# Effect of GABA Treatment on Heat Stress Mitigation in Lentils

By

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# ABSTRACT

Environmental factors impact the growth, development, and productivity of crop yields. Unfavourable environmental conditions can result in a decline in crop yield due to irreversible damage, and even mortality. Responses of plants to different environmental stresses are highly variable and complex. Plant growth and development are adversely affected by abiotic stresses, including extreme temperatures, water deficiency or excess, high salinity, heavy metals, and ultraviolet radiation, resulting in significant crop yield losses globally. Although it is difficult to estimate the effects of abiotic stresses on agricultural lands, FAO reports estimate that about 96.5% of global rural land is affected by abiotic stresses. A rise in temperature has negative consequences on plant growth. GABA is a natural signalling molecule in plants that helps regulate their growth and development. It not only helps plants grow better but also reduces the negative effects of stress by boosting the antioxidant defence system. A high GABA concentration is known to enhance plant stress tolerance by improving photosynthesis, inhibiting the ROS level, activating antioxidant enzymes, and regulating stomatal opening during heat stress. Lentils, which are grown in the winter, are especially sensitive to heat stress. Lentil plants subjected to heat stress experience a range of negative effects. Several beneficial effects were observed by applying GABA to lentils under heat stress. GABA improved the stress tolerance in lentils promoting growth and development and thereby reducing oxidative damage. The study showed that foliar application of GABA in lentils promoted more positive and better results than the seeds pretreated with GABA. Therefore, the supplementation of GABA through the foliar spray method enhanced lentils' physiological and biochemical performance, thus leading to improved growth and yield. Moreover, applying GABA to lentils under heat stress can significantly enhance their resilience and growth. GABA treatment helps mitigate the adverse effects of high temperatures by regulating the GABA level, and starch contents promoting the photosynthetic efficiency in plants. Research shows that GABA can improve seed germination, root development, and overall plant health during heat stress, leading to better yield and quality. Therefore, GABA applications in lentil cultivation can be a promising strategy to ensure productivity and sustainability in the face of rising temperatures.

# DECLARATION

I certify that this thesis does not incorporate without acknowledgement any 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 reference is made in the text.

Signed: Megha Eldho

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| GABA | Gamma-Aminobutyric Acid |
| ROS | Reactive Oxygen Species |
| GAD | Glutamate Decarboxylase |
| GHB | Gamma-Hydroxybutyrate |
| SSA | Succinic Semialdehyde |
| SSADH | Succinic Semialdehyde Dehydrogenase |
| TCA | Tricarboxylic Acid |
| AMADH | Aminoaldehyde Dehydrogenase |
| MDA | Malondialdehyde |
| TBARS | Thiobarbituric acid reactive substances |

# CHAPTER 1: LITERATURE REVIEW

## 1.1. Introduction

Environmental stresses play crucial roles in the productivity, survival, and reproductive biology of plants as well as crops. It is believed that severe environmental stresses may be faced by approximately 90% of the world's arable land due to factors such as soil degradation, water scarcity, salinity, climate change, and extreme temperatures(Fathi and Tari, 2016). Plants are subjected to many forms of environmental stresses, which can be grouped into biotic and abiotic stresses. Abiotic stress refers to plants encountering difficulties from the non-organisms in their environment which include water availability, extreme temperatures and high salt concentrations. Biotic stress refers to living organisms such as pest, diseases and competing plants that pose challenges to other plants (Anzano et al., 2022). Abiotic stress is a primary factor contributing to crop yield loss worldwide, reducing average yields for many major crop species by more than 50%(Alcázar et al., 2006). Among the various abiotic stresses, high temperatures and low water availability are two of the most significant factors that adversely impact crop growth and yield. Many metabolic reactions and bioprocesses, which control plant growth and development, are adversely impacted by temperature. Continuing climate change without crop improvement will cause extensive economic loss in agricultural and horticultural crops (Beck et al., 2007).

Lentil is a cool-season crop legume that is cultivated to diversify the cereal-based cropping system to enhance land productivity. This makes lentils one of the healthier pulse crops since they contain proteins (22%–25%), fibres (0. 7%), minerals (2. 4%), and vitamins (Kumar et al., 2016). This cool-season food legume is cultivated in several countries including India, Middle Eastern countries, northern and eastern Africa, southern Europe, North and South Americas, Australia and western Asia. Lentil is a significant legume crop utilized for human consumption and livestock feed, with the proximate composition of lentil seeds being influenced by both genetic factors and environmental conditions (Ninou et al., 2019). Lentils are planted in the cool season and are very sensitive to temperature increases (Bhandari et al., 2020). Furthermore, it has been recognized that the duration of chilling periods is decreasing

while the duration of heat periods is increasing, resulting in cool-season crops being subjected to heat stress, particularly during their reproductive phase (Bhardwaj et al., 2021). In 2009, southeastern Australia experienced a heat wave (35°C for six continuous days), and this caused the yield of lentil crops to decrease by 70% (Sita et al., 2017). Similarly, in India, farmers do not prefer to delay the sowing of lentils in rice fallow areas because high-temperature stress during flowering and podding results in early maturity. However, short periods of winter and fluctuation in temperatures do not favour lentil cultivation and production. Lentils are vulnerable to heat stress especially during the mid to late reproductive stage causing a maximum reduction in biomass and seed yield (Kumar et al., 2021).

Plant hormones, organic acids, signalling molecules, and other trace factors are examples of exogenous protectants that are used in experiments to enhance tolerance to abiotic stresses, such as metal toxicity (Sharma et al., 2022). An effective approach to stabilize heat-sensitive plant species involves utilizing biomolecules that support plant defence under stress conditions. Nonprotein amino acids are said to be involved in plant functioning more so in stressful conditions e.g., in osmoregulation, metal binding, antioxidant defence and signalling (Sharma and Dietz, 2006).

GABA is a non-protein amino acid found in living organisms (Nayyar et al. , 2014) ) that acts as an osmoprotectant, and a stress signalling molecule (Li et al., 2021). It is found across diverse life forms, including microbes like bacteria and fungi, as well as in plants and animals. GABA has been largely treated as a metabolite for decades. Recent evidence from Arabidopsis functional genomic tools suggests a new role for GABA as a signal molecule, offering insights into its metabolic pathways related to stress and the metabolism of carbon and nitrogen (Bouché and Fromm, 2004). In plants, GABA has been known to play roles in controlling carbon and nitrogen content, pH of the cell, osmotic potential and stress response (Zeng et al., 2021).

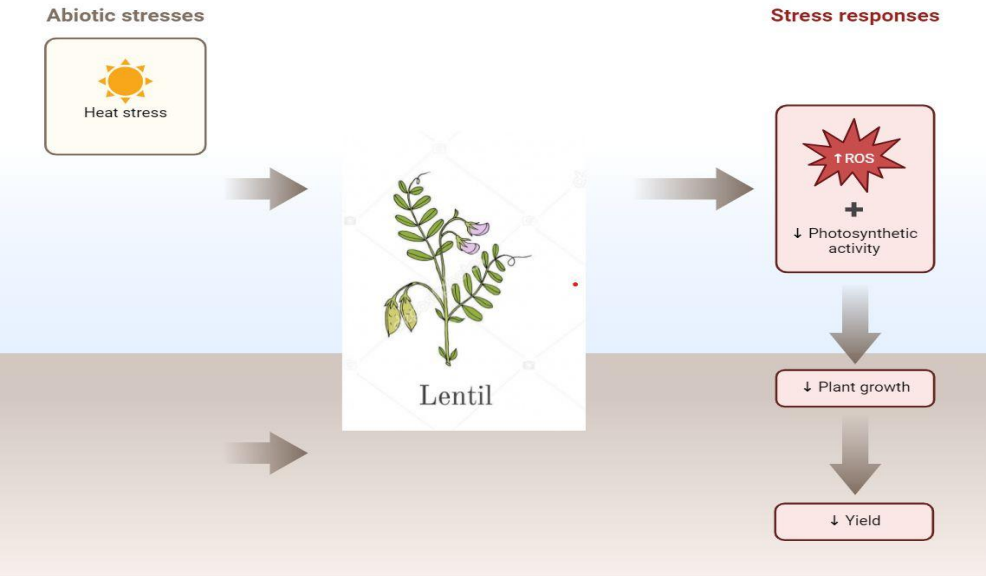


**Figure 1**: Heat stress increases ROS production and decreases photosynthetic activity in lentil plants (Image made with BioRender).

### 1.2. Abiotic stresses and the effect on plant growth

As sessile organisms, plants are continually subjected to fluctuating environmental conditions that can be less than ideal or stressful for their growth and development. Climate factors can greatly influence the development and yield of crop plants and sometimes, these unfavourable conditions may even decline the yield to half or even to two-third. Stunted growth due to drought or heat stress is a frequent and very common occurrence in many regions across the world. Whereas, if both the stresses are imposed, their impact on the yield of the crop is even higher than that of the individual stress (Dreesen et al., 2012). Above moderate stresses significantly reduce crop yield and quality. In abiotic stress, the availability of one or more factors is limited or the nutrient composition in the plant body has an imbalance, which ultimately results in low yield and poor quality of products.

These abiotic stresses considerably reduce plant growth, and development and as a result yield. This is largely due to the production of Reactive Oxygen Species (ROS), which in turn damage proteins and lipids and reduce photosynthesis, stomatal conductance, transpiration rate, plant biomass, and chlorophyll content.

Plant production, growth and figure distribution are also limited by an imperative factor which is temperature. Since there is always fluctuation in climate conditions, heat stress is a

real threat to sustainable agriculture due to the frequent increase in the global air temperature (Rai et al., 2020). Heat stress may impact growth, development, metabolism and plant productivity. Heat stress induces several changes in plant organs including leaf and stem scorching, leaf abscission and senescence, inhibited shoot and root growth, reduced number of flowers, bad pollen tube germination, low pollen viability, and damages to the fruits resulting in severe crop yield losses (Hasanuzzaman et al., 2013). Plant responses can vary based on the sensitivity of each developmental stage. During the vegetative phase, heat stress may reduce growth rates and cause symptoms like chlorosis, scorching, necrosis, and, in severe cases, even lead to plant death. On a cellular level, heat stress can result in membrane damage, protein denaturation, enzyme inactivation within mitochondria and chloroplasts, reduced protein synthesis, and disruptions in carbon metabolism.

Some of the important growth parameters which are relevant in the evaluation of crop growth are the plant height, leaf area, root growth, biomass production, etc. Plant growth is influenced by the nutrients and water availability in the environment, the biotic and abiotic factors. Cell growth is known to be one of the most stress-reactive biological processes due to the decrease in turgor pressure. During water stress, cell elongation in higher-order plants is inhibited because water is no longer transported effectively by the xylem to the expanding cells. This limits the potential for plant height, leaf area, and biomass production, ultimately impacting the yield and quality of crops under abiotic stress conditions (Kaya et al., 2006). Environmental stresses not only influence the yield of the crop but also impact the production and quality of the yield.

### 1.3. Abiotic stress and effect at the cellular level

Membrane disorders, ROS production, protein coagulation, and osmotic stress are often included among common cellular disruptions and secondary stresses that are caused by abiotic stresses such as extreme temperatures, drought, salt stress, and flooding (de Melo et al., 2022). The ability to respond to the various unfavourable conditions in plants is associated with a complex of different levels including cellular, physiological and morphological defence systems. When the harmful effects of stress, particularly from ROS generation, are not effectively minimized by stress defence mechanisms, environment-induced cell death processes are triggered, leading to plant senescence.

### 1.4. Effect of GABA on different plants under different stress conditions

As found in plants, the possible roles of GABA are also multiple depending on whether the plants are in a non-stressed or stressed situation. Exogenous application of GABA greatly alleviates the negative effects of various biotic and abiotic stress factors by enhancing the water content, photosynthesis and exhibiting antioxidant potentiality in plants (Ansari et al., 2021). The external application of GABA has been shown to alleviate oxidative damage caused by chilling stress in tomatoes and wheat, as well as to reduce heat stress in rice, drought stress in black pepper, aluminium toxicity in barley, and chromium stress in mustard. Five-day-old tomato seedlings were exposed to chilling stress (2±0.05 °C for 48 h) and the plants were treated with the control and different GABA concentrations (100, 250, 500 and 750 μmolL−1). Antioxidant enzyme activity, electrolyte leakage and proline were significantly declined in seedlings. Further, GABA-treated seedlings contained higher levels of sugar and proline thereby showing that the improvement of antioxidant enzymes by GABA helps the seedlings to survive chilling stress(Malekzadeh et al., 2014, Malekzadeh et al., 2012).

In barley (*Hordeum vulgare L.*) the antioxidative property of GABA was evaluated against the oxidative stresses induced by Proton ($H^+$) and aluminium ($Al^{3+}$) toxicities. The barley seedlings were treated for 24 hours under $H^+$, $Al^{3+}$ and combined stresses with and without GABA (10 μmol $L^{-1}$), and morphological and biochemical assays were conducted. As a result, GABA significantly mitigates root elongation caused by $Al^{3+}$ and $H^+$ toxicities, reduces carbonylated proteins, enhances antioxidant enzyme activities, reduces malondialdehyde content, and decreases reactive oxygen species accumulation. This suggests that GABA can help reduce oxidative damage in barley seedlings(Song et al., 2010).

Another study emphasizes how GABA enhances the physiological mechanisms of mustard seedlings when exposed to chromium stress. Eight-day-old mustard (*Brassica juncea L.*) seedlings were treated with chromium (0.15 and 0.3 mM $K_2CrO_4$, 5 days) and GABA (125 μM) in a semi-hydroponic medium. The seedlings accumulated Cr, leading to increased oxidative damage and disrupted antioxidant defence. The addition of GABA decreased chromium uptake and increased the activities of non-enzymatic antioxidants as well as enzymatic antioxidants. It also increased leaf relative water content, and chlorophyll content, and restored plant growth (Mahmud et al., 2017).

Four-day-old rice (*Oryza sativa L.*) seedlings were exposed to varying temperatures30/20, 35/25, and 42/37 C [light/dark (15/9 h); for 10 days, either without or with GABA (1mM). The temperature increased shoot and root length, leading to decreased survival. Endogenous GABA content increased in moderately stressed plants, while it decreased in severely stressed plants. Exogenous application of GABA improved growth and survival, reduced membrane damage, improved cellular reducing ability, chlorophyll content, and photochemical efficiency. Leaf water content and stomatal conductance also improved. GABA also suppresses the activities of enzymatic antioxidants, indicating that it offers partial protection against heat stress by increasing leaf turgor and minimizing oxidative damage (Nayyar et al., 2014) .

Another study examined the impact of pre-treating two varieties of black pepper, Panniyur 1 and Panniyur 5, with GABA at a concentration of 2 mM. Both varieties showed positive physiological and biochemical changes, and Panniyur 5 performed better. GABA-primed plants showed enhanced leaf relative water content, faster cell osmotic potential reduction, and increased antioxidant enzyme activity. GABA priming also reduced lipid peroxidation and photosynthetic and mitochondrial activity. The study observed an increased GABA content in plants treated with PEG (polyethylene glycol 6000; 10% w/v) (Vijayakumari and Puthur, 2016).

### 1.5. Role of GABA in reducing the ROS level during stress

ROS are partially reduced or activated forms of atmospheric oxygen ($O_2$) and are produced normally in association with the survival of aerobic organisms. These molecules mostly work as signal molecules involved in signalling during plant stress response but are also products of stress metabolism which has toxic effects(Bor and Turkan, 2019). In plants, reactive oxygen species (ROS) can be highly damaging as they adversely affect the structures and functions of biomolecules within the cells. When ROS levels surpass the capacity of defence mechanisms, they become toxic and lead to oxidative damage in plant cells (Sachdev et al., 2021). GABA a vital part of the defence system, is present in all cells, from prokaryotic to eukaryotic. In plants, GABA reduces ROS, and the GABA shunt pathway is essential for several regulatory processes under stress, either as endogenous signalling molecules or as metabolites (Sharma et al., 2012). The GABA shunt generates NADH and/or succinate during stress conditions, which inhibits the Krebs cycle, disrupts respiration, and increases

the production of ROS (Bouché et al., 2003). This mechanism is backed by research conducted on mammalian brain nerve terminals, which found that hydrogen peroxide (H2O2) inhibits the Krebs cycle enzyme aconitase by blocking α-ketoglutarate dehydrogenase. This inhibition reduces the availability of NADH for the respiratory chain and disrupts mitochondrial function during oxidative stress (Alzheimer et al., 2000). Other components of the GABA shunt exhibit scavenging activity for ROS under stress conditions. The activity of glutamate decarboxylase (GAD), which is regulated by cytosolic calcium ($Ca^{2+}$) levels, enables plant cells to tolerate various stress conditions by modulating GABA synthesis (Mazzucotelli et al., 2006). Metabolites from the GABA shunt play a crucial role in osmoregulation, nitrogen and carbon metabolism, and signalling in response to salt and oxidative stresses. The high accumulation of GABA and proline serves as an osmoprotectant during oxidative and osmotic stress, promoting cell synthesis and minimizing degradation. Additionally, GABA and proline are vital in decreasing ROS production in plants during various environmental stressors, with their overproduction enhancing tolerance to osmotic stress (Liu et al., 2011).

### 1.6. Biosynthetic Pathway of GABA in Plants

Plant growth regulators are commonly utilized to promote plant growth and increase resilience to stress. GABA is a non-protein endogenous amino acid found in various organisms. In plants, GABA was first discovered in the tuber of potato (*Solanum Tuberosum*) more than 70 years ago (Zeng et al., 2021).

In plants, GABA is primarily synthesized and broken down through two main pathways: the irreversible decarboxylation of glutamate by glutamate decarboxylase (GAD) in the cytoplasm and the degradation of polyamines. GABA primarily moves into the mitochondria for catabolism via the GABA shunt pathway, where GABA or GHB is converted to SSA. SSA is then oxidized by SSADH to form succinic acid, which enters the TCA cycle (Zheng et al., 2024).

***GABA synthesized through glutamate decarboxylation and polyamine degradation***

GABA is an amino acid formed from glutamate which is itself produced in the cytoplasm through an irreversible decarboxylation reaction. This process is affected by factors such as glutamate concentration, unfavourable environmental conditions, pH and gene regulation. It

is found that glutamate content is directly proportional to GABA content and production is found in various cell regions (Bouche et al., 2004). The nitrogen from ammonium ions can be incorporated into glutamate and other amino acids, by the action of the GS/GOGAT cycle. The extracellular stimuli can influence the calcium concentration which ultimately activates the calmodulin gene and forms an active complex with $Ca^{2+}$/CaM. This complex can activate GAD in vitro by interacting with its C-terminal domain, thereby increasing the rate of GABA synthesis. Stress can also cause increased hydrogen ion production in the cytoplasm, which in turn helps in GABA synthesis (Yuan et al., 2023).

Polyamine degradation is a key process that contributes to GABA synthesis. It begins with the breakdown of polyamines into 4-aminobutyraldehyde, followed by conversion to 4-aminobutyraldehyde dehydrogenase. This process is catalysed by the enzymes diamine oxidase (DAO) and polyamine oxidase (PAO). DAO activity is enhanced by $Cu^{2+}$ treatment and inhibited by EDTA. While PAO depends on flavin adenine dinucleotide (FAD), the enzyme quinone is displaced from its active site, and AMADH utilizes nicotinamide adenine dinucleotide ($NAD^{+}$) as its coenzyme. Other factors affecting the efficiency of polyamine degradation are also the environmental conditions that turn unfavourable, the main reason for this being the elevated polyamine concentration (Skopelitis et al., 2006). For instance, the anaerobic stress in the broad beans leads to the enhancement of the enzyme activity for polyamine synthesis while the salt stress in the soybean root causes an elevation on the content of free polyamines. However, when considering the roles of the polyamine degradation pathway, its ability to synthesize GABA in monocotyledons is less effective than that of the identified GABA shunt (Podlešáková et al., 2019).

***GABA catabolism produces succinate and γ-hydroxybutyric acid (GHB)***

The GABA is converted into succinate in the matrix of mitochondria. GABA is metabolised to SSA through GABA transaminase (GABA-T) and SSA can alternatively be metabolised through succinic semialdehyde dehydrogenase (SSADH) metabolites and finally enters the TCA cycle in the form of succinate. This process is referred to as the GABA shunt, which helps prevent the accumulation of toxic by-products in cells during normal mitochondrial function. To illustrate this, GABA bypasses direct entry into the TCA cycle by utilizing the GABA shunt, involving two enzymatic steps that convert α-ketoglutarate to succinyl-CoA and then to succinate. Two isoforms of GABA transaminase (GABA-T) participate in the conversion of succinic semialdehyde (SSA), primarily involving GABA-TK and GABA-TP.

GABA-TK uses α-ketoglutarate as the amino group donor to produce glutamate, while GABA-TP uses pyruvate as the amino group donor to generate alanine. Additionally, GABA-TP functions as GABA-TG, where glyoxylic acid serves as an amino group donor to form glycine. All these reactions are reversible (Bouché et al., 2003). Another metabolite obtained after the breakdown of GABA is gamma hydroxybutyric acid commonly referred to as GHB. SSA can be oxidized to generate GHB through the Succinate reductase (SSR), and this process is reversible (Chen et al., 2022).

### 1.7. Function of GABA under the abiotic stress in plants

Plants also have several strategies that initiate the synthesis of signalling biomolecules on stress tolerance. Out of these, GABA is one of the significant biomolecules among the stress-responsive metabolites. GABA plays a vital role in the growth, development, and stress resilience of plants (Abdel Razik et al., 2021). In most plants, GABA seems to provide partial protection from several abiotic stresses by enhancing leaf turgidity, accumulation of osmotic solutes, and reduction in oxidative injury, all through the stimulation of antioxidant enzymes(Shelp et al., 2021). GABA is involved in the reduction of ROS in plants, and GABA shunt is involved in several regulatory processes in plants as metabolites or messengers during stress reactions. Temperature stress is one of the major factors that affect plant growth and development and, generally, high amounts of GABA have been detected in plant species under low temperatures. GABA at high levels has also been linked to plant tolerance to low temperatures and can prevent low-temperature injury through strengthening plant antioxidant system as well as through accumulation of proline to regulate osmotic pressure or osmoregulation (Wang et al., 2014)

Heat stress can have negative effects on plant growth and development, and some studies have examined the link between heat and GABA in multiple plant species. Drought is one of the factors that has great impact on crop development and production, thus stimulating GABA production. Some of the specific plants include Turnip leaves and bean leaves, Soybean leaves and sesame leaves and stems all have been documented to raise GABA levels when the leaves are excised. The results showed that the exogenous application of GABA enhanced white clover drought tolerance which can be due to the upregulation of GABA cycle, polyamines and proline. GABA helps improve nitrogen use efficiency, protect the photosystem II, and prevent the decline of cell elongation, wax formation, fatty acid

desaturation, and retardation of leaf ageing under water deficit conditions (Filho et al., 2018). GABA is synthesized in high amounts in the root systems of soybean as well as grape plants where it regulates growth by suppressing the enzymes which produce Reactive Oxygen Intermediates, activating the antioxidant enzymes, and enhancing the chloroplast structure and functions to a large extent. These effects worsen when the environment leads to hypoxic conditions due to waterlogging by restricting the permeability of the soil to water and causing a build-up of GABA in plants (Jackson and Colmer, 2005). Salinity is one of the most detrimental factors affecting plant growth and production of cropped plants all over the world. Three cellular responses of salt tolerance in plants are proposed that is osmotic stress tolerance, or resistance to osmotic stress, $Na^+$ exclusion capacity, or the ability to reduce the import of $Na^+$ into a tissue, and tissue tolerance, or the ability of a tissue to withstand high levels of Na presence inside the tissue. Exogenous GABA application to Maize, white clover, muskmelon, germinated hull-less barley and tomato enhances the endogenous GABA content, increases antioxidant activity, reduces salt damage and increases in plant salt tolerance as described in (Renault et al., 2010)Heavy metals, which are among the influential pollutants of soil, pose a potential threat to food through bioaccumulation in the edible parts of crop plants. There has been an increase in GABA content in the following cases of chromium stress in rice roots, zinc and copper stress in soybeans and in arsenic stress in rice seedlings (Kumar et al., 2017). The GABA application leads to the expression of genes involved in stress tolerance, activation of antioxidant enzymes and a reduction in as accumulation which helps to develop the tolerance level. Therefore, long-term accumulation of a substance such as GABA is more effective in the development of tolerance, rather than short-term toxicity.

### 1.8. Effect of GABA on Seed Germination.

In seeds germination process, water is an essential factor, and this is the reason why seeds germination is very sensitive to drought stress. In previous studies, seed priming was considered to induce tolerance to various abiotic stresses that cause changes in the physiological, molecular, and biochemical properties during seed germination. For instance, seed priming with spermidine and 5-aminolevulinic acid enhanced the improvement of amylolysis, antioxidant defences, and polyamine metabolisms in chilling-stressed rice (*Oryza sativa*) seed germination (Sheteiwy et al., 2017). It has been observed that seed coating with

GABA was effective in reducing the impacts of salt stress on germination inhibition of white clover as due to alterations in physiological, metabolic, and molecular processes (Cheng et al., 2018). It is also important to understand the effect and the underlying mechanism of seed priming using different chemicals in reducing stress damage under different abiotic stresses. GABA is an essential non-protein contributing amino acid that is developed in animals and plants (Kinnersley and Turano, 2000) Normally, the plant tissues have low content of GABA, while in this case, it is elevated in different plant species at different stressed conditions for example, reduced temperature stress in soybean (*Glycine max*) leaves, drought and heat stress in creeping bentgrass (*Agrostis stolonifera*), and drought stress in white clover (Yong et al., 2017). This could be because GABA engaged in stress tolerance of plants by controlling the tricarboxylic acid cycle, nitrogen meter, cytoplasmic pH antioxidant defense, and osmotic potential, (Li et al., 2021). These reactions indicated that the exogenous GABA application could enhance the hypoxic tolerance in muskmelon seedling. Moreover, GABA application increases the peroxidase and ascorbate peroxidase activity and osmolytes content, which could protect perennial ryegrass (*Lolium perenne*) from oxidative stress and achieve the purpose of reducing water deficit in leaf samples (Krishnan et al., 2013). The present study found that GABA improved antioxidant activity and reduced oxidative stress, which has potential applications in enhancing drought tolerance of creeping bentgrass (Tang et al., 2020). However, GABA is useful in plant's adaptation to abiotic stresses.

### 1.9. Hypothesis

Supplementing lentil plants with GABA will enhance their resilience to heat stress, leading to improved growth, physiological, and biochemical performances and yield compared to untreated plants.

### 1.10. Experimental Aims

AIM 1: To assess the effect of GABA treatment on the physiological and biochemical responses of lentils subjected to heat stress.

AIM 2: To determine the optimal application rate and timing of GABA treatment for maximum effectiveness in reducing temperature stress.

AIM 3: To compare the effects of foliar treatment and seed priming with GABA in the two varieties of lentils.

# CHAPTER 2: MATERIALS AND METHODS

## 1. Materials

Various lentil varieties, including L13 (AGG70778, ILL5725), L16 (71645), and Thunder, GABA (A2129, Sigma Aldrich), distilled water, and Silwet at a concentration of 0.03%. The TBARS assay employs 5% TCA along with two solutions: Solution A containing 20% TCA, 0.01% BHT, and 0.5% TBA, and Solution B containing 20% TCA and 0.01% BHT, with standards prepared using 0.1 mM MDA added to 5% TCA. For the GABase enzyme assay, a 0.5 M potassium pyrophosphate buffer at pH 9.0 (adjusted with KOH) is used alongside 100% 2-mercaptoethanol, 20 mM α-ketoglutarate, 10 mM NADP, and the GABase enzyme. The starch assay consists of 80% v/v ethanol, 1.7 M sodium hydroxide, a pH 3.8 sodium acetate buffer, amylase, amyloglucosidase enzymes, GOPOD, and glucose standards.

## 2. Methods

### 2.1.Treatment of two lentil seed varieties with GABA and Distilled water

The seeds from two lentil varieties L13(AGG70778) (ILL5725) and L16(71645) were taken for undergoing the pilot experiment. Forty seeds were taken from both the lines collected and weighed. The seeds were sterilized with 100% bleach solution (v/v, 6% sodium hypochlorite) for 1 minute. Ten seeds of one lentil variety were treated in distilled water (control), likewise, the three sets of ten seeds were treated in 10 µM,100 µM and 1000 µM GABA concentrations separately. Likewise, the other lentil variety were also treated. The seeds were plated in four Petri dishes (control, 10 µM,100 µM and 1000 µM GABA) with filter paper as a growth medium and filled with 10ml of the concentrated solutions prepared. The plates were foiled and stored in a controlled environment at 28/18 °C (day/night) and incubated for 2 days.

**Table1**: From 1mM GABA stock solution, 10 µM,100 µM and 1000 µM concentrated GABA solutions were prepared. The volume of GABA for sample preparation were estimated by the equation (C1V1=C2V2)

| GABA concentration | 0 (control) | 10 µM | 100 µM | 1000 µM |
|---|---|---|---|---|
| Volume of GABA (ml) | 0 | 0.01 | 0.1 | 1 |
| Volume of Distilled water(ml) | 10 | 9.99 | 9.9 | 9 |
| Total Volume (ml) | 10 | 10 | 10 | 10 |

**Germination percentage (G%) = ((number of seeds germinated /**

**total number of seeds) × 100%)**

In this experiment, germinated seeds were transferred to pots filled with bio-grow soil from the University of Adelaide. The 24 germinated seeds of one lentil variety treated with distilled water (control) 10 µM,100 µM and 1000 µM GABA were transferred to the pots (2 seeds in one pot). Likewise, 3 pots were treated with one treatment (control, 10 µM,100 µM,1000 µM). A total of 48 seeds from both varieties were set for growing inside the pots for 14 days in greenhouse 6. The plants were watered thrice a week, and the growth was observed. The plants were harvested on day 15 to estimate the fresh biomass(g) of shoot and root. Thereafter, the shoot and roots are kept in the oven under 60 ° C for three days to obtain the dry biomass(g).

From the pilot experiment, by calculating the fresh biomass, dry biomass, root length and germination percentage the best lentil variety and the best GABA concentration were obtained.1000 µM was considered the best GABA concentration and from the two lentil varieties taken, the L16 variety showed more significance when compared to the plants treated with control. So, for the actual experiment, L16 lentil variety and the wild variety of lentil Thunder were taken.

## 2.2.Seed priming with GABA and distilled water

The GABA solution was prepared using 1 mM stock solution. The volume of GABA for sample preparation was calculated and each GABA volume from the stock solution was transferred into a 50ml sterile falcon tube and topped with distilled water. Forty seeds from each lentil variety, L16 and Thunder were taken. From the prepared 50ml 1mM GABA, 20 ml of the sample's solution were transferred to each Petri dish with double-layered filter papers. Twenty seeds from one lentil variety were soaked in 30ml of 1mM GABA for 1hour. After 1hour, the soaked seeds were plated in the Petri dishes with double-layered filter papers, moistened with 20ml 1mM GABA-concentrated solution and distilled water (control). The plates were covered using aluminium foil paper and incubated for 3 days in a controlled condition at 28/18 °C (day/night). Four plates were taken with filter papers in them as a growth medium. Two plates were prepared for each variety containing one with 1mM GABA concentrated solution and one with control (distilled water). 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 bio grow soil and grown at greenhouse 3 at 17° C for 34 days (5 weeks)

**Figure 2**: Seed priming with GABA and distilled water in both the lentil varieties (L16 and Thunder) for 1 hour and transferred them to plates for incubation (Image made with BioRender).

## 2.3.Foliar Treatment

The best germinated seed was selected and transferred into 12 pots and grown in the greenhouse at 17°C. On 31 days from seed potting, plants were exposed to foliar spray treatment with GABA twice before heat stress application. The first foliar treatment was done 3 days before the heat treatment and the second foliar treatment was done one day before the heat treatment. For this, a 5 mM GABA solution was prepared for foliar treatment. An

amount of 0.515g GABA powder was dissolved in 1000 ml distilled water (5 mM GABA) and 0.3 ml of silwet (0.03%) surfactant was added to promote better adhesion and reduce surface tension on foliar surfaces. After the final foliar treatment, plants were transferred into a heat bay in the greenhouse for heat treatment under 27°C for 24 and 72 hours by the next day.


**Figure 3**: Foliar treatment on leaf surface of lentil varieties with 5mM GABA using a spray bottle(Image made with BioRender).

### 2.4.Sampling and Harvesting

Following 24 h heat stress for GABA-treated (control and 1mM) plants and foliar-treated plants, leaf samples were collected from each plant. The young leaves were taken from the plant's apex and transferred into a falcon tube. Falcon tubes were kept in the liquid nitrogen container right after the sampling. Subsequently, all samples were stored in a -80 C freezer for biochemical examination. Sampling and harvesting were conducted at two time points ($4^{th}$ week and $12^{th}$ week) intervals.

The plant parts, roots and shoots were gently taken out from the pots. Excess soil particles from the roots were washed off and dried. Once the roots and shots were clean, root and shoot fresh biomass was determined. Then the shoots and roots were marked and put into different paper bags individually. Subsequently, the labelled bags were put in an oven at 60° C for three days to get dry biomass.

## 2.5.Biochemical assays

### 2.5.1. TBARS (Thiobarbituric Acid Reactive Substances) assay (For determining the ROS level)

The basic and preparatory step of the experiment was to make 5% TCA, Solution A (20% TCA, 0.01% BHT, 0.5% TBA), Solution B (20% TCA, 0.01% BHT) and the standards which were prepared using 0.1mM MDA + 5% TCA. The plant samples collected and stored in -80°C fridge was ground and weighed to 50-100mg tissue into 1.5ml tubes in liquid nitrogen. Thereafter, into the plant tissues, 1 ml of 5%TCA is added and vortexed. Subsequently the sample tubes are allowed to spin at maximum speed for 15 minutes in a centrifuge machine. Following, 400µl of supernatant is pipetted to new two centrifuge tubes with labels A and B. To each of the samples labelled as 'A', solution A is added while to the samples labelled 'B', solution B is added. Then, the lid open tubes were placed in heat block which is set to 96°C for 30 minutes. Afterward, the samples are taken from the heat block and were immediately cooled on ice for nearly 5 minutes. After the samples has been cooled, the tubes were centrifuged at 9500g for ten minutes. Finally, 100µM of the sample solutions is added to the 96 well plate (duplicate or triplicate) and the microplate-reader is used to get the reading at an absorbance range of 440nm,532nm and 600nm (Singh et al., 2012, Hodges et al., 1999).

Calculation for MDA equivalent value.

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

Table 2: Standards preparation for TBARS

| Standard concentration(nmoles) | Amount of 0.1mM MDA to add to TCA | Amount of 5% TCA to add |
|---|---|---|
| 0 | 0 | 1000 |
| 1 | 10 | 990 |
| 3 | 30 | 970 |
| 4 | 40 | 960 |

### 2.5.2. GABA Extraction

The stock solutions, 100% Methanol, 70 mM Lanthanum Chloride and 1.0 M Potassium hydroxide were made for the assay. 50mg of Plant tissues were weighed into a 2.0ml microfuge tubes. Afterward, 400µl of methanol were added incubated at 25°C for 10minutes in a heat block. Following, the plant tissues are dried in speed vacuum for 2 hours. Thereafter, 500µl of 70mM Lanthanum chloride is added to the dried samples. Shake the samples at 100rpm for 15 minutes in a shaker. After that, centrifuge the tissue samples at 12000 rpm for 5 minutes. Into the centrifuge tubes containing 160 µl 1.0 M KOH, 400 µl of supernatant were transferred. The microfuge tubes are inverted and shake at 100rpm for 5 minutes. Once again, the samples are centrifuged at 12000rpm for 5 minutes. At last, the supernatant is transferred to new 2.0ml centrifuge tubes and store the extracted samples at -80°C.

### 2.5.3. GABase Enzyme Assay

The stock solutions to be prepared were 0.5M Potassium pyrophosphate with a pH of 9.0 with KOH, 100% 2-Mercaptoethanol, 20Mm α-ketoglutarate, 10mM Nicotinamide adenine dinucleotide phosphate (NADP) and GABase enzyme.

Table3: Preparation of Master mix

| 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 |

A 96 well plate is taken, into which 45.2 µl of sample extract were added. Then 52.8 µl of master max were added to each well. Thereafter, the pre-reaction absorbance at 340nm for 3 cycles at 25°C were measured. After taking that, 2 µl of GABase enzyme (5 U/ml) were added

to each well. Lastly, the reaction absorbance at 340nm for 30 cycles at 25°C were measured(Ramesh et al., 2015).

