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Effect of Methyl Salicylate on the Physiological and Biochemical Response of Wheat To Cold Stress

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TABLE OF CONTENTS

List of Figures and Table

List of Abbreviations

ABA	Abscisic acid
AOX	Antioxidant enzymes
APX	Ascorbate peroxidase
CAT	Catalase
GPX	Guaiacol peroxidase
GR	Glutathione reductase
HIPVs	Herbivore-induced plant volatiles
JA	Jasmonic acid
MeSA	Methyl salicylate
POD	Peroxidase
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
VOC	Volatile organic compound
NADPH	Nicotinamide adenine dinucleotide phosphate
CDNB	1-chloro-2,4-dinitrobenzene
GSSG	Glutathione disulfide

1.0 INTRODUCTION

1.1 Background Study

Sustainable crop yield and food security are the two most important challenges in agriculture because their precise management helps to satisfy the hunger of the world's ever-growing population (Xiao et al. 2013, Kou et al. 2018). The primary focus of such sustainable agriculture is typically kept on cereal crops, which produce the staple food for the entire world's population (Sarwar et al. 2013). All cereal crops are members of the Poaceae plant family (Gramineae). Apart from their nutritional value, Laskowski et al. (2019) claim that cereals also regulate the highest proportion of the agricultural economy in all countries. According to Arvanitoyannis & Tserkezou (2008), *Oryza sativa* (rice), *Triticum aestivum* (wheat), *Zea mays* (maize), *Hordeum vulgare* (barley), *Sorghum bicolor* (sorghum), *Setaria italica* (millets), and *Avena sativa* (oats) produce nearly the entire world's staple food. Mohammed et al. (2020) opine that these crops are more widely cultivated than any other group of crops worldwide. Cereal grains are high in carbohydrates, fiber, and essential amino acids such as methionine and threonine, which are found in storage proteins (Arvanitoyannis & Tserkezou, 2008).

Climate change is among the core problems of recent times, as it is threatening global food security (FAO, 2020). According to the Intergovernmental Panel on Climate Change, extreme temperature events have significantly increased over the past few decades (IPCC, 2014). Yadav (2010) claims persistent cold extremes halt plant growth by causing mechanical injury and metabolic dysfunction through ice crystallization. Most of the wheat-growing areas of the world often undergo low-temperature stress, such as China (Xiao et al. 2018), the United States (Holman et al. 2011), Europe (Trnka et al. 2014), and Australia (Zheng et al. 2015, Crimp et al. 2016). Even though some regions noticed reduced winter duration because of global warming, plant ecologists revealed a paradoxical connection between plant growth and climatic variations, confirming that an upsurge in warm climate increased the risk of cold injury to plants (Gu et al. 2008).

With 225 million ha under cultivation worldwide, wheat (*Triticum aestivum*) is the crop with the largest global land area (FAO STAT 2009). Wheat is widely cultivated in temperate regions of China, the United States, India, and Russia with 30-90 cm of rainfall, and is used to produce flour, bread, malt, dextrose, gluten, and alcohol (Shewry 2009). Wheat provides nearly 20% of the total

dietary calorie and protein requirements for 4.5 billion people, with a total harvesting area of 215.9 million hectares (FAO 2019). Dinu et al. (2018) claim that because unpolished wheat is high in thiamine, niacin, and riboflavin, it is widely regarded as a nutritious food crop.

1.2 Problem Statement

Despite the numerous benefits of wheat, the plant is always significantly affected by stress conditions, especially abiotic stressors. These stressors adversely affect the morphological, physiological, and biochemical processes in the wheat plant. This, when prolonged, results in the death of plant cells and consequently reduced yield. Notably, the sensitivity of plants to cold led to over 78 percent yield decline in wheat and 30–40 percent in rice. Disturbance in reproductive processes which precedes the damage of grain formation process is said to be the cause of these losses (Andaya & Mackill 2003). In 2009, Japan suffered a severe ruin in crop production worth 58 billion yen. This was attributed to the adverse impact of climate change (Rahman 2013).

Abiotic stressors, such as cold stress, adversely affect the growth and yield of plants as well as their distribution by causing oxidative stress, which is characterized by the overproduction of reactive oxygen species (ROS) such as superoxide radicals (O2-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and singlet oxygen (1O2) (Ding et al. 2019, Waszczak et al. 2018). Zandalinas et al. (2020) added that during abiotic stress, increased activity of the membrane-bound enzyme nicotinamide adenine dinucleotide phosphate (NDPH) oxidase causes the production of reactive oxygen species, which then disrupts cellular membranes (plasma membrane, chloroplast membrane, mitochondrial membrane, and nuclear membrane) by accelerating lipid peroxidation. The loss of plasma membrane integrity invariably results in electrolyte loss and cell death (Kollist et al. 2019, Decker et al. 2020).

The synthesis of antioxidants in plants in response to oxidative stress is a natural defense mechanism that can be artificially influenced using external hormones and elicitors. It is worth mentioning that this natural mechanism has not been found efficient enough in protecting plants against stressful conditions. Salicylic acid (SA) is considered a plant hormone as well as an elicitor. SA plays a significant role in developmental/physiological events like germination, ethylene synthesis, fruit ripening, systemic acquired resistance (SAR), and stomatal closure (Miura & Tada, 2014, Khan et al. 2015, Klessig et al. 2016). Multiple derivatives of SA are found in plants.

Examples are methyl salicylate (MeSA), amorfrutins, benzyl salicylate, and isoamyl salicylate. Methyl salicylate (MeSA) is a known volatile biological compound synthesized from salicylic acid (SA) and a plant hormone that helps defend against pests and pathogens. However, little is known about its role in inducing defense mechanisms in plants against abiotic stressors like cold stress.

1.3 General Objective

This work, therefore, sought to unearth the effect of methyl salicylate (MeSA) on wheat's (*Triticum aestivum*) physiological and biochemical responses to cold stress. Special attention was given to the measurement of some antioxidants such as glutathione reductase (GR), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), glutathione s-transferase (GST), as well as malondialdehyde (MDA) content as an indicator of lipid peroxidation.

1.4 Specific Objectives

1. To assess the effect of methyl salicylate on the physiological response of wheat to cold stress.
2. To assess the effect of methyl salicylate on the biochemical response of wheat to cold stress.

1.5 Hypothesis

1. Methyl salicylate can reduce lipid peroxidation in wheat under cold stress.
2. Methyl salicylate can enhance the production of antioxidant enzymes in wheat under cold stress.

2.0 LITERATURE REVIEW

2.1 Wheat (*Triticum aestivum*) Production

2.1.1 Origin and Botanical Description

The most widely grown cereal crops were wheat, maize, and rice, accounting for nearly 90% of all cereal crops produced globally (FAOSTAT 2021) and providing roughly two-thirds of the calories in human diets (Cassman 1999). Wheat (*Triticum aestivum*) is an annual grass that has a long stalk that ends in a closely packed cluster of plump kernels surrounded by a beard of bristly spikes. It grows between 500 and 125 centimeters in height (Smith 2010). It is a member of the Poaceae family, which comprises between 330 and 360 species of several valuable cereal crops like wheat, barley, and rye (Watson & Dallwitz 1994). Around 9600 BCE, according to the archeological record, wheat cultivation began in the Fertile Crescent. The wheat kernel is a type of fruit known as caryopsis, in botany. The Tigris- Euphrates drainage basin of South Asia is where wheat originates. The genus Triticum includes numerous types of wheat, with common wheat being the most extensively cultivated (T. aestivum). Common wheat, commonly known as bread wheat (Triticum aestivum), durum wheat (*Triticum turgidum* ssp. Durum), einkorn (*Triticum monococcum*), emmer (*Triticum dicoccum*), and spelt (*Triticum spelta*) are the main forms of wheat, according to purpose.

According to Jolankai (2004), most of the winter wheat seeds produced in Hungary are sown by domestic farmers, but export revenue is also substantial. Germination is an important phase in the life of wheat plants. It is a physiological process that involves a series of biological and biochemical events that start and nurture a seedling. It controls seedling establishment and ensures that nutrients and water resources are used effectively (Kende et al. 2017, Shah et al. 2019, Tarnawa et al. 2021). The meteorological conditions of the specific crop year, particularly the amount and distribution of precipitation and the actual temperature have a significant impact on the quantity and quality of grain production of winter wheat *Triticum aestivum* L. (Gyri 2008, Pepó 2010).

2.1.2 Ecological Requirement

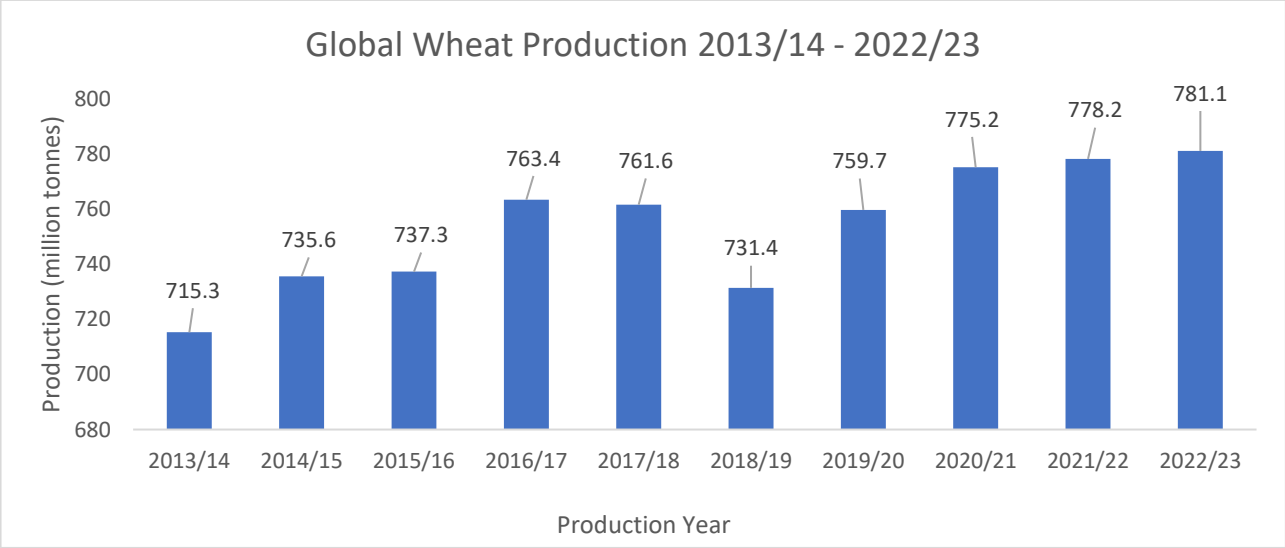
Sowing is usually done in mid-October-mid-November when the temperature is between 10°C and 15°C and harvested in March at the temperature range of 21°C and 26°C. Wheat grows best in

well-drained loamy soil under cool, moist climates and ripens in warm, dry climates. As a Rabi crop, wheat prefers those areas where rainfall occurs in winter. Annual average rainfall of 50cm to 100cm can ensure optimum growth. However, to attain full maturity with a better-quality yield, wheat requires a cloudless sky with bright sunshine during ripening and harvesting periods.

2.1.3 Production Trend

According to OECD/FAO (2021), the global wheat output is anticipated to rise from 87 million tons (Mt) to 840 Mt by 2030, a growth that will occur at a moderate rate compared to the previous ten years. In response to national plans to increase self-sufficiency in wheat, India, the third-biggest producer of wheat in the world, is anticipated to provide the largest portion of the increased wheat supply, expanding its wheat production to 18 Mt by 2030. However, it is projected that China will produce the most wheat by 2030. In comparison to the base period of 2018–2020, wheat consumption is anticipated to rise by 12% by 2030. Nearly half of this rise comes from four nations: India (+18 Mt), China (+15 Mt), Pakistan (+6 Mt), and Egypt (+4 Mt). The expected increases in the global usage of wheat for food and feed are 58 Mt and 22 Mt, respectively. By 2030, it is anticipated that wheat exports will increase from 36 Mt to 220 Mt, with 14% of global commerce coming from the European Union, the second-largest wheat exporter.

