



**RESPONSE OF SAFFLOWER TO CHILLING TEMPERATURE AND
DURATION OF EXPOSURE**

**A thesis submitted in fulfilment of the requirements for the award of
degree of Doctor of Philosophy in Crop Science (Horticulture Stream)**

By

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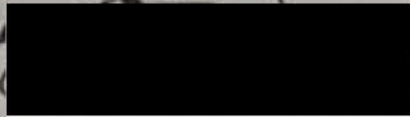
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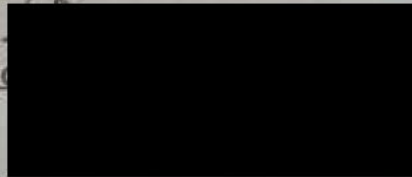


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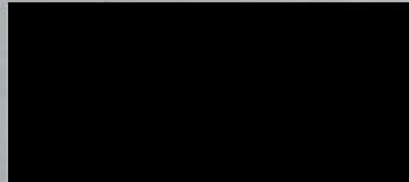
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STATEMENT OF ORIGINALITY

The work contained in this thesis was compiled by the author at the Botswana University of Agriculture and Natural Resources (BUAN) between October 2021 and March 2026. To the best of my knowledge, this work contains no work previously published by any author or materials credited for any degree, diploma or certificate of any other University except where due acknowledgement and reference has been made in text.

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DEDICATION

This thesis is dedicated to my late father, Lieutenant Kebalaole Wire Phuduhudu, who always urged me to aim for greater heights academically. Completion of my thesis writing and PhD studies commemorates the motivational words and incentives he provided to encourage me to work harder in my studies and to be tough in all life situations. I will always be proud of you dad, may your soul rest in eternal peace.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AP 2/ ERF	Apetala 2/ Ethylene responsive factor
APX	Ascorbate peroxidase
ASALs	Arid and semi-arid lands
ATP	Adenosine triphosphate
BR	Brassinosteroids
BUAN	Botswana University of Agriculture & Natural Resources
Ca ²⁺	Calcium
CaMs	Calmodulins
CaMV35S	Cauliflower mosaic virus 35S
CAT	Catalase
CBF	Cold binding factor
CBF1, CBF2, CBF3	Cold binding factor 1,2 & 3
CBLs	Calcineurin B- like proteins
cDNA	Complementary deoxyribonucleic acid
Chl a, b & a+b	Chlorophyll, Chlorophyll a, b, a+b
CI	Chilling injury
CISP1, CISP2 & CISP3	Cold induced small protein 1,2 &3
CK	Cytokinin
CO ₂	Carbon dioxide
COR	Cold responsive
CRT/DRE	C-repeat/ dehydration responsive element
CuZnSOD	Copper- Zinc superoxide dismutase
DAG	Diacylglycerol kinase
DHN	Dehydri
DNA	Deoxyribonucleic acid
DREB a, b & c	Dehydration responsive element a, b & c
DGDG	Digalactosyldiacylglycerol
ETR	Electron transport rate
FA	Fatty acid
FABI	Fatty acid biosynthesis
FBPase	Fructose-1,6-bisphosphatase
FA	Fatty acid desaturase
GA	Giberellic acid
GPAT	Glycerol-3-phosphate acyltransferase
GR	Glutathione reductase
gs	Stomatal conductance
H ⁺	Hydrogen ion
H ₂ O ₂	Hydrogen peroxide
IAA	Indole-3-acetic acid
JA	Jasmonate acid
K ⁺	Potassium ion
LE	Late embryogenesis abundant
LSD	Least significant difference
LT	Low temperature
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MEcPP	Methylerythritol cyclodiphosphate
MeJa	Methyl jasmonate

mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitrite
NR	Nitrogen reductase
$^1\text{O}_2$	Singlet oxygen
O^{2-}	Superoxide
OH	Hydroxyl radical
PA	Phosphatic acid
PM	Plasma membrane
POD	Peroxidase
PPFD	Photosynthetic photon flux density
PSU	Photosynthetic unit
PVP	Polyvinylpyrrolidone
ϕ II	Quantum yield of photosystem II
ϕ NO	Non-regulated energy for photosystem II
ϕ NPQ	Non-photochemical quenching
q RT- PCR	Quantitative Real time -polymerase chain reaction
RAB	Responsive to abscisic acid
RBD1	RNA-binding domain 1
RBP _s	RNA-binding proteins
RGR	Relative growth rate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SBPase	Sedoheptoluse-1,7-bisphosphatase
SOD	Superoxide dismutase
TF	Transcription factor

LIST OF PUBLICATIONS AND PRESENTATIONS

1. Dineo Kereilwe, Vallantino E. Emongor, Utlwang Batlang and Goitseone Malambane. (2026). Photosynthetic parameters as possible indicators of chilling injury in safflower (*Carthamus tinctorious* L.). *Journal of Phytology*. Under review.
2. Dineo Kereilwe, Vallantino E. Emongor, Utlwang Batlang and Goitseone Malambane. (2025). Linking photosynthetic dynamics to stress resilience in safflower (*Carthamus tinctorious* L.). A paper orally presented at YouSustain Conference Botswana, held on the 29-30th of May 2025. University of Botswana, Business Block 245, Gaborone, Botswana.
3. Dineo Kereilwe, Goitseone Malambane, Vallantino E. Emongor and Utlwang Batlang. (2024). Cold-induced biochemical and molecular responses of Safflower to cold stress. *Plant Science Today*, 12(1), 1-10.
4. Dineo Kereilwe, Vallantino E. Emongor, Goitseone Malambane and Utlwang Batlang. (2025). Photosynthetic parameters as stress indicators in safflower. A poster presented at Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) 2nd Triennial Conference. Held on the 12-16, 2024. Mecure Hotel, Windhoek, Republic of Namibia.
5. Dineo Kereilwe, Vallantino E. Emongor and Goitseone Malambane (2023). Temperature and duration of chilling injury of Safflower. *European Journal of Agricultural and Food Sciences*, 5(2):660.
6. Kereilwe et al. (2022). Temperature and duration of chilling injury of Safflower. A paper orally presented at the Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) 18th Annual General Meeting. December 12-16, 2022. Harare International Conference Center, Harare, Zimbabwe.

ABSTRACT

Cold stress causes substantial losses in global agricultural productivity by impairing plant growth, physiology, and survival. Safflower (*Carthamus tinctorius* L.), an ancient, marginalized, neglected yet multipurpose domesticated oilseed crop, is known for its cold and drought tolerance. However, the crop has shown phenological stage-dependent susceptibility to cold stress, especially under Mediterranean type climates, posing a major threat to safflower growers. Moreover, the underlying physiological, biochemical, and molecular mechanisms of cold signal transduction in different developmental stages of safflower are poorly understood due to a lack of systematic research on cold signalling pathways and gene regulatory networks. To address this knowledge gap and issues of chilling injury (CI), this study investigated chilling incidence, physiological, biochemical, and molecular responses of safflower to chilling stress at two phenological stages (rosette and bolling) under both controlled (artificial) and natural (field) conditions. The experimental designs were 2 x 4 x 4 factorial design in artificial chilling condition and 2 x 4 factorial laid down in a completely randomized block design (RCBD) in natural conditions. The results demonstrated that chilling injury incidence (CII) occurred when safflower plants at the bolling stage were exposed to 0 and 4°C for durations of 8 and 12 hours, respectively under artificial chilling stress (refrigerator). While safflower plants at the rosette stage under artificial chilling stress induction did not show any symptoms of CI. On the other hand, plants naturally chilled did not exhibit any signs of CII at either the rosette or bolling stage. Moreover, safflower plants chilled at 4°C for 8 to 12 hours showed moderate increases in relative electrolyte leakage (REL), up to 17.1% and reduced proline levels from 4.79 to 5.40 $\mu\text{mol/g}$ FW under artificial cold stress. While at 0°C for 12 hours, plants at the bolling stage experienced severe membrane damage as indicated by a sharp increase in REL to 65.7%, compared to 12.0% rise in rosette plants. This was accompanied by a drastic decline in total chlorophyll content (Chl a+b), from 2.64 to 1.19 mg/g FW and stomatal conductance (gs) reducing from 518.23 to 9.20 $\text{mmol m}^{-2} \text{s}^{-1}$ in plants at the bolling stage, while rosette plants maintained higher pigment levels and gs (199.07 $\text{mmol m}^{-2} \text{s}^{-1}$). Conversely, proline accumulation peaked at 8.84 $\mu\text{mol/g}$ FW in rosette plants but remained low (~ 4.9 $\mu\text{mol/g}$ FW) in bolling plants. Carotenoids increased in rosette plants from 1.04 to 1.77 mg/g FW but decreased in bolling plants from 0.91 to 0.37 mg/g FW. For the field experiment, exposure of plants to 2°C at night in the bolling stage increased REL (20.18%) compared to rosette plants (6.81%), with gs declining significantly but partially recovering by mid-day from 311.35 to 512.67 $\text{mmol m}^{-2} \text{s}^{-1}$ due to rise in air temperature. Safflower plants at the rosette stage exposed