### 2.5.4. Starch Extraction

About 50mg of homogenized tissue was weighed and taken in 2.0ml tube and stored at -80 °C. 150µL of 80% v/v ethanol was added to each frozen sample, and vortexed for some time. Then refrigerated chilled 1.7M sodium hydroxide was added to the solution and it was vortex again for few seconds. That was followed by sample shaking for 15 minutes at 100 rpm. Supernatant was prepared by centrifuging the samples at 10000g for 2 minutes.

### 2.5.5. Starch Assay

150 µl of supernatant was taken in two microcentrifuge tubes and named as unknown sample extracts (UK) and unknown sample blank (UKB). After that, 400 µl of pH 3.8 sodium acetate buffer was added to both UK and UKB samples. To each UK samples, 5µl of amylase and 5µl of amyloglucosidase was added, while to each UKB samples, 10µl of pH 3.8 sodium acetate buffer was added. Then all the samples were vortex for 5 seconds and heat treatment at 50 °C for 30 min. Incubation samples were then allowed to cool for min and mixed gently by inverting the tubes. 200µl each of the UK and UKB extracts were transferred to two new microcentrifuge tubes. All the samples were then centrifuged at 13 000 rpm for 5 min. Another 100 µl from each sample was then gently pipetted into new micro centrifuge tubes after performing centrifugation was done. UK and UKB were pipetted into 96-well microplate (triplicates) 5µl from each sample. Further, triplicate of 5 µl glucose standards and 5 µl distilled water were pipetted into the micro well plate. When all the sample and blanks have been pipetted into the 96 well plate, 150 µl of GOPOD regent was then added to each well. Subsequently, the 96 microwell plate was covered with aluminium foil to protect from light and incubated at 50° C for a period of twenty minutes. Finally, after incubation the absorbance of each microplate wells was measured at 510 nm using microplate reader. Before measure absorbance, mixing with micro plate reader shake function on, mixing time was 5 s after incubation (McCleary et al., 2019) (Kumari et al., 2020).

# CHAPTER 3: RESULTS

## 1. Treatment of lentil seed varieties with GABA and Distilled water

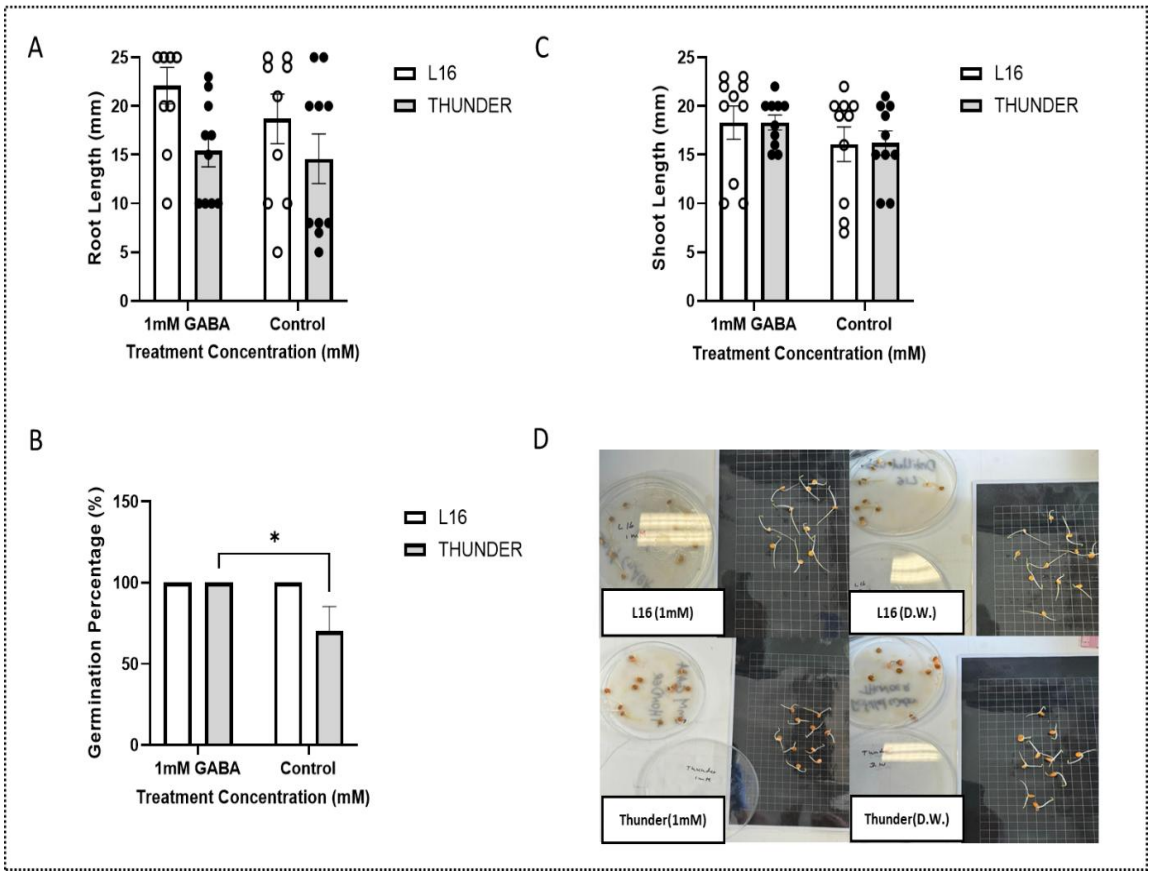


**Figure 4: The germination percentage, root length and shoot length of the lentil lines L16 and Thunder were displayed.** (A) The Figure shows the root length (mm) of seeds germinated of the lentil lines under the treatments 1mM GABA and control. (B) Germination percentage (%) of the lentil lines, L16 and Thunder, under treatments 1mm GABA and control. (C) The shoot length (mm) of the germinated lentil seeds treated with 1mM GABA and control. (D) The image shows the seed germination of both the lentil lines, L16 and Thunder, under the treatments 1mM GABA and control. n=10±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

Figure 4 depicts the effects of 1 mM GABA and control treatments on root length, shoot length, and germination percentage in two lentil varieties, L16 and Thunder. In Figure 4 A, it is shown that the root length of L16 significantly increases with GABA treatment, whereas the root length of Thunder remains nearly unchanged between GABA and control treatments.

In Figure 4 B, the germination percentage for L16 under both 1 mM GABA and control conditions remains the same, while a slight difference is observed for Thunder, with GABA-treated seeds showing a 15% increase in germination compared to those treated with distilled water (control). Figure 4 C illustrates a minimal variance between treatments; however, both lentil lines treated with GABA exhibit greater shoot length compared to control-treated seeds. Figure 4 D shows the germinated seeds of the lentil varieties after three days of incubation. From the Figures 4 A and C, it is evident that the lentil line L16 demonstrates greater growth in root and shoot length than Thunder.

**2. Harvesting was conducted for 4-week-old 36 plant growths.**

Out of a total of 72 plants, a combined total of 36 plants from both lentil lines was harvested by day 38. Heat stress was applied for 24 hours and 72 hours from the day 35. Of the 36 plants harvested, 12 were pretreated with 1 mM GABA and control (distilled water), and 12 were treated with a foliar spray application of 5 mM GABA. The foliar spray was applied twice: once three days before and once one day before the heat stress application. This treatment was performed for both the L16 and Thunder varieties.

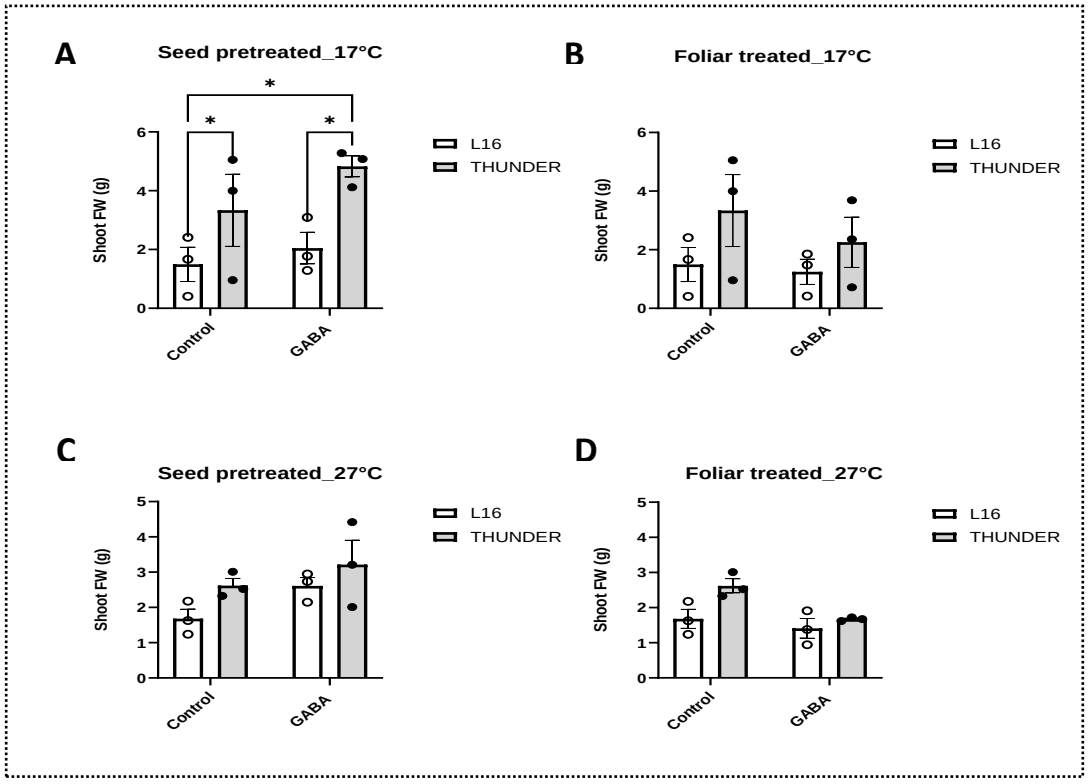


**Figure 5: The shoot fresh weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The shoot fresh weight of L16

and Thunder at 17°C after 4 weeks of plant growth which was treated with 1mM GABA and distilled water (control). (B) The shoot fresh weight of both the lentil lines at 17°C treated by foliar application of GABA (5mM) twice (three days and one day before heat stress application for 24 hours and 72 hours) along the leaf surface by day 31 and day 33 of plant growth. (C) The shoot fresh (g) weight of L16, and Thunder pretreated with 1mM GABA and control at a heat temperature of 27°C (D) The Shoot FW(g) of the lines L16 and Thunder after undergoing the foliar treatment twice with a GABA concentration 5mM and with pretreated control. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

Figure 5 compares the effects of GABA pretreatment and foliar treatment on the shoot fresh weight (FW) of two plant varieties, L16 and Thunder, at 17°C and 27°C. At 17°C (Figure 5 A), a significant increase in shoot FW(g) of Thunder variety pretreated with GABA, indicating that plant growth is greatly enhanced by this treatment. In contrast, the foliar treatment at the same temperature has a smaller impact and is not marked as significant (Figure 5 B). At 27°C, an increase in shoot FW (g) is observed in both plant varieties with GABA pretreatment, although the changes are not marked as significant, suggesting a more moderate effect (Figure 5 C). A consistent but less pronounced increase in shoot FW (g) is observed with foliar treatment at 27°C for both varieties (Figure 5 D), like its impact at 17°C. Therefore, a stronger influence of GABA pretreatment on shoot FW (g) is indicated, especially for the Thunder lentil variety at 17°C, while smaller, more consistent improvements are observed with foliar treatment at both temperatures (17°C and 27°C).

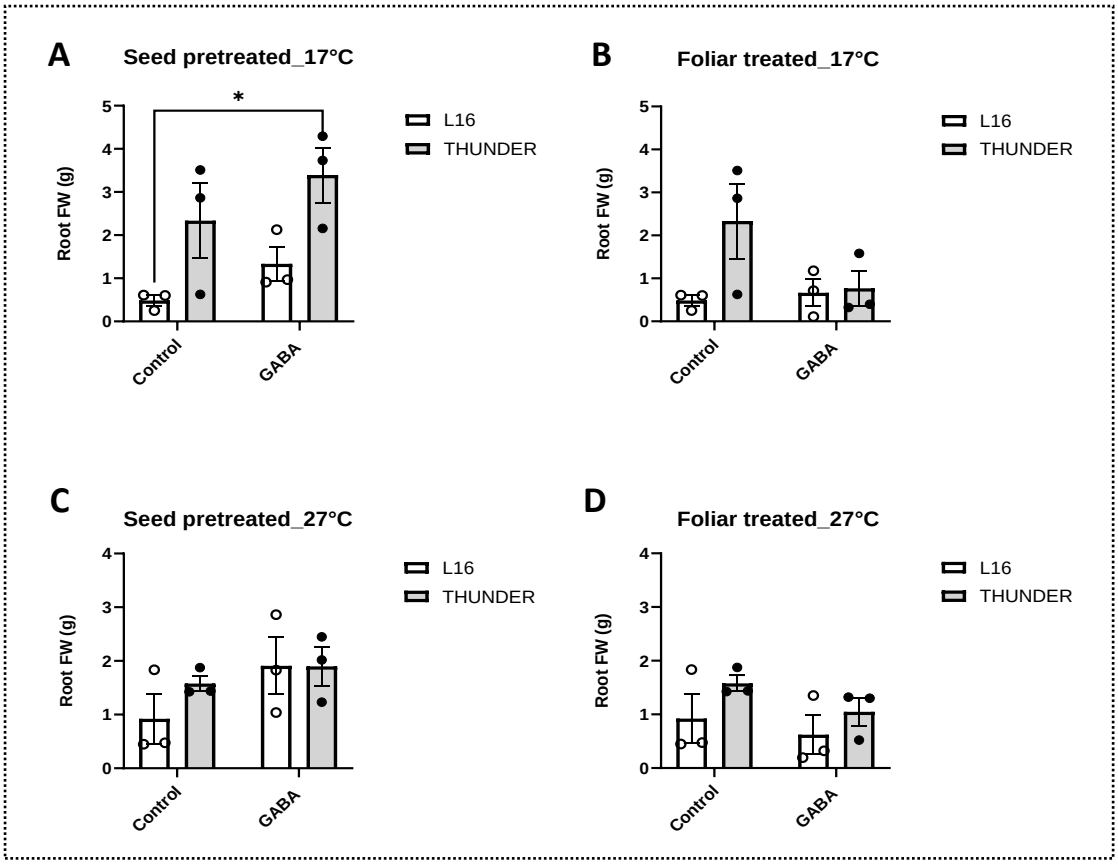


**Figure 6: The root fresh weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The root fresh weight (g) of L16 and Thunder variety at 17°C which was treated with 1mM GABA and distilled water (control). (B) The figure shows the root fresh weight(g) of both the lentil lines at 17°C treated with 5mM GABA through foliar application (three days and one day before heat stress application for 24 hours and 72 hours) along the leaf surface. (C) The root fresh weight (g) of L16, and Thunder pretreated with 1mM GABA and control at a heat temperature of 27°C (D) Figure illustrating the root FW(g) of the lines L16 and Thunder after undergoing the foliar treatment twice with a GABA concentration 5mM and with pretreated control. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

Figures 6 (A, B, C, and D) illustrate the root fresh weight (FW) in grams (g) of two plant varieties, L16 and Thunder, under varying treatment conditions and temperatures. The most noticeable change in the graphs is observed in Figure 6 A, where a significant increase in the root fresh weight (FW) is seen in the Thunder variety pretreated with GABA (1 mM) compared to the L16 variety pretreated with the control at 17°C. This observation suggests that, at the lower temperature of 17°C, a strong positive effect of GABA on the root growth

of the Thunder variety is present. In the other Figures 6 (B, C, and D), which display results at both 17°C and 27°C under GABA and control treatments, some changes in root FW are noted; however, none are as pronounced as those in Figure 6 A. This emphasizes that the largest impact on root growth in the Thunder variety at 17°C is attributed to GABA pretreatment, highlighting how this plant variety's response to GABA differs based on temperature. In addition, the GABA pretreatment in both the lentil lines seems to be more effective than the foliar treatment at both temperatures (17°C and 27°C).

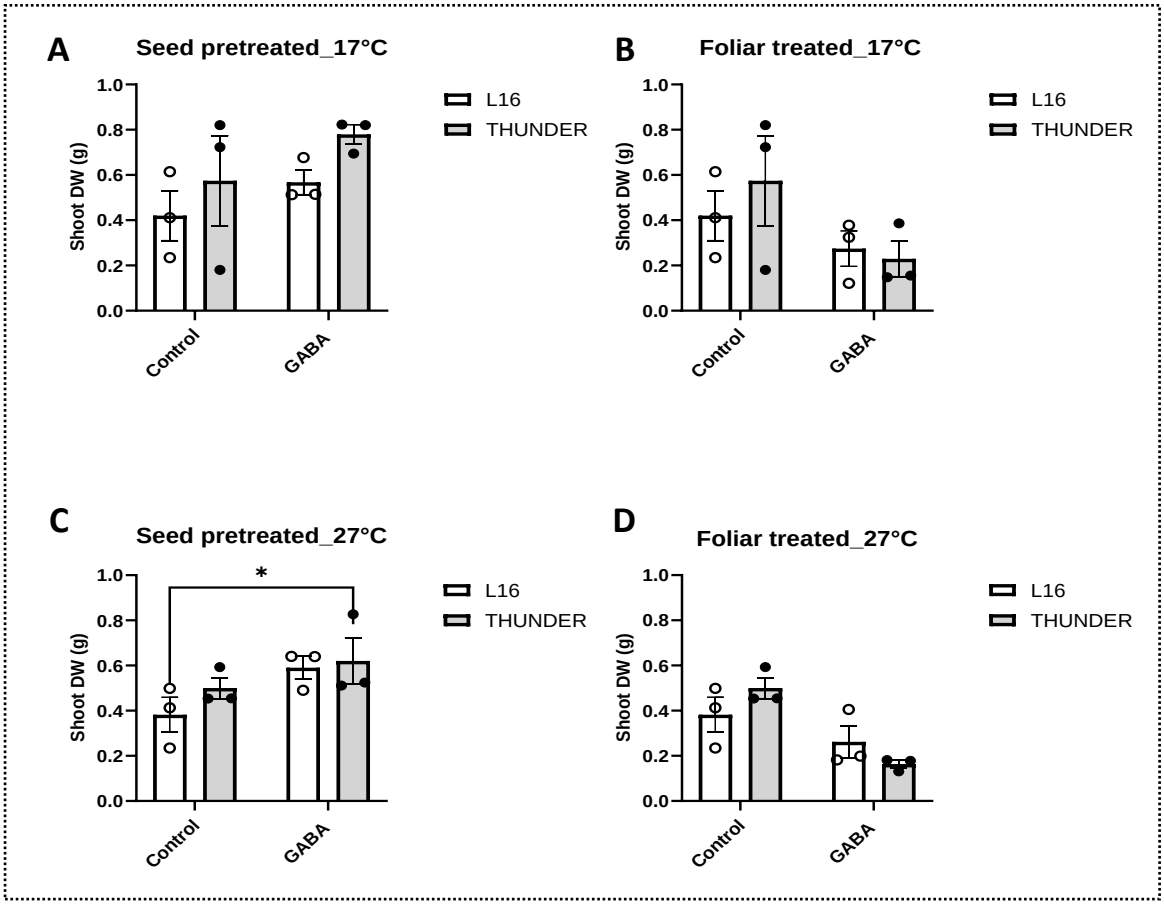


**Figure 7: The shoot dry weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The shoot dry weight (g) of L16 and Thunder variety at 17°C which was treated with 1mM GABA and distilled water (control). (B) Figure shows the shoot dry weight (g) of both the lentil lines at 17°C treated by foliar application of GABA with a concentration of 5mM twice (three days and one day before heat stress application for 24 hours and 72 hours) along the leaf surface by day 31 and day 33 of plant growth. (C) The shoot dry weight (g) of L16, and Thunder pretreated with 1mM GABA and control at a heat temperature of 27°C (D) Figure illustrating the shoot dry

weight (g) of the lines L16, and Thunder treated through foliar GABA treatment twice with a concentration 5mM and with pretreated control. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

Figure 7 displays the shoot dry weight (DW) for the plant varieties L16 and Thunder under different treatments (GABA and control) and temperatures (17°C and 27°C), the most notable change is observed in Figure 7 C. At 27°C, a significant increase in shoot DW (g) is seen with GABA pretreatment in both L16 and Thunder compared to the control, with a particularly strong effect in the Thunder variety. This indicates that, at the higher temperature, shoot dry weights in both the varieties, especially Thunder, are greatly enhanced by GABA pretreatment (1mM). In comparison, Figures 7 A and B show only minor changes in both lentil lines at 17°C with GABA and foliar treatments. Additionally, GABA pretreatment appears to be more effective for both lentil lines than foliar treatment. Similarly, in Figure 7 D, both L16 and Thunder show higher dry weights with 1 mM GABA pretreatment compared to foliar treatment with 5 mM GABA at both temperatures (17°C and 27°C). This presents the effectiveness of GABA pretreatment at both temperatures for increasing dry weights (g).

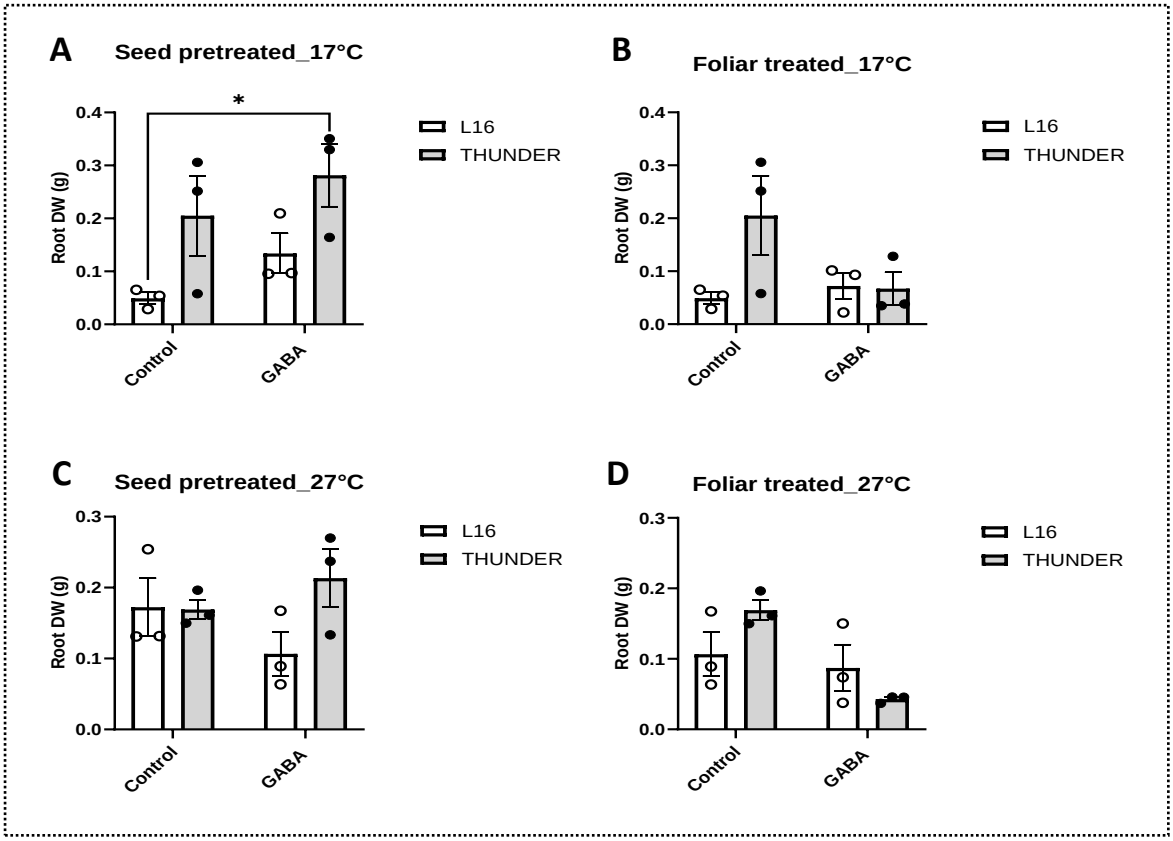

**Figure 8: The root dry weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The Figure depicts the root dry weight (g) of L16 and Thunder at 17°C which was treated with 1mM GABA and distilled water (control). (B) The root dry weight (g) of both the lentil lines at 17°C treated by foliar application of GABA with a concentration of 5mM twice (three days and one day before heat stress application for 24 hours and 72 hours) along the leaf surface by day 31 and day 33 of plant growth. (C) The root dry weight (g) of L16, and Thunder pretreated with 1mM GABA and control at a heat temperature of 27°C (D) Figure illustrating the root DW (g) of the lines L16 and Thunder after undergoing the foliar treatment twice with a GABA concentration 5mM and with pretreated control. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

Figures 8 (A, B, C, and D) illustrate the root dry weight (DW) in grams (g) of two plant varieties, L16 and Thunder, under varying treatment conditions and temperatures. The most noticeable change in the graphs is observed in Figure 8 A, where a significant increase in the root dry weight (DW) is seen in the Thunder variety pretreated with GABA (1 mM) compared to the L16 variety pretreated with the control at 17°C. This observation suggests that, at the lower temperature of 17°C, a strong positive effect of GABA on the root growth of the Thunder variety is present. In Figures 8 (B, C, and D), which display results at both 17°C and 27°C under GABA and control treatments, some changes in root DW are noted; however, none are as pronounced as those in Figure 8 A. This emphasizes that the largest impact on root growth in the Thunder variety at 17°C is attributed to GABA pretreatment, highlighting how this plant variety's response to GABA differs based on temperature. In addition, the GABA pretreatment in both the lentil lines seems to be more effective than the foliar treatment at both temperatures (17°C and 27°C).

## 3. Biochemical analysis

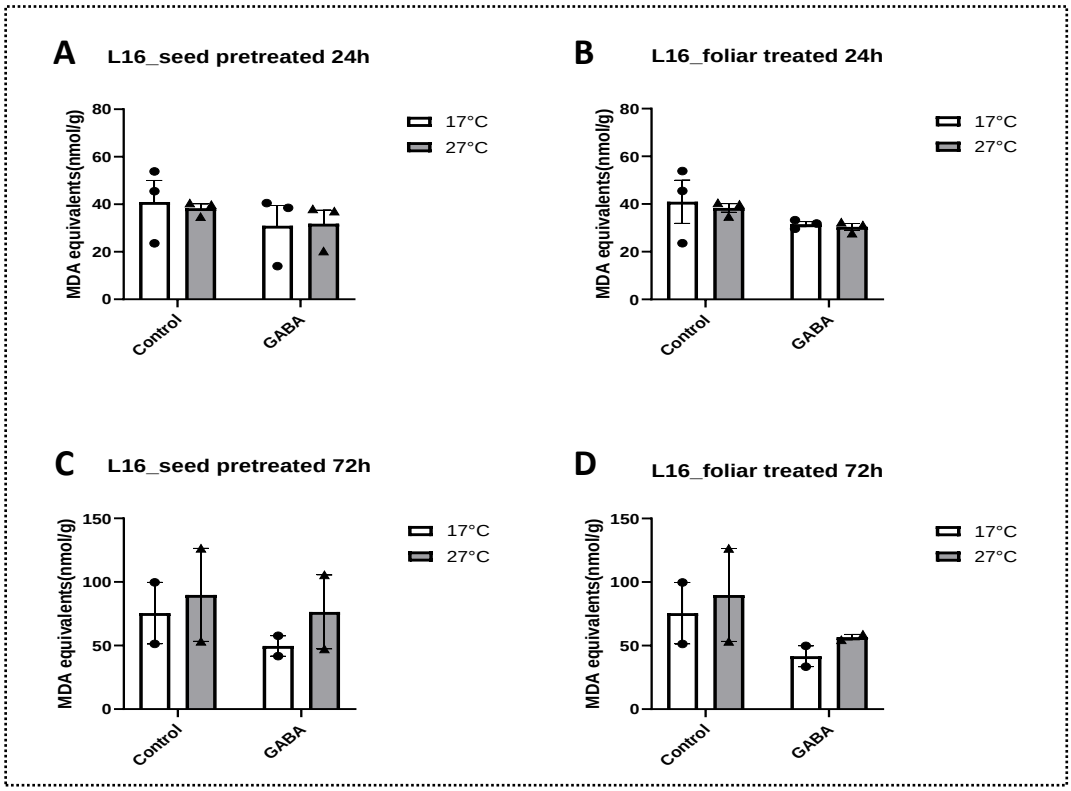


**Figure 9: Indicating the MDA level of the lentil variety L16 treated with control and GABA (seed pretreatment and foliar treatment) under the influence of 24hours and 72hours of heat stress.** (A) Figure depicts the MDA levels(nmol/g) in control and GABA pretreated L16 lentil variety after 24 hours of heat stress. (B) Figure reveals the MDA equivalents(nmol/g) of control and foliar treated L16 lentil variety after 24 hours of heat stress. (C) The MDA levels(nmol/g) of control and GABA pretreated L16 lentil varieties after 72 hours of heat stress application. (D) The MDA levels(nmol/g) of control and foliar-treated L16 lentil varieties after 72 hours of heat stress application.

The Figure 9 show MDA levels, which indicate oxidative stress, in the L16 plant variety under heat stress and normal temperature (17°C and 27°C) for 24 and 72 hours. In Figure 9 A, for seed-pretreated plants after 24 hours, the untreated plants exhibit slightly higher MDA levels (around 40 nmol/g) than GABA pretreated plants at 17°C and 27°C. Figure 9 B, GABA treated plants show less MDA level than the GABA untreated plants, even though there is not much significance. In Figure 9 C, after 72 hours, there can see higher MDA levels for control treated plants than the L16 plants pretreated with GABA at 27°C compared to 17°C with values between 50-100 nmol/g. Meanwhile, Figure 9 D indicates that control-treated plants exhibit higher MDA levels at 27°C after 72 hours of heat stress, with values between 50 and

100 nmol/g. These findings suggest that MDA levels and thus oxidative stress tend to increase over the duration of heat stress (72 hours) and are more pronounced at 27°C in control-treated plants. Moreover, both GABA-pretreated and foliar-treated L16 plants display comparatively lower MDA levels than control treated plants. However, foliar treatment appears to be more effective, as a more noticeable decrease in MDA levels which means less oxidative stress is observed compared to GABA-pretreated plants.

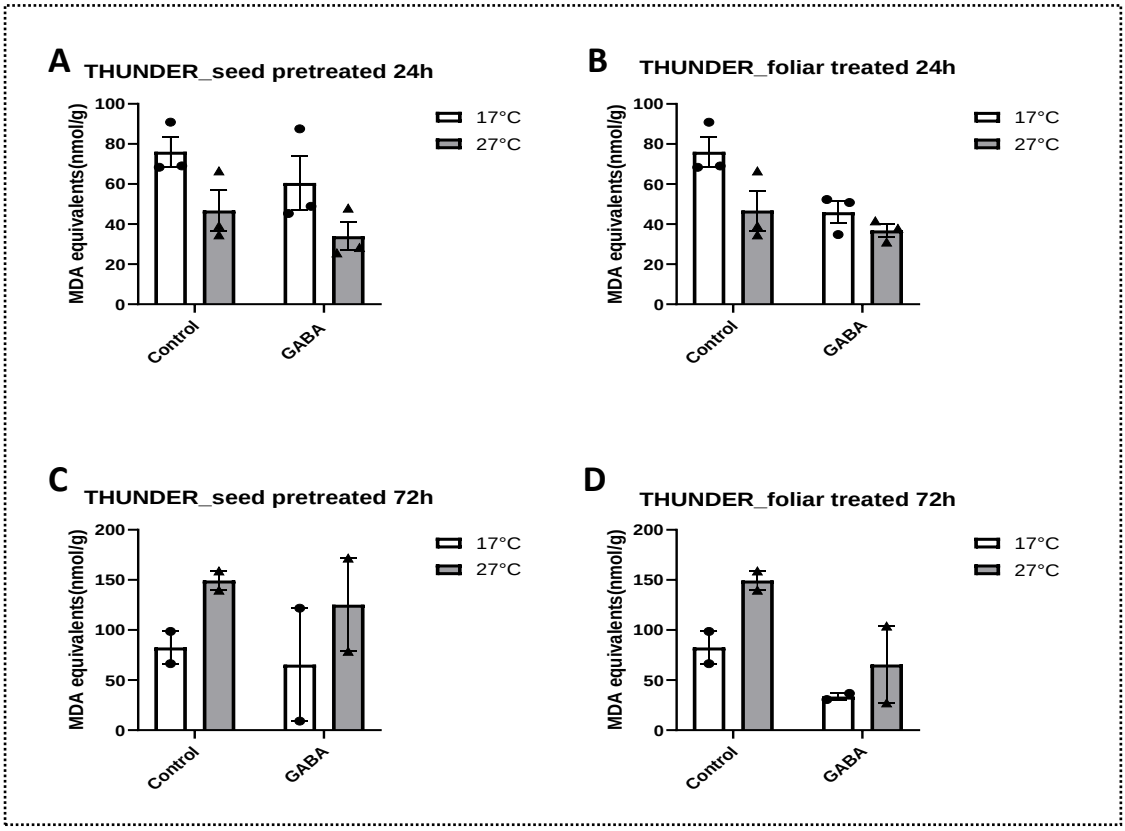


**Figure 10: Depicting the MDA level of the lentil variety Thunder treated with control and GABA (seed pretreatment and foliar treatment) under the influence of 24 hours and 72 hours of heat stress.** (A) The MDA levels (nmol/g) in control and GABA pretreated Thunder lentil variety after 24 hours of heat stress. (B) Figure reveals the MDA equivalents(nmol/g) of control and foliar-treated Thunder lentil variety after 24 hours of heat stress. (C) The MDA levels (nmol/g) of control and GABA pretreated Thunder plants after 72 hours of heat stress application. (D) The MDA levels (nmol/g) of control and foliar-treated Thunder lentil varieties after 72 hours of heat stress application.

Figure 10 illustrates the MDA levels in the Thunder lentil variety treated with GABA and control at 17°C and 27°C for 24 and 72 hours. When comparing Figures 10 A and B, a notable increase in MDA levels is observed in plants untreated with GABA, with values ranging between 40 and 80 nmol/g. In contrast, foliar-treated plants exhibit visibly lower

MDA levels compared to GABA-pretreated plants. Similarly, in Figures 10 C and D, control-treated plants display higher MDA content than GABA-treated plants. Additionally, foliar-treated Thunder plants show lower MDA levels than GABA-pretreated plants. This suggests that GABA treatment through foliar application in plants seems to be more effective than priming the seeds with GABA. In addition, a noticeable increase in oxidative stress is present in control-treated Thunder plants exposed to 72 hours of heat stress compared the plants treated with GABA.

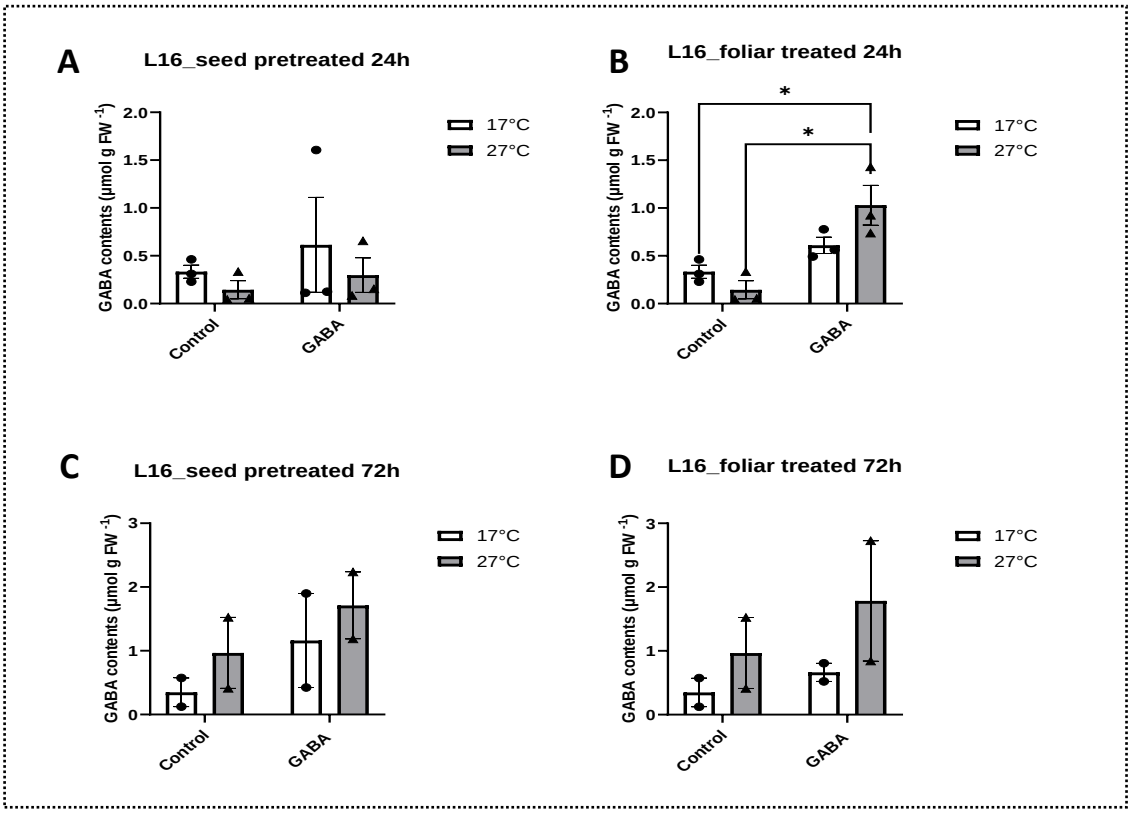


**Figure 11: Indicates the GABA level of the lentil variety L16 treated with control and GABA (seed pretreatment and foliar application) under the influence of 24 hours and 72 hours of heat stress and control condition** (A) The GABA contents (µmol/g) in control and GABA pretreated L16 lentil variety after 24 hours of heat stress. (B)The GABA contents (µmol/g) of control and foliar-treated L16 lentil variety after 24 hours of heat stress. (C) The figure indicates the GABA level (µmols/g) of control and GABA-pretreated L16 lentil varieties after 72 hours of heat stress application. (D) The GABA level (µmols/g) of control and foliar-treated L16 lentil varieties after 72 hours of heat stress application. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

The Figure 11 shows how GABA contents change in the L16 plant variety at heat stress and control condition applied for 24 and 72 hours. In Figure 11 A, for seed-pretreated plants after 24 hours, both the control and GABA-treated plants have similar GABA levels at 17°C and 27°C. In Figure 11 B, after 24 hours, foliar-treated plants have a big increase in GABA content in GABA-treated L16 replicates, especially at 27°C compared to the control treated plants. Whereas the Figure 11 C indicates that after 72 hours, seed-pretreated plants treated with GABA have higher GABA levels, particularly at 27°C. Also, in Figure 11 D, after 72 hours, foliar-treated plants exhibit higher GABA levels especially at 27°C than at 17°C. Overall, the graphs show that GABA treatment increases GABA levels in the L16 variety, which means that GABA boost the survival rates of the L16 plants with stronger effects at 27°C after 72 hours.

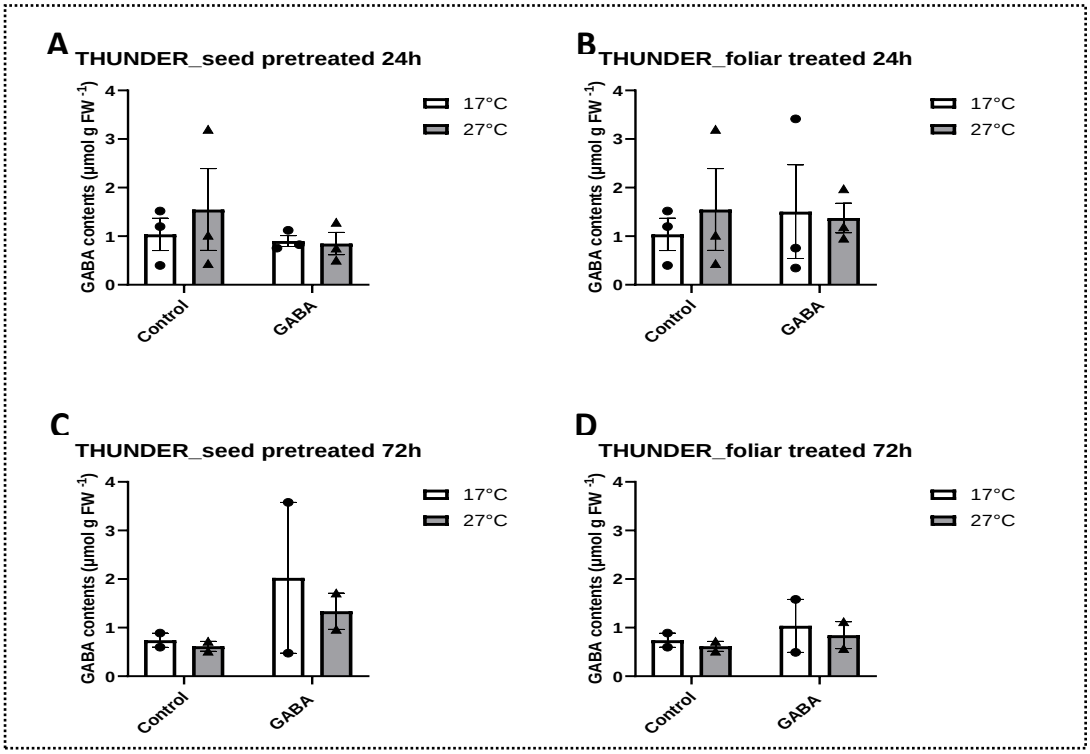


**Figure 12: Illustrates the GABA level of the lentil variety Thunder under the treatments control, GABA pretreated and Foliar treated at 17°C and 27°C for 24 hours and 72 hours.** (A) The figure depicts the GABA contents (µmol/g) in control and GABA pretreated Thunder lentil variety after 24 hours of heat stress. (B) The GABA contents (µmol/g) of control and foliar-treated Thunder lentil variety after 24 hours of heat stress. (C) The figure indicates the GABA level (µmols/g) of control and GABA-pretreated Thunder lentil varieties

after 72 hours of heat stress application. (D) Figure delivers the GABA level (µmols/g) of control and foliar-treated Thunder lentil varieties after 72 hours of heat stress application.

In Figure 12, the GABA levels in the Thunder plant variety under different treatments and temperature conditions (24 hours and 72 hours) were shown. Figure 12 A, after 24 hours, for the seed-pretreated plants there were not any visible changes at 27°C and 17°C, whereas a small increase in GABA level for the control-treated plants was noted at 27°C. In Figure 12 B, after 24 hours of foliar treatment, GABA levels were higher at 17°C than at 27°C, with only a slight difference between both the treated plants. Figure 12 C shows that after 72 hours, seed-pretreated plants have much higher GABA levels at 17°C compared to 27°C in GABA-treated plants. In Figure 12 D, after 72 hours, GABA levels are slightly higher in Foliar-treated plants at 17°C compared to 27°C. Moreover, the graphs show that GABA treatment generally increases GABA levels in Thunder plants, especially after 72 hours and more strongly at 17°C for GABA-pretreated plants.

**4. Harvesting is done for 12 weeks old 36 plant growths.**

Heat stress was applied for 72 hours and 144 hours. Out of 36 plants harvested, 12 plants were GABA pretreated (1mM),12 plants were pretreated with control (distilled water) and 12 plants were treated through foliar spray application of 5mM GABA twice that is one foliar treatment three days before and another foliar treatment was one day before heat stress application which was done for both the varieties, L16 and Thunder.

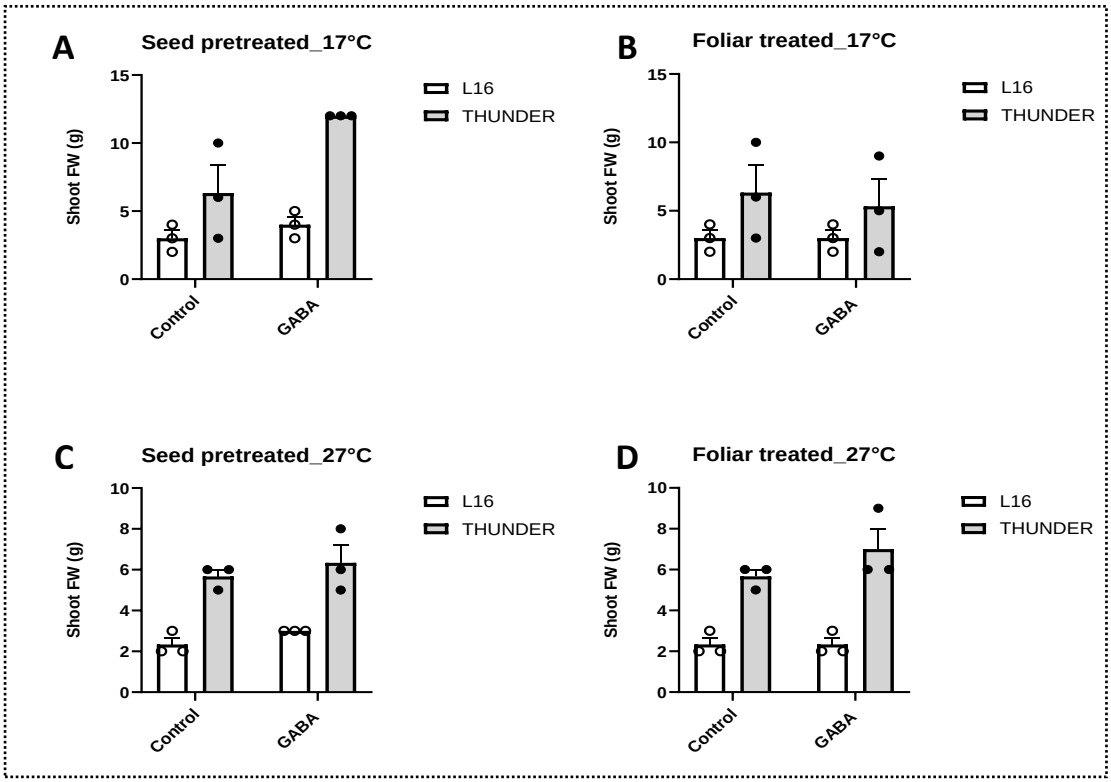

**Figure 13: The shoot fresh weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The Figure depicts the shoot fresh weight of L16 and Thunder at 17°C which was pretreated with 1mM GABA and distilled water (control). (B) The shoot fresh weight of both the lentil lines at 17°C treated by foliar application of GABA with a concentration of 5mM twice (three days and one day before heat stress application for 72 hours and 144 hours) along the leaf surface of the plants. (C) The figure presents the shoot fresh weight of L16, and Thunder pretreated with 1mM GABA and control at a heat temperature of 27°C (D) The Figure illustrating the Shoot FW(g) of the lines L16 and Thunder after undergoing the foliar treatment twice with a GABA concentration 5mM and with pretreated control.

The Figures depict shoot fresh weight (FW) in grams (g) for the L16 and Thunder plant varieties under different temperatures at 17°C and 27°C. For the Figure 13 A the Thunder variety has significantly shown higher shoot FW(g) compared to L16 variety when pretreated with GABA at 27°C. Figure 13 B shows a higher shoot FW (g) for Thunder variety when compared to L16 under foliar treatment, though no significant difference marked between the temperatures. Figure 13 C (27°C, GABA pretreatment) demonstrates that Thunder maintains a higher shoot FW (g) compared to the L16 variety with GABA pretreatment at the higher temperature (27°C), but the differences were not statistically significant. Lastly, Figure 13 D (27°C, foliar treatment) shows similar results, with Thunder having a higher shoot FW (g) than L16 under foliar treatment, but again with no significant differences. Overall, the most notable change is the significant increase in shoot FW (g) for Thunder replicates with GABA pretreatment at 17°C, highlighting the influence of both treatment and temperature on shoot growth.

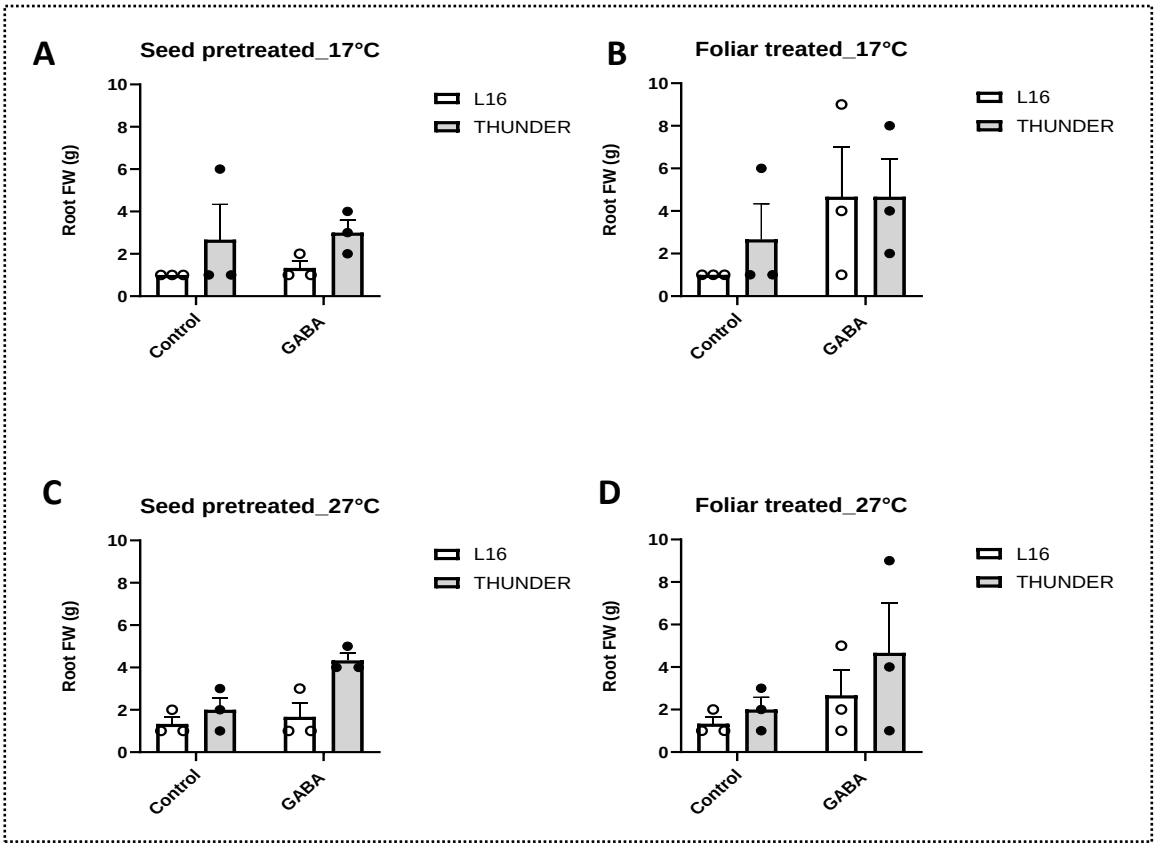


**Figure 14: The root fresh weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The Figure depicts the root fresh weight (g) of L16 and Thunder at 17°C which was treated with 1mM GABA and distilled water (control). (B) The figure shows the root fresh weight(g) of both the lentil lines at 17°C treated by foliar application of GABA with a concentration of 5mM twice (three days and one day before heat stress application for 72 hours and 144 hours) along the leaf surface of the plants. (C) The root fresh weight (g) of L16, and Thunder pretreated with 1mM GABA and control at a heat temperature of 27°C (D) The Figure illustrating the root FW(g) of the lines L16 and Thunder after undergoing the foliar treatment twice with a GABA concentration 5mM and with pretreated control.

The Figures illustrate root fresh weight (FW) in grams (g) for the L16 and Thunder plant varieties under different conditions (GABA pretreated and Foliar treated) at two temperatures (17°C and 27°C). Figure 14 A shows that at 17°C, GABA pretreatment slightly enhances root FW (g) for Thunder compared to L16. Figure 14 B at 17°C shows that foliar treatment increases root FW (g) for both Thunder and L16 than the plants treated with distilled water but there was not any statistical significance. Figure 14 C at 27°C demonstrates that GABA pretreatment boosts root FW (g) for Thunder, but the increase in L16 were not more pronounced. Finally, for the Figure 14 D at 27°C foliar treatment results in higher root FW (g) for Thunder compared to L16, although these changes were not significant as compared to

the control treated plants. Moreover, there were a visible change in Thunder replicates exhibiting elevated root FW(g) under all treatment conditions, with GABA pretreatment at 17°C and 27°C showing the most significant increase for Thunder, indicating a stronger response to these treatments compared to L16.

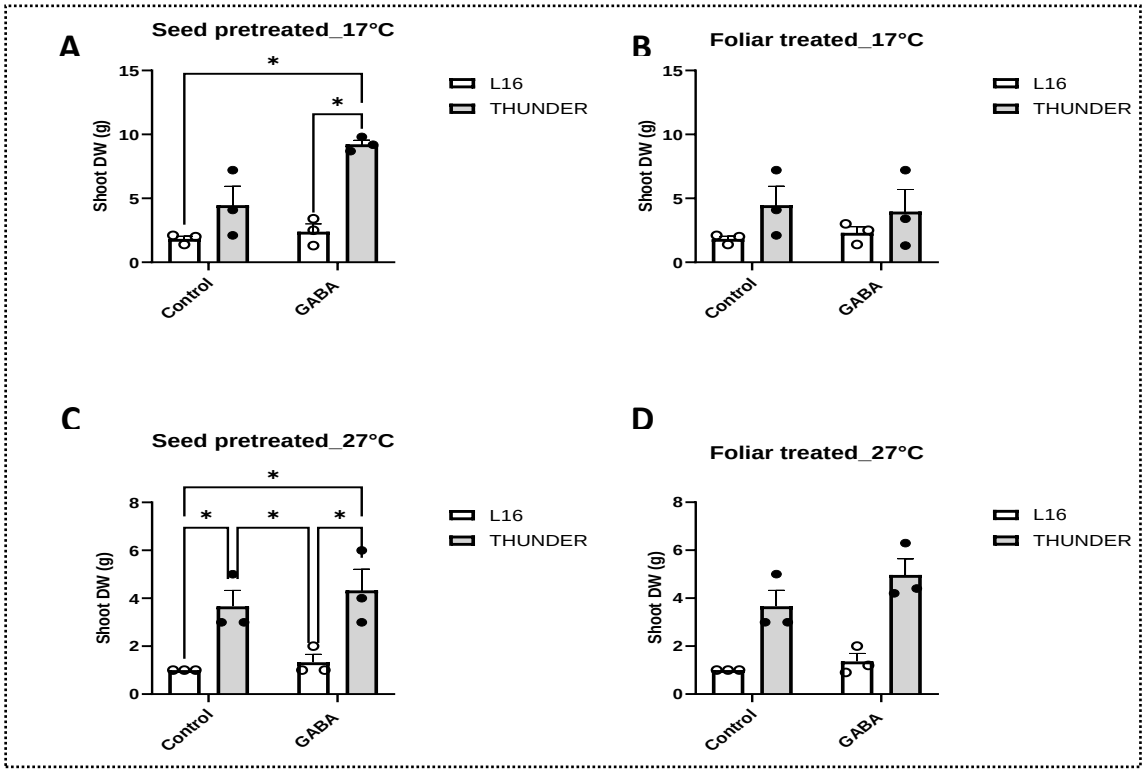


**Figure 15: The shoot dry weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The Figure depicts the shoot dry weight of L16 and Thunder at 17°C which was pretreated with 1mM GABA and distilled water (control). (B) The figure shows the shoot dry weight of both the lentil lines at 17°C treated by foliar application of GABA with a concentration of 5mM twice (three days and one day before heat stress application for 72 hours and 144 hours) along the leaf surface of the plants. (C) The shoot dry weight of L16, and Thunder pretreated with 1mM GABA and control at a heat temperature of 27°C (D) Figure illustrating the Shoot DW(g) of the lines L16 and Thunder after undergoing the foliar treatment twice with a GABA concentration of 5mM and with pretreated control. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

The Figures depict shoot dry weight (DW) in grams (g) for the L16 and Thunder plant varieties under different temperatures at 17°C and 27°C. For Figure 15 A and C, the Thunder variety has significantly shown higher shoot DW(g) compared to L16 variety when pretreated

with GABA at both the temperatures 17°C and 27°C. Figure 15 B and D shows a higher shoot DW (g) for Thunder variety when compared to L16 under foliar treatment, but there was no significant difference marked between the temperatures. Therefore, the most notable change is the significant increase in shoot DW (g) for Thunder replicates pretreated with GABA at 17°C and 27°C, highlighting the influence of both the treatments at the temperatures (17°C and 27°C) whereas GABA pretreated plants show rise shoot dry weight(g) than the lentil lines pretreated with control.

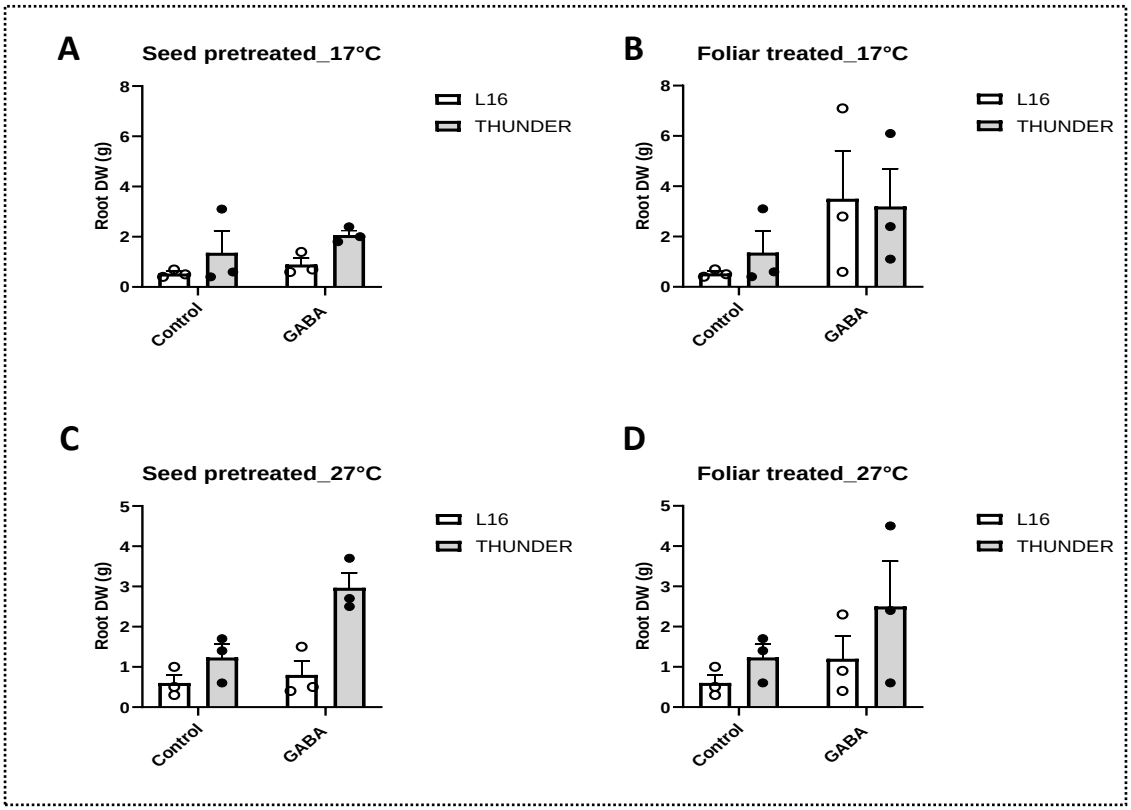


**Figure 16: The root dry weight of both lentil lines L16 and Thunder after GABA pretreatment and Foliar treatment at 17°C and 27°C.** (A) The Figure depicts the root dry weight (g) of L16 and Thunder at 17°C which was treated with 1mM GABA and distilled water (control). (B) The figure shows the root dry weight(g) of both the lentil lines at 17°C treated by foliar application of GABA with a concentration of 5mM twice (three days and one day before heat stress application for 72 hours and 144 hours) along the leaf surface of the plants. (C) The root dry weight (g) of L16, and Thunder was pretreated with 1mM GABA and control at a heat temperature of 27°C (D) The root DW(g) of the lines L16 and Thunder after undergoing the foliar treatment twice with a GABA concentration 5mM and with pretreated control.

The Figures illustrate root dry weight (DW) in grams (g) for the L16 and Thunder plant varieties under different treatment conditions (GABA pretreated and Foliar treated) at two temperatures (17°C and 27°C). Figure 16 A shows that at 17°C, GABA pretreatment slightly enhances root DW (g) for Thunder compared to L16. Figure 16 B at 17°C shows that foliar treatment increases root DW (g) in both Thunder and L16 than the plants treated with distilled water but there was not any statistical significance. Figure 16 C at 27°C demonstrates that GABA pretreatment boosted the root DW (g) for Thunder. Finally, for Figure 16 D at 27°C foliar treatment results in higher root DW (g) for Thunder compared to L16, although these changes were not significant as compared to the control-treated plants. Therefore, both L16 and Thunder treated through the foliar application (5mM GABA) revealed rise in dry weights of root.

## 5. Biochemical analysis

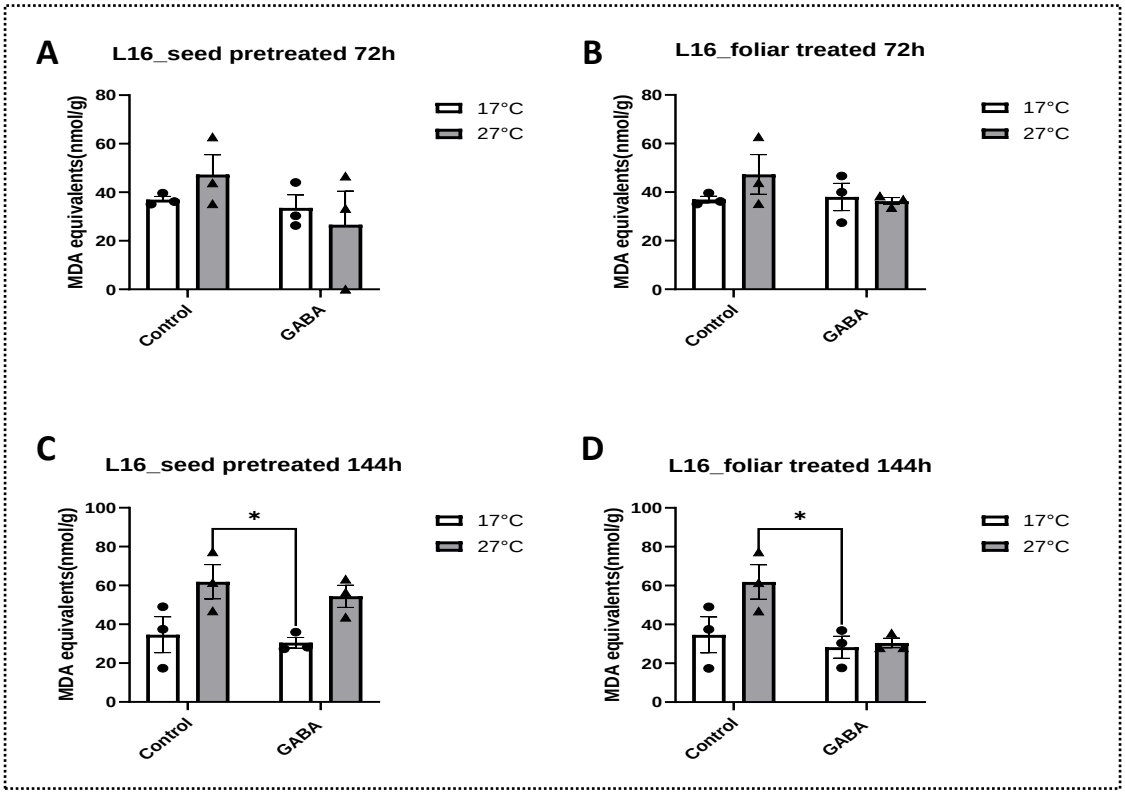


**Figure 17: The MDA level in lentil variety, L16 under control, GABA pretreated and Foliar treated under the influence of 72 hours and 144 hours of heat stress.** (A) The figure depicts the MDA levels (nmol/g) in the control and GABA-pretreated L16 lentil variety after 72 hours of heat stress. (B) The figure reveals the MDA equivalents(nmol/g) of

the control and foliar-treated L16 variety after 72 hours of heat stress. (C) The MDA levels (nmol/g) of control and GABA-pretreated L16 lentil varieties after 144 hours of heat stress application. (D) The MDA levels (nmol/g) of control and foliar-treated L16 varieties after 144 hours of heat stress application. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA).

Figure 17 shows MDA levels, which indicate oxidative stress, in the L16 plant variety under heat stress and normal temperature (17°C and 27°C) for 72 and 144 hours. In Figure 17 A and B, the L16 plants treated with control exhibited higher MDA levels (30-60nmol/g) than the GABA-treated plants after 72 hours of heat stress. In Figure 17 C and D, the control-treated plants delivered higher MDA levels at 27°C with MDA equivalents around 60nmol/g and 20-40nmol/g at 17°C (144 hours of heat stress application). These findings suggested that MDA levels and thus oxidative stress tend to increase over the duration of heat stress (144 hours) and are more pronounced at 27°C in control-treated plants. Moreover, both GABA-pretreated and foliar-treated L16 plants display comparatively lower MDA levels than control treated plants. However, foliar treatment appears to be more effective, as a more noticeable decrease in MDA levels which means less oxidative stress is found in foliar treated plants exposed with 144 hours of heat stress.

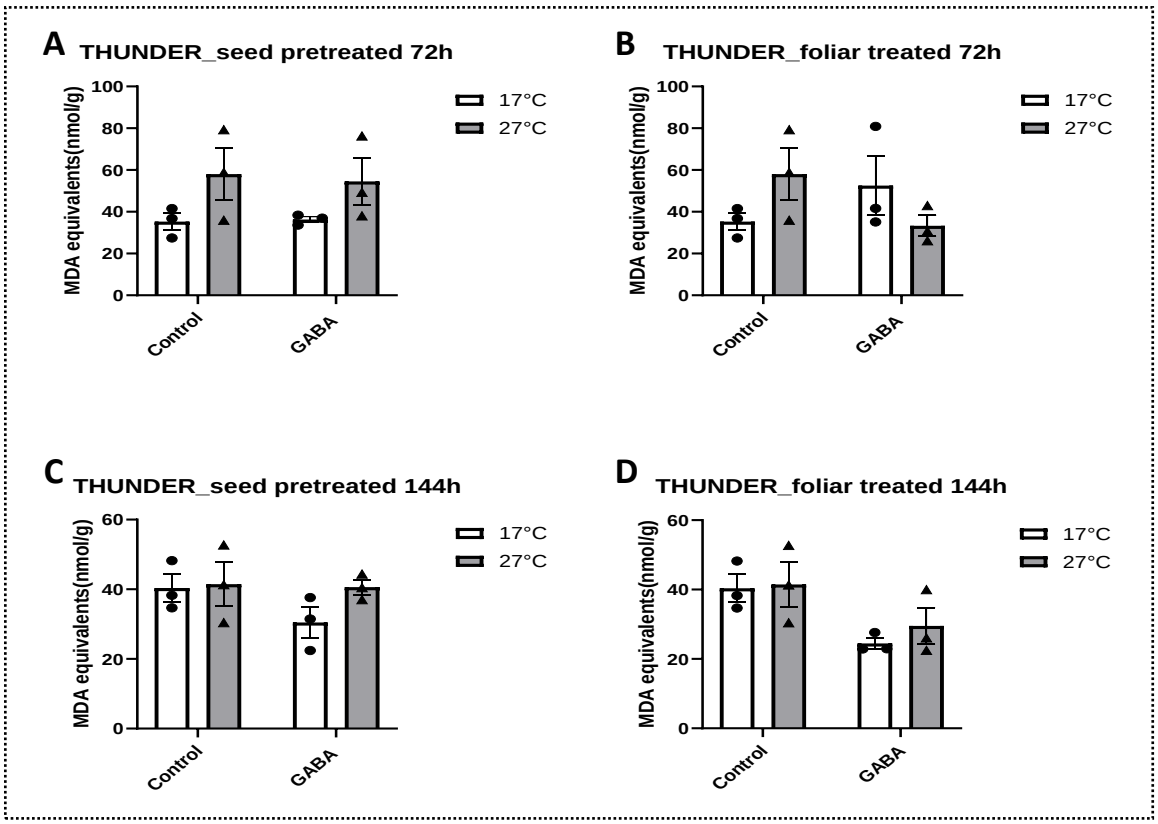

**Figure 18: The MDA level in Thunder variety treated with control and GABA (seed pretreated and foliar treated) for 72 hours and 144 hours of heat stress.** (A) The MDA levels (nmol/g) in control and GABA pretreated Thunder variety after 72 hours of heat stress. (B) The MDA equivalents(nmol/g) of control and foliar treated Thunder variety after 72 hours of heat stress. (C) The MDA level (nmol/g) of control and GABA pretreated Thunder plants after 144 hours of heat stress application. (D) The MDA levels (nmol/g) of control and foliar-treated Thunder varieties after 144 hours of heat stress application.

Figure 18 illustrates the MDA levels in the Thunder lentil variety treated with GABA and control at 17°C and 27°C for 72 and 144 hours. When comparing Figures 18 A and B, a notable increase in MDA levels is observed in plants untreated with GABA, with values ranging between 30 and 60 nmol/g. In contrast, foliar-treated plants exhibit visibly lower MDA levels compared to GABA-pretreated plants at 27°C. Similarly, in Figures 18 C and D, control-treated plants display higher MDA content than GABA-treated plants. Additionally, foliar-treated Thunder plants show lower MDA levels than GABA-pretreated plants at 17°C and 27°C. This indicated the effect of GABA treatment through foliar application in plants rather than pre-treating the seeds with GABA.

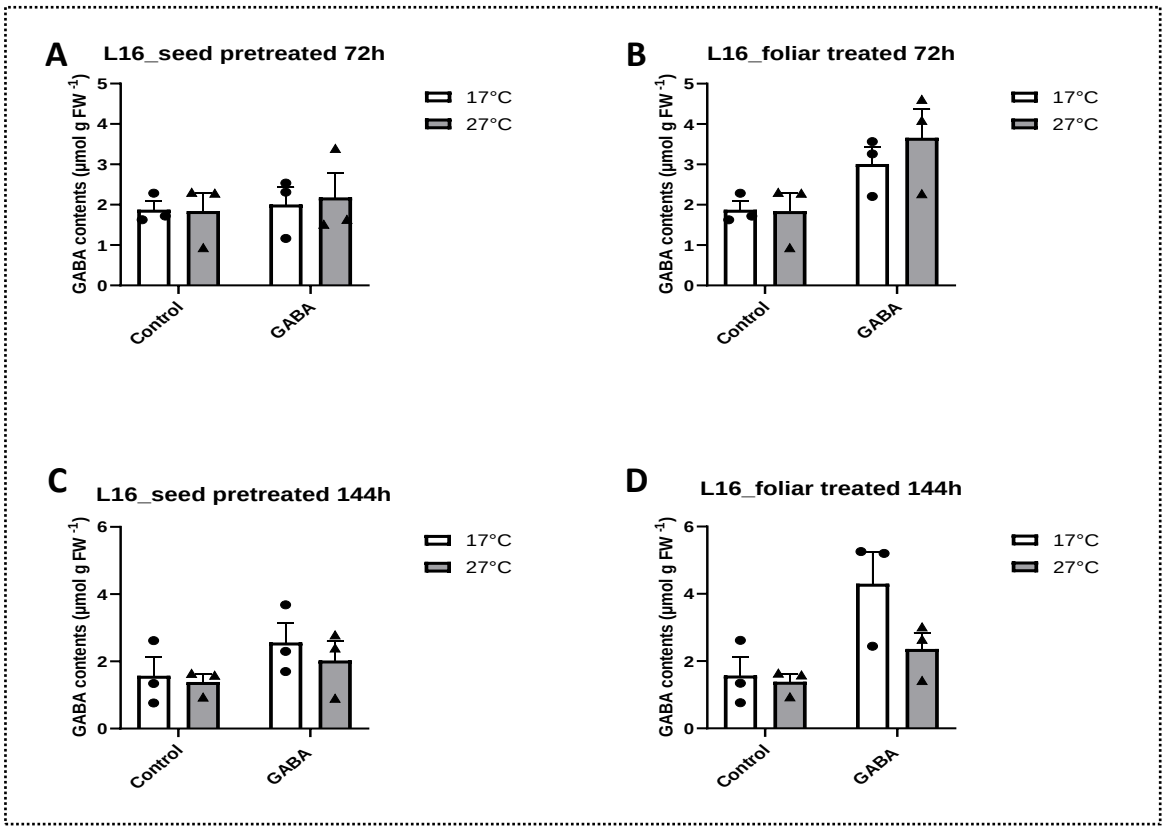

**Figure 19: The GABA level in L16 variety treated with control and GABA under the influence of 72 hours and 144 hours of heat stress.** (A) Depicts the GABA contents (µmol/g) in control and GABA-pretreated L16 lentil variety after 72 hours of heat stress. (B) Reveals the GABA contents (µmol/g) of control and foliar-treated L16 lentil variety after 72 hours of heat stress. (C) Indicates the GABA level (µmols/g) of control and GABA-pretreated L16 lentil varieties after 144 hours of heat stress application. (D) The GABA level (µmols/g) of control and foliar-treated L16 lentil varieties after 144 hours of heat stress.

Figure 19 shows how GABA contents change in the L16 plant variety at heat stress and control conditions applied for 72 and 24 hours. In Figure 19 A, both control and GABA-pretreated plants have similar GABA levels at 17°C and 27°C. In Figure 19 B, foliar-treated plants experienced higher GABA contents, especially at 27°C compared to the control-treated plants. Whereas Figure 19 C exhibited higher GABA levels in L16 replicates, particularly at 17°C when compared to the L16 plants treated with control for 144 hours of heat stress. Also, in Figure 19 D, foliar-treated plants exhibited higher GABA levels especially at 17°C than 27°C. Overall, this figure revealed an increase in GABA levels for the L16 varieties treated through the foliar spray technique (5 mM GABA). Moreover, GABA treatment enhanced the survival rates of the L16 plants with stronger effects at 27°C for 72 hours and at 17°C for 144 hours of heat application respectively.

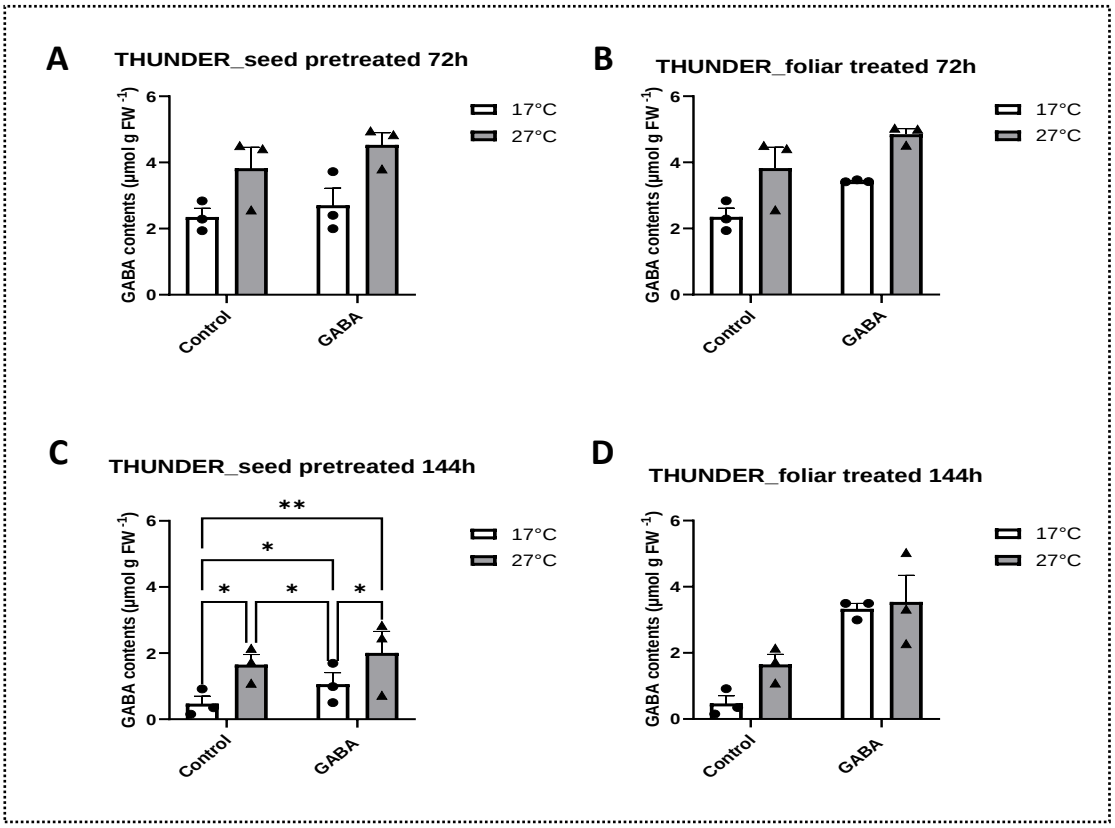

**Figure 20: The GABA level of Thunder varieties treated under control, GABA pretreated and Foliar treated for 72 hours and 144 hours of heat stress.** (A) Depicts the GABA contents (µmol/g) in control and GABA pretreated Thunder variety after 72 hours of heat stress. (B) The GABA contents (µmol/g) of control and foliar treated Thunder variety after 72 hours of heat stress. (C) The GABA level (µmols/g) of control and GABA pretreated Thunder varieties after 144 hours of heat stress application. (D) The GABA level (µmols/g) of control and foliar-treated Thunder varieties after 144 hours of heat stress application. n=3±SEM, *P<0.05, **P<0.01 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA)

In Figure 20, the GABA levels in the Thunder plant variety under different treatments and temperature conditions (72 hours and 144 hours) were shown. Figure 20 A, after 72 hours, for the Thunder plants pretreated with GABA, the GABA levels indicated were higher at 27°C when compared to 17°C. In Figure 20 B, as compared to Thunder replicates pretreated with distilled water(control), the foliar spray treatment (5mM GABA) exhibited higher GABA levels between 3.0 to 6.0 µmols/g especially more at 27°C. The GABA contents (µmols/g) are significantly elevated in Thunder plants treated with GABA, experiencing a higher GABA level at 27°C than at 17°C (Figure 20 C). Even though there is not any significance marked, the Thunder replicates treated through foliar GABA application elucidated a rise in GABA level(µmols/g) than the replicates treated with control (Figure 20 D). Therefore, the figure indicates higher GABA contents for foliar-treated Thunder plants at both the temperatures in case of 72 hours and 144 hours of heat application.

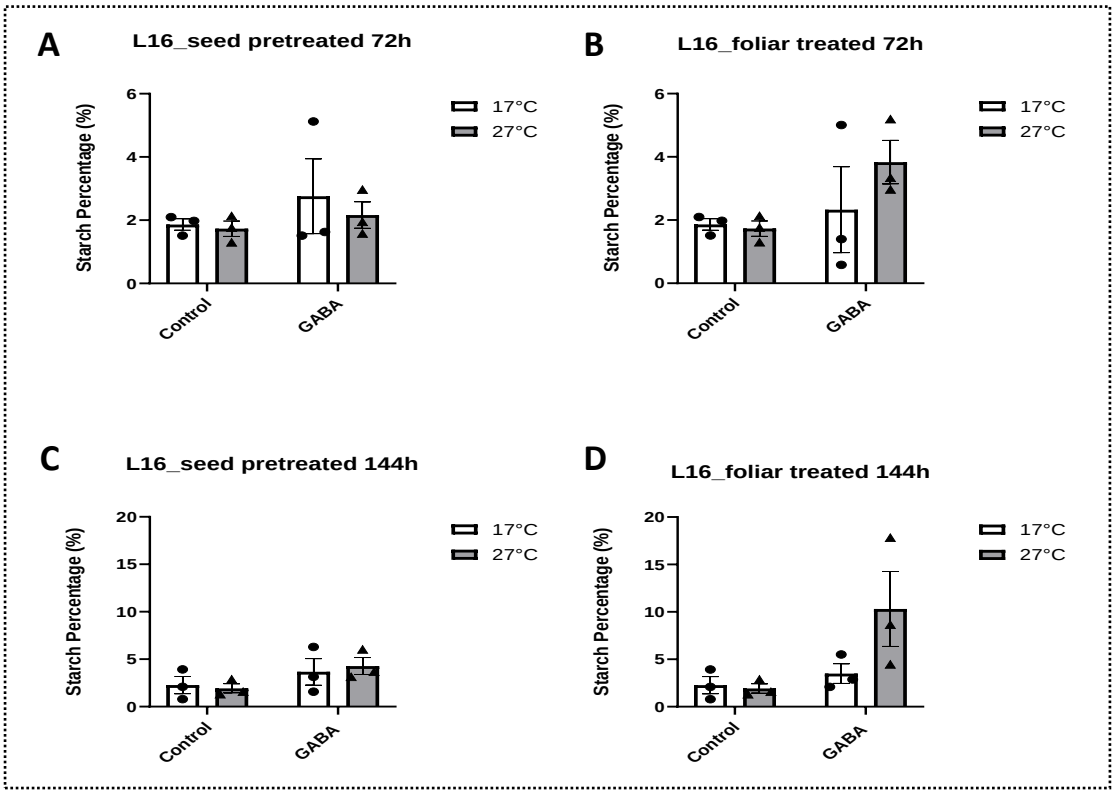


**Figure 21: The Starch percentage of L16 variety treated with control and GABA under the influence of 72 hours and 144 hours of heat stress application.** (A) The starch percentage (%) in control and GABA-pretreated L16 lentil variety after 72 hours of heat stress. (B) The starch percentage (%) of control and foliar-treated L16 lentil variety after 72 hours of heat stress. (C) The starch percentage (%) of control and GABA-pretreated L16 lentil varieties after 144 hours of heat stress application. (D) The starch percentage (%) of control and foliar-treated L16 lentil varieties after 144 hours of heat stress application.

The starch percentage (%) in the L16 varieties after being treated with GABA and control at 17°C and 27°C for 72 and 144 hours is denoted in the figure. When comparing Figures 21 A and B, a notable increase in starch content is observed in L16 plants treated with GABA at both temperatures. Whereas, when the GABA treatments are compared, the foliar application (5mM GABA) tends to show increased starch content than the seed pretreatment (1mM GABA). In contrast, when the L16 plants undergoes 144 hours of heat stress, the starch percentage increased (Figures 21 C and D). Here also, foliar treated plants with increased starch percentage (%) is visible, particularly more at 27°C.

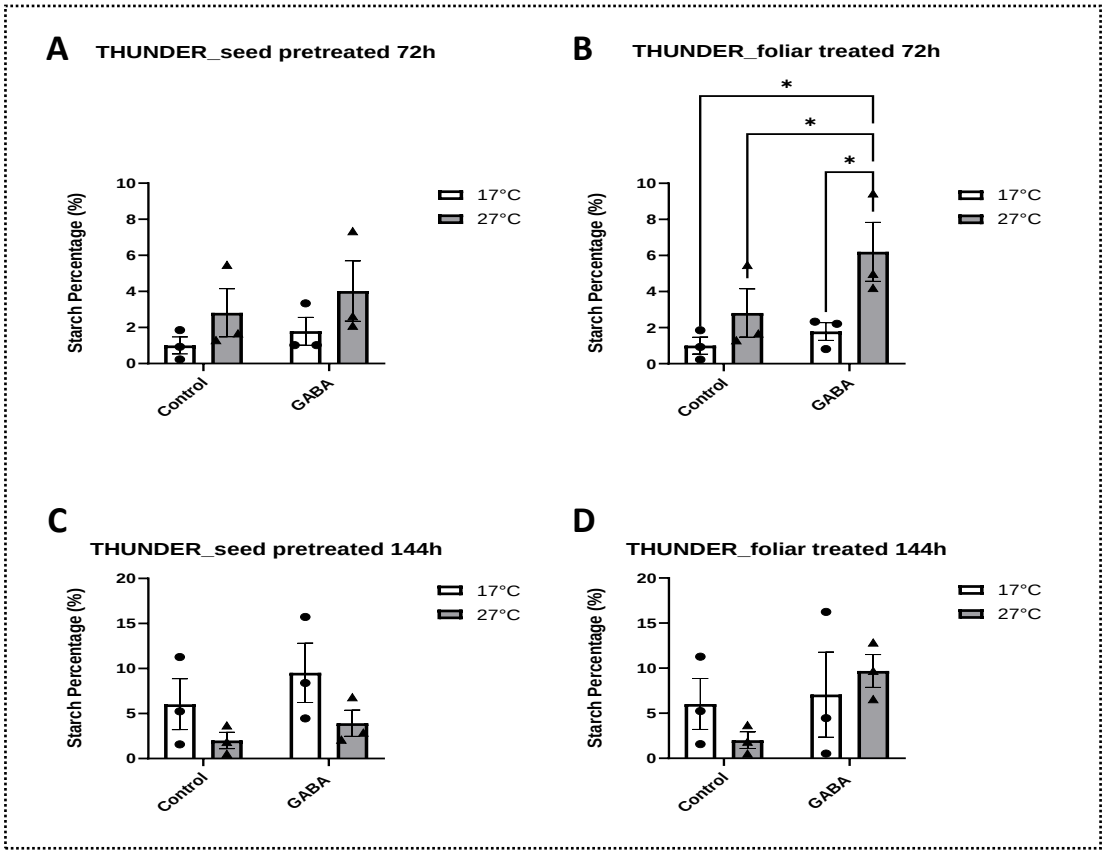


**Figure 22: The starch percentage of Thunder variety treated with control and GABA under the influence of 72 hours and 144 hours of heat stress.** (A) The starch percentage (%) in control and GABA pretreated Thunder variety after 72 hours of heat stress. (B) The starch percentage (%) of control and foliar treated Thunder variety after 72 hours of heat stress. (C) The starch percentage (%) of control and GABA-pretreated Thunder replicates after 144 hours of heat stress application. (D) The starch percentage (%) of control and foliar-treated Thunder varieties after 144 hours of heat stress application. n=3±SEM, *P<0.05 (Figure made with the help of Figure pad prism. Statistical analysis was done by using two-way ANOVA)

The starch percentage (%) in the Thunder varieties after being treated with GABA and control at 17°C and 27°C for 72 and 144 hours is denoted in the figure. When comparing Figures 22 A and B, a significant increase in starch content is marked in Thunder plants treated with GABA through foliar technique at the temperature of 27°C. Whereas Thunder plants treated with GABA indicated higher starch content than the replicates treated with control, it did not show any significance like foliar-treated plants. For the 144 hours, the starch percentage (%) in foliar treated plants was raised at 27°C and the GABA pretreated plants exhibited higher starch contents at 17°C.

# CHAPTER 4: DISCUSSIONS

From the pilot experiment, the optimal GABA concentration and the most effective lentil variety were identified. It was determined that 1 mM GABA was the most effective concentration, compared to other concentrations (10 µM and 100 µM). When both lentil lines were compared, a significant difference was observed in L16 between the GABA-treated and untreated plants. The L16 plants treated with 10 µM, 100 µM, and 1000 µM of GABA showed a significant increase in root fresh weight (g) compared to the control-treated plants.

It has been reported in similar studies that the germination rate in lettuce, citrus and wheat is positively affected by exogenous GABA treatment (Kalhor et al., 2018, Ziogas et al., 2017) (Suhel et al., 2023). While a range of GABA concentrations was tested in this study, it remains unknown whether a higher concentration of GABA could produce even more favourable conditions for germination and early growth; this should be explored in future work.

For the main experiment, the L16 lentil variety, previously used in the pilot experiment, was selected for further treatment. The wild lentil variety, Thunder, was chosen instead of L13, with the expectation of better growth after GABA treatment, as shown in Figure 4 B. Plants, 4 weeks old, were harvested after exposure to heat stress and control conditions (17°C and 27°C). From the harvested plants, the fresh weight (FW) and dry weight (DW) of roots and shoots were measured to assess plant responses under heat stress and control conditions.

As shown in Figure 5 A, GABA pretreatment significantly increased shoot fresh weight (FW) in the Thunder variety at 17°C, promoting plant growth. Both lentil lines pretreated with GABA demonstrated increased shoot FW compared to the control plants at both temperatures. The effect of foliar treatment at 27°C was observed to be smaller and less significant. GABA pretreatment was found to have a stronger impact on shoot FW, especially for Thunder at 17°C.

For root fresh weight, as seen in Figure 6 A, a notable and significant increase was observed in GABA-pretreated Thunder replicates compared to the control-treated L16 replicates. When examining dry weight (Figure 7 C), the Thunder variety pretreated with GABA showed significant increases in shoot DW at 27°C when compared with the L16 variety treated with

control. Root DW also showed a greater increase in the GABA-pretreated Thunder variety than in the control-treated L16 at 17°C (Figure 8 A).

The studies indicate that the root length, shoot length, and both fresh and dry weights of maize (*Zea mays L.*) seedlings were greatly enhanced by exogenous GABA when subjected to different abiotic stresses (Wang et al., 2017). Pre-treatment with GABA-enhanced seed germination in pepper (*Capsicum annuum L.*) by increasing the germination percentage, germination rate, seedling length, fresh and dry weights of seedlings (Al-Quraan et al., 2024).

Therefore, while observing the FW and DW of 38-day-old plants undergoing heat and control, the Thunder variety seems to show higher FW and DW than L16 at both temperatures. Also, GABA-pretreated plants showed better results than the Foliar treated plants when compared with control at both temperatures (17°C and 27°C). The fresh weight and dry weight of a plant's root and shoot were measured under heat stress and control conditions to assess the response of plants towards stress. Fresh weight is recorded to show the actual size and water content of the plant, while dry weight is measured to determine the actual mass of the plant without water, helping to evaluate its growth and biomass (Rivero et al., 2004).

The collected tissue samples were used to analyse the ROS and GABA levels in the plants. Therefore, the TBARS assay was conducted to examine ROS levels, and the GABA assay was performed to assess GABA content. The TBARS assay is used to measure lipid peroxidation, indicating oxidative stress in cells. When lipids in cell membranes are attacked by ROS, byproducts like MDA are produced. These byproducts, particularly MDA, are detected in the TBARS assay by being reacted with TBA (Jane and Knight, 2002).

The decrease in MDA level in late-sown lentils foliar treated with micronutrients (Zn, Fe, and B) suggests that the potential to alleviate stress exists through the activation of the enzymatic response (Venugopalan et al., 2022). The combined treatment of heat priming and GABA on lentil (*Lens culinaris*) significantly upregulated the photosynthetic function, endogenous GABA, proline, glycine betaine, their biosynthetic enzymes, sucrose, and its biosynthetic enzyme (Bhardwaj et al., 2021). In mungbean (*Vigna radiata L.*) plants, a significant reduction in MDA and $H_2O_2$ levels was observed with GABA treatment, along with improved antioxidant activities in anthers and leaves. Additionally, the synthesis of osmolytes was upregulated, and carbon fixation and assimilation were enhanced to help maintain leaf water status under heat stress(Priya et al., 2019).

There was no significant difference in MDA levels between the GABA-treated and control plants for both the L16 and Thunder variety. The foliar-treated L16 and Thunder plants had lower MDA levels compared to the GABA-treated ones. Which means the foliar application of GABA along the leaf surface makes the plant to experience lower oxidative stress than the seeds which were treated with GABA before planting. The L16 and Thunder plants which undergoes 24 hours of heat stress exhibited lower MDA levels compared to those exposed to 72 hours of heat stress. In the case of both the treatment periods of plant growth (4 weeks and 12 weeks) for both the lentil lines. As expected, both L16 and Thunder treated with 5mM GABA (foliar application) inhibiting the MDA level in plants points out, that GABA treatment was effective in reducing oxidative stress and preventing cellular damage caused by heat stress.

The GABase enzyme assay was conducted to evaluate the GABA (Gamma-Aminobutyric Acid) level inside the plants. As GABA is a natural signalling molecule that is found in plants, it helps the plants to tolerate adverse stress conditions such as extreme temperatures, high salinity, drought and so on(Ren et al., 2021). High GABA levels are beneficial for plants as they reduce the ROS level in plants boosting the growth and the survival rates of the heat-stressed plants.

There can be a significant increase in GABA levels for the foliar-treated L16 plants when compared with the control-treated plants. Whereas there was not much significance between the GABA pretreated and control. In the case of the Thunder variety, there were not many changes between the control and GABA treated. Moreover, foliar-treated ones show higher GABA levels than the Thunder seeds pretreated with GABA. In addition, for the 12 weeks, lentil lines were treated with GABA and control exposed for 72 and 144 hours of heat stress. A visible significant change was seen in Thunder varieties treated with GABA at 27°C. Moreover, the Foliar GABA application is an effective method for enhancing the endogenous GABA contents in plants which promotes growth and reduces the stress-induced damages in plants.

An increased level of internal GABA was observed in wheat (*Triticum aestivum L.*) seeds that were primed with exogenous GABA under heat and salinity stresses (Yu et al., 2023). Similarly, an increase in endogenous GABA and glutamate contents was caused by the exogenous treatment of apple (*Malus domestica, c.v. Cripps Pink*) fruit with GABA under drought stress (Cheng et al., 2023). GABA content in kiwifruits (*Actinidia spp*.) was

decreased by heat stress, while an application of exogenous GABA resulted in elevated GABA levels up to 3.36 times higher than the untreated fruits. Although the glutamate level remained unchanged under normal conditions, it was significantly increased following the application of GABA in response to heat stress (Huo et al., 2023).

Starch is a key molecule that modulates plant responses under heat stress. Under such stress conditions, the photosynthetic rate, starch accumulation, and expression of enzymes involved in starch biosynthesis are altered in plants (Thalmann and Santelia, 2017).Both the lentil lines treated with foliar GABA application exhibited a rise in starch content, especially the Thunder replicates (Figure 22 ). Moreover, foliar-treated lentil lines showed high starch percentage at heat temperature which means by foliar application the GABA is easily absorbed by plants and improves the stress tolerance thereby enhancing the growth and yield.

Several limitations were identified in the study, indicating the need for further investigation. Results from biochemical assays indicated that the foliar application method was more effective than GABA seed priming. However, further studies should be conducted to determine the practical and effective GABA concentration for foliar treatment for crop yields. Also, the ideal frequency of application is also important for growth improvements of plants under heat stress.

The findings from this research indicate that, in comparison to untreated plants, the application of GABA to lentil plants effectively increased their resistance to heat stress, enhancing growth, physiology, and yield. This effect became evident through the measurements of germination percentage, root length, shoot length, fresh weight, dry weight, ROS levels, GABA contents, and starch percentage in GABA-treated plants. When comparing the effects of foliar treatment and seed priming with GABA across two lentil varieties, the assays demonstrated that foliar treatment was more effective than seed pretreatment. Thus, the lentil plants supplemented with GABA enhance their tolerance to heat stress as stated in the hypothesis.

Future research should focus on optimizing GABA concentration and application methods in lentils to enhance resilience and yield. Exploring molecular and physiological pathways activated by GABA under stress could guide genetic or biochemical strategies for improved heat tolerance. Longer-term studies under field conditions should assess GABA's efficacy in diverse environmental settings, identifying lentil varieties most responsive to treatment. Examining interactions between GABA and other bio stimulants or micronutrients could

provide greater protection against heat stress. Assessing crop yield, quality, and nutritional composition can also help support resilient crop production under variable climates.

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# APPENDIX

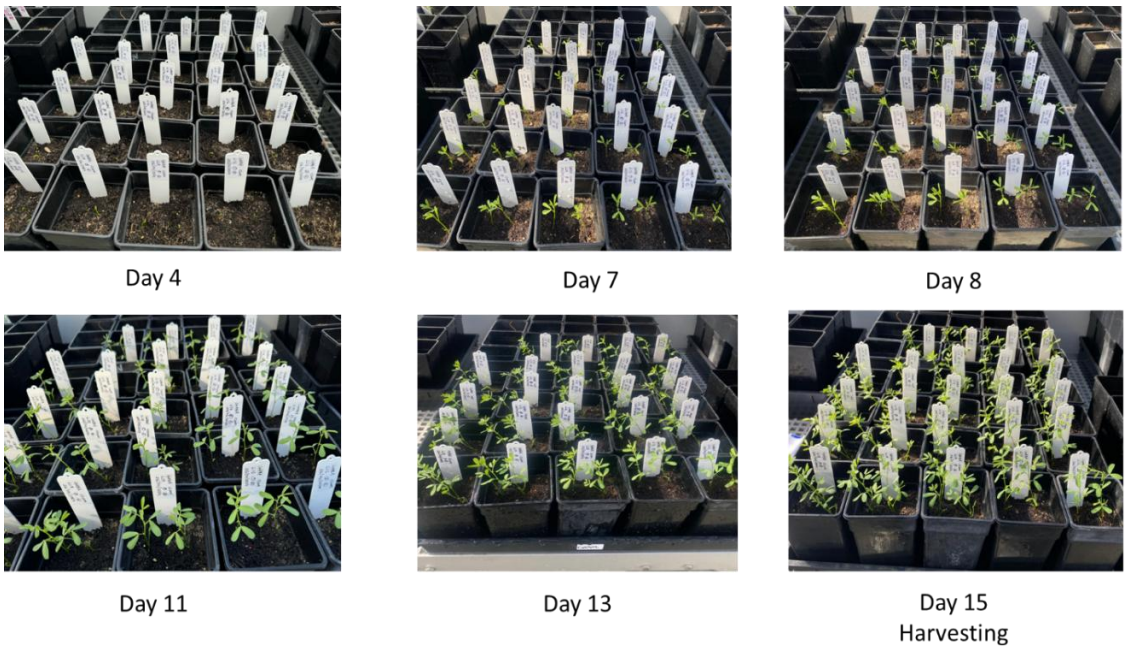


**Figure 1:** Images show the growth of L13 and L16 varieties treated with GABA (10µM,100µM,1000µM) and control.

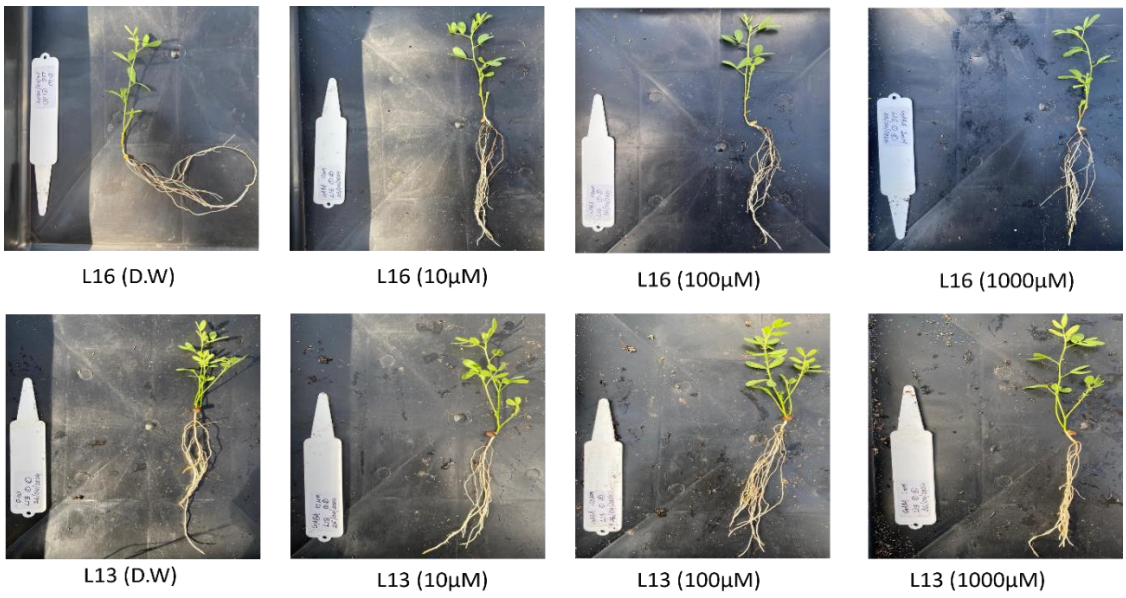


**Figure 2:** Plants harvested for measuring the fresh biomass and dry biomass of shoot and root of both the varieties L16 and L13.

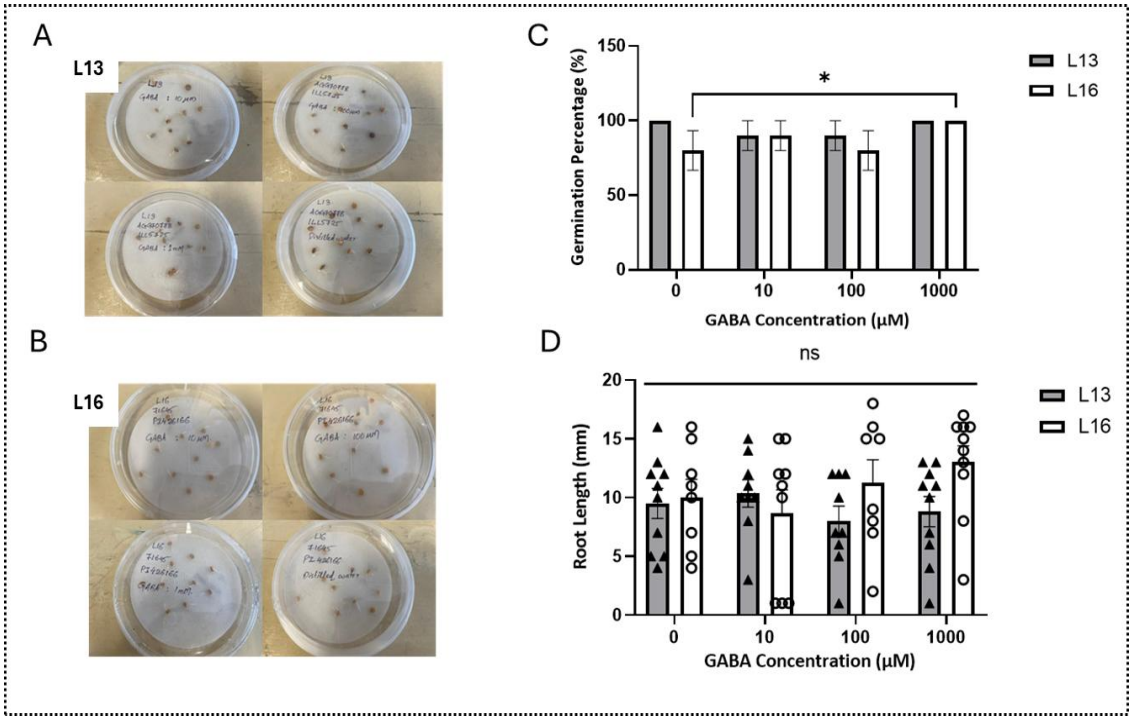


**Figure 3:** Germinated lentil seed varieties after GABA (10µM,100µM,1000µM) and control (distilled water) treatments.

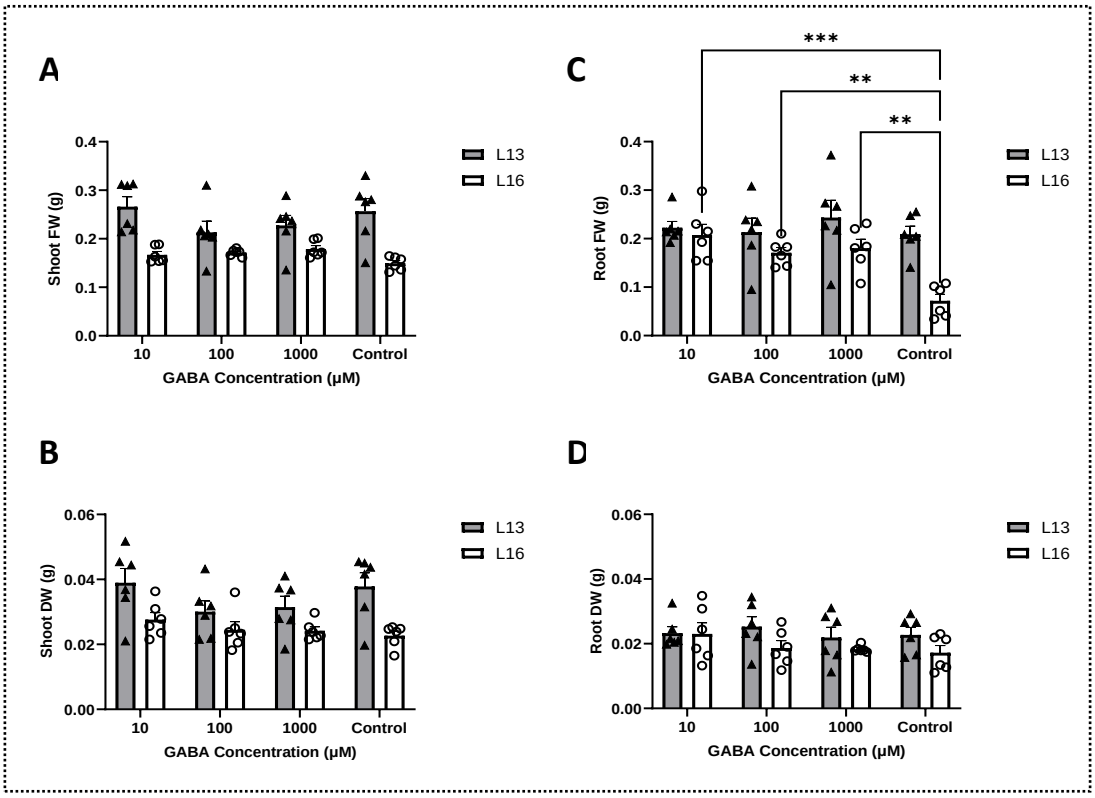


**Figure 4:** The fresh and dry weights of the roots and shoots of the harvested lentil lines L13 and L16 were shown.

**Figure 5:** Images showing the plant growth for 4 weeks and 12 weeks.


**Figure 6:** Plants harvested for measuring the fresh biomass and dry biomass of shoot and root of both the varieties L16 and Thunder treated with GABA and control.

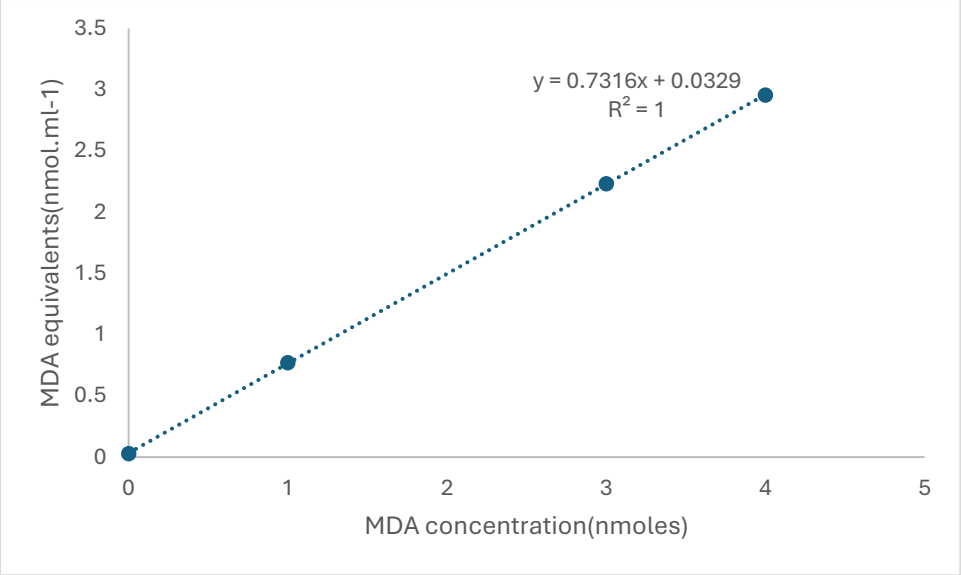


**Figure 7:** The standard curve with MDA equivalents(nmol.ml$^{-1}$) plotted against MDA concentration(nmoles).

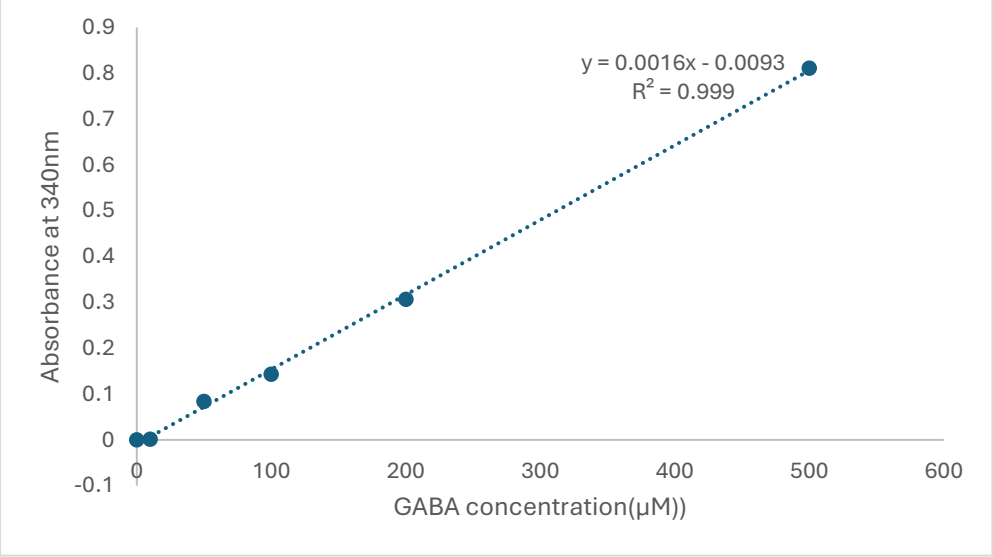


**Figure 8:** The standard curve with absorbance at 340nm plotted against GABA concentration(µM).