Figure 1: Global Wheat Production 2013/2014 – 2022/2023



Data from the Food and Agriculture Organization of the United Nations, FAO, December 2022 https://www.fao.org/worldfoodsituation/csdb/en/

2.2 Abiotic Stressors

Abiotic stresses can be edaphic, such as salt, drought, and heavy metal toxicity, or they can be atmospheric, such as heat, cold, and UV radiation (Wani & Gosal, 2011, Surekha et al. 2015). In steep terrains, the most significant environmental issue limiting agricultural expansion and crop output is cold stress (Sanghera et al. 2011). By causing oxidative stress which is characterized by an excess of reactive oxygen species (ROS) such as superoxide radicals (O2), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and singlet oxygen (1O2). Waszczak et al. (2018) argue that these stressors can significantly slow crop growth and development.

The length and severity of the stress, the plant's growth stage, and the timing of the stress exposure all affect how the plant reacts to it (Gupta & Sheoran 1983). According to Ruelland et al. (2009), plants have an amazing ability to adjust to challenging environmental circumstances and survive in environments with abiotic challenges like temperature extremes. According to Bohnert et al. (1995), plants adapt to these pressures through a variety of processes, including modifications to their morphological and developmental patterns as well as alterations to their physiological and biochemical responses.

2.2.1 Cold Stress

Spring frost often occurs in March and April during the early booting stage and affects 85% of the wheat planted worldwide each year (Yue et al. 2016). In the spring, wheat suffers significant frost damage when the canopy temperature drops below 0°C or lower (Frederiks et al. 2015, Zheng et al. 2015). Origin of the crop (tropical or temperate), species type, development stage (vegetative or reproductive stage), plant organs (shoots or roots), length of cold stress, and other environmental factors all affect how severe the impacts of cold stress are on crops (Leipner & Stamp 2009, Farooq et al. 2009/a, 2009/b).

Low temperatures that are not below freezing impair plant development physiology by causing chilling injuries such as photosynthesis-related harms, chlorosis, uncontrolled apoptosis, loss of membrane fluidity, and finally wilting (Wani et al. 2016). According to the degree of sensitivity, Wani et al. (2013) separated cold stress into two categories: chilling stress, which is defined as temperatures between 0°C and 15°C, and freezing stress, which is defined as temperatures below 0°C. Compared with their tropical and subtropical counterparts, temperate climatic plants show a

wider range of cold tolerance under cold acclimation and associated changes at the molecular and biochemical levels (Yamaguchi-Shinozaki & Shinozaki 2006). This cold acclimation in wheat plants involves modifications to a range of physical and physiological processes that enable survival at low temperatures, including the induction of antifreeze proteins (Yeh et al. 2000) and alterations to membrane composition (Wang et al. 2006). According to Thakur et al. (2010), springtime low-temperature periods frequently harm the micro-organelles of the cell severely, which leads to an abundance of reactive oxygen species (ROS) and the incidence of lipid peroxidation.

In addition, it has been noted that key growth and development processes such as seed germination, seedling hardiness, and establishment are affected by cold stress (Hussain et al. 2018), as well as inadequacies in water uptake and minerals nutrients (Aroca et al. 2003), declined photosynthetic capacity, which is linked to perturbations in source-sink exchanges of assimilates, and the overaccumulation of reactive oxygen species in several crops (2009, Li et al. 2017, Hassan et al. 2021, Zhou et al. 2022)

in response to chilling (10°C /3°C, day/night) in wheat, it was observed by Zhang et al. (2015) that some growth indices including shoot and root length as well as fresh and dry weights, demonstrated a notable reduction. Root growth can be compromised by cold stress in relation to root elongation and biomass accumulation, which heavily rely on cell wall expansion (Sanders & Markhart, 2001). Under cold stress, plant root growth, water uptake capacity, and the capacity for mineral nutrients (N, P, and K) to contribute to plant growth are all significantly impacted (Yan et al. 2012). In some cases, cold stress does not only obstruct seedling growth but also acts as a catalyst for pathogen attack by reducing the seedlings' resistance to pathogen attack (Juurakko et al. 2021).

2.2.2 Cold Sensing in Plants

Comprehension of how plants sense and interpret cold signals using tiny signaling molecules has advanced significantly in recent years. Plants first detect temperature changes through changes in membrane fluidity and cytoskeleton rearrangement during the transduction of cold signals. Then, calcium influx causes a variety of downstream reactions (Guo et al. 2018, Ding et al. 2019, 2020). This process causes significant changes in practically every observable physiological, biochemical, and molecular feature, which results in alteration in gene expression and may increase plants'

ability to withstand low temperatures (Theocharis et al. 2012, Rihan et al. 2017). Small signaling molecules control signaling pathways and are crucial in a variety of stressful situations (Jain et al. 2018). Small signaling molecules, including calcium (Ca2+) (Steinhorst & Kudla, 2014), reactive oxygen species (ROS) (Sharma et al. 2012), hydrogen sulfide (H_2S) (Zhang et al. 2021), and hydrogen peroxide (H_2O_2) have been found to work synergistically through cross talking (Jain et al. 2018).

2.3 Role of Small Signaling Molecules in Plant Stress

2.3.1 Calcium (Ca^{2+})

In plant cells, nearly all abiotic stimuli, including cold stress, can quickly activate cytosolic Ca^{2+} signals (Yuan et al. 2018). Ca^{2+} signature creation, signal detection, and signal transduction are the three stages of the Ca^{2+} signal transduction pathway (Reddy & Reddy, 2004). In typical conditions, the amount of free Ca^{2+} in the cytosol is kept at a low level. Contrarily, when plants are subjected to adverse environmental circumstances, the cytosolic Ca^{2+} level rapidly increases and Ca^{2+} transients, also known as Ca^{2+} signatures, are formed (Bose et al. 2011).

Different environmental conditions lead to the generation of Ca^{2+} signals in plant cells. Numerous Ca^{2+} sensors, including calmodulins (CaMs), CaM-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CPKs/CDPKs), and calcineurin B-like proteins (CBLs), are phosphorylated during the perception and transmission of Ca^{2+} signals (Guo et al. 2018). These Ca^{2+} sensors work in conjunction with other Ca^{2+} signaling components to convert Ca^{2+} signals into downstream signaling events such as kinase cascade activation, transcriptional reprogramming, and the accumulation of reactive oxygen species or nitric oxide (Yuan et al. 2018).

2.3.2 Hydrogen Peroxide (H_2O_2)

Reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) are produced during oxidation-reduction reactions (Huang et al. 2019). Plant cells generate reactive oxygen species (ROS) by enzymatic processes in the peroxisomes and apoplasts, as well as electron transport in the chloroplasts and mitochondria (Wrzaczek et al. 2013). Hydrogen peroxide (H_2O_2) causes oxidative damage to biomolecules in high quantities, which can cause cell death (Zheng et al. 2021). According to Cerny et al. (2018), hydrogen peroxide (H_2O_2) however functions as a signaling

molecule at low concentrations, modifying plant growth and development in response to various conditions.

H_2O_2 may activate mitogen-activated protein kinase (MPK1/2) to generate responses to cold stress because low temperature increases H_2O_2 levels, NADPH oxidase activity, and respiratory burst oxidase homolog (RBOH1) gene expression modestly (Zhou et al. 2012, 2014). The nucleotide diphosphate kinase 2 (NDPK2) gene was significantly upregulated in response to H_2O_2 stress in Arabidopsis, acting as negative feedback for H_2O_2 buildup and promoting plant tolerance to low-temperature stress (Moon et al. 2003). Increased levels of H_2O_2 in wheat caused the synthesis of abscisic acid (ABA), which in turn encouraged H_2O_2 production by activating NADPH oxidase, resulting in longer stress tolerance. In the end, elevated levels of H_2O_2 and ABA activate their corresponding defense pathways, which increase the expression of the genes that respond to cold and the activities of the antioxidant enzymes, minimize electrolyte leakage and cell membrane peroxidation, and enhance photosynthesis capacity (Fv/Fm and ETR) under freezing stress (Wang et al. 2018).

2.3.3 Nitric Oxide (NO)

A series of derived compounds known as reactive nitrogen species (RNS) are produced by the free radical nitric oxide (NO), including nitrogen dioxide (NO_2), peroxynitrite anion (ONOO-), and S-nitrosothiols (SNOs) (Corpas et al. 2021). Depending on the cell concentration, nitric oxide has different biological effects: at low concentrations, it works as a signal molecule, while at large concentrations, it damages cells and induces nitro-oxidative stress (Asgher et al. 2017). Nitric oxide performs a signaling role in plants, where it is engaged in several physiological processes including seed germination, root development, stomatal closure, senescence, and reaction to abiotic and biotic stressors (Domingos et al. 2015).

It is believed that exposure to cold increases endogenous nitric oxide production and that nitric oxide modifies proline synthesis in Arabidopsis, which is crucial for plant freezing tolerance (Zhao et al. 2009). Additionally, nitric oxide supplementation improves the ability to withstand chilling by increasing the levels of soluble sugar, proline, total phenol, γ-Glutamyl-cysteinyl-glycine (GSH), and GSH/GSSG to reduce electrolyte leakage, lipid peroxidation, and the decrease of photosynthetic efficiency brought on by chilling stress (Dong et al. 2018). By lowering

malondialdehyde (MDA) levels and electrolyte leakage, increasing antioxidant enzyme activities like superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT), recovering photosystem II, and inducing the expression of cold-responsive genes, exogenous nitric oxide was found to protect bermudagrass (*Cynodon dactylon* L.) from cold stress (Fan et al. 2015). However, research using mutants with nitric oxide deficiency suggests that nitric oxide controls constitutive freezing tolerance in Arabidopsis by lowering the production of osmoprotectant metabolites, flavonoids, anthocyanins, sugars, polyamines, and stress-related hormones (abscisic acid and jasmonates) (Zheng et al. 2021).

2.3.4 Hydrogen Sulfide (H_2S)

Various physiological processes may be modulated by hydrogen sulfide (H_2S), which functions as a second messenger (Li et al. 2020). Physiological processes, including seed germination, lateral root development, stomatal movement, photosynthesis, fruit ripening, and plant senescence are regulated by H_2S. (Xuan et al. 2020). H_2S increases antioxidant processes, which reduces oxidative cellular damage and promotes stress tolerance (Li et al. 2016, Zhang et al. 2021). H_2S has been demonstrated to have a significant function in plant cell signaling. Treatment with sodium hydrosulfide, an H_2S donor, at 4 °C enhanced superoxide dismutase (SOD) activity and plasma membrane integrity while lowering levels of the superoxide anion radical and malondialdehyde (MDA) (Fu et al. 2013). By decreasing electrolyte leakage and enhancing antioxidant activity, such as that of catalase (CAT), peroxidase (POD), and glutathione reductase (GR), exogenously administered sodium hydrosulfide (NaHS) increased freezing tolerance in bermudagrass by scavenging the overproduced ROS (Shi et al. 2013).

Additionally, under low-temperature stress, hydrogen sulfide (H_2S) increased total phenolic content as well as the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and the phenylalanine ammonia-lyase, improving antioxidant ability (Luo et al. 2015). According to Zheng et al. (2021), exogenous H_2S administration activated the downstream defense response, leading to a significant buildup of proline and soluble sugars, and reduced reactive oxygen species (ROS) and RNS damage by enhancing antioxidant enzyme and S-nitroso glutathione reductase (GSNOR) activity. These findings demonstrate that H_2S is crucial for plants' ability to tolerate cold temperatures (Ma et al. 2015).

Guanylate cyclase produces cyclic guanosine monophosphate (cGMP), which is a catalytic byproduct of guanosine triphosphate (GTP). It has been demonstrated that cGMP serves as a secondary messenger that is essential for both abiotic stress responses and plant growth and development (Dubovskaya et al. 2015, Isner & Maathuis 2018). Low temperatures led to a rise in cGMP levels in Arabidopsis seedlings, and cold stress led to nitric oxide-induced cGMP production. Cold tolerance was increased by cGMP's interaction with nitric oxide and Ca^{2+} (Bakakina et al. 2014).

2.4 Physiological Effects of Cold Stress on Plants

2.4.1 Photosynthesis

The physiological and biochemical processes in the plant cell are greatly slowed down by cold stress, which causes leaf chlorosis, wilting, and even necrosis of plant cells (Ruelland & Zachowski 2010). Photosynthesis and respiration in plants are particularly susceptible to low-temperature stress (Yadav 2010). According to Ploschuk et al. (2014), photosynthesis is a key physiological activity that includes various sub-processes such as CO_2 reduction pathways, photosystems, and the electron transport system, all of which are vulnerable to harm brought on by cold. Several studies have reported that the suppression of photosynthesis by cold stress depends on low carbon dioxide availability due to stomatal restrictions under chilling (4–12 degree Celsius) and freezing (1–3 degree Celsius) (Ploschuk et al. 2014, Bilska-Kos et al. 2018). Hussain et al. (2018) found that several factors, including decreased chlorophyll synthesis, poor chloroplast development, decreased efficiency of the photosynthetic apparatus, limited carbohydrate transport, restricted stomatal conductivity, suppressed Rubisco activity during carbon assimilation, disrupted electron transport chain, and decreased energy stock, contribute to cold-induced photosynthetic inhibition.

Low temperatures damage the structure of the chloroplasts and loosen the thylakoids, which ruin the photosynthetic apparatus (Zhang et al. 2020). Reactive oxygen species (ROS) concentration may rise due to growth at low temperatures, which can also result in excessive stimulation of the electron transport systems (Janda et al. 2007). In addition, according to Basu et al. (2016), cold weather causes drought stress in plants, which lowers molecular oxygen levels and generates reactive oxygen species (ROS) that seriously harm the photosynthetic system. Plants may damage membrane lipids, nucleic acids, and proteins which could result in cell death if they are unable to control the intracellular reactive oxygen species (ROS) level (Suzuki & Mittler, 2006).

2.4.2 Respiration

Several modifications to the wheat plant's biological and biochemical processes are brought on by cold stress, including decreased respiration rate, decreased enzyme activity, oxidative stress, and loss of seed reserves (Li et al. 2013, Esim et al. 2014). In the end, the respiration rate is reduced as a result of the extended cold stress period; significant damage to the mitochondrial structure, slowing the passage of kinetic energy, and disrupting enzymatic activity are observed (Ikkonen et al. 2020). Calegario et al. (2003) claim that a drop in ATP synthase yield brought on by the stimulation of respiration is associated with a rise in heat dissipation. According to Theocharis et al. (2012), cold stress also alters membrane fluidity, and lipid composition, and causes low water potential, reduced ATP supply, accumulation of hazardous chemicals, unbalanced ion supply, and solute leakage, all of which lead to damage to plant cell membranes. Investigations have shown that, in wheat and maize, cooling activates the alternate respiratory systems. Such alternate modes of respiration are essential for decreasing mitochondrial structural damage and cooling stress (Feng et al. 2008).

2.4.3 Cellular Effects

Studies have shown that induced ultrastructural alterations in cold-sensitive wheat plants cause an unbalanced composition and lower permeability of membrane fluid, which then impairs several physiological and biochemical processes related to membrane function (Bohn et al. 2007, Los et al. 2013). According to Salinas (2002), ice formation which builds up in the intracellular spaces and physically disrupts cells and tissues is frequently linked to freezing stress, which results in membrane damage, solute precipitation, and protein denaturation brought on by extreme cellular dehydration. By creating an osmotic gradient between the fluid-filled cell cytoplasm and the ice-filled intercellular space, below-freezing temperatures cause cell contraction (Li & Palva 2012, Fujikawa & Kuroda 2000). Increased oxidative stress and reactive oxygen species (ROS) generation, delayed metabolism, increased dehydration, poor nutrient absorption, and decreased photosynthesis are additional biological impacts of cold stress on plant cells (Dreyer & Dietz, 2018/a). The effects of low, non-freezing temperature on the anatomy and morphology of plants include altered stomatal frequency (Equiza et al. 2001), decreased epidermal cell size, increased mesophyll cell size, and suberization (Hudak & Salaj 1999). Phytohormones such as salicylic acid

(SA), brassinosteroids (BRs), jasmonic acid (JA), auxins (IAA), gibberellins (GA), ethylene (ET), and strigolactones are involved in mediating cold stress signaling and controlling the transcription of specific cold-responsive (COR) genes (Deng et al. 2018). When plants are exposed to cold stress, their levels of abscisic acid (ABA) and jasmonic acid (JA) rise while their levels of cytokinin, ethylene, and gibberellic acid fall. According to Zhang et al. (2006), ABA stimulates antioxidant defense to scavenge ROS and aids in lowering the rate of ROS production.

2.4.4 Lipid Peroxidation

Lipid peroxidation (LPO) is the term used to describe the oxidative degradation of membrane lipids (Horton & Fairhurst 1987). Several physiological processes, including increased membrane rigidity, decreased cellular deformities, and lipid fluidity in erythrocytes, have been linked to lipid peroxidation (LPO) of the cell membrane (Matkovics et al. 1998). Lipid peroxidation occurs spontaneously when the generation of reactive oxygen species (ROS) in cellular and organellar membranes exceeds the equilibrium level, which affects normal cellular function. Lipid peroxidation generates lipid free radicals, which interact with biomolecules and cause damage to DNA and proteins (Hakeem et al. 2014). It has been discovered that in stressed plants, there is an increased rate of lipid breakdown and an increased production of reactive oxygen species (ROS) (Mishra & Dubey 2011). One of the by-products of lipid oxidation called malondialdehyde (MDA) causes cell membrane damage (Halliwell 1989). Therefore, MDA is utilized as an indicator to measure the level of lipid peroxidation in plants.

2.5 Biochemical Effects of Cold Stress on Plants

2.5.1 Reactive Oxygen Species (ROS)

According to research on cellular biological systems, the interaction between excited pigments and oxygen (O_2) in chloroplasts leads to the production of reactive oxygen species (Reddy et al. 2004). In addition, Moller (2001) hypothesized that a series of interactions in mitochondria involving O_2 and a few elements of the electron transport chain led to the production of reactive oxygen species (ROS). By preventing the synthesis of the D1 protein needed to repair photodamage in the PSII complex under cold stress, reactive oxygen species indirectly harm the PSII complex (Banerjee & Roychoudhury 2019). Reactive oxygen species, including superoxide (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH), formed as a result of abiotic stressors like cold, are harmful

chemicals that should be scavenged, according to Dreyer and Dietz's opinion in 2018/b. However, it is important to note that ROS are not inherently toxic; rather, it is their buildup to high concentrations that harms the plant. Higher reactive oxygen species (ROS) amounts, according to Soualiou et al. (2022), impair cell processes by causing DNA damage, protein oxidation, and lipid peroxidation (base deletion and alteration). Extreme ROS production and a changed electric charge make proteins more susceptible to proteolysis (Hakeem et al. 2014). Chi et al. (2013) assert that plants have a variety of antioxidant redox proteins, including superoxide dismutase, catalase, glutaredoxin, thioredoxin reductase, protein disulfide reductase, thioredoxin (Trx), and other types of peroxidases, to protect plant cells in response to cold stress.

Despite this, it is impossible to overstate how important reactive oxygen species (ROS) are in controlling plant stress. In plants, where they can influence gene expression, growth, and development, ROS functions as signaling molecules at lower concentrations (Cern et al. 2018). Low levels of ROS trigger defense mechanisms against stressors, while elevated levels of ROS cause cell damage and cold injury, according to Gechev et al. (2006). ROS is a key regulator of rice's response to cooling temperature (2°C) according to metabolomic and RNA-seq investigations (Zhang et al. 2016). 60% of the genes involved in cold tolerance were activated by H_2O_2, and cold stress has the highest evidence for H_2O_2-regulated signaling (Yun et al. 2010). According to Gill and Tuteja (2010), the levels of ROS generation must be carefully controlled by the scavenging system of antioxidants, which includes both enzymatic (such as superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), catalase (CAT), and glutathione reductase (GR) and non-enzymatic such as ascorbic acid, ASA. The discovery of Mi et al. (2021) claim that cold stress-induced formation of ROS is swiftly followed by enhanced activities of these antioxidants to regulate the level of ROS toxicity, preventing oxidative damage, and activating signaling pathways to boost cold tolerance.

2.5.2 Osmotic Adjustment

Osmotic adjustment lowers the osmotic potential that results from solute accumulation in response to various stimuli, such as cold and drought (Hussain et al. 2018). Carbohydrates (starch and soluble sugars) and proline are a few of the important and crucial compounds involved in osmotic adjustment in plants (Soualiou et al. 2022). According to Pirzadah et al. (2014), these osmolytes all have a brief shelf-life, which means that they start to degrade once the stress conditions get

better. However, they function by enabling the plants to effectively absorb water (Flowers 2004, Ashraf & Foolad 2007), remove free radicals (Okuma et al. 2004, Banu et al. 2010), and serve as a reservoir of nitrogen and energy (Pirzadah et al. 2014). Proline, glycerophosphocholine, trimethylamine oxide (TMAO), myoinositol, sarcosine, GB, and taurine, are natural osmolytes that act as osmo-protectants (Neuhofer & Beck 2006). These osmolytes defend plants from stressors by eliminating ROS, stabilizing protein and enzymes, adjusting cellular osmotic pressure, and preserving membrane integrity (Verbruggen & Hermans 2008).

It has been discovered that compared to any photosynthetic pigment, carbohydrate metabolism demonstrates higher and faster low-temperature sensitivity (Fernandez et al. 2012). These carbohydrates stabilize the membranes, according to studies using mechanical methods, by interacting with polar groups of phospholipids and forming hydrophobic bonds with proteins thus, protecting the membranes' natural structure and functioning state, including that of chloroplasts and mitochondria, which are the main sources of active forms of oxygen Pirzadah et al. (2014).

According to Soualiou et al. (2022), starch is recognized to deliver carbon and energy in situations where plants' ability to synthesize food may be restricted. Additionally, Thalmann & Santelia (2017) noted that investigations have revealed higher leaf starch concentrations in plants under stress conditions. Mozgova et al. (2019) also found that the accumulation of cryoprotectants such as soluble sugar, prolines, and flavonoids, is a part of cold acclimation in plants. In addition to performing an energy function for photosynthetic plants, sugars serve a crucial regulatory role in many essential processes and are regarded as important signals that control plant metabolism and development Pirzadah et al. (2014). According to Welling & Palva (2006), sugars (glucose, sucrose, and fructose) build up in the leaves of cold-adapted plants and function as osmoregulators. Sugars have a variety of functions in low-temperature tolerance; as common compatible osmolytes, they help keep water in plant cells, which reduces the availability of water and ice nucleation in the apoplast (Ruelland et al. 2009). According to a recent study on Petunia hybrida, cold-induced sugar buildup in source leaves may serve as a defense against freezing temperatures, which limit the transport and use of carbohydrates in growth sinks (Bauerfeind et al. 2015). Sugars may protect plant cell membranes from damage caused by cold by forming hydrogen bonds with lipid molecules instead of water molecules (Ruelland et al. 2009). According to Hurry et al. (1995), winter wheat has a higher amount of sugar (sucrose, fructose, and glucose) than spring wheat.

Trehalose is a nonreducing disaccharide that plays a significant role in bacteria, yeast, and plant, as an osmoprotectant. According to Pirzadah et al. (2014), trehalose sugar also has a unique ability to maintain the loss of fluidity in cell membranes, which occurs when temperature decreases. In response to chilling stress (12 °C), there was a fleeting increase in both trehalose concentration and trehalose-6-phosphate phosphatase activity. This indicates that the early chilling stress response in rice involves a brief stimulation of trehalose biosynthesis (Pramanik & Imai 2005). Trehalose is not the only oligosaccharide that accumulates under stress; others include raffinose, stachyose, verbascose, fructans, and sucrose which are also more useful in protecting plants from abiotic stresses like cold stress (Usadel et al. 2008, Pirzadah et al. 2014).

As a proteogenic α-amino acid, proline plays a crucial role in plants' fundamental metabolism Pirzadah et al. (2014). Proline (Pro) is known to have a variety of roles in plants, including controlling osmotic adjustment, activating osmotic stress-related genes, stabilizing proteins and membranes, and scavenging reactive oxygen species (ROS) (Verbruggen & Hermans 2008, Szabados & Savoure 2010). It has been discovered that proline plays a crucial function in enhancing the pentose phosphatase pathway, which is a crucial component of the antioxidant defense system (Hare-Cress 1997). Improved cold tolerance was positively connected with increases in endogenous proline levels, especially in chilling-tolerant plants including barley, winter wheat, potato, and chickpea (Szabados & Savoure 2010, Kaur et al. 2011, Cao et al. 2017). Ding et al. (2019) equally claim that proline, soluble sugar, and protective proteins (late embryogenesis abundant proteins, antifreeze proteins, and cold shock proteins) are all produced by plants to boost their tolerance to cold stress. Additionally, proline may activate genes associated with osmotic stress as well as act as a potential inhibitor of apoptosis (Theocharis et al. 2011, Pirzadah et al. 2014). In recent reports, it has been shown that plants exposed to stressful circumstances like cold stress produce more proline and degrade less of it (Jonytiene et al. 2012).

According to Soualiou et al. (2022) the buildup of abscisic acid (ABA), a stress hormone that can start numerous processes involved in the adaptation to various pressures, is the primary characteristic of the stress response in several plant species. Early research demonstrated that abscisic acid (ABA) treatment given to chilling-sensitive plants, such as maize and rice, before, during, or after a cold temperature treatment could significantly lessen chilling damage in these species (Prasad et al. 1994). It is interesting to note that Guo et al. (2021) found that pre-treating maize seedlings with exogenous ABA 3 days before beginning cold stress could prime the

seedlings and help them resist chilling temperature (8°C /4°C) by inducing higher endogenous ABA levels. Theocharis et al. (2012) and Rihan et al. (2017) also found that ABA-regulated pathways are involved in the development of cold tolerance or acclimation. The rise in antioxidant activity and decline in reactive oxygen species (ROS) accumulation after abscisic acid (ABA) treatment help to explain how cold injury is mitigated (Liu et al. 2012).

2.5.3 Antioxidant Defense Responses

It is generally recognized that most stress causes, whether biotic or abiotic, are typically linked to oxidative stress and may cause the antioxidant systems to become active (Janda et al. 2007). Increased antioxidant enzyme activity and higher levels of antioxidant metabolites have long been linked to antioxidant defense responses (Hegedus et al. 2001, Kocsy et al. 2001). According to recent studies, the ability of plants to combat environmental stress is increased by boosting the activity of the antioxidant defense system's enzymes, which is only feasible by maintaining an intense antioxidant activity to quench the poisonous reactive oxygen species (ROS) (Q. Chen et al. 2011). Plants' reaction to abiotic stressors involves both enzymatic (superoxide dismutase, catalase, glutathione reductase, guaiacol peroxidase, and ascorbate peroxidase) and non-enzymatic (ascorbate, glutathione) antioxidants.

The superoxide anion is changed into oxygen and hydrogen peroxide by the enzyme superoxide dismutase (SOD). There are three different forms of SOD: extracellular SOD, Cu/Zn-dependent SOD, and Mn-containing SOD (Racchi et al. 2001). In all aerobic species, SOD plays a crucial part in the defense system against oxidative stress (Scandalios 1993). Mn-SOD is concentrated in the chloroplast, and Cu/Zn SOD is distributed throughout the cytosol, chloroplast (Jackson et al. 1978), peroxisomes, and mitochondria (Del Rio et al. 1998). Increased SOD production has been linked to increased oxidative stress tolerance in plants (Gupta et al. 1993).

The tetrameric enzyme catalase is primarily found in peroxisomes, while mitochondria and cytoplasm also contain small amounts of it. By converting H_2O_2 to water and oxygen, catalase aids in cellular detoxification and shields cells from internal H_2O_2 generation (Hakeem et al. 2014). Based on the strength and type of stress, it has been discovered that environmental stress either increases or decreases the activity of catalase (Moussa & Abdel-Aziz 2008). In transgenic tobacco plants with 10% wild type, catalase activity indicates the production of GSSH and a 4-fold

reduction in ASA, indicating that catalase is important for controlling the redox equilibrium during oxidative stress (Willekens et al. 1997).

As an antioxidant, glutathione reductase (GR) is crucial to the enzymatic and nonenzymatic redox cycles that result in the oxidation of GSH to GSSH. It can be found in chloroplasts, cytosol, mitochondria, and peroxisomes. Photosynthetic tissues and some chloroplast isoforms are known to have 80% of the glutathione reductase activity (Edwards et al. 1990). The activity of glutathione reductase is increased by environmental stressors (Maheshwari & Dubey 2009).

Under stressful circumstances, it has been discovered that guaiacol peroxidase (GPX) functions effectively by removing O_2 and peroxyl radicals, earning it the nickname "stress" enzyme (Vangronsveld & Clijsters, 1994). Plants respond to biotic and abiotic stressors by rapidly increasing their GPX activity (Moussa & Abdel-Aziz 2008). According to one of the research works done by Radotic et al. (2000), enhanced guaiacol peroxidase (GPX) activity during oxidative stressors in response to metal toxicity can be employed as a biomarker for less-lethal metal toxicity in plants. Recent findings have shown that guaiacol peroxidase (GPX) plays a crucial function in reducing the salt resistance of safflower plants and that this effect is enhanced by boosting guaiacol peroxidase (GPX) activity in the plants (Tayef-Nasrabadi et al. 2011).

According to Danna et al. (2003), ascorbate peroxidases (APX) in the chloroplast are crucial for preventing oxidative damage to the photosynthetic machinery, keeping the processes effective enough to supply energy for the development of cold resistance. An association between frost resistance and the activity of the protein APX in the leaves of cold-hardened plants of various cereal species, varieties, and chromosome replacement lines was found in an earlier study (Janda et al. 2003).

The most prevalent low molecular weight antioxidant that is crucial in preventing oxidative damage brought on by an increase in reactive oxygen species (ROS) production is ascorbate (ASA). Additionally, it has been pivotal for the development, differentiation, and metabolism of plants. Different plant cell types, organelles, and apoplast contain ascorbate (Smirnoff et al. 2004). Apoplastic ASA has been reported to have basal protection against external oxidants (Barnes et al. 2002). Pinto et al. (2003) add that ASA performs a vital role in removing H_2O_2 via the ascorbate-glutathione (AsA-GSH) cycle. A balance between the rate and capacity of ASA accumulation and the output of antioxidant requirement under stress conditions has been found to determine the

content of ASA (Chaves et al. 2002). According to a recent study, potato plants overexpressing strawberry d-galacturonic acid reductase produce more ASA and are more resistant to abiotic stress (Hemavathi et al. 2009). Similar to this, increasing ASA content in Arabidopsis has demonstrated the plant's stress tolerance (Wang et al. 2010).

A nonprotein thiol with a low molecular weight called -Glutamyl-cysteinyl-glycine (GSH) is crucial in the fight against oxidative stress brought on by reactive oxygen species (ROS). It is almost always found in the cytosol, mitochondria, and the ER's chloroplast (Foyer & Noctor, 2003). Due to its reducing capability, GSH is crucial for a variety of biological functions, including signaling, metabolite conjugation, enzymatic control, protein, and nucleic acid accumulation, and the expression of genes that respond to stress (Foyer et al. 1997). Because of its chemical interactions with O_2, •OH, and H_2O_2, it serves as a scavenger. Proteins, lipids, and DNA can be protected by GSH by producing adducts or by acting as a proton donor when GSSH is present and producing reactive oxygen species (ROS) (Asada 1994). It has been discovered that altered ratios of GSH/GSSH are present in plants under a variety of stressors, including salinity (Hefny & Abdel-Kader 2009), cold (Radyuk et al. 2009), and metal toxicity (Mishra & Dubey 2011).

Flavonoids, tannins, hydroxyl cinnamate esters, and lignin are only a few examples of the diverse secondary metabolites known as phenolic chemicals that are present in plant tissues and have antioxidant properties (Grace & Logan 2000). Additionally, polyphenols alter the packing of lipids and lessen membrane permeability (Arora et al. 2000).

2.6 Morphological Effect of Cold Stress on Plants

2.6.1 Assimilate Partitioning

The effect of cold stress on plant morphology can result from the alteration of assimilate partitioning. The biomass allocation to roots rises during cold stress, whereas under normal circumstances, more biomass is given to the shoots for optimal crop growth, and just a tiny portion is provided to their roots (Bowen 1991). As a result, Li et al. (2017) assessed how different wheat genotypes responded to chilling stress (10°C /5°C) by partitioning photo-assimilates. They discovered that more biomass was devoted to the roots, enhancing their potential to grow and take in more nutrients under cold circumstances. In different wheat genotypes, it has been discovered that decreasing leaf area is directly associated with poor assimilate production and eventually bad

yields because leaves are the primary source for the synthesis and buildup of carbohydrates in plants (Li et al. 2017). Additionally, cold stress alters the metabolism of carbohydrates (sugar and starch) and lowers the sucrose concentration, which lowers the rate at which assimilates are exported to reproductive sinks like pollen grains and the tapetum (Ji et al. 2013, Niu et al. 2013)

2.7 Effect of Cold Stress on Plant Microbiome

Cold stress has a big impact on plant microbial activity. For instance, low-temperature stress increases resistance to grapevine powdery mildew and lowers the risk of fungal infections by accumulating antifreeze proteins, which also function as antifungal substances (Weldon et al. 2020, Moyer et al. 2016). Additionally, some endophyte bacteria resist abiotic stressors like the cold (Miura & Furumoto 2013).

Endophytes have frequently been employed as sustainable methods to handle abiotic challenges like cold in the field because they help host plants maintain fitness, nutrient availability, and stress tolerance (Verma et al. 2021). It has been discovered that facultative fungal endophytes are effective at enhancing plant agronomic features and granting tolerance to environmental challenges, such as cold (Sword 2017).

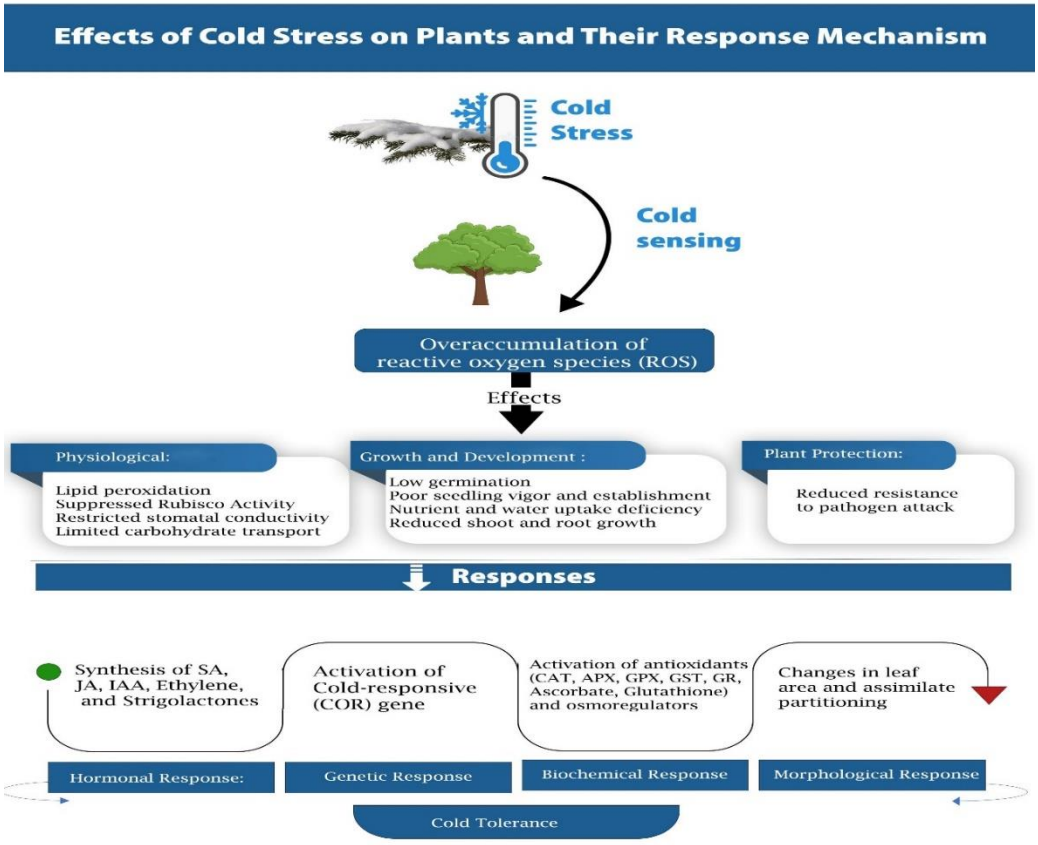


Figure 2: Effects of Cold Stress on Plants and Their Response Mechanism (Designed by: Adu Donyina 2023)

2.8 Systemic Acquired Resistance (SAR)

The plant defense mechanism known as systemic acquired resistance (SAR) is functional against a variety of biotic stressors. The SA-mediated and pipecolic acid-dependent pathways are the most significant SAR processes. These two branches can work separately or in concert because they are parallel and connected (Bernsdorff et al. 2016, Wang et al. 2018).

One of the first validated volatile organic compound (VOC) signal molecules in systemic acquired resistance (SAR) was methyl salicylate (MeSA), which also acts as a plant-plant signal (Shulaev et al. 1997 reviewed in Singewar et al. 2021). When sweet pepper is exposed to herbivore-induced plant volatiles (HIPVs), such as MeSA, it upregulates the Jasmonic Acid and Salicylic Acid

signaling pathways in healthy plants, which activates the immunological defense system (Riahi et al. 2022).

2.8.1 Plant-Plant Communication

Volatile Organic Compounds (VOCs) may also trigger defense responses in surrounding plants and serve as external signals within-plant communication (Heil & Bueno 2007). Due to their high volatility, several VOCs, including methanol, isoprene, the phytohormone ethylene, and some monoterpenes, are only used in proximity plant interactions. Terpene, MeJA, MeSA, or green leaf volatiles (C6 aldehydes, alcohols, and their esters), which are less volatile VOCs, can function across greater distances War et al. (2011). According to Gondor et al. (2022), methyl salicylate could act as a signal both within and between plants because it is a volatile substance.

Abiotic stress can also cause the formation of volatile organic compounds (VOCs), which nearby plants may be able to detect, and VOCs may trigger pathways connected to salicylic acid (SA) that help nearby plants defend themselves against abiotic stress (Gondor et al. 2022). Under cold stress, tea plants have been shown to release geraniol, linalool, and MeSA, and these VOCs were able to produce cold tolerance (Zhao et al. 2020). Recently, it was proposed that plant VOCs may also participate in below-ground plant-plant interactions, even though they have primarily been investigated as above-ground chemical signals (Gfeller et al. 2019).

2.9 Salicylic Acid

Salicylic acid (SA), a phenolic substance and plant hormone, improves plant growth and immunity by functioning as a signal molecule that modifies the plant's reactions to stressful situations (Lattanzio et al. 2006, Dempsey et al. 2011, Yan et al. 2018). The esters of SA were used as pharmacological agents and fragrance material (Klessig et al. 2016, Kamatham et al. 2016). Acetylsalicylic acid (ASA), commonly called aspirin, has been a classic pharmaceutical agent. methyl, benzyl, and isoamyl salicylic acid derivatives were used as fragrances (Deng et al. 2017). It is also crucial for a variety of physiological and developmental activities in plants, including seed germination, blooming, fruit production, cell proliferation, nodulation in legumes, stomatal closure, and the control of biotic resistance and abiotic stress tolerance (Gondor et al. 2016, Koo et al. 2020, Sharma et al. 2020). In a physiological and molecular research, it was discovered that SA administration during cooling treatment reduced the formation of active oxygen species (AOS),

blocked electrolyte leakage, improved the activity of cell-protective enzymes, and increased photosynthetic efficiency (Kang et al. 2004). By activating the antioxidant enzyme system to guard plants against oxidative damage, SA, which functions as a signal molecule, can improve the chilling tolerance of plants throughout the growth phase or during cold storage (Kim et al. 2017, Miura & Tada 2014).

Bean seeds immersed in 0.1 mM SA dramatically increased germination rates and percent germination under ideal and low-temperature conditions (Gharib & Hegazi 2010). In addition, SA seed priming increased seedling emergence, root and shoot length, fresh and dry mass of seedling and leaf, and both at optimal and chilling temperatures in maize, as compared to the control (Farooq et al. 2008). As evidenced by the increase in both their masses and lengths, the pre-sowing treatment of wheat seeds with SA increased the growth of shoots and roots in the ensuing seedlings (Rakhmankulova et al. 2010). Amorfrutins, benzyl salicylate, isoamyl salicylate, methyl salicylate (MeSA), acetylsalicylic acid, and other derivatives of salicylate acid have also been discovered. MeSA, a molecule generated from salicylic acid, is still being researched and developed as a potential agricultural product that can stimulate plant growth regulators to boost growth and development or strengthen defense responses (Kalaivani et al. 2016).

2.10 Methyl Salicylate

Methyl salicylate (MeSA), a volatile organic molecule (VOC), is the methyl ester version of salicylic acid (SA). In 1843, MeSA was first isolated. Because it is the most prevalent component in wintergreen oil and was historically isolated from wintergreen plants, it is also known as the oil of wintergreen. This ester has a high boiling point (223°C), a low molecular weight (152.149 g/mol), is colorless, and has a pleasant aroma Gondor et al. (2002).

Salicylic acid (SA) is less phloem mobile than Methyl Salicylate (MeSA), which is more hydrophobic (Lim et al. 2020). Methyl salicylate can function as a long-distance signal for distant tissues of the infected plant as well as a signal for plant-to-plant communication because it is extremely volatile and can be transmitted through the phloem (Oelmüller 2021). It has been determined to be a constituent of the herbivore-induced plant volatiles (HIPVs) released by several crop species, including hops (Humulus lupulus L.), tomatoes, and cucumbers (Van Den Boom et al. 2004).

2.10. 1 Effects of Methyl Salicylate (MeSA) on Plant Biotic Stress Tolerance

According to Gondor et al. (2022), an increase in herbivore resistance to the rice leafroller which resulted from induced peroxidase (POD) was observed due to exogenous methyl salicylate (MeSA) treatment of rice plants (Cnaphalocrocis medinalis Guenee). Salicylic acid, volatile organic compounds, and reactive oxygen species were all increased by MeSA spraying, and the expression of PR and defense-related genes was also increased (Kalaivani et al. 2016, 2018). Following Xanthomonas oryzae infection and MeSA solution treatment, peroxidase (POD) gene expression was controlled, and the antioxidant enzyme system's activity was elevated in rice seedlings (Kalaivani et al. 2021). However, MeSA enhanced Scymnus (Pullus) sodalis insect visits, which reduced crop output losses by controlling the aphid population (Dong & Hwang 2017). The SA and MeSA treatments decreased survival, lengthened the nymphal stage, and increased wing production in Faba bean seeds, indicating that the chemicals boosted the beans' resistance to the Aphis craccivora pest (El-Solimany 2020). Methyl salicylate improved grape quality and raised quantities of ascorbic, succinic, and fumaric acids by increasing phenolic compound levels and the activity of the antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POD) (Garca-Pastor et al. 2020a).

2.10.2 Effects of Methyl Salicylate (MeSA) on Plant Abiotic Stress Tolerance

As a seed-soaking therapy for cucumber plants, methyl salicylate (MeSA) was also suggested as a signal molecule in plant responses to abiotic stresses since it increased plant tolerance to chilling injury (Seydpour & Sayyari, 2016). After full bloom, MeSA was sprayed on Prunus armeniaca L. cv. Kate, and the soluble sugar content and associated enzyme activity increased. These changes appeared to be related to induced freezing tolerance (Fan et al. 2021). In horticultural crop plants, the vapor treatment boosted antioxidant enzyme activity, produced heat shock proteins, and decreased the amount of lipid peroxidation, resulting in less severe membrane breakdown (Ding et al. 2001, Asghari & Aghdam 2010). Following salinity stress, soaking seeds of several rice varieties in MeSA solutions raised the growth rate, phytic, total phenolic, and flavonoid contents, and elevated -amylase activity, which improved the plants' physiological and biochemical features (Thu et al. 2020).

Methyl salicylate (MeSA) treatment of rice seeds greatly boosted the peroxidase enzyme activity, which changed plant physiology and improved crop protection against the disease known as bacterial blight (Kalaivani et al. 2021). Rice's physiological and biochemical characteristics under salt stress were similarly improved by seed soaking with MeSA, and this was associated with increased seed development, -amylase activity, phytic acid, and flavonoid levels (Thu et al. 2020).

Methyl salicylate (MeSA) enhanced the concentration of benzenoids, monoterpenes, and chemicals generated from fatty acids (Liu et al. 2018). The activity of PAL, chalcone synthase and isomerase, and flavone-3-hydroxylase, however, was boosted when apple trees (Malus domestica; 'Topaz') were sprayed with MeSA during fruit development, leading to elevated levels of flavanols and hydroxycinnamic acids but lower fruit quality (Gacnik et al. 2021).

The age of the leaf may also have an impact on how MeSA therapy affects it (Gondor et al. 2022). In a wheat experiment, MeSA was applied to either the first or second to third leaves. In the case of the oldest (1st) leaves, the free SA content increased while the bound form and catalase activity remained constant. However, treatment of younger leaves increased the bound form and catalase activity (Janda et al. 2021).

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted at the Department of Plant Physiology and Metabolomics of the ATK Centre For Agricultural Research, 2462 Martonvasar, H-2462 Hungary. After thorough discussion and planning of the experiment, seeds were sowed, and the experiment spanned from 12th August 2022 to 4th November 2022.

3.2 Seed Treatment

The type of wheat variety used is the Gk-Szeged. Gk-Szeged has good performance. However, it is sensitive to cold stress. An equal number of seeds for control and treatment were selected. Seeds for control were soaked in 500ml of distilled water, whiles the treated seeds were soaked in 500ml solution containing 40µl methyl salicylate (MeSA). The soaking period lasted four (4) hours. After 4 hours, seeds from the control and treatment were put in pots containing sandy loam soil. Sowing was done on the same day and time to avoid varying growth stages. The pots were then put into the phytotron.

Figure 3: Preparatory Stages of The Experiment (Photo by: Adu Donyina 2022).

3.3 Growth Conditions

Table 1: The growth conditions of the phytotron were set at

	Parameter	Time	Intensity
1.	Temperature	Day	20
		Night	18
2.	Humidity	Day	70
		Night	75
3.	Light Intensity	Day	180

Figure 4: Measurement of Light Intensity of the Phytotron Using a Photometer (Photo by: Adu Donyina 2022)

3.4 Sample Collection

Sample collection for laboratory analysis was performed in two stages. The first collection was done 14 days after sowing after which the plants were subjected to cold stress for 4 hours in a different phytotron. The second and last collection was done after the 4-hour stress condition. About 10 grams of leaves were collected from each treatment for sample preparation and analysis. Compounds measured include malondialdehyde (MDA), and antioxidant enzymes including glutathione reductase (GR), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione s-transferase (GST).

Figure 5: Seed Germination and Growth on 14th Day. Yellow And Pink Pots Representing Control and Treated Plants Respectively. (Photo by: Adu Donyina 2022)

Figure 6: Sample Collection for Preparation and Laboratory Analysis (Photo by: Adu Donyina 2022)

3.5 Experimental Design

The experiment was laid out in a completely randomized design (CRD) with two factors, and two levels each. Due to the use of the phytotron and a common soil type, there was complete uniformity in the experiment.

3.6 Antioxidants Measured

3.6.1 Measurement of lipid peroxidation level (MDA Content)

0.2g of the plant sample was rubbed with 900l of 0.1% (w/v) trichloroacetic acid (TCA) in a ribbing mortar and centrifuged at 10000g for 10mins. Afterward, 2 ml of 20% (w/v) TCA containing 0.5% (w/v) Thiobarbituric acid (TBA) was added, and the mixture was incubated at 90°C for 30 minutes. With the help of a spectrophotometer, the MDA content was determined. The specific and non-specific absorbances were set at 532nm and 600nm, respectively. The concentration of lipid peroxides was calculated from the malondialdehyde (MDA) content using an extinction coefficient of 155 mM-1 cm-1 and expressed as pmol g -1 fresh weight.

3.6.2 Measurement of Glutathione Reductase Activity

0.5g of plant sample (leaves) was homogenized with liquid nitrogen and extracted with 2.5 ml of isolation solution in a refrigerated mortar. The extract was then centrifuged at 10,000rpm for 20 minutes in a refrigerated centrifuge. The supernatant was separated and stored on ice until measurement. Using a pipette, the following samples: 750 µl Na-K phosphate, 100 µl DTNB, 50 µl NADPH, and 50 µl GSSG were weighed into 2 different glass cuvettes. The wavelength of the spectrophotometer was set at 412nm. The reaction mixture (blank) was placed in the spectrophotometer and then zeroed. Then 50 µl of the plant sample was added to one of the cuvettes, shaken, and then 1-minute kinetics was measured at 10 seconds lag time with the spectrophotometer.

3.6.3 Measurement of Ascorbate Peroxidase

0.5g of plant sample (leaves) was homogenized with liquid nitrogen and extracted with 2.5 ml of isolation solution in a mortar. At 10,000 rpm, the extract was centrifuged for 20 minutes in a refrigerated centrifuge. The supernatant was separated and stored on ice until measurement. With

the help of the pipette, the following samples: 2 ml of 0.2 M TRIS-HCl, 100 µl of 5.625 mM ascorbate solution, and 50 µl plant sample were weighed into two different glass cuvettes. The wavelength of the spectrophotometer was set at 290nm. The reaction mixture (blank) was placed in the spectrophotometer and then zeroed. Then 100 µl of H_2O_2 was added to one of the cuvettes (in place of the plant sample), shaken, and then 1-minute kinetics measured at 10 seconds lag time with the spectrophotometer.

3.6.4 Measurement of Glutathione S-transferase Activity

0.5g of plant sample (leaves) was homogenized with liquid nitrogen and extracted with 2.5 ml of isolation solution in a refrigerated mortar. For 20 minutes, the extract was then centrifuged at 10,000 rpm. The supernatant was separated and stored on ice until measurement. With the help of the pipette, the following samples: 2 ml phosphate buffer, 150 µl 1-chloro 2,4-dinitrobenzene (CDNB), and 500 µl GSH were weighed into 2 different glass quartz cuvettes. The wavelength of the spectrophotometer was set at 340nm. The reaction mixture (blank) was placed in the spectrophotometer and then zeroed. Then 100 µl of plant sample was added to one of the cuvettes (in place of the plant sample), shaken, and then 1-minute kinetics measured at 10 seconds lag time with the spectrophotometer.

3.6.5 Measurement of guaiacol peroxidase activity

0.5g of plant sample (leaves) was homogenized with liquid nitrogen and extracted with 2.5 ml of isolation solution in a refrigerated mortar. For 20 minutes, the extract was then centrifuged at 10,000 rpm. The supernatant was separated and stored on ice until measurement. With the help of the pipette, the following samples: 2,65 ml Na acetate, and 50 µl plant sample were weighed into two different glass cuvettes. The wavelength of the spectrophotometer was set at 470nm. The reaction mixture (blank) was placed in the spectrophotometer and then zeroed. Then 300 µl of H_2O_2 was added to one of the cuvettes (in place of the plant sample), shaken, and then using the spectrophotometer 1-minute kinetics was measured at 10 seconds lag time.

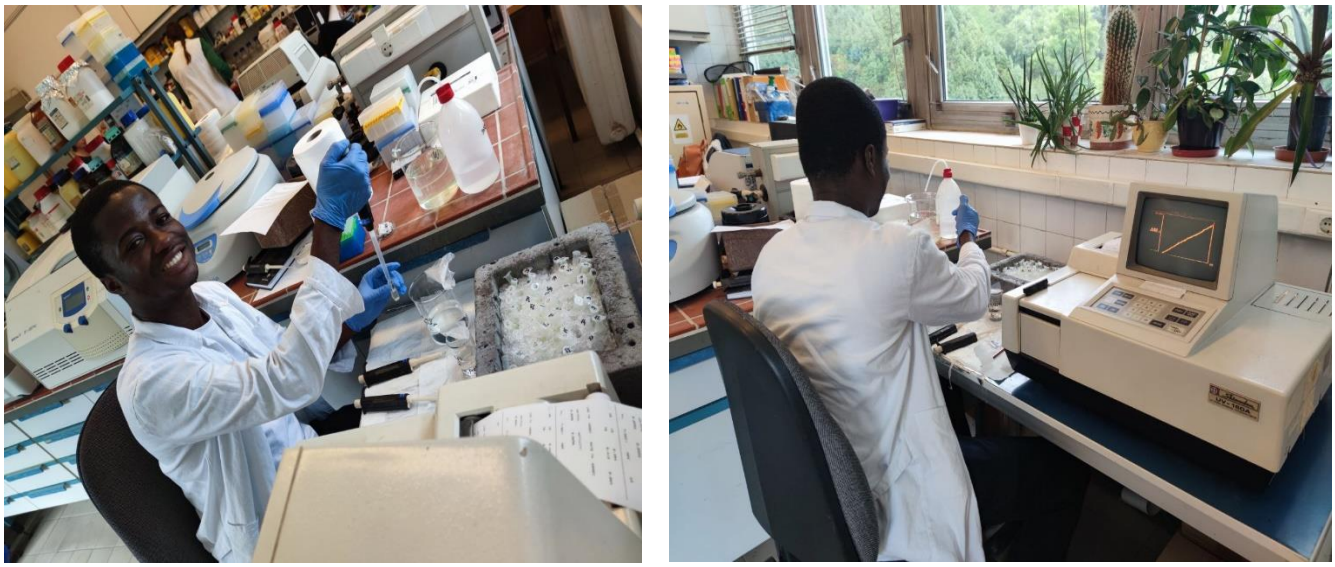


Figure 7: Measurement of Antioxidants Using the Spectrophotometer (Photo by: Adu Donyina 2022)

3.7 Data Analysis

Data obtained from the analysis of the various antioxidant enzymes and malondialdehyde (MDA) was carefully organized and subjected to the two-way multivariate analysis of variance (MANOVA) procedure in IBM SPSS (v 27) at a significance level of 5%. The normality of the model residuals was proved by the Kolmogorov-Smirnov test ($p>0.05$). The homogeneity of variances was checked by Levene's test ($p>0.05$). In case the overall result was significant, univariate two-way ANOVA was run with Bonferroni's correction to avoid the familywise error rate inflation.

4.0 RESULTS AND DISCUSSION

This section presents the main results of the study, as well as its interpretation, and discussion.

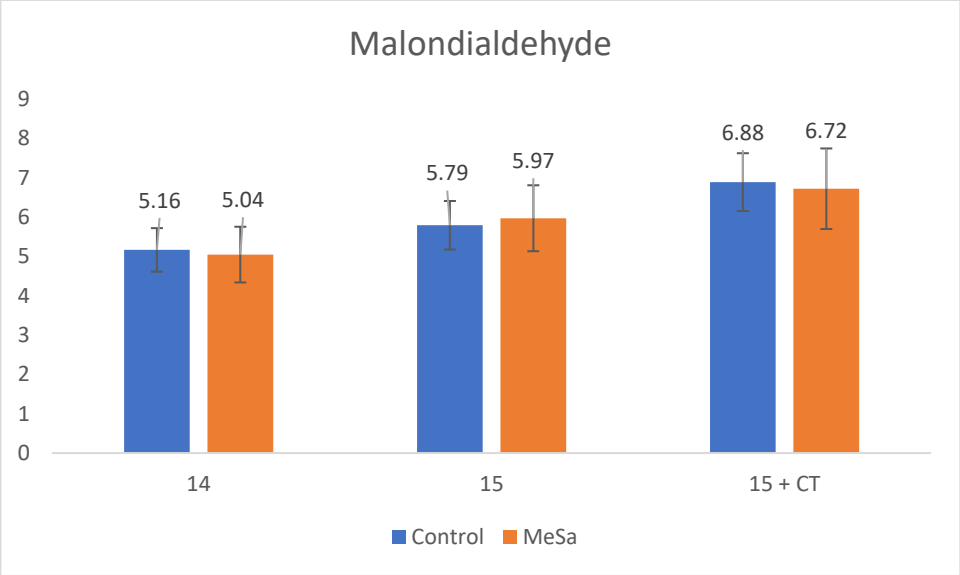


Figure 8: Mean and standard deviation of malondialdehyde (MDA) content in control and MeSA-treated plants at 14 days old, and 15 days old (with and without cold treatment (CT).

From Figure 8.0, it can be observed that the control plant (at 15+ CT) and MeSA-treated plant (at 14 days old) recorded the highest and lowest amount of malondialdehyde (MDA) respectively. Also, MDA content in the plants increased with time, as can be seen from the figure above. Since aging is associated with the death of living cells, it can be said that the increased MDA content in aging plants shows the involvement of lipid peroxidation in plants' cell degradation and death. This agrees with the findings of Hakeem et al., (2014) which indicate that lipid peroxidation generates lipid free radicals, which interact with biomolecules and cause damage to DNA and proteins which consequently leads to cell death. Again, it can be observed from Figure 8 that the highest MDA content was measured in plants that were subject to cold stress (i.e., Control and MeSA-treated). This also agrees with the assertion of Mishra & Dubey (2011) that an increased rate of lipid breakdown is associated with stressed conditions in plants. The increased MDA content under cold stress also indicates an increased production of reactive oxygen species which again confirms the findings of Mishra & Dubey (2011) which claim that under stressed conditions, there is increased production of ROS in plants. Again, it can be observed that under the stress condition, the MeSA-

treated plant had a relatively less MDA content than that of the control plant. This suggests increased activity in antioxidant enzymes due to the application of MeSA, which reduced the effect of ROS in the MeSA-treated plant. This also falls in line with the assertion of Asghari & Aghdam (2010) who recorded in their experiment that MeSA-treated plants had boosted enzyme activity and reduced rate of lipid peroxidation, resulting in less severe membrane breakdown. These differences are however said to be statistically insignificant ($p>0.05$), according to the analysis. This observation could be attributed to either the short duration of cold stress or the lower sample size used for the laboratory analysis.

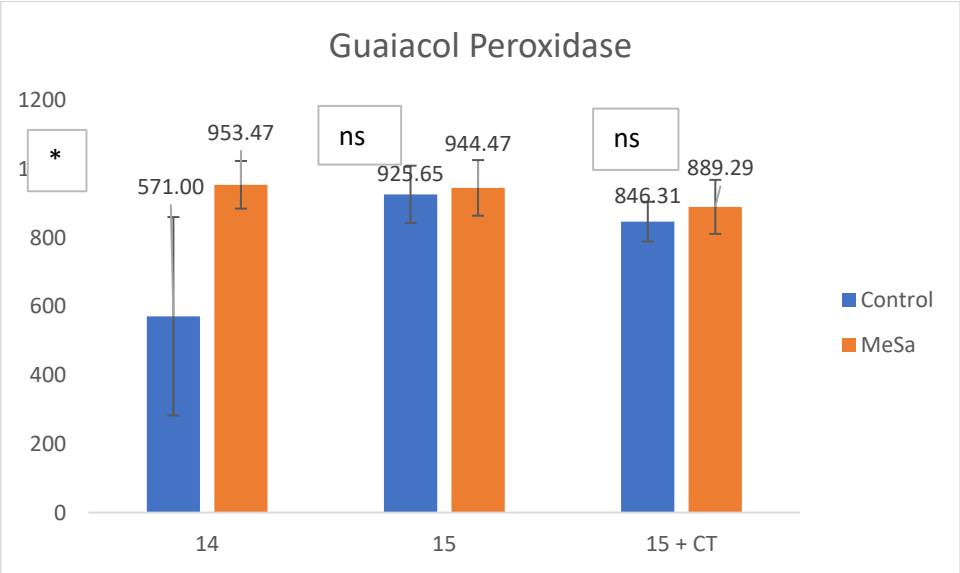


Figure 9: Mean and standard deviation of guaiacol peroxidase (GPX) content in control and MeSA-treated plants at 14 days old, and 15 days old (with and without cold treatment (CT).

Different letters indicate significantly different samples ($p<0.05$). *Significant at $p<0.05$ in comparison of Control and MeSA-treated samples, pair wisely.

From Figure 9, it can be seen that the control and treated plants (at 14 days old) recorded the lowest and highest amount of guaiacol peroxidase, respectively. It can also be observed that there is a significant difference ($p<0.05$) between the control plant (14 days old) and all MeSA-treated plants. Again, it can be observed that all the MeSA-treated plants exhibited higher guaiacol peroxidase content than the control plants. This agrees with the assertion of Kalaivani et al. (2021) which state that treatment of rice seeds with MeSA can greatly boost the peroxidase enzyme activity of treated rice. Again, it can be seen from Figure 9 that, between the last two groups (i.e. 15 days old without cold stress and 15 days old with cold stress) the plants that were subjected to cold stress showed relatively less amount of GPX than the unstressed ones. This, however, contradicts the assertion of Moussa & Abdel – Aziz (2008) that plants respond rapidly to biotic and abiotic stressors by increasing GPX activity. As an explanation for this observation, it can be said that the plants without cold stress experienced an unknown stress condition which could trigger the increased GPX content. These differences are, however, said to be statistically insignificant ($p>0.05$), according to the analysis. Either the short duration of cold stress or the lower sample size used for the laboratory analysis can be the cause of this observation.

The significant ($p < 0.05$) increase in GPX content in MeSA-treated plants (at14 days old) can be attributed to the role of MeSA in enhancing antioxidant enzyme synthesis and activities, which also agrees with the work of Kalaivani et al., (2021) who found that treatment of rice seeds with MeSA can greatly boost the peroxidase enzyme activity in the plant.

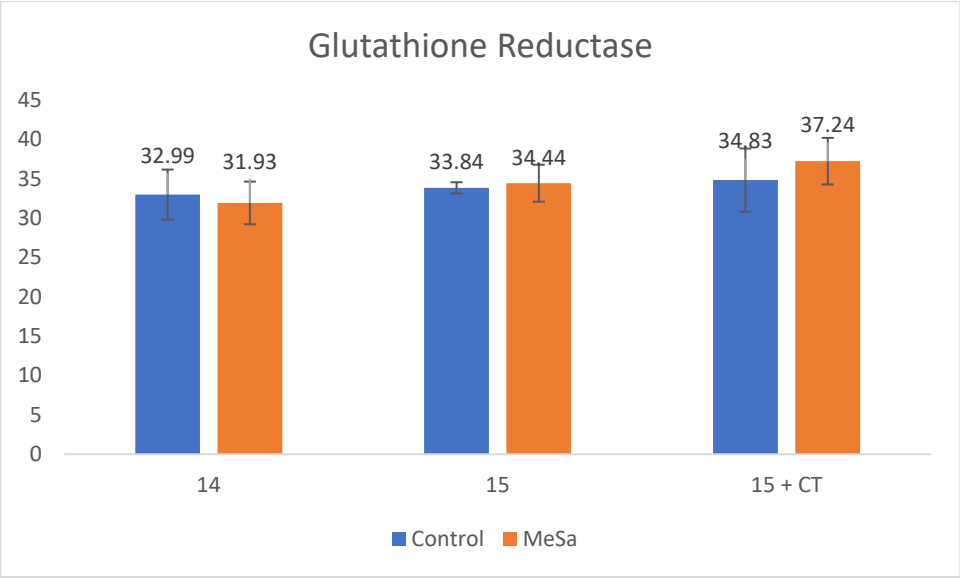


Figure 10: Mean and standard deviation of glutathione reductase (GR) content in control and MeSA-treated plants at 14 days old, and 15 days old (with and without cold treatment (CT).

From Figure 10, it can be observed that glutathione reductase (GR) content in the plants increased with increasing plant age, with the methyl salicylate (MeSA) treated plants having a higher increase rate than the control plants. It can also be observed that under cold stress, the MeSA-treated plant had a higher GR content than the control plant.

Maheshwari & Dubey (2009) found in their experiment that the activity of glutathione reductase enzyme in plants is increased by environmental stressors. Q. Chen et al. (2011), according to their experimental finding, also make a similar claim that the ability of plants to withstand environmental stress is increased by boosting the activity of the antioxidant enzyme defense system. In agreement with these claims, it can be said that the highest amount of GR recorded in the control plant which was subjected to cold stress (i.e., 15+CT), is the result of the plants' own systemic acquired strategy for resisting cold stress. Furthermore, the high amount of GR in the MeSA-treated plant (15+CT) compared with that of the untreated plant (15+CT) can be attributed to the effect of MeSA in enhancing antioxidant enzyme synthesis in plants under stress conditions. This agrees with the experiment of Fan et al. (2021) who recorded an increased enzyme activity and soluble sugar in MeSA-treated Prunus armeniaca L cv. Kate. These differences are, however, statistically insignificant ($p>0.05$), according to the data analysis. Either the short duration of cold stress or the lower sample size used for the laboratory analysis can be the cause of this observation.

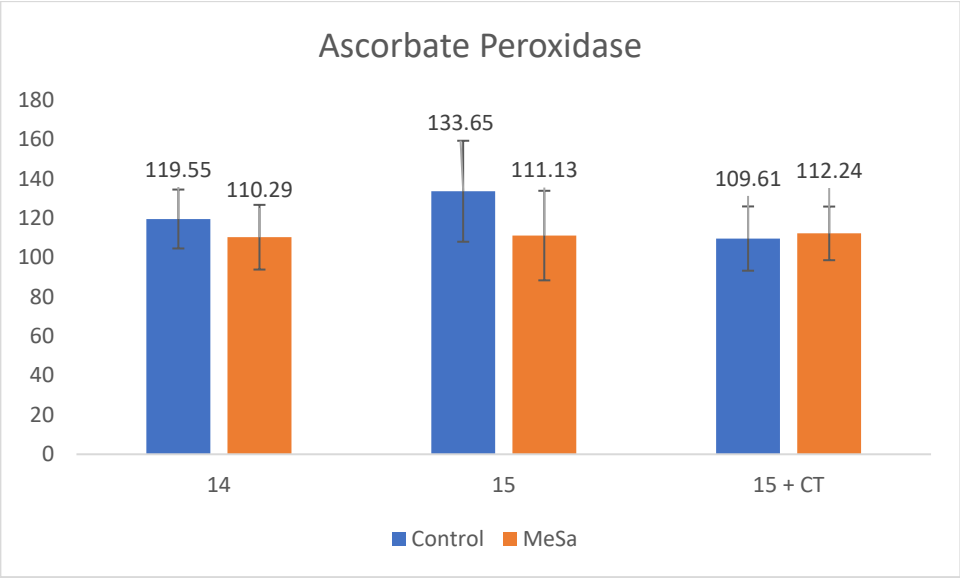


Figure 11: Mean and standard deviation of ascorbate peroxidase (APX) content in control and MeSA-treated plants at 14 days old, and 15 days old (with and without cold treatment (CT).

From Figure 11, it can be observed that the control plant (at 15 days without cold stress) recorded the highest amount of ascorbate peroxidase (APX) while the control plant (at 15 days +CT) had the least. While the APX content in MeSA-treated plants increased with plant age, it can also be remarked that under cold stress, MeSA-treated plants had a higher amount of APX than the control plant. These observations can be attributed to the role of MeSA in enhancing antioxidant enzyme activity in plants. This agrees with the findings of Kalaivani et al. (2021) which claim that treatment of rice seeds with MeSA greatly boosted the peroxidase enzyme activity. These differences are, however, statistically insignificant ($p>0.05$), according to the data analysis. Either the short duration of cold stress or the lower sample size used for the laboratory analysis can be the cause of this observation.

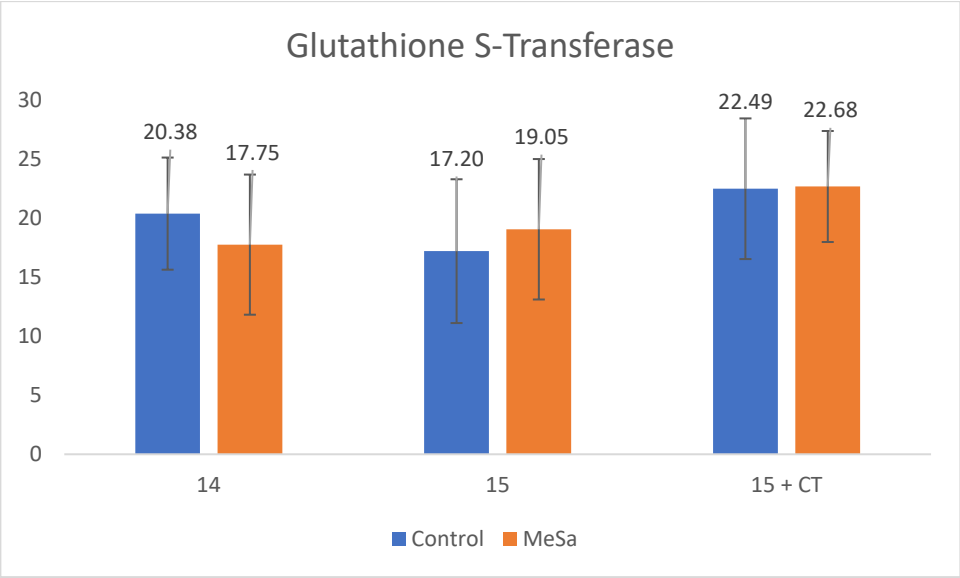


Figure 12: Mean and standard deviation of glutathione s-transferase (GST) content in control and MeSA-treated plants at 14 days old, and 15 days old (with and without cold treatment (CT).

From Figure 12, it can be observed that the highest amount of glutathione s-transferase was recorded in the MeSA-treated plant (at 15 days+CT) while the lowest was recorded in the control plant (at 15 days without CT). It can also be observed that the glutathione s-transferase enzyme increased steadily in the MeSA-treated plants. Again, it can be seen that among the control plants, control 15+CT recorded the highest amount of glutathione s-transferase, likewise the MeSA-treated plant (at15 days +CT) recorded the highest amount of glutathione s-transferase among all the MeSA-treated plants. The highest amount of GST recorded in the control plant (15 days +CT) can be due to the increased synthesis of antioxidant enzymes to scavenge the reactive oxygen species produced as a result of the cold stress. This agrees with the findings of Q. Chen et al. (2011), which claim that the ability of plants to withstand environmental stress is increased by boosting the activity of the antioxidant enzyme defense system. Similarly, the highest amount of GST recorded in the MeSA-treated plant (at 15 days +CT) can be attributed to the role of MeSA as a plant hormone that can enhance the synthesis of antioxidant enzymes. This agrees with the assertion of Fan et al. (2021) who recorded an increased enzyme activity and soluble sugar in MeSA-treated Prunus armeniaca L cv. Kate. These differences are, however, statistically insignificant ($p>0.05$), according to the data analysis. Either the short duration of cold stress or the lower sample size used for the laboratory analysis can be the cause of this observation.

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The results of this study do not provide enough evidence to accept the alternate hypothesis.

Firstly it has been revealed through this study that presoaking wheat seeds in 40µl of methyl salicylate (MeSA) solution may not significantly reduce lipid peroxidation in plants under cold stress.

It was also revealed that seed soaking treatment of wheat with methyl salicylate (MeSA) may not significantly enhance the synthesis of antioxidant enzymes in plants under cold stress.

5.2 Recommendations

With the advent of climate change and its adverse effect on crop production, there is a need to explore various means of enhancing crop resilience against abiotic stressors.

Further research may be conducted to assess the effect of methyl salicylate (MeSA) on plants' response to a longer duration of cold, drought, heat, or salinity stress. These experiments may include the assessment of plants' morphological and genetic responses to abiotic stressors under the influence of MeSA.

SUMMARY

Title: **Effect of Methyl Salicylate on The Physiological and Biochemical Response of Wheat to Cold Stress**

Author: **Gideon Adu Donyina**

Course: MSc. Crop Production Engineering

Institute: Institute of Agronomy

Thesis Advisor: Dr. Jolánkai Marton, Emeritus Professor (Lecturer/MATE)

Independent Consultant: Dr. Orsolya Kinga Gondor (Senior Research Associate/ATK Centre For Agricultural Research)

Wheat (*Triticum aestivum*) is one of the most important cereal crops with the largest area of cultivation, globally. Despite the increasing rate of wheat production, climate change and its resultant abiotic stressors like cold stress tend to significantly affect the crop's performance. Methyl salicylate (MeSA) is a volatile organic compound and herbivore-induced plant volatile (HIPV) whose role in plant abiotic stress response has not received enough investigation.

This study, therefore, sought to unearth the effect of methyl salicylate (MeSA) on the physiological and biochemical response of wheat to cold stress. The Department of Plant Physiology and Metabolomics at the Centre for Agricultural Research - Martonvásár, was the study area. The experiment was laid out in a completely randomized design (CRD). Seeds of the Gk-Szeged variety were soaked in a MeSA solution for 4 hours before sowing. Sample collection for laboratory analysis was performed in two stages: before cold treatment and after cold treatment. The cold treatment also lasted 4 hours. With the help of the spectrophotometer, the guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione s-transferase (GST), glutathione reductase (GR) as well as malondialdehyde (MDA) content in plants were measured and statistically analyzed using the two-way multivariate analysis of variance (MANOVA) procedure in IBM SPSS (v 27) at a significance level of 5%.

The study could not provide enough evidence to accept the alternate hypothesis. It was, therefore, concluded that MeSA had no significant effect on lipid peroxidation reduction as well as the synthesis of antioxidant enzymes in plants, under cold stress. Therefore, further research geared towards the assessment of MeSA's effect on crop response to longer durations of abiotic stressors is recommended.

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To God be the glory, great things He has done. Herein is the scripture (Psalm 37:4) fulfilled which says, “Delight yourself also in the LORD: and he shall give you the desires of your heart.”

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APPENDIX

Descriptive Statistics			Control		MeSA				
x	time	coldtreat	Mean	Std. Deviation	Mean	Std. Deviation		MeSa effect	time effect
APX	0	no	119.55	14.97	110.29	16.43		ns	ns
GPX	0	no	**571.00**	288.54	953.47	69.29		GPX MeSatreated is sig higher at 0	GPX control sig lower at 0
GR	0	no	32.99	3.18	31.93	2.71		ns	ns
GST	0	no	20.38	4.75	17.75	5.93		ns	ns
MDA	0	no	5.16	0.55	5.04	0.71		ns	ns
APX	14	no	133.65	25.66	111.13	22.76	cold treatment is ns	no sig MeSa treatment effect	ns
GPX	14	no	925.65	83.42	944.47	80.89			GPX control sig higher at 14
GR	14	no	33.84	0.71	34.44	2.35			ns
GST	14	no	17.20	6.09	19.05	5.95			ns
MDA	14	no	5.79	0.62	5.97	0.84			ns
APX	14	col	109.61	16.33	112.24	13.62			
GPX	14	col	846.31	57.69	889.29	78.52			
GR	14	col	34.83	4.02	37.24	2.96			
GST	14	col	22.49	5.96	22.68	4.70			
MDA	14	col	6.88	0.73	6.72	1.02			

Multivariate Testsa						
Effect		Value	F	Hypothesi	Error df	Sig.
Intercept	Pillai's Tra	0.998	1469.990b	5	12	0
	Wilks' Lam	0.002	1469.990b	5	12	0
	Hotelling's	612.496	1469.990b	5	12	0
	Roy's Larg	612.496	1469.990b	5	12	0
time	Pillai's Tra	0.588	3.427b	5	12	0.037
	Wilks' Lam	0.412	3.427b	5	12	0.037
	Hotelling's	1.428	3.427b	5	12	0.037
	Roy's Larg	1.428	3.427b	5	12	0.037
MeSa	Pillai's Tra	0.432	1.825b	5	12	0.182
	Wilks' Lam	0.568	1.825b	5	12	0.182
	Hotelling's	0.761	1.825b	5	12	0.182
	Roy's Larg	0.761	1.825b	5	12	0.182
time * Me	Pillai's Tra	0.368	1.396b	5	12	0.293
	Wilks' Lam	0.632	1.396b	5	12	0.293
	Hotelling's	0.582	1.396b	5	12	0.293
	Roy's Larg	0.582	1.396b	5	12	0.293
a Design: Intercept + time + MeSa + time * MeSa						
b Exact statistic						

Tests of Normality						
	Kolmogorov-Smirnova			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Residual for APX	0.133	20	.200*	0.94	20	0.24
Residual for GPX	0.191	20	0.055	0.823	20	0.002
Residual for GR	0.117	20	.200*	0.945	20	0.299
Residual for GST	0.113	20	.200*	0.961	20	0.568
Residual for MDA	0.156	20	.200*	0.903	20	0.047
* This is a lower bound of the true significance.						

Tests of Between-Subjects Effects							
MeSa	Source	Dependen	Type III Su	df	Mean Squ	F	Sig.
CONTROL	Corrected	APX	497.166a	1	497.166	1.127	0.319
		GPX	314445.10	1	314445.1	6.971	0.03
		GR	1.840c	1	1.84	0.347	0.572
		GST	25.249d	1	25.249	0.847	0.384
		MDA	.980e	1	0.98	2.858	0.129
	Intercept	APX	160273.1	1	160273.1	363.275	0
		GPX	5599888	1	5599888	124.143	0
		GR	11165.62	1	11165.62	2106.064	0
		GST	3531.017	1	3531.017	118.465	0
		MDA	299.756	1	299.756	874.575	0
	time	APX	497.166	1	497.166	1.127	0.319
		GPX	314445.1	1	314445.1	6.971	0.03
		GR	1.84	1	1.84	0.347	0.572
		GST	25.249	1	25.249	0.847	0.384
		MDA	0.98	1	0.98	2.858	0.129
	Error	APX	3529.512	8	441.189		
		GPX	360865.6	8	45108.2		
		GR	42.413	8	5.302		
		GST	238.45	8	29.806		
		MDA	2.742	8	0.343		
	Total	APX	164299.7	10			
		GPX	6275199	10			
		GR	11209.88	10			
		GST	3794.716	10			
		MDA	303.478	10			
	Corrected	APX	4026.678	9			
		GPX	675310.7	9			
		GR	44.254	9			
		GST	263.7	9			
		MDA	3.722	9			
MeSA	Corrected	APX	1.747f	1	1.747	0.004	0.949
		GPX	202.770g	1	202.77	0.036	0.855
		GR	15.700h	1	15.7	2.442	0.157
		GST	4.225i	1	4.225	0.12	0.738
		MDA	2.134j	1	2.134	3.547	0.096
	Intercept	APX	122571.5	1	122571.5	311.148	0
		GPX	9005460	1	9005460	1587.816	0
		GR	11011.12	1	11011.12	1712.575	0
		GST	3387.072	1	3387.072	95.962	0
		MDA	302.94	1	302.94	503.495	0
	time	APX	1.747	1	1.747	0.004	0.949
		GPX	202.77	1	202.77	0.036	0.855
		GR	15.7	1	15.7	2.442	0.157
		GST	4.225	1	4.225	0.12	0.738
		MDA	2.134	1	2.134	3.547	0.096
	Error	APX	3151.463	8	393.933		
		GPX	45372.82	8	5671.602		
		GR	51.437	8	6.43		
		GST	282.368	8	35.296		
		MDA	4.813	8	0.602		
	Total	APX	125724.7	10			
		GPX	9051035	10			
		GR	11078.25	10			
		GST	3673.665	10			
		MDA	309.888	10			
	Corrected	APX	3153.21	9			
		GPX	45575.59	9			
		GR	67.137	9			
		GST	286.593	9			
		MDA	6.948	9			

STUDENT DECLARATION

Signed below, **Gideon Adu Donyina**, student of the Szent István Campus of the Hungarian University of Agriculture and Life Science, at the **MSc Course of Crop Production Engineering** declare that the present thesis is my own work and I have used the cited and quoted literature in accordance with the relevant legal and ethical rules. I understand that the one-page summary of my thesis will be uploaded on the website of the Campus/Institute/Course and my Thesis will be available at the Host Department/Institute and in the repository of the University in accordance with the relevant legal and ethical rules.

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As the primary supervisor of the author of this thesis, I hereby declare that the review of the thesis was done thoroughly; the student was informed and guided on the method of citing literature sources in the dissertation, and attention was drawn to the importance of using literature data in accordance with the relevant legal and ethical rules.

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STATEMENT ON CONSULTATION PRACTICES

As a supervisor of **Gideon Adu Donyina (DJ5NJZ)**, I hereby declare that the final essay/thesis/**master's thesis**/portfolio has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

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