to chilling stress at 0°C for 12 hours significantly ($P < 0.05$) produced higher proline content of 8.84 $\mu\text{mol/g}$ FW than plants in both development stages exposed to low temperatures of 0 and 4°C for 8 and 12 hours and control. Proline levels increased to 11.65 $\mu\text{mol/g}$ FW in rosette and 10.70 $\mu\text{mol/g}$ FW in bolting plants under field conditions. Safflower genotypes in both phenological stages exposed to chilling stress of 2°C at night under had low leaf chlorophyll content in the morning than control plants. The reduction in leaf chlorophyll content in all genotypes was much higher in plants at the bolting stage compared to the rosette stage. The genotype Sina had higher leaf chlorophyll contents of 73.87 and 61.97 (SPAD) at rosette and bolting stages, respectively than other genotypes under field conditions. In artificial cold induced stress experiments, safflower plants at the rosette stage exhibited enhanced photochemical performance under cold exposure, with quantum yield of PSII (ΦII) increasing from 0.35 (control) to 0.71 after exposure to 4°C for 12 hours, while plants at the bolting stage showed a decrease from 0.40 to 0.19 under the same conditions. Similarly, non-photochemical quenching (ΦNPQ) increased from 0.56 to 0.66 in plants at the rosette stage but dropped sharply from 0.56 to 0.23 in plants at the bolting stage exposed to 4°C for 8 hours. At 0°C for 8 hours, the bolting stage plants displayed a substantial increase in ΦNPQ (from 0.56 to 2.26), but this was later reversed with a 45.42% decrease after 12 hours. Non-regulated energy loss (ΦNO) declined in safflower plants at the rosette stage from 0.21 (control) to 0.15 and 0.18 at 4°C and 0°C, respectively, but increased in plants at the bolting stage from 0.21 to 0.31 and 0.26. Cold stress drastically reduced ETR II from 220.17 (control) to 0.22 at both 0°C and 4°C across all stages. Under field conditions, morning cold exposure at 2°C led to an increase in ΦII from 0.46 to 0.57 for plants at the rosette stage, while plants at the bolting stage experienced a reduction from 0.57 to 0.51. Concurrently, plants at the rosette stage plants maintained lower ΦNO (from 0.16 to 0.17) than plants at the bolting stage (from 0.16 to 0.28). While mid-day recovery showed higher ETR II in plants at the rosette stage (312.23) than plants at the bolting stage (237.92). Among genotypes, Sina exhibited the highest ETR II (300.97), followed by Gila (272.02), PI 537636 (265.77), and Turkey (261.54). Antioxidant enzyme activities (CAT, SOD, POD) also varied significantly. Under artificial cold stress, plants at the rosette stage showed peak activities at 0°C for 12 hours: CAT (1.80 U/mL FW), POD (2.29 U/mL FW), and SOD (4.45 U/mL FW). In contrast, plants at the bolting stage under prolonged cold exposure of 0°C for 12 hours led to a sharp decrease in CAT (0.31 U mL⁻¹ FW) and SOD (0.41 U mL⁻¹ FW) activities, and a significant reduction in POD activity (0.27 U mL⁻¹ FW), indicating differential stress responses across growth stages. Meanwhile, under natural cold stress, CAT, SOD, and POD activities were notably influenced by genotype and time of day.

Gila demonstrated the highest CAT activity of 1.58 U/mL FW in plants at the bolting stage, while Sina exhibited the highest mid-day SOD (3.21 U/mL FW) and POD (0.92 U/mL FW) activities. At the molecular level, expression analysis identified three cold-responsive genes: two E3 ligase genes (CtAH10T0001700.1, CtAH10T0001500.1) with RING finger domains, and a GRAS family gene (CtAH10T0000500). These genes successfully exhibited similar cold responses as per their respective family. Overexpression of some E3 genes like CIP8 and HOS 1 could be associated with the cold susceptibility of plants at the bolting stage. Additionally, CtAH10T0001700.1 expression was found to be positively correlated with electrolyte leakage and proline content in safflower plants at the rosette stage. Meanwhile CtAH10T0001500.1, a negative cold regulator significantly correlated with electrolyte leakage and proline content. While CtAH10T0000500, a positive cold regulator significantly correlated with electrolyte leakage and proline at the rosette stage but negatively associated with the two parameters in plants at the bolting stage. Overall, the genes were found to play a significant role in cold responsiveness of safflower plants at the rosette and bolting phenological stages. In conclusion, safflower exhibited developmental stage-specific responses to cold stress, with plants at the rosette stage demonstrating better physiological resilience, antioxidant defence, and gene regulation than plants at the bolting stage. These findings provide novel insight into the cold signal transduction pathways and identified molecular markers for breeding cold-tolerant safflower genotypes. Therefore, the study recommended that future research and breeding efforts should focus on enhancing safflower cold tolerance, particularly at the bolting stage, by targeting physiological, biochemical, and molecular traits associated with resilience at this stage. Strengthening these mechanisms will be vital for improving safflower's performance and yield stability under chilling conditions.

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 General information

Safflower (*Carthamus tinctorius* L.) is an herbaceous, oilseed, annual plant in the Asteraceae family. Safflower is highly suited for the arid and semi-arid lands (ASALs) for dry land crop production (Öztürk et al., 2008; Janmohammadi, 2015; Eslam et al., 2024). It has a sturdy taproot that penetrates deep into the soil layers (2-3 m) where most cereal and other oil crops cannot reach, hence its drought tolerance derived from that character (Janmohammadi et al., 2016; Emongor & Oagile, 2017; Eslam et al., 2024). Safflower is tolerant to water deficit, salinity, extreme low and high temperature conditions (-15 to 40°C), and soils with poor fertility (El-Bey et al., 2021; Koç, 2021; Mosupiemang et al., 2022; Eslam et al., 2024), which contributes to its good seed and oil yield in the ASALs. In the ASALs safflower is grown as an alternative oil crop (Gecgel et al., 2007; Emongor & Emongor, 2023) rich in essential fatty acids; oleic, linoleic, and linolenic (Brenna et al., 2009; Mišurcová et al., 2011; Glick & Fischer, 2013; Katkade et al., 2018; Moatshe et al., 2020a), which are beneficial to human health (Mani et al., 2020; Emongor & Emongor, 2023). Furthermore, safflower cultivation is favourable in most soils. However, for increased production deep, lush soils with good drainage are preferable (Armad-Agyeman et al., 2002). The adaptable characteristics of safflower have so far qualified it as a promising crop to counteract the negative repercussions of climate change (low and unreliable rainfall, heat, hailstorm) and alleviate poverty in the ASALs, which are faced with hot summers and cold winters (Armad-Agyeman et al., 2002; Gecgel et al., 2007; Mosupiemang, 2024). Although safflower can withstand a wide range of temperature extremes, it remains vulnerable to low temperatures (<-6.2°C at night) at the bolling stage, which can restrict its performance (Kolanyane, 2022; Mosupiemang, 2024). Future research should focus on improving its cold tolerance through genetic and agronomic interventions to maximize its potential as a climate-resilient crop.

1.2 The geographical distribution of Safflower

Safflower is naturally adapted to regions with latitudes between 45°S (Argentina and Australia) and 60°N (Russia) (Esendal, 2001). Furthermore, it is cultivated at elevations below 900 m above sea level. However, in the torrid weather, it can be cultivated in altitudes of 1400–2000 m above sea level (Ethiopia, Kenya) (Khalili et al., 2014; Emongor & Emongor, 2023). The

native distribution of safflower is in areas surrounded by the eastern Mediterranean and Persian Gulf, Eastern Europe, Central Asia and Abyssinia (Ethiopia and Eritrea) (Zohary et al., 2012; Khalili et al., 2014). Remnants of safflower seeds were found in Egyptian burial sites dating back more than 4,000 years, and the Chinese have been using it for over 2,200 years (Weiss, 2000). To date it is cultivated in many regions of the world, with Kazakhstan, the USA, Russia, Mexico, China, India, Argentina, Turkey, Tanzania, and Australia being the top ten leading seed producers (FAO, 2020; Tridge, 2022). Generally, safflower is adapted to diverse climatic areas of the world, such as regions with Mediterranean climate, which experience humid winters and arid summers. It is also adapted to regions with weather like the Northern Great Plains of the USA and Canada, where safflower is grown in the spring and harvested in the fall season. Furthermore, safflower is adapted to regions with high autumn precipitation, which results in high soil moisture content favourable for autumn planting and spring harvesting, like in India (Knowles, 1989). Recently, due to its adaptability to various environments, safflower cultivation has been introduced to Southern African countries like Angola, Botswana, Namibia, South Africa, and Eswatini, in which it is irrigated in winter and rainfed in summer (Emongor & Oagile, 2017; Emongor & Emongor, 2023).

1.3 Effect of temperature on safflower developmental stages

Safflower plant growth is composed of four main important developmental stages, namely, seed germination, rosette, stem elongation, and bloom stages. Then, the plant reaches its physiological maturity (Moatshe et al., 2020b; Emongor & Emongor, 2023). Safflower can be cultivated in temperatures between -15°C and 40°C, provided it is not extremely cold when the plant is in elongation and bolting stages of plant growth (Kolanyane, 2022; Mosupiemang, 2024). Usually, seed germination and seedling emergence take place within 3 to 14 days from planting, depending on temperature and if moisture is available. Seed germination can take place at low temperatures of 2-5°C, depending on the soil moisture (Torabi et al., 2013; Emongor & Oagile, 2017; OECD, 2020). After seedling emergence, the next developmental stage is the rosette, which is accompanied by slow growth during which numerous leaves sprout close to the ground level and a strong taproot develops (Dajue & Mündel, 1996; Emongor & Oagile, 2017). The rosette stage is considered the cold hardy stage, as safflower plants can endure chilling temperatures as low as -15°C depending on genotype (Li et al., 1997; Carapetian, 2001; El-Bassam, 2010). Safflower can be in the rosette stage for 20 to 39 days, depending on genotype, light duration, temperature after emergence, and management practices (El-Bassam, 2010; Kolanyane, 2022; Mosupiemang, 2024). However, the rosette

phase can be prolonged by cold acclimation, which is associated with increased survival rate from chilling injury and higher grain/oil yields (Emongor et al., 2013; Landry et al., 2017). Conversely, the rosette stage can be shortened from 39 days to 23 days by increasing photoperiod from 10 hours to 14 hours (Weiss, 2000). Air temperature and short days (photoperiod) of less than 20°C and 10 hours, respectively, have been reported to delay rosette, elongation, flowering, and physiological maturity stages of safflower by 5.2, 10, 24.4, and 35.3 days, respectively, in Botswana (Moatshe, 2019; Moatshe et al., 2020b). Immediately after the rosette stage, stem elongation begins forming extensive branches about 45-75 cm long, depending on the growing season (Dajue & Mündel, 1996; Singh & Nimbkar, 2006; Emongor & Oagile, 2017). Rosette stage is then followed by the flowering period, which lasts 4 - 6 weeks as influenced by agronomic practices and weather conditions, particularly temperature (Weiss, 2000; Moatshe et al., 2020b). High temperature in conjunction with long days accelerates flowering time (Gilbert, 2008). From the late stem elongation phase through to the start of the flowering stage, safflower is cold sensitive in winter (-2°C or less) and plants tend to suffer high mortality due to chilling injury (El-Bassam, 2010; Kolanyane, 2022; Mosupiemang, 2024). At these stages (stem elongation and flowering), plants require about 20-30°C temperature for the development of a strong stem, bolling, and seed filling (Mündel et al., 1992; El-Bassam, 2010). Cultivars with a shorter stem elongation period are a better choice in cold winter months (Johnson, 2006; Landry et al., 2017). These studies suggested that there is an interesting phenomenon about safflower development and low temperatures.

1.4 Problem statement

Safflower awareness and cultivation is growing worldwide mainly due to its capability to produce high seed yields and quality oil, used for cooking, making cosmetics, paints, and varnishes (Ekin, 2005; Singh & Nimbkar, 2006; Mailer et al., 2008; Khalid et al., 2017). The nutritional value of safflower oil is the same as that of olive oil (Kumar et al., 2015; Moatshe et al., 2020a; Esmailzadeh et al., 2022), and on this basis, the crop has recently been noticed (Aghamohammadreza et al., 2013; Kumar et al., 2015; Bella et al., 2019; Emongor & Emongor, 2023). The versatile adaptability of safflower to various environments has made it a potential crop for diversification for many economies especially in the ASALs (Mailer et al., 2008; Khalid et al., 2017; Phuduhudu et al., 2018; Kereilwe et al., 2020).

Safflower can be grown as a cut flower, vegetable, birdseed, medicinal plant, livestock feed, as well as industrial applications such as in the production of high-quality vegetable oil and

paints, varnishes, biodiesel, ethanol, natural food, and pharmaceutical colourants, and production of pharmaceuticals (Bar-Tal et al., 2008; Rudolphi et al., 2012; Phuduhudu et al., 2018; Mani et al., 2020). The cultivation of cold tolerant safflower genotypes could contribute greatly to winter grain crop rotations in the ASALs of Southern Africa. However, the crop tends to suffer from chilling injury at the bolling developmental stage (just before the onset of flowering) (El-Bassam, 2010; Emongor, 2010; Landry et al., 2017; Mosupiemang, 2024). Chilling injury (CI) is undesirable as it is known to reduce crop productivity and quality of most crops (Lukatkin et al., 2012; Bortolheiro & Silva, 2017; Kereilwe et al., 2023). In countries like Botswana, which at times experience a radical drop in temperature in late winter (-10°C) and frequent night frosts in some parts of the country (Dube & Chiguvi, 2017; Mosupiemang, 2024), CI can be a major threat to safflower planted between March to end of April in Southern Botswana because the bolling stage and start of flowering will coincide with the lowest temperatures in July-August (Kolanyane, Korononeo, 2023; Mosupiemang, 2024). Late planting is not always a solution, as farmers can miscalculate and plant too late, thus causing more harm than it would have been caused by early planting (GRDC, 2017). Several studies recommend early safflower planting so that the stage of flowering and seed development is not synchronized with the time of extreme cold winter (Bergman & Kandel, 2013; Korononeo, 2023; Mosupiemang, 2024). Up until now, there is no scientific knowledge base on the specific low temperature that causes CI in safflower plants. Moreover, there is no scientific information about morphological, physiological, biochemical, and molecular changes induced by CI in safflower and causes differential responses of safflower in different developmental stages when exposed to chilling low temperatures.

1.5 Rational of the research

The research sought to better comprehend the phenomenon of chilling injury in safflower plants. That includes,

- i. The chilling injury symptoms
- ii. The temperature and duration of exposure that cause chilling injury in safflower
- iii. The physiological, biochemical, and molecular (gene expression) alterations that take place within safflower plant cells/tissues that have been exposed to low temperature stress in the later stages of plant growth and development, and how cold acclimation and tolerance are attained.

1.6 Research question

Which actual temperature, growth stage, and duration of exposure induces chilling injury in safflower, and what mechanisms are involved in cold acclimation that brings about morphological/physiological disorders in response to chilling stress?

1.7 Objectives

1.7.1 General objective

The general objective of the research was to understand the phenomenon of CI in safflower plants to maximize crop production and crop health under cold conditions.

1.7.2 Specific objectives

The specific objectives were to,

1. Determine the specific low temperature and duration of exposure that induces chilling injury in safflower and recovery.
2. Screen for the physiological, biochemical and molecular (gene expression) changes that occur in safflower plants exposed to chilling temperatures.

1.8 Hypothesis

The hypotheses that were tested included,

1. Ho: Low temperature and duration of exposure do not induce chilling injury in safflower and there is no significant difference in its recovery response.
Ha: Low temperature and duration of exposure do induce chilling injury in safflower plants, hence affecting its recovery response.
2. Ho: Physiological, biochemical, or molecular (gene expression) responses are not altered during exposure of safflower plants to chilling temperatures.
Ha: Physiological, biochemical, or molecular (gene expression) responses are altered during exposure of safflower plants to chilling temperatures.

1.9 Significance of the study

Avoidance of cold stress factors aids in the production of quality safflower crop hence increased seed yields, excellent oil levels, and other by-products of safflower (Kolanyane, 2022; Korononeo, 2023; Mosupiemang, 2024). Improved production of safflower could provide the anticipated economic advancements, especially for developing countries that need

to diversify their economies. Farmers also need a reliable database that will guide them not to synchronize the most sensitive stage of safflower plant growth with frost time (GRDC, 2017; Kolanyane, 2022; Korononeo, 2023; Mosupiemang, 2024). Hence, this study will add value to the body of knowledge on the physiological, biochemical, and molecular basis of chilling injury in safflower. The results of this study will be applied in selection and breeding programmes for cold tolerance and acclimation in safflower.

CHAPTER 2

2.0 LITERATURE REVIEW

Plants encounter significant challenges in field conditions due to various abiotic stresses, which can severely impact their growth and productivity. Among these stresses, chilling stress poses a particular threat to plant development and yields. Low temperatures can cause physical damage, disrupt physiological and biochemical processes, and trigger molecular changes, all of which negatively impact plant performance (Sagehashi, 2023; Oyebamiji et al., 2024; Wang et al., 2024). To tolerate chilling stress, plants employ a range of protective mechanisms, including morphological adjustments, physiological adaptations, and molecular responses. These strategies are critical for mitigating the damaging effects of chilling stress on crop production (Raza et al., 2022a; Oyebamiji et al., 2024; Wang et al., 2024).

The literature reviewed in this chapter focuses on the concept of CI in safflower and other crops. It provides insights into what CI is, the crops that are affected, and mechanisms of cold acclimation and tolerance. These mechanisms include morphological, physiological, biochemical and molecular responses of plants to CI. Furthermore, the chapter discusses cold signal perception and the responsive pathways in different crops. It also outlines management strategies to mitigate chilling stress, which is crucial for enhancing crop resilience against low temperature stress.

2.1 Theory of Chilling Injury

Low temperature is a major abiotic stress that limits the growing season, productivity, and geographical distribution of many agricultural crops (Guan et al., 2023; Jan et al., 2024, Qian et al., 2024, Wang et al., 2024). Approximately 57% of the world's land and 26% of rural areas are affected by chilling stress, respectively (Crammer et al., 2011). Different plants have different capacities to tolerate chilling stress. Many tropical and subtropical plants such as tomato (*Solanum lycopersicum*), rice (*Oryza sativa*), and maize (*Zea mays* L.) are susceptible to chilling temperatures, whereas arabidopsis (*Arabidopsis thaliana*) and some overwintering cereals are tolerant to chilling (Ahmad & Prasad, 2012; Wang et al., 2016a; Sagehashi, 2023). Cold stress sensitive plants suffer damage at temperatures lower than 15°C, while cold tolerant plants survive at temperatures as low as 5°C (Rao, 2004). Theocharis et al. (2012a) reported that a series of physiological and cellular changes occur in plants under chilling conditions, including alterations in the membrane structure, photosynthesis, calcium signals, and

metabolism. These alterations make the plant to either adapt or suffer chilling stress. Therefore, tolerance to chilling stress is an important factor to be considered in crop production.

Chilling injury is defined as the physiological changes that are induced by exposure of the susceptible tissues to low temperatures, typically between 0 and 15°C, but above their freezing point (Raza et al., 2023; Sagehashi, 2023; Chang and Brecht, 2024). This implies that CI is a distinct phenomenon from freezing injury, which occurs when tissue freezes and ice crystals form below the freezing point. The physiological changes in CI are classified as either primary or secondary injuries (Loay, 2005). Those considered as primary injuries only cause transitory changes and are immediately shifted back if the temperature is changed to a warmer condition. While the secondary injuries are irreversible and lead to permanent plant damage or necrosis (Theocharis et al., 2012a; Chang and Brecht, 2024). There is what is referred to as cold shock, which often occurs a few minutes after a sudden cold, evident by minor CI symptoms (Pikuta et al., 2007). However, low temperature stress can severely suppress physiological and biochemical reactions in plant cells, resulting in reduced seedling emergence, leaf chlorosis, disordered root cell division, reduced root growth, poor seed filling and development, wilting, and necrosis of plant cells (Hassan et al., 2021; Wu et al., 2022; Chang and Brecht, 2024).

Chilling stress has been reported to adversely affect the growth and development of several economically important crops such as tomato (Starck et al., 2000; Raza et al., 2023), soyabeans (Thakur et al., 2010), potato (Svensson et al., 2002; Yan et al., 2021), wheat (Jan et al., 2024), rice (Hussain et al., 2016; Sagehashi, 2023; Jan et al., 2024), cotton (Thakur et al., 2010; Zhao et al., 2012), maize (Raza et al., 2023; Jan et al., 2024; Wang et al., 2024) and sugarcane (Anjum et al., 2011; Zhu et al., 2013). Sagehashi (2023) reported that six Hokkaido rice cultivars exposed to 4°C had zero survival after 10 days of cold exposure. Meanwhile, those initially cold acclimated at 12°C for 5 days had 88-100% survival rates. Yan et al. (2021) reported that the leaves of potato plants (*Solanum tuberosum*) can survive low temperatures of 0°C, but lower temperatures of -2°C cause detrimental damages such as leaf wilting, dropping, and even total death of the whole plant. These findings highlight the importance of understanding crop-specific responses to chilling stress and developing strategies to improve cold tolerance in susceptible species.

2.2 Mechanisms of cold adaptation and tolerance

Cold acclimation and tolerance mechanisms in plants involve a complex interplay of structural, physiological, biochemical, and molecular adaptations that enable survival in low-temperature environments. Together, these mechanisms not only protect plants from cellular damage caused by chilling but also prepare them for improved performance under fluctuating or recurrent cold conditions (Raza et al., 2023; Wang et al., 2024).

2.2.1 Morphological adjustment during chilling stress and symptoms

Cold stressed plants undergo morphological adjustments such as reduction in leaf size and surface area to minimize water loss, thickening of cell walls to enhance structural integrity, development of clustered growth forms, or altered root architecture for improved resource allocation (Adhikari et al., 2022; Soualiou et al., 2022). For instance, many cold-acclimated crops such as winter wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) develop smaller, thicker leaves with reduced surface area to minimize water loss and reduce exposure to chilling temperatures (Soualiou et al., 2022; Xu et al., 2022).

The compact growth habit observed in some cold-tolerant species, such as kale (*Brassica oleracea* var. *acephala*) and rye (*Secale cereale*), helps conserve energy and protect vulnerable tissues (Bauer et al., 2022). Similarly, root system modifications, such as enhanced root density and reduced elongation, are observed in crops like kale and sugar beet (*Beta vulgaris* L.), allowing them to improve water and nutrient uptake under low-temperature conditions (Bauer et al., 2022; Li et al., 2023; Kulan and Kaya, 2024). These morphological changes are vital in protecting plants from cold-induced dehydration, mechanical damage, and reduced metabolic activity, enabling them to maintain growth and productivity in adverse environments.

Chilling stress often manifests a range of detrimental morphological symptoms in plants such as leaf surfaces may develop necrotic lesions, which are dark, dead patches that can hinder photosynthesis and overall vigor. The process of leaf development may be delayed, resulting in stunted growth and reduced foliage. Plants may exhibit wilting, characterized by drooping leaves that signal a lack of water uptake, alongside browning tissues that indicate cellular damage (Goering et al., 2021; Adhikari et al., 2022; Chang and Brecht, 2024). Additionally, chilling stress can cause off flavors in fruits and vegetables, negatively affecting their taste and marketability. Fruits may develop sunken spots and surface pitting, which can detract from their visual appeal (Perkins-Veazie et al., 1993). The ripening process may become uneven,

leading to fruits with varying levels of maturity. Translucency in plant tissues may occur, making them appear waterlogged, while water-soaked lesions can develop, creating a breeding ground for pathogens. Overall, plants experiencing chilling stress become increasingly vulnerable to infections and diseases, compromising their health and productivity (Rymen et al., 2007; Hussain et al., 2018; Shah et al., 2024). Moreover, it is crucial to note that the symptoms exhibited during CI differ among species and cultivars of different crops (Chang & Brecht, 2024). Karim & Yusof (2021) reported loss of flavour, wilting of leaves, surface pitting, and browning of leaf margins as symptoms of CI in spinach while Adhikari et al. (2021) reported stem cracking, lack of vigour, leaf wilting, and necrosis on seedlings of alfalfa (*Medicago sativa* L.) affected by chilling temperatures. Jan et al. (2024) reported low germination rate, decreased seedling vigour, stunted growth, smaller leaves, leaf yellowing and wilting, reduced tillering, limited root development, increased seed abortion, and reduced seed size as CI symptoms in maize, rice and wheat plants. These symptoms slow crop growth and ultimately decrease crop yield and quality.

2.2.2 Physiological changes during chilling stress

Based on the model for temperature sensing, thermal variations cause alterations in membrane fluidity, re-structuring of the cytoskeleton and invasion of calcium ions (Ca^{2+}) which activate downstream responses to bring about cold tolerance (Ma et al., 2015; Zhu, 2016).

2.2.2.1 Changes in the cell membranes

Plant cells play a crucial role in detecting changes in their environment. In response to these changes, signals are transmitted throughout the cell to initiate the appropriate physiological response. Although the exact mechanism(s) by which plants sense temperature is not yet fully understood (Penfield, 2008), it has been suggested that variations in membrane fluidity due to temperature changes may be the primary way that plants perceive thermal stimuli (Das & Roychoudhury, 2014; Jan, 2024). Thus, there is a change in the physical arrangement of the cell membrane brought about by cold temperatures. One of the significant implications of temperature decrease is a decrease in membrane fluidity, which affects membrane functions, and the plasma membrane (PM) has been suggested as a primary sensor of low-temperature stress (Wei et al., 2021). Recent studies investigating cold stress signal transduction mechanisms have confirmed PM as a key sensor of low temperature stress in crops such as rice, maize, wheat and arabidopsis (Wu et al., 2022; Jan, 2024). For instance, Cano-Ramirez et al. (2021) found that PM demonstrated highly fluidity and underwent numerous phase

transitions and hysteresis, whereas microsomal fraction and vacuole membrane did not exhibit these characteristics in arabidopsis plantlets subjected to a temperature range of -5 to 41°C. These findings suggest that the PM serves as a crucial cellular hub, capable of rapidly modifying its fluidity in response to slight temperature fluctuations within the ranges typically experienced in natural environments.

2.2.2.2 Change in the physiological structure and function of the chloroplasts

Chloroplasts play an important role in the process of plant adaptation to low temperature. During this process, plants need to coordinate the relationship between low-temperature adaptation and photosynthetic efficiency to obtain better growth and more production. Therefore, there is a trade-off between chilling tolerance and photosynthesis. The regulatory mechanism which seeks to maintain the normal physiological functions of chloroplasts includes chloroplast development retardation in cold tolerant plants. In cold-sensitive plants, low temperatures promote cool-temperature-induced-chlorosis (CTIC) symptoms in newly emerging leaves or result in different degrees of an albino phenotype (Song et al., 2014; Wu et al., 2016). This has been corroborated by *wlp1* (White Leaf and Panicles 1) mutant gene in rice, which expressed white albino leaves and immature panicle at an early seedling stage, with severe symptoms strongly expressed at low temperatures (Song et al., 2014). In another study, a new rice pentatricopeptide repeat (PPR) protein mutant gene called thermo-sensitive chlorophyll-deficient mutant 10 (*tcd10*) was characterized, that also expressed the albino phenotype, malformed chloroplast, and could not survive after the 5th leaf stage when grown at 20°C. However, the mutant gene displayed a normal phenotype when grown at a warmer temperature of 32°C (Wu et al., 2016). Various RNA-binding proteins (RBPs) like pentatricopeptide repeat protein (PPR) and ribonucleoproteins (RNPs), are needed for the response to chilling stress to maintain chloroplast development. The loss of RBP genes results in yellow or bleached leaves, and some mutants even exhibit delayed seed germination accompanied by pale primary leaves (Wang et al., 2016b; Wu et al., 2016; Zhang et al., 2016; Cui et al., 2019). Some proteins have been reported to influence the development of chloroplasts by disturbing photosynthetic electron transfer, regulating the transcript levels of genes involved in chloroplast transcription/translation and photosynthesis, and interfering with RNA processing (Gu et al., 2014; Wang et al., 2016b; Morita et al., 2017). Mutations in these genes result in a deficiency in chlorophyll synthesis that causes chlorotic, white, or dead leaves to varying degrees at low temperatures, and some mutations even cause severe growth inhibition, such as the temperature-sensitive chlorophyll deficient rice mutant *tcd5*, cold

sensitive virescent1 (CSV1) and DEAD-box RNA helicase AtRH3 genes (Gu et al., 2014; Wang et al., 2016b; Morita et al., 2017). The decrease in chlorophyll content will further affect the ability of chloroplasts to capture light signals. Since chilling stress impacts chloroplast function in various species, it is crucial to explore whether safflower exhibits similar cold-sensitive mutations or regulatory pathways that influence chlorophyll biosynthesis. Understanding this could help maintain chloroplast function, thereby enhancing cold tolerance and preventing yield losses in safflower during the winter season or in cooler regions.

2.2.2.3 Changes in the photosystems

Plants can differentiate variations in day length, light quality, and light intensity. Light plays a significant role in the detection of thermal variations (Ensminger et al., 2006; Kocova et al., 2009). Light plays a major role in photosynthesis, which is mostly affected by temperature changes (Ensminger et al., 2006; Kocova et al., 2009; Wu et al., 2022). It is in the presence of light that photosynthesis can strike a balance between the energy utilized by photosystems (PSI and PSII) and that consumed by plants metabolic reactions (Winfield et al., 2010; Wu et al., 2022). The photophysical and photochemical reactions of PSI and PSII are tremendously fast and do not depend on temperature (Winfield et al., 2010; Lambrev et al., 2012; Tikkanen et al., 2013; Garab et al., 2023). In contrast, biochemical reactions are extremely dependent on temperature and are reduced by chilling temperatures. Reduction in the rate of biochemical reactions at low temperatures is known to uncouple PSI and PSII (Winfield et al., 2010; Wu et al., 2022). When the two systems are uncoupled, electrons from PSI are usually converted to oxygen to form undesirable reactive oxygen species (ROS) such as singlet oxygen ($^1\text{O}_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\text{OH}^\cdot$) (Pospíšil, 2009; Winfield et al., 2010). The uncoupling of the two systems was shown to increase accumulation of $^1\text{O}_2$ and H_2O_2 in cold sensitive silage corn (*Zea mays* L.) seedlings exposed to chilling temperatures of 5°C (Wu et al., 2022).

Reactive oxygen species are responsible for DNA, proteins, carbohydrates, and lipids damage related to cold stress (Kocova et al., 2009). They may also be involved in defensive mechanisms which try to bring tolerance in cold-stressed plants. The rate of light-induced ROS production plays a key role in cold stress perception (Suzuki & Mittler, 2006). Earlier studies have reported that cold treatment in the light triggers the up-regulation of about twice the number of cold-responsive genes as compared to the number induced under the same conditions in the dark (Soitamo et al., 2008). Kosmala et al. (2009) reported that close to 50% of the proteins that

change in high levels during cold acclimation are involved in photosynthesis in meadow fescue (*Festuca pratensis*). Furthermore, chloroplasts employ light as a source of energy and respond to differences in light intensity by acclimatizing metabolism to the redox state of the electron transport chain (Pfannschmidt et al., 2001). One of the primary stimuli promoting expression of cold regulated genes is increased PSII stimulation pressure (Gray et al., 1997; Ndong et al., 2001). Moreover, exposure to cold in dark conditions reduces the induction of numerous COR genes (Crosatti et al., 1999; Kobayashi et al., 2004). As shown by barley albino mutants that poorly developed chloroplasts under-expression of cold-responsive genes (Svensson et al., 2006). Since there is no data relating to safflower regarding changes in the photosystem due to low temperature, there is therefore a need for further studies to discover the reactions of safflower photosystems in response to CI.

2.2.2.4 Influence of cold stress on the physiology of cold-sensitive plants

2.2.2.4.1 Water regime

Low temperature stress causes dehydration and subsequent wilting of the leaves in cold sensitive plants. Bloom et al. (2004) observed wilting in the shoot of the cold susceptible tomato after exposure to chilling temperatures of 5°C. Adhikari et al. (2021) reported leaf wilting on alfalfa (*Medicago sativa* L.) seedlings exposed to chilling temperatures of 0°C. The wilting may be attributed to failure of roots to absorb water from the soil and transport it to the shoots (Lukatkin et al., 2012). One other factor that may contribute to plant wilting is decreased ability of the stomata to close in response to subsequent water deficit. As a result of poor water supply to the leaves, water potential is lost and this usually occurs in the first hours of low chilling temperature exposure to plants (Bloom et al., 2004; Hussain et al., 2018).

2.2.2.4.2 Mineral nutrients

Low temperature stress influences cellular turgidity and initiates drought stress (Yadav, 2010; Kumar et al., 2021). Drought stress induced by low temperature stress reduces root hydraulic conductivity, restricts root growth, and reduces leaf turgidity in plants (Siddique et al., 2000; Hussain et al., 2018), resulting in leaf wilting. Consequently, water relations, nutrient uptake, carbohydrate metabolism, and translocation of photoassimilates are significantly disrupted (Hussain et al., 2018; Wei et al., 2021). Nutrients uptake from the soil as well as their distribution to upper plant parts become difficult under chilling conditions. This situation leads to nutrient deficiencies as plants are not receiving adequate nutrients (Lukatkin et al., 2012).

Of the affected nutrients components due to chilling stress include decreased activity of nitrate reductase, reduction in nitrogen incorporation into amino acids and proteins, a drop in the proportion of organic phosphorus, and an increase in inorganic P content (Zia et al., 1994), which is a consequence of a breach of phosphorylation and enhanced decomposition of organic P compounds (Bose & Srivastava, 2001). In relation to ammonium absorption, a study with sensitive (*Lycopersicon esculentum* Mill. cv. T-5) and cold-resistant (*Lycopersicon hirsutum* LAI 778) tomato plants showed absorption inhibition of this ion up to 7 hours after 4 hours exposure to a temperature gradient from 15 to 5°C in susceptible plants, whereas reabsorption in resistant plants occurred soon after increase in temperature (Nievola et al., 2017). The mechanisms involved in the reduction of absorption of nutrients by plants induced by low temperatures include depression of respiration and/or oxidative phosphorylation, impaired enzymatic transport systems associated with conformational proteins changes in membranes, changes in membrane potential, reduced supply of adenosine triphosphate (ATP) to H⁺-transporting ATPase, as well as lowered permeability coefficients for ions (Ruelland & Zachowski, 2010; Zhang et al., 2011b; Lukatkin et al., 2012).

2.2.2.4.3 Respiration rate

One of the negative effects of low temperature in cold-sensitive plants is a change in respiration rate. Low temperatures disrupt mitochondrial structure, thereby lowering overall kinetic energy and suppressing enzymatic activity, resulting in a decreased respiration rate in plants (Li et al., 2013; Kumar et al., 2018; Hassan et al., 2021). Munro et al. (2004) reported 85-92% reduction in ubiquinone pool, comprised of ubiquinol (UQH₂), ubiquinone (UQ) and total ubiquinone content, in mung beans (*Vigna radiata* L.) that were cold acclimated at 10°C for 3 days, followed by exposure to 5°C for an additional 3 days. This reduction in the ubiquinone pool led to decreased respiration rate, indicating mitochondrial dysfunction under chilling stress. Since ubiquinol (UQH₂) and ubiquinone (UQ) are key components of the mitochondrial electron transport chain (ETC), their depletion disrupted electron transfer efficiency, impairing mitochondrial function. Similarly, mitochondrial dysfunction under chilling temperatures was observed in wheat (*Triticum aestivum*) seedlings exposed to 4-12°C for 12 hours (Li et al., 2013; Hassan et al., 2021). In wheat, chilling stress led to reduced energy flow kinetics, altered gaseous exchange, decreased enzymatic activity, ATP depletion, and overall metabolic dysfunction, further confirming the impact of low temperatures on mitochondrial integrity and respiratory efficiency. These studies collectively highlight how chilling-induced disruptions in

mitochondrial activity can compromise energy production and stress resilience in different plant species.

In contrast, Yadegari et al. (2008) found that chilling temperatures and prolonged duration of exposure resulted in an increased respiration rate and membrane permeability in soybean (*Glycine max*) seedlings. Non-acclimated plants (at 25°C) exhibited an oxygen (O₂) concentration of approximately 0.9 mg/L against 0.4 mg/L of previously acclimated plants (at 15°C) after both plants were subjected to chilling temperature of 4°C for 24 hours. These results may suggest irreversible metabolic dysfunction and accumulation of incompletely oxidized intermediates. Although the mode of stimulation was unclear, Kumar et al. (2018) and Ikkonen et al. (2020) proposed that the increase may be due to uncoupling of oxidative phosphorylation. Because of decreased respiration and increased consumption of energy-rich phosphates at low chilling temperatures, there is a reduction in ATP production.

The above findings highlight that chilling stress disrupts mitochondrial function, leading to reduced respiration rates in cold-sensitive plants like mung bean and wheat, primarily due to ubiquinone depletion, impaired electron transport, and ATP deficiency. However, in some species like soybean, chilling may trigger an increase in respiration, possibly due to metabolic imbalance and oxidative stress. Given safflower's susceptibility to chilling injury, similar mitochondrial dysfunction and altered respiration dynamics may contribute to its reduced stress resilience, particularly during the flowering stage. Therefore, in-depth investigation of strategies that could help improve safflower's metabolic stability, mitochondrial integrity, and overall resilience under chilling stress should be taken into consideration.

2.2.2.4.4 Photosynthesis rates

In grain crops such as safflower, photosynthesis and biomass accumulation are significant for grain production and important physiological processes in the crop's phenological stages, and these processes are vulnerable to low temperature stress (Leonardos et al., 2003; Valluru et al., 2012; Rinalducci et al., 2011; Emongor et al., 2017; Khan et al., 2017; Moatshe et al., 2020). Reduction in photosynthetic rate is one of the adverse problems encountered by plant exposure to chilling temperatures (Leonardos et al., 2003; Valluru et al., 2012; Nievola et al., 2017; Wei et al. 2021). The reduction in photosynthesis is thought to be attributed to the inhibition of phloem transport of carbohydrates from the leaves, stomatal limitation, destruction of the photosynthetic apparatus, damage to water-splitting complex of photosystem I, reduced chlorophyll synthesis, poor chloroplast development, inhibition of electron transport system,

and uncoupling of electron transfer and energy storage, changes in the enzyme activity, suppressed Rubisco activity during carbon assimilation, and inhibition of synthesis of other key enzymes of the Calvin cycle and C₄-way (Terashima et al., 1998; Kingston-Smith et al., 1999; Venema et al., 1999; Van-Heerden et al., 2003; Bota et al., 2004; Garstka et al., 2007; Hussain et al., 2018; Wei et al., 2021). Recent studies on the role of photoreceptors as temperature-sensitive components have reported that the photoreceptor phyB and phototropins act as thermosensory molecules that perceive fluctuating ambient temperatures (Jung et al., 2016; Legris et al., 2016; Fujii et al., 2017). Phototropin 2, a blue light receptor, was found to direct the position of chloroplasts to optimize photosynthesis in cold-avoidance response in plants such as arabidopsis, green alga (*Mougeotia scalaris*), fern (*Adiantum capillus-veneris*) and moss (*Physcomitrella patens*) (Kodama et al., 2008). Decrease in photosynthesis at chilling temperatures may be a consequence of photooxidative damage to the photosystems in chloroplast membranes, manifested by increased lipid peroxidation and degradation of chlorophyll, carotene, and xanthophylls in maize, wheat, and rubber plants (Fryer et al., 1998; Kingston-Smith & Foyer, 2000). Venzhik et al. (2011) reported reduction in photosynthesis rate (18%) and electron transport rate (7%) in wheat plants after five hours of cold hardening at 4°C. Meanwhile, the maximum efficiency of photosystem II (PSII) and chlorophyll content decreased by 8% and 11% after 24 hours and one hour, respectively. In contrast, non-photochemical quenching increased by 12% within five hours of cold hardening, indicating an enhanced photoprotective response under cold stress.

A study on the rubber plant (*Hevea brasiliensis* Muell. Arg.) demonstrated that photosynthetic injury can take place within a few hours of cold exposure. The plant exhibited photosynthetic damage after being exposed to chilling temperatures of 10°C for four hours (Clark et al., 2000). However, those plants returned to their previous values of photosynthetic rates when transferred to temperature of 28°C. Recovery took place in less than 24 hours (Clark et al., 2000). Reduction in photosynthetic rate may also be associated with reduction in photosynthetic pigments content induced by cold stress. A study on corn showed that cold sensitive genotypes grown at 14/12°C (day/night) had low photosynthetic pigments content and, consequently low photosynthetic rate when compared to tolerant genotypes (Nievola et al., 2017). These studies imply that chilling can induce a series of physiological and biochemical events in chloroplasts, and both photoreceptors and membrane can perceive temperature changes (Liu et al., 2018).

2.2.2.4.5 Metabolic rate

Mostly in cold sensitive plant tissues, protein synthesis is inhibited due to low temperatures. This scenario usually leads to elevated contents of free amino acids, especially proline (Gilmour et al., 2000). Accumulation of proline is regarded to be the key factor of mechanism of cold hardening (Sanghera et al., 2011). During chilling of sensitive plants, toxic products of metabolism are accumulated due to the imbalances triggered during cold stress, and this is one of the main factors contributing to chilling injury, which often occurs after the return of chilled plants to warmer environments (Sanghera et al., 2011; Lukatkin et al., 2012). Alterations in metabolic activities only take place after a long exposure to chilling condition. Therefore, metabolic changes together with changes in water regimes are not the primary cause of chilling injury (Loay, 2005; Theocharis et al., 2012b). In general, low temperature stress significantly suppresses plants metabolic rates by disrupting enzyme activity, hindering key physiological processes such as photosynthesis and respiration (Kumar et al., 2018; Fürtauer et al., 2019; Zhang et al., 2020; Wei et al., 2021). Reduction in photosynthesis and respiration due to low temperature stress leads to reduction in growth and development (Kumar et al., 2018; Fürtauer et al., 2019; Zhang et al., 2020) which may result in altered gene expression, changes in membrane fluidity, and accumulation of protective compounds to mitigate the effects of cold stress (Kumar et al., 2018; Fürtauer et al., 2019; Zhang et al., 2020; Wei et al., 2021).

2.2.3 Biochemical changes during chilling stress

Plants use various biochemical mechanisms to combat the negative effects of low temperature stress. These mechanisms include changes in the lipid composition of cellular membranes, the accumulation of cryoprotectants and osmolytes, increased enzyme activity, and the regulated production of ROS. Together, these adaptations allow plants to adjust and survive in cold stress conditions (Horvath et al., 1998; Orvar et al., 2000; Sanghera et al., 2011; Theocharis et al., 2012; Longo et al., 2017; Nievola et al., 2017; Hussain et al., 2018; Liu et al., 2018; Wang et al., 2018; Sun et al., 2019). These adaptations are further elaborated below.

2.2.3.1 Change in the lipid composition

Change in the lipid composition is one of the outstanding documented cold acclimation mechanisms. Since a change in the membrane fluidity due to low temperature is perceived as a potential site of CI (Horvath et al., 1998; Orvar et al., 2000), adaptation of cells to chilling temperatures is a function of alteration in the membrane lipid composition by increased fatty

acid (FA) unsaturation. Monogalactosyldiacylglycerols (MGDGs, digalactosyldiacylglycerol (DGDG), sulphoquinovosyldiacylglycerols (SQDGs and phosphatidylcholine (PC) are major lipids in the thylakoid membrane (Pribil et al., 2014; Li & Yu, 2018). Low temperatures reduce the levels of MGDGs and SQDGs and an increase in lutein (Skupien et al., 2017). Levels of unsaturated FAs are regulated by FA-desaturase (FAD) activities (D'Angeli & Altamura, 2016). Researchers have reported that the extent of the unsaturation of FAs in Phosphatidylglycerol (PG) in the thylakoid membrane can protect the photosynthesis system against chilling-induced photo-inhibition (Liu et al., 2008).

Overexpression of the LeGPAT gene in tomatoes increased cis-unsaturated fatty acid content in phosphatidylglycerol (PG) of thylakoid membranes under chilling stress conditions. This modification enhanced tolerance to chilling stress by improving photosystem II (PSII) efficiency and antioxidant enzyme activities (Sun et al., 2011). In another experiment, exposing tobacco plants to low temperature of 8°C for several days elevated the relative levels of unsaturated FAs, specifically 18:3n-3 FA. Hence, that altered membrane fluidity to accommodate the high-level functioning of the photosynthetic apparatus (Sakamoto et al., 2004; Khodakovskaya et al., 2006; Popov et al., 2017). As evident above, the presence of high levels of unsaturated FAs in chloroplasts increases the tolerance of plants to chilling.

2.2.3.2 Accumulation of cryoprotective polypeptides and osmolytes

In cold stress-tolerant plants, many genes are involved in the synthesis of osmoprotectant organic compounds, such as amino acids like proline, quaternary and other amines like glycinebetaine, and polyamines. These compounds, along with various sugars and sugar alcohols such as mannitol, trehalose, and galactinol, accumulate during osmotic adjustment (Sanghera et al., 2011). It has been reported that sugar content in *Sansevieria trifasciata*, *Yucca aloifolia*, *Echinocactus grusonii*, *Euphorbia resinifera*, *Bryophyllum pinnatum*, *Aloe vera*, and *Crassula lactea* increased after cold stress (Khan et al., 2015). Plants that tolerate cold stress tend to accumulate more carbohydrates in their underground tissues, including starch, amylose, maltose, fructans, D-Glucose, and D-Glucose-6-Phosphate (Janská et al., 2010; Londo et al., 2018). Sucrose, composed of monosaccharides such as glucose and fructose, play a key role in growth, development, storage, signalling, and stress adaptation (Janská et al., 2010). Leaf starch may contribute carbon to carbohydrate accumulation in response to low temperatures (Sicher, 2011). Fructans are often accumulated during cold acclimation in cereals and grasses like ryegrass (*Lolium perenne*) and wheat, serving to stabilize cell membranes (Livingston &

Heyer, 2009). Additionally, trehalose, a non-reducing disaccharide of glucose, has been linked to increasing freezing tolerance and may also be involved in starch accumulation (Theocharis et al., 2012).

2.2.3.3 Change in enzymatic activities

The rate of carbon dioxide (CO₂) utilisation in plants is reduced under chilling stress. It has been reported that the activity of two key carbon reductive cycle enzymes, a crucial pathway for photosynthetic carbon fixation where atmospheric CO₂ is converted into organic compounds, fructose-1,6-bisphosphatase (FBPase) and sedoheptoluse-1,7-bisphosphatase (SBPase) substantially decreased under light-induced chilling (Hutchison et al., 2000). Additionally, the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) also declines under low temperatures. Low temperatures may also damage the Rubisco protein or affect the redox regulation of the larger Rubisco activase isoform (Liu et al., 2018). Naturally, plants adapt to chilling stress by production of high number of photosynthetic enzymes, specifically those involved in carbon reduction cycle and sucrose synthesis (Savitch et al., 2001). Those are synthesized for the compensation of reduced activity of other enzymes like Rubisco, stromal FBPase, SBPase, sucrose phosphate synthase and cytosolic FBPase, under cold stress (Savitch et al., 2001; Liu et al., 2018). An increase in the activity of the enzyme sucrose-phosphate synthase was verified in *Arabidopsis* after cultivation at 4°C for 24 hours (Nägele et al., 2011) and in cabbage (*B. oleracea*) at 5°C for 72 hours (Sasak et al., 2001). The increase in this enzyme shows an important role in sucrose accumulation and acquisition of cold tolerance (Nievola et al., 2017).

2.2.3.4 Manipulation of ROS-scavenging mechanisms in chloroplasts (Antioxidants)

Since ROS are one of the main causes of cold stress-induced damage to DNA, proteins and lipids, the use of ROS-scavenging mechanisms is very important for maintaining normal function in chloroplasts under chilling stress, in turn cold acclimation. The levels of ROS and the activities of ROS-scavenging enzymes have been shown to increase in chloroplasts of cold sensitive silage corn and cucumber grown in low temperatures (Choi et al., 2002; Hu et al., 2008; Wu et al., 2022). Zhou et al. (2006) reported that a chilling-tolerant cucumber cultivar had higher H₂O₂-scavenging activity in chloroplasts than a chilling-sensitive cultivar under low temperature conditions. Londo et al. (2018) also reported that over production of H₂O₂ in grapevine (*Vitis vinifera*) plants resulted in necrotic plants, even after 4 days of recovery. The high level of H₂O₂ in cold stressed plants was attributed to increased oxygenation reaction in

the chloroplasts, which leads to an increased glycolate content. The glycolate was converted to glyoxylate in peroxisomes by glycolate oxidase, hence consequence accumulation of H₂O₂ (Londo et al., 2018). The physiological process of ROS toxic concentration in plants could be relieved by developing efficient ROS scavenging and antioxidant defence system. Decreasing ROS toxicity can be achieved by increasing the activity of antioxidant enzymes, like as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and peroxidase (POD), while decreasing malondialdehyde (MDA) content and Ca²⁺ in the cytosol, as these was found to reduce lipid degradation (Longo et al., 2017; Hussain et al., 2018; Wang et al., 2018; Sun et al., 2019; Wu et al., 2022). Overexpressing both copper zinc superoxide dismutase (CuZnSOD) and ascorbate peroxidase (APX) in the chloroplasts of transgenic tobacco (*Nicotiana tabacum*) and sweet potato (*Ipomoea batatas*) enhanced plant tolerance to high light and chilling stress (Kim et al., 2003; Lim et al., 2007). Jing et al. (2006) identified a gene encoding a chloroplast-localized peroxiredoxin Q, Suaeda salsa Peroxiredoxin Q (SsPrxQ), in seepweed (*Suaeda salsa* L.). The overexpression of SsPrxQ in Arabidopsis enhanced plant tolerance to low temperatures (Shu et al., 2011; Ding et al., 2012).

In another study, it was revealed that cold tolerance in watermelon plants (*Citrullus lanatus* (Thomb.) Mansf. cv. *dulce maravilla*), grown at 10°C for 30 days, was due to higher H₂O₂ content and reduced activity of APX, CAT and GR cultivated when compared to plants grown at 35°C (Rivero et al., 2002). The rapid rise in the enzyme activities in response to cold is thought to be associated with gene expression regulation. It has been reported that rubber plants cultivated at 10°C have a 2.5-fold increase in APX gene expression after 24 hours, and the activity levels of this enzyme also increased after one hour of cold exposure (Mai et al., 2009), indicating that responses of the antioxidant system may occur hastily. Furthermore, the findings from studies on watermelon and rubber plants indicate that antioxidant enzyme activity varies across species. Therefore, since safflower enzyme activity under cold stress has not yet been established, there is a need to investigate the specific activity of antioxidant enzymes in safflower when exposed to chilling temperatures.

2.2.4 Molecular changes during chilling stress

2.2.4.1 Cold Responsive Genes

To understand the mechanisms involved in chilling stress of cold acclimation plants, studying transcriptional alterations is very important. As of today, several cold responsive genes and their corresponding regulatory networks have been identified and characterized in plants (Lee

et al., 2005; Tsuda et al., 2000; Zhang et al., 2011a; Ren et al., 2021; Wang et al., 2024). These genes are termed as Late Embryogenesis–Abundant (LEA), Dehydrin (DHN), Responsive to Absciscic Acid (RAB), Low Temperature–Responsive (LT), and Cold-Responsive (COR) genes (Winfield et al., 2010). Most of these genes encode highly hydrophilic proteins, and they belong to the LEA family, hence; they are collectively termed COR/LEA genes or COR genes (Jan, 2024; Qian et al., 2024; Wang et al., 2024).

Some of these genes are structural proteins, and they provide a defence mechanism in cold-stressed plants. Such proteins act as protein chaperones, cryoprotectants, and ice-binding proteins. On the other hand, others are regulatory genes, which control expression levels of structural genes (Winfield et al., 2010; Ritonga & Chen, 2020). The regulatory genes are also transcription factors that control many target genes. They achieve that by directly binding the cis-acting elements in the promoter regions of the target genes. The transcription factors and the target genes are collectively referred to as a Regulon. One of the most studied regulons involved in cold responses is the C-repeat binding factor (CBF) regulon driven by CBF transcription factors (Jia et al., 2016; Wang et al., 2016a; Zhao et al., 2016; Ren et al., 2021; Wang et al., 2024). A common characteristic of cold acclimation is the rapid induction of genes encoding CBF-like transcription activators. In Arabidopsis, these are simply called CBF1, CBF2, and CBF3 or dehydration-responsive elements DREB1b, DREB1c and DREB1a, respectively (Wang et al., 2016a; Ren et al., 2021; Jan, 2024; Wang et al., 2024).

The CBF transcription factors belong to the family of DNA-binding proteins commonly known as APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) (Campoli et al., 2009; Sanghera et al., 2011; Borba et al., 2018; Hussain et al., 2018; Qian et al., 2024; Wang et al., 2024). Those transcription factors identify the cold- and dehydration-responsive DNA regulatory element nominated as the C-repeat/dehydration-responsive element (CRT/DRE). C-repeat binding factors (CBFs), serve as master transcription factors which stimulates the expression of many downstream cold responsive (COR) genes (Chinnusamy et al., 2007; Wang et al., 2016a; Zhu, 2016; Borba et al., 2018; 2016; Shi et al., 2018; Wu et al., 2022; Jan, 2024). It is reported that nearly 10-20% of the COR genes are controlled by the CBF transcription factors (Rihan et al., 2017b; Kosova et al., 2021; Qian et al., 2024). CBFs activate the expression of COR genes via binding to cis-element in the promoter of COR genes (CRT: TGGCCCGAC) (Zhen & Ungerer, 2008, Rihan et al., 2017a; Hussain et al., 2018; Jan, 2024). The CBF genes are induced within 15 minutes in plants being exposed to low chilling temperatures. After about two hours one begins to observe the induction of cold-regulated

genes that contain the CRT /DRE regulatory element (Chinnusamy et al., 2007; Zhu, 2016; Shi et al., 2018). The CBF3 and COR genes are sensitive to the redox state of chloroplasts (Kurepin et al., 2013). Moreover, the expression of some COR genes is regulated by CBF-independent transcription factors (Shi et al., 2018), such as RNA-binding domain 1 (RBD1) (Wang et al., 2016a). These changes in chloroplasts are communicated to the nucleus through retrograde signals, including singlet oxygen (Kim et al., 2012; Mullineaux et al., 2018) and methylerythritol cyclodiphosphate (MEcPP) (Xiao et al., 2012), in *Arabidopsis* and H₂O₂ in *Nicotiana benthamiana* (Exposito-Rodriguez et al., 2017). Retrograde-signalling molecules further activates a broad range of responses and elicit the expression of stress-responsive nuclear-encoded proteins. There may be multiple retrograde signals from chloroplast to nucleus involved in chilling stress and they may work together in the plant (Liu et al., 2018). CBFs also play important roles in other abiotic stresses (Qian et al., 2024; Wang et al., 2024). Some of the COR genes have an ABA responsive element and dehydration responsