4.1.3.: Effect of Ethephon pre-treatment on root and shoot length of wheat seedling after 3 days of germination. Plants were treated with different concentrations of ETH (0, 5, 50, 500, and 1000 µM). (A) Scepter shoot. (B) E62 shoot. (C) Scepter root. (D) E62 root. Length was reported in mm. Data represent means ± SE of ten biological replicates (n = 10). (*) indicate significant differences between ethylene-treated and untreated plants (*$p<0.05$, ANOVA).

Ethephon priming markedly enhanced biomass production in wheat lines Scepter and E62 following two weeks of growth in controlled conditions. Plants pre-treated with ethephon exhibited increased biomass relative to untreated controls in both cultivars. Optimal biomass production for the E62 cultivar was achieved at a concentration of 5 µM ethephon. The shoot fresh weight was approximately 1.0 g, indicating a 2.5-fold increase relative to the control (0.4 g) **(Figure 4.1.4.a).** The root fresh weight measured 1.1 g, indicating a 2.2-fold increase relative to the control value of 0.5 g **(Figure 4.1.4.b).** The shoot dry weight measured 0.13 g,

representing a 2.6-fold increase relative to the control (0.05 g), while the root dry weight was 0.08 g, indicating a 2.7-fold increase compared to the control (0.03 g) **(Figure 4.1.4.c & d).** Optimal biomass production for the Scepter cultivar was achieved at a concentration of 10 µM ethephon. The shoot fresh weight attained approximately 0.9 g, indicating a 1.8-fold increase relative to the control (0.5 g) **(Figure 4.1.5.a).** The root fresh weight measured 0.8 g, indicating a 1.6-fold increase relative to the control value of 0.5 g **(Figure 4.1.5.b).** The shoot dry weight measured 0.1 g, indicating a 2.0-fold increase relative to the control (0.05 g), while the root dry weight was 0.07 g, reflecting a 2.3-fold increase compared to the control (0.03 g) **(Figure 4.1.5.c & d).**

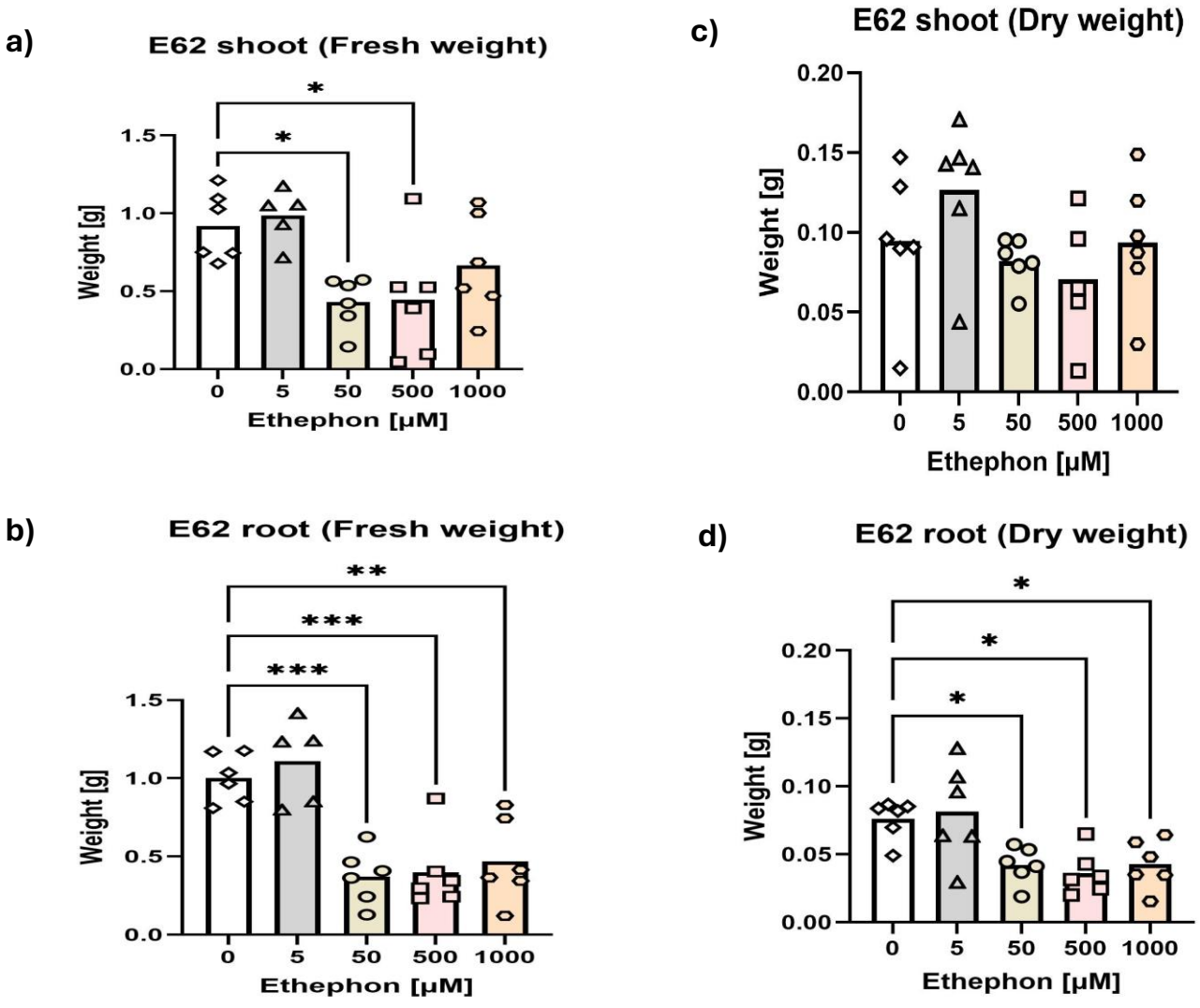


Figure 4.1.4.: Effect of Ethephon pre-treatment on fresh and dry weight of wheat plants. Fresh and dry weights of shoots and roots were measured in ETH-treated and untreated wheat plants of two varieties (E62) after 2 weeks of pot transfer. Plants were treated with different concentrations of ethephon (0, 5, 50, 500, and 1000 µM). (A) Fresh shoot weight. (B) Fresh root weights. (C) Dry shoot weight. (D) Dry root weights. Weights were reported in grams. Data represent means ± SE of six biological replicates (n = 6). (*) indicate significant differences between ethylene-treated and untreated plants (*p<0.05, ANOVA).

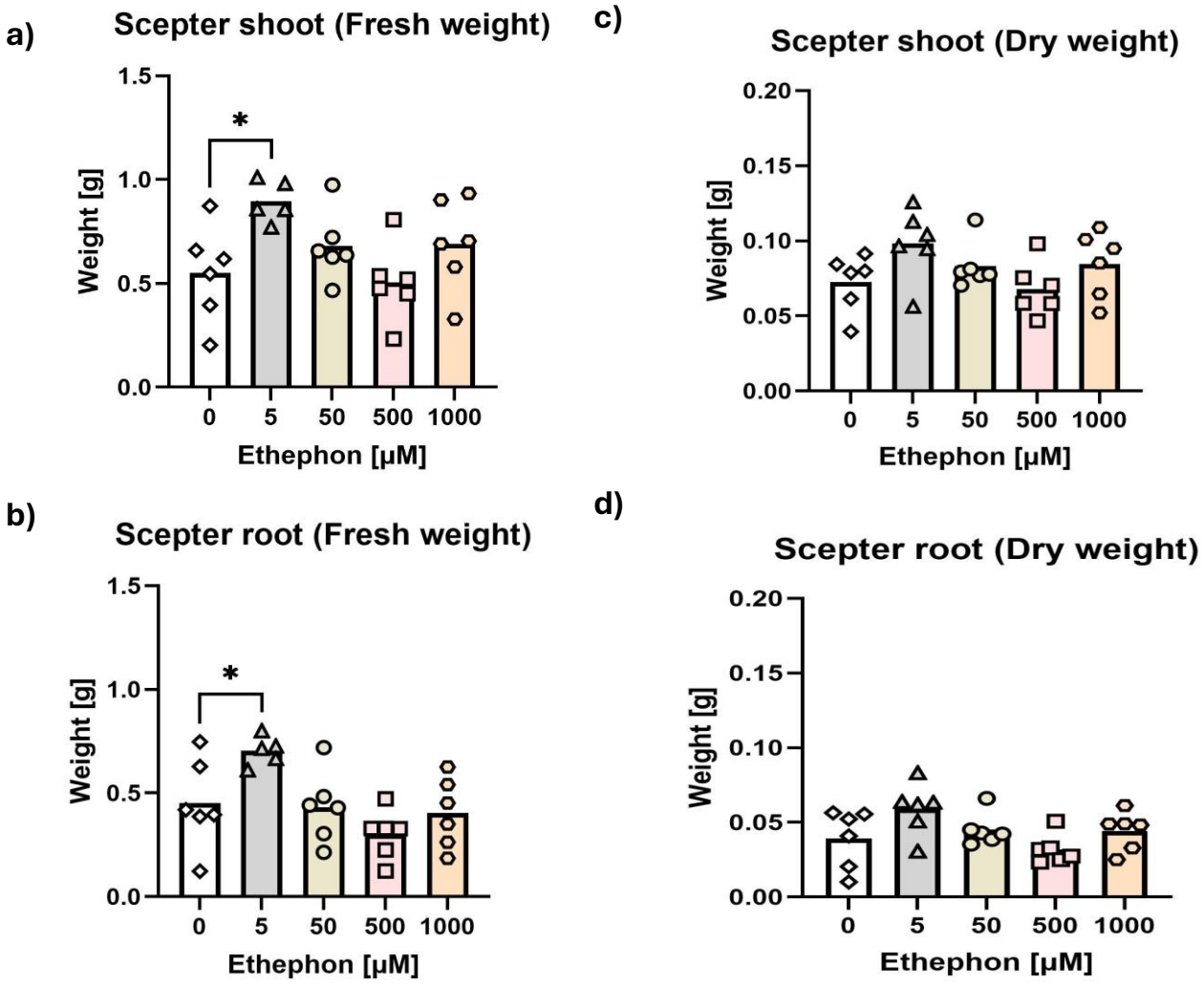


Figure 4.1.5.: Effect of Ethephon pre-treatment on fresh and dry weight of wheat plants. Fresh and dry weights of shoots and roots were measured in ETH-treated and untreated wheat plants of two varieties (Scepter) after 2 weeks of pot transfer. Plants were treated with different concentrations of ethephon (0, 5, 50, 500, and 1000 µM). (A) Fresh shoot weight. (B) Fresh root weights. (C) Dry shoot weight. (D) Dry root weights. Weights were reported in grams. Data represent means ± SE of six biological replicates (n = 6). (*) indicate significant differences between ethylene-treated and untreated plants (*p<0.05, ANOVA).

4.2. MDA level

The T-bar assay was employed to investigate the impact of heat stress on malondialdehyde (MDA) concentrations in two wheat cultivars, E60 and Scepter. Heat stress was administered following 6 weeks of growth for durations of 24 and 72 hours. MDA levels for E60 were consistent at 50 nmol/g under both heat stress (27°C) and optimal temperature (17°C) for untreated samples, yielding no fold change (1.0-fold) **(Figure 4.2.1.c).** Ethylene pre-treatment resulted in minor changes, with MDA levels reducing to 45 nmol/g at 17°C (a 0.9-fold decrease) and stabilising at 50 nmol/g under heat stress (1.0-fold) **(Figure 4.2.1.c).** MDA levels for Scepter were approximately 25 nmol/g under optimal conditions for both treated and untreated plants, indicating no change (1.0-fold). Under heat stress, ethylene pre-treatment decreased MDA levels from 30 nmol/g in untreated plants to 25 nmol/g, representing a 0.83-fold reduction **(Figure 4.2.1.a).** In E60, untreated plants exhibited notable variation in MDA levels under heat stress compared to optimal conditions, rising from 20 nmol/g at 17°C to 48 nmol/g at 27°C, representing a 2.4-fold increase **(Figure 4.2.1.d).** Plants pre-treated with ethylene exhibited stable MDA levels of 30 nmol/g across both conditions, demonstrating no fold change (1.0-fold) **(Figure 4.2.1.d).** In the case of Scepter, ethylene pre-treated plants exhibited consistent MDA levels of 45 nmol/g under both heat stress and optimal temperature, reflecting no change (1.0-fold). MDA levels in untreated plants decreased from 40 nmol/g at 17°C to 30 nmol/g at 27°C, indicating a 0.75-fold reduction **(Figure 4.2.1.c).**

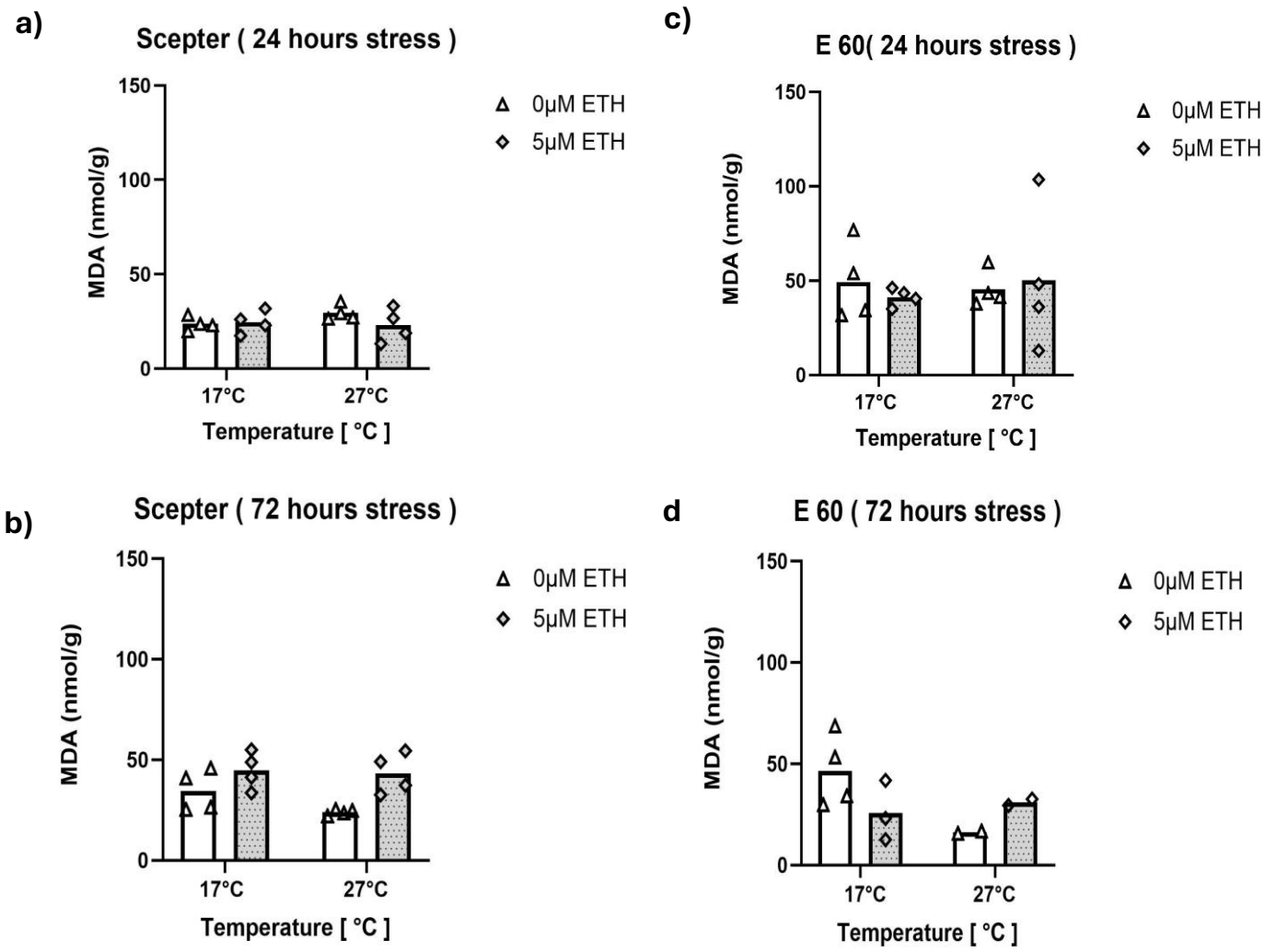
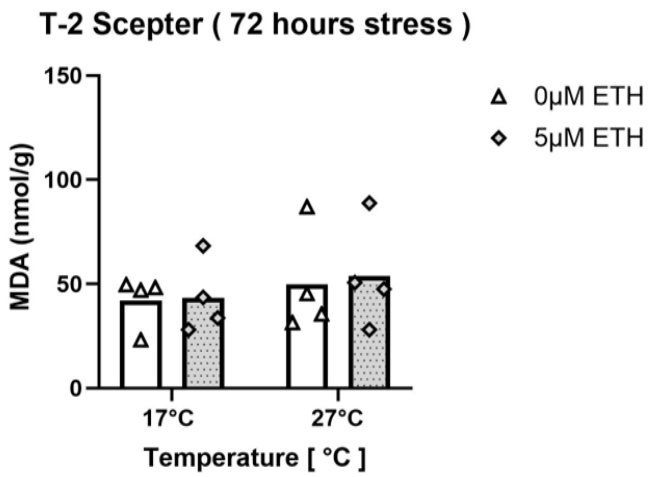


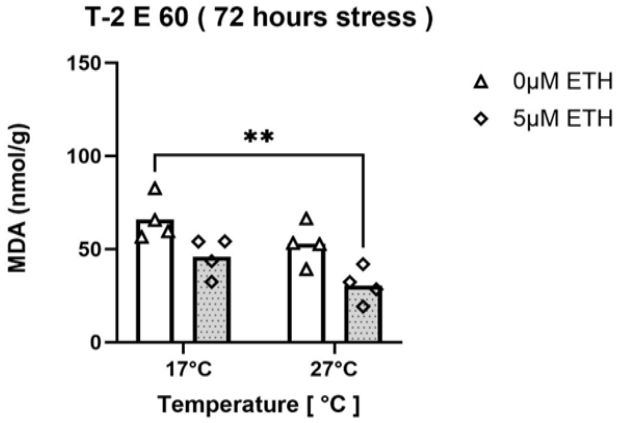
FIGURE 4.2.1: Effect of ethylene pre-treatment on malondialdehyde (MDA) content in wheat plants after heat stress conditions. MDA levels were measured in ethylene-treated and untreated wheat plants of two varieties (Scepter and E60) subjected to heat stress(27°C) for 24 and 72 hours. (A) MDA content in scepter variety after 24 hours of heat stress. (B) MDA content in scepter variety after 72 hours of heat stress. (C) MDA content in E60 variety after 24 hours of heat stress. (D) MDA content in E60 variety after 72 hours of heat stress. MDA values are expressed as nmol/g. Data represent means ± SE of four biological replicates (n = 4). (*) indicate significant differences between ethylene-treated and untreated plants (*$p<0.05$, ANOVA).

The second heat stress was given after 12 weeks of plant growth for 72 and 144 hours. Following 72 hours of heat stress, the E60 cultivar showed a significant protective response to ethylene pre-treatment plants. Untreated plants had MDA levels of 60 nmol/g at control temperatures (17°C). However, ethylene pre-treated plants had lower levels of 40nmol/g indicating a 33% reduction (0.67-fold). **(Figure – 4.2.2. c)** Under heat stress (27°C) untreated E60 plants showed MDA levels of 45nmol/g. At the same time, ethylene pre-treatment lowered MDA levels by 30nmol/g. There is a 33% decrease (0.67-fold). **(Figure – 4.2.2. c)** In contrast, the Scepter cultivar displayed MDA levels of 45nmol/g in both pre-treated and untreated plants at 17°C. **(Figure – 4.2.2. a)** Under heat stress, both groups showed higher levels of 55nmol/g. **(Figure – 4.2.2 a)**

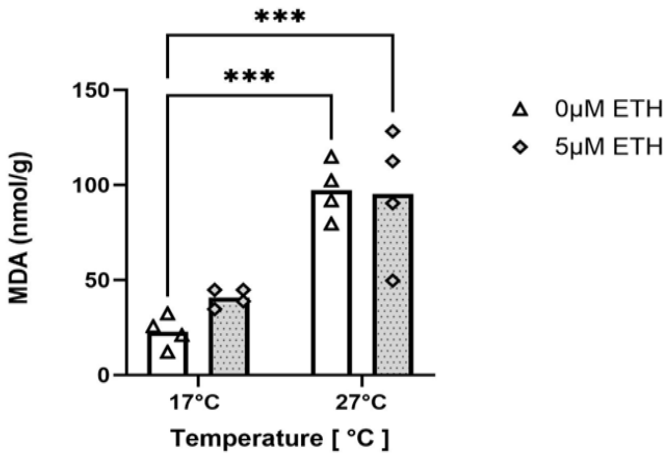
a)



c)



b)



d)

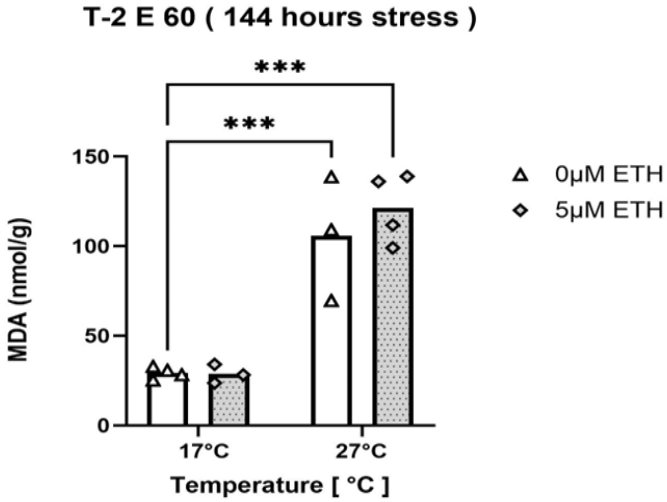


FIGURE 4.2.2: Effect of pre-ethylene treatment on malondialdehyde (MDA) content in wheat plants after heat stress. MDA levels were measured in ethylene-treated and untreated wheat plants of two varieties (Scepter and E60) subjected to heat stress(27°C) for 72 and 144 hours. (A) MDA content in Scepter variety after 72 hours of heat stress. (B) MDA content in Scepter variety after 144 hours of heat stress. (C) MDA content in E60 variety after 72 hours of heat stress. (D) MDA content in E60 variety after 144 hours of heat stress. MDA values are expressed as nmol/g. Data represent means ± SE of four biological replicates (n = 4). (*) indicate significant differences between ethylene-treated and untreated plants (*$p<0.05$, ANOVA).

Heat stress extended to 144 hours had a greater impact on oxidative damage. At 17°C untreated plants in Scepter had MDA levels of 25nmol/g. However, ethylene pre-treated plants had levels of 45 nmol/g. **(Figure – 4.2.2. b)** Under heat stress (27°C) both pre-treated and untreated Scepter plants increased MDA levels to 100nmol/g. **(Figure – 4.2.2. b)** At 17°C, ethylene pre-treated and untreated E60 plants-maintained MDA levels of 30nmol/g. However, during heat stress (27°C) MDA levels increased in untreated plants to 100nmol/g, whereas ethylene pre-treated plants showed unexpected to 120 nmol/g. **(Figure – 4.2.2 d)**

4.3. GABA concentration

The concentrations of GABA in wheat cultivars Scepter and E60 were analysed under control (17°C) and heat stress (27°C) conditions, demonstrating the substantial impact of ethylene pre-treatment. Compared to untreated plants, which had a GABA concentration of 2.5 µmol/g FW under control conditions, ethylene-pretreated Scepter plants had a GABA concentration of 1 µmol/g FW after 24 hours of thermal stress, a 60% decrease (0.4-fold). Nevertheless, the GABA concentrations of both ethylene-pretreated and untreated Scepter plants were equivalent at 2.5 µmol/g FW (1.0-fold) during heat stress. (Figure - 4.3.1 a) In the E60 cultivar, the concentration of GABA was reduced by 20% (0.8-fold) under control conditions by ethylene pre-treatment, with a value of 2 µmol/g FW compared to 2.5 µmol/g FW in untreated plants. In contrast, E60 plants that were pretreated with ethylene demonstrated a 100% increase in GABA concentrations (2.0-fold) during heat stress, with a value of 3 µmol/g FW, as opposed to 1.5 µmol/g FW in untreated plants. **(Figure - 4.3.1 c)** The GABA concentrations in the Scepter cultivar decreased significantly in both groups after 72 hours of thermal stress. The ethylene pre-treated plants demonstrated a 70% decrease (0.3-fold) in FW

from untreated plants at 1 µmol/g FW under the same conditions, resulting in an FW of 0.3 µmol/g. **(Figure 4.3.1 a & b)** Under protracted thermal duress, the E60 cultivar exhibited a distinct pattern. In comparison to untreated plants, which had 4 µmol/g FW, ethylene-pretreated plants had 2 µmol/g FW GABA at 17°C, a 50% reduction (0.5-fold change). Untreated E60 plants exhibited a GABA concentration of 1 µmol/g FW under heat stress conditions (27°C), whereas ethylene pre-treated plants exhibited a GABA concentration of 2 µmol/g FW, indicating a 100% increase (2.0-fold). **(Figure - 4.3.1 d)**

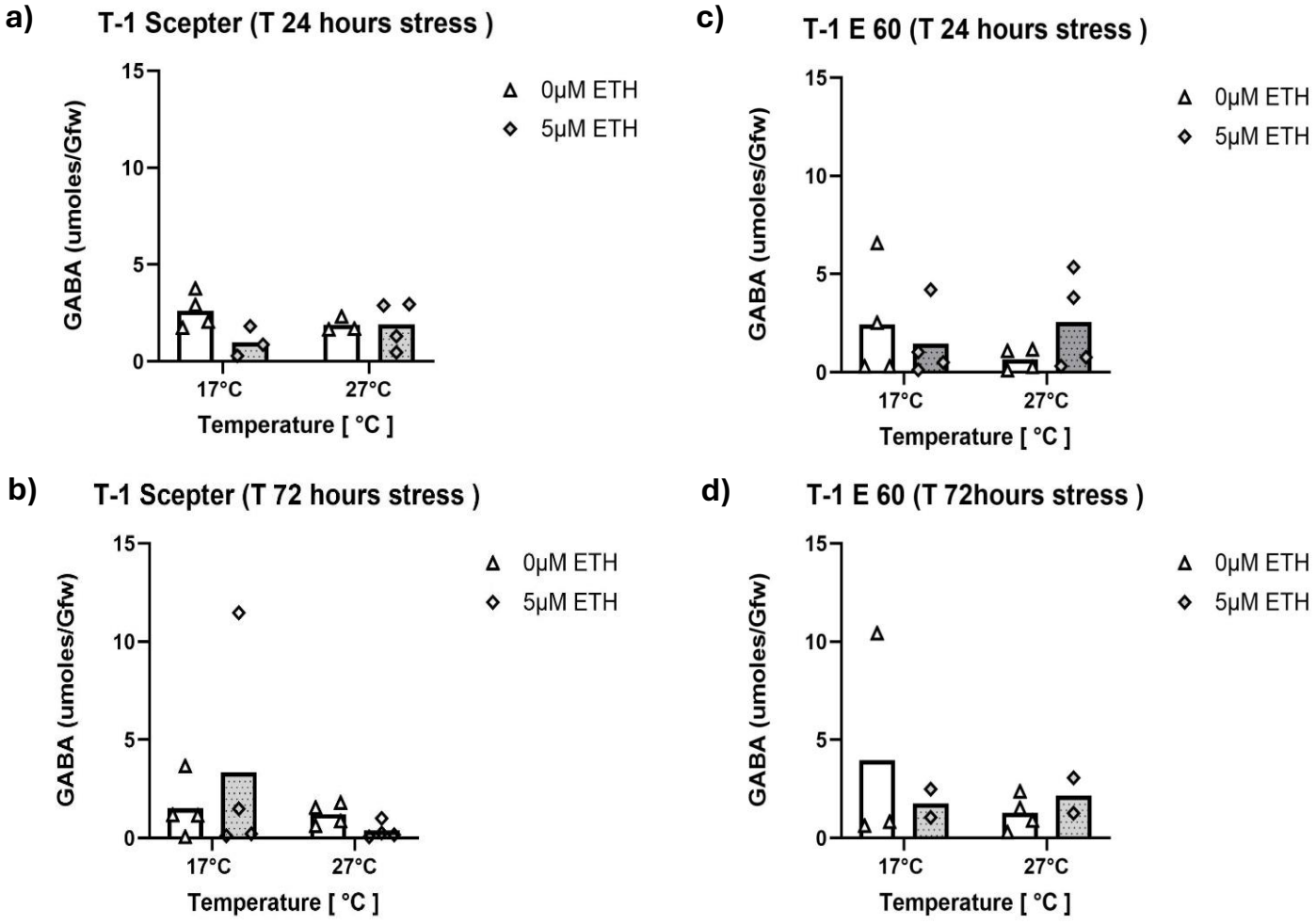


FIGURE 4.3.1: Effect of pre-ethylene pre-treatment on Gamma-Aminobutyric Acid (GABA) content in wheat plants after heat stress. GABA concentrations were measured in ethylene-treated and untreated wheat plants of two varieties (Scepter and E60) subjected to heat stress(27°C) for 24 and 72 hours. (A) GABA content in Scepter variety after 24 hours of heat stress. (B) GABA content in scepter variety after 72 hours of heat stress. (C) GABA content in E60 variety after 24 hours of heat stress. (D) GABA content in E60 variety after 72 hours of heat stress. GABA values are expressed as nmol/g. Data represent means ± SE of four biological replicates (n = 4). (*) indicate significant differences between ethylene-treated and untreated plants (*p<0.05, ANOVA).

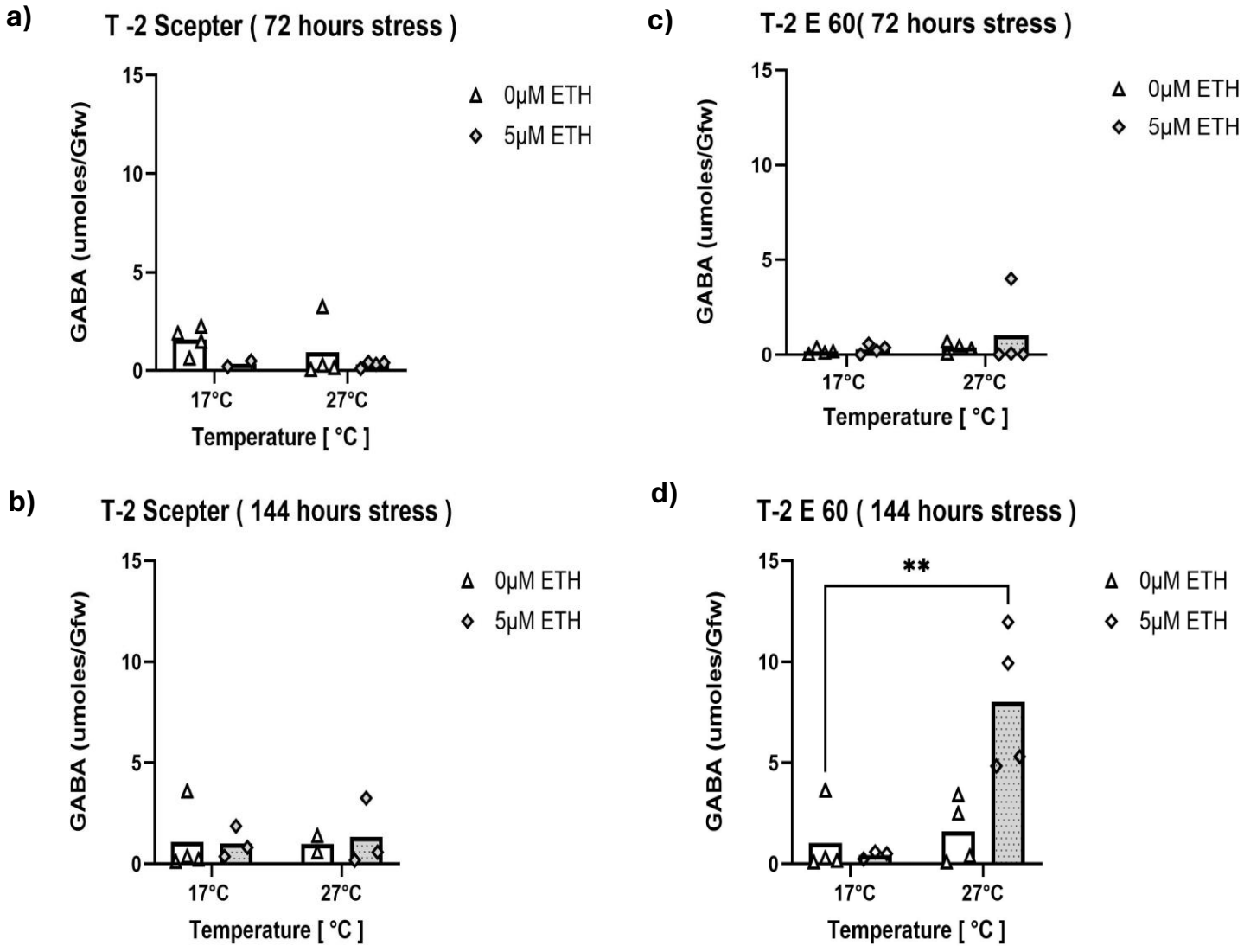


FIGURE 4.3.2: Effect of pre-ethylene treatment on Gamma-Aminobutyric Acid (GABA) content in wheat plants after heat stress conditions. GABA concentrations were measured in ethylene-treated and untreated wheat plants of two varieties (Scepter and E60) subjected to heat stress(27°C) for 72 and 144 hours. (A) GABA content in Scepter variety after 72 hours of heat stress. (B) GABA content in scepter variety after 144 hours of heat stress. (C) GABA content in E60 variety after 72 hours of heat stress. (D) GABA content in E60 variety after 144 hours of heat stress. GABA values are expressed as nmol/g. Data represent means ± SE of four biological replicates (n = 4). (*) indicate significant differences between ethylene-treated and untreated plants (*$p<0.05$, ANOVA).

After 72 hours, Scepter cultivar plants that were pre-treated with ethylene showed a 7.5-fold decrease in GABA concentrations at 17°C (0.2 µmol/GFW) relative to untreated plants (1.5 µmol/GFW). Under heat stress (27°C), ethylene pre-treated Scepter plants sustained low GABA concentrations (0.2 µmol/GFW), indicating a 5-fold reduction relative to untreated plants, which exhibited a rise to 1 µmol/GFW. **(Figure 4.3.2 a)** At 72 hours, both ethylene pre-treated and untreated E60 cultivar plants exhibited identical GABA concentrations of 0.1 µmol/GFW at 17°C. Under heat stress (27°C), ethylene pre-treated plants exhibited marginally reduced GABA levels (0.11 µmol/GFW), reflecting a 0.55-fold drop relative to untreated plants, which measured 0.2 µmol/GFW**.(Figure 4.3.2 c)**
After 144 hours of heat stress, both ethylene pre-treated and untreated Scepter plants had similar GABA concentrations of 1 µmol/GFW at 17°C and 27°C. **(Figure 4.3.2 b)**
Conversely, significant alterations were seen in the E60 cultivar after 144 hours. At 17°C, ethylene pre-treated plants exhibited a 3.3-fold decrease in GABA concentration (0.3 µmol/GFW) relative to untreated plants (1 µmol/GFW). Under heat stress (27°C), ethylene pre-treated E60 plants demonstrated a 3.75-fold elevation in GABA concentrations (7.5 µmol/GFW) relative to untreated plants, which displayed just 2 µmol/GFW. **(Figure 4.3.2 d)**

4.4. Starch level:

Following 72 hours of heat stress, Scepter plants exhibited a threefold decrease in starch content at 17°C when subjected to ethephon priming (5%) in contrast to untreated plants (15%). Under heat stress (27°C), untreated plants exhibited a threefold reduction in starch levels, decreasing from 15% to 5%. In contrast, ethephon pre-treated plants demonstrated a 1.5-fold increase in starch content, reaching 7.5% compared to untreated plants. **(Figure 4.4 a)** At 72 hours in E60, ethephon pre-treatment resulted in a 1.75-fold increase in starch levels

at 17°C, rising from 10% in the untreated sample to 17.5%. Following exposure to heat stress (27°C), untreated plants showed a 50% decrease in starch levels, dropping from 10% to 5%. In contrast, ethylene pre-treated plants retained higher starch levels at 6%, representing a 1.2-fold increase relative to untreated plants**. (Figure 4.4c)** Following 144 hours of heat stress, Scepter starch levels at 17°C were 1.36 times greater in ethephon pre-treated plants (15%) than in untreated plants (11%). Under heat stress (27°C), starch levels in ethephon-treated plants were 1.1 times greater (11%) compared to untreated plants, which exhibited 10% starch levels. **(Figure 4.4b)** In E60 at 144 hours, starch levels decreased below 17°C with ethephon pre-treatment, demonstrating a 1.6-fold reduction from 8% (untreated) to 5%. Under heat stress (27°C), untreated plants had 5% starch, while ethephon pre-treated plants showed a 100% increase to 10% starch. **(Figure 4.4 d)**

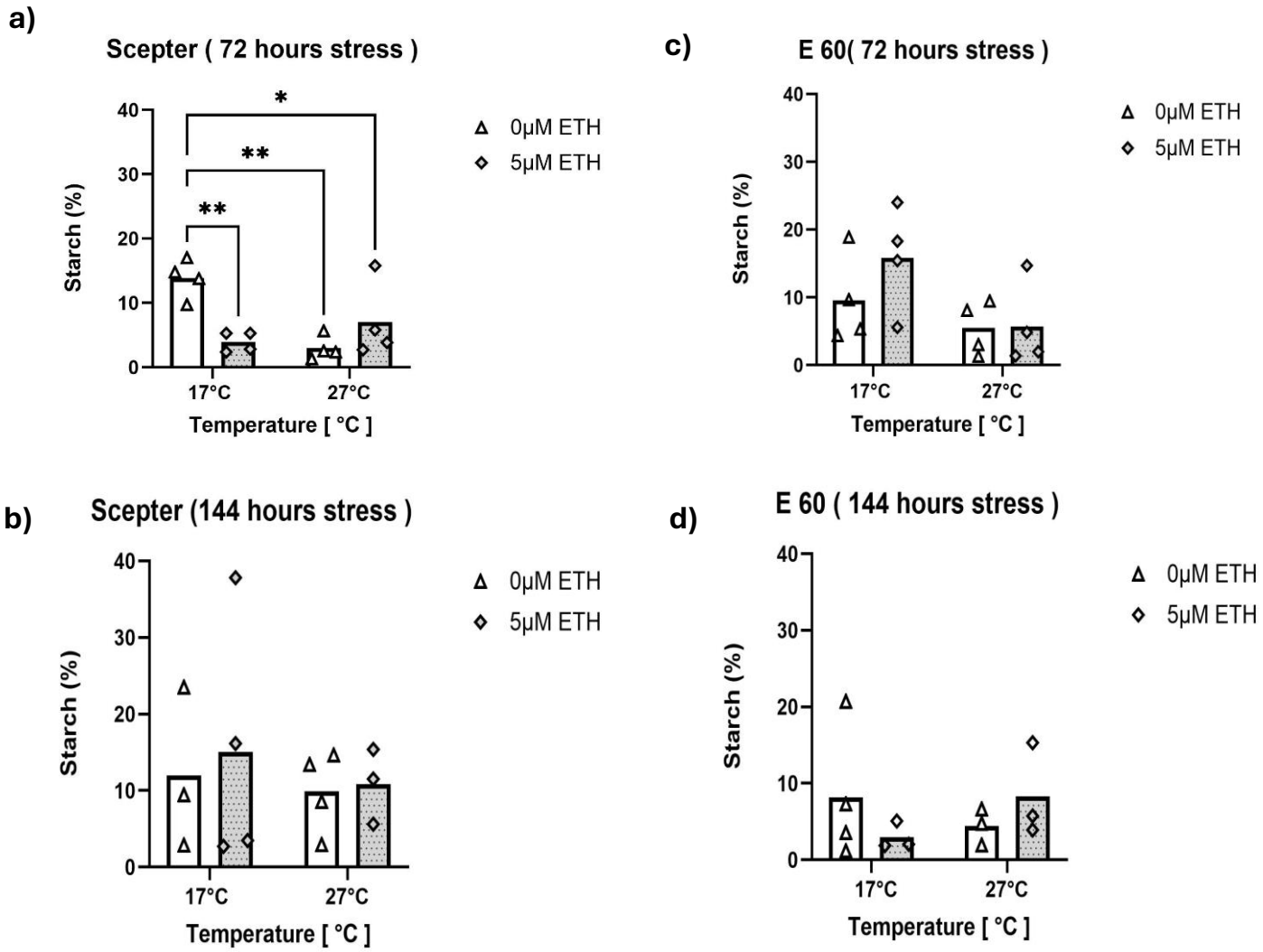


FIGURE 4.4: Effect of ethylene pre-treatment on Starch content in wheat plants after heat stress conditions. Starch levels were measured in ethylene-treated and untreated wheat plants of two varieties (Scepter and E60) subjected to heat stress(27°C) for 72 and 144 hours. (A) Starch content in Scepter variety after 72 hours of heat stress. (B) starch content in scepter variety after 144 hours of heat stress. (C) Starch content in E60 variety after 72 hours of heat stress. (D) Starch content in E60 variety after 144 hours of heat stress. Data represent means ± SE of four biological replicates (n = 4). (*) indicate significant differences between ethylene-treated and untreated plants (*$p<0.05$, ANOVA).

5. Discussion

5.1. Pilot study:

Heat stress is a major environmental issue that harms wheat (Triticum aestivum L.) growth, development, and production around the world. The study investigated the effects of ACC seed priming on the growth of wheat seedlings. The hypothesis is that ACC treatment would promote faster growth in early seedling development. High temperatures can disturb important physiological processes, resulting in lower yield and quality. To prevent these deleterious effects seed priming with ethylene is a viable approach. 1-aminocyclopropane-1-carboxylic acid (ACC) is the immediate precursor of ethylene. This pilot study investigates the effects of ACC seed priming on early wheat seedling growth and determines the best concentration for seed priming.

We investigated the effects of ACC seed prim`ing on the growth of two wheat cultivars Beckom and E62. Our findings demonstrated that ACC seed priming had no significant effect on seedling growth. As shown in **(Figure – 4.1.2. a)** root length of ACC pre-treated seedlings was comparable to untreated seedlings. Biomass levels were similar in both ACC pre-treated and untreated groups **(Figure – 4.1.1.)**. This study found no significant effect of ACC pre-treatment on early growth stages in wheat cultivars with tested concentrations.

The second pilot study examined how ethephon pre-treatment affected germination and early seedling growth in two wheat cultivars (Scepter and E62). Initial studies with ACC pre-treatment revealed no significant effects on seedling growth. **(Figure – 4.1.1.)** As a result, we used an ethephon water-soluble chemical that emits ethylene gas to prime the seeds. Various concentrations of ethephon were investigated to discover the optimum concentration. Low concentrations of ethephon (5µM) significantly increase early biomass accumulation in Scepter and E62 cultivars **(Figure – 4.1.3.)** indicating low concentration stimulate growth and higher concentrations inhibit it. This result is consistent with earlier research which has shown that ethylene-related seed priming treatments enhance germination percentage and seedling growth.(Li et al., 2023)

Our research found that ethephon concentration is critical in determining the impacts on root development and overall plant growth. A lower concentration of ethephon (5µM) resulted in longer root lengths than a higher concentration. Shiono et al. (2019) found that ethephon pretreatment at lower concentrations (0.1 and 1µM) resulted in longer roots than higher

values (37.5µM). (Shiono et al., 2019) Our observations of increased root length and biomass at low (5µM) ethephon concentrations indicate that this is the optimum concentration for seed priming. High concentration (500µM and 1000µM) decreases root growth, suggesting that lower concentration is more effective for seed priming. **(Figure 4.1.3.)**

5.2. MDA content

Ethylene pre-treatment is shown to enhance wheat plant resilience to oxidative stress under heat stress conditions. One key marker of oxidative damage is malondialdehyde (MDA). Which is a byproduct of lipid peroxidation that tends to accumulate under stress. (Sehar et al., 2023). Ethylene pre-treatment helps reduce initial MDA levels by stimulating antioxidant enzyme activities that combat reactive oxygen species (ROS). Enzymes like superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) become more active in response to ethylene. Those enzymes efficiently reduce lipid peroxidation and promote cellular stability.

Ethylene influences stress-response genes ERFs (ethylene response factors), and ethylene receptors (ERS1 and ETR1). Biosynthetic enzymes such as ACS (1-aminocyclopropane-1-carboxylate synthase) and ACO (1-aminocyclopropane-1-carboxylate oxidase). The upregulation of ERFs triggers stress-responsive genes that bolster plant capacity to withstand oxidative stress. Additionally, the upregulation of ACS and ACO amplifies ethylene synthesis and ensures sustained response to oxidative stress.

Antioxidant enzymes including SOD, CAT, and GST are critical as they directly scavenge ROS and prevent membrane damage. SOD converts harmful superoxide radicals to hydrogen peroxide. That hydrogen peroxide further detoxifies by CAT and GST. Ultimately lowering MDA levels and protecting cellular structures. These antioxidant defences collectively preserve membrane integrity, reducing cellular leakage and maintaining stability under stress. (Hameed et al., 2012; Hong et al., 2023). MDA levels in both E60 and Scepter were steady during the first 24 hours of heat stress. **(Figure – 4.2.1. a & c)** This shows that wheat plant has strong antioxidant defence mechanisms that can mitigate early oxidative damage caused by heat stress.

The result indicates that the E60 cultivar with ethylene pre-treated plant effectively reduced MDA level after 72 hours of heat stress. This indicates that ethylene pre-treatment can enhance a plant's ability to manage oxidative damage. **(Figure – 4.2.2. c)** However, the

scepter cultivar gave different responses to ethylene pre-treatment with slightly higher MDA levels compared to untreated plants. This suggests that ethylene pre-treatment did not help the scepter to mitigate heat stress. **(Figure – 4.2.2. a)** This outcome suggests that ethylene pre-treatment gave different responses to different cultivars. These findings match with previous research indicating that different wheat cultivars with ethylene treatment respond differently to heat stress(Hays et al., 2007). heat-sensitive cultivar 'Karl 92' was found to increase ethylene production under stress whereas heat-tolerant 'Halberd' did not. This cultivar-specific response is caused by genetic differences in stress response control such as ethylene signalling and antioxidant defences.

A considerable increase in MDA level was reported in both wheat cultivars (E60 and Scepter) after 144 hours of heat stress at 27°C. **(Figure – 4.2.2. b & d)** the plant antioxidant systems may become overwhelmed as ROS levels rise. Under extended stress ethylene's ability to mitigate oxidative damage diminishes. Sustained ROS production exhausts antioxidant capacity leading to increased lipid peroxidation and MDA accumulation. Prolonged exposure to ethylene can drive senescence-related processes accelerated respiration which increases ROS generation. The overproduction of ROS under prolonged stress conditions could also explain the higher MDA levels in ethylene-treated E60 plants. These results match previous findings on waterlogging stress. Where prolonged exposure to stress can lead to excessive H2O2 production. Overwhelming plant antioxidant defences and causing lipid peroxidation.(Rauf et al., 2021)

5.3. GABA concentration:

High temperatures have a substantial impact on wheat growth and production, lowering crop productivity and quality. Gamma-aminobutyric acid (GABA), a non-protein amino acid, is vital for protecting plants from heat stress. GABA levels rise in response to abiotic stimuli like as heat stress, which are produced by the enzyme glutamic acid decarboxylase (GAD). In this investigation, ethylene pre-treatment increased GABA production in the E60 cultivar, resulting in increased GABA accumulation under heat stress conditions **(Figure 4.3.1.d).** Ethylene seems to alter this route via increasing GAD activity or expression, which is consistent with Yuan et al.'s (2023) findings on the connection between ethylene signalling and GABA metabolism.(Yuan et al., 2023) GABA is a protective substance that reduces oxidative stress by scavenging reactive oxygen species (ROS) that build during heat stress. This ROS-scavenging action lowers lipid peroxidation, strengthens cellular structures, and

aids in maintaining physiological activities such as photosynthesis (Priya et al., 2019). The elevated GABA levels in E60 after ethylene pre-treatment indicate a stronger antioxidative defence, which is most likely mediated by ethylene-induced activation of stress-responsive pathways. These protective actions increase metabolic stability and maintain photosynthetic efficiency under stress.

The results of this study are consistent with earlier research that has shown GABA's function in enhancing stress tolerance via increased enzymatic activity and decreased ROS-induced damage (Abdel Razik et al., 2021). GABA also helps with cellular pH stability, osmotic balance, and signalling, strengthening its role in heat stress tolerance. However, reactions differed across cultivars, with Sceptre exhibiting less severe GABA buildup under identical circumstances. This emphasises the genetic variation in stress signalling pathways and metabolite production, as previously shown (Hays et al., 2007).

5.4. Starch content:

Heat stress inhibits wheat growth and starch formation which is critical for energy storage. Ethephon pre-treatment stimulates ethylene synthesis. It has shown the potential to mitigate the effects of heat stress on wheat plant starch levels. Untreated plant starch level rapidly drops starch level after heat stress. High temperatures hinder photosynthesis and alter starch metabolism by decreasing enzyme activity required for starch synthesis. The results show **(Figure – 4.4)** that wheat cultivars pre-treated with ethephon mitigate the negative effects of heat stress on starch levels. By preserving or even increasing starch reserves in comparison to untreated counterparts.

Ethylene helps to stabilize or even activate key enzymes involved in starch biosynthesis, such as ADP-glucose pyrophosphorylase (AGPase).(Cuesta-Seijo et al., 2019) Which ensures starch synthesis continues despite high temperatures. Ethylene can help plants allocate carbon more efficiently by redirecting resources from growth toward the survival pathway. Ethylene helps to preserve carbohydrates under stress conditions. This ability to shift metabolic prioritise means that more carbon is available for starch synthesis. Even when the photosynthesis process is compromised.

Furthermore, ethylene may inhibit the activity of starch-degrading enzymes like amylases, helping retain existing starch stores. By limiting degradation ethylene pre-treated plants conserve starch reserves. Which serves as an energy source during prolonged stress periods.

In wheat cultivar E60 ethephon pre-treated plants showed higher starch levels after 144 hours of heat stress. **(Figure – 4.4 d)** suggesting that ethylene primes plant metabolic pathways to sustain carbohydrate storage under stressful conditions. The ability of ethephon pre-treated plants to maintain starch levels during heat stress highlights ethylene's role in improving heat resilience in wheat.

This research on the effects of ethylene pre-treatment in wheat under heat stress provides valuable insight but it is constrained by several limitations. While malondialdehyde (MDA) is typically a reliable marker for oxidative damage. After 24 hours of heat stress no significant changes were observed in malondialdehyde (MDA) levels in ethylene pre-treated and un-treated plants. **(Figure – 4.2.1. a & c)** This may be due to initial oxidative stress responses not pronounced or immediate as anticipated. However, 144 hours of heat stress and the significantly high MDA level show that ethylene pre-treatment did not help to mitigate oxidative damage. **(Figure – 4.2.2. B & D)**

The results reveal differential responses of two wheat cultivars regarding MDA, gamma-aminobutyric acid (GABA), and starch levels. Such variability highlighted the influence of genetic and physiological differences on stress responses. Another critical limitation stems from the lack of uniform growth among the plants during the experiment. Variations in plant size, leaf area, and overall vigour could lead to inconsistencies in metabolic responses and stress tolerance. Potentially did not give true effects of ethylene pre-treatment.

6. Conclusion

The results of this study help to understand the role of ethylene pretreatment of seeds with its precursors (ACC / Ethephon) under heat stress. Pre-treatment ethylene to wheat cultivars Scepter and E62 to improve development and stress responses is a complex and promising process. The ethylene-releasing ethephon treatment effectively improved biomass accumulation with low concentrations. This shows that ethylene signalling has a favourable effect on plant development in non-stressed conditions with low concentrations. However, the inhibition of development with higher ethephon concentrations emphasises the importance of optimising treatment to harness ethylene's positive benefit while avoiding its negative consequences.

Under heat stress differences in cultivar responses highlight ethylene's subtle involvement in controlling oxidative stress and metabolic pathways. Short-term heat stress boosted antioxidant defences with ethylene-treated plants showing lower MDA levels indicating less oxidative damage. However, extended exposure resulted in increasing MDA levels showing limitations of ethylene protective potential under long-term stress. The opposing effects on MDA buildup in Scepter and E60 highlight cultivar-specific sensitivity to ethylene signalling during long-term stress. Furthermore, differential regulation of GABA and starch metabolism during ethylene treatment demonstrates the complexity of wheat stress responses. The capacity of ethylene to stabilise starch levels under heat stress notably in E60. It shows the potential to retain energy stores and increase stress tolerance through enhanced metabolic performance. These results emphasise the need to use genotype-specific ethylene treatment to improve wheat heat stress resistance. Future research should concentrate on understanding molecular mechanisms behind this interaction to optimise ethylene-based treatment and improve wheat resilience under stress conditions.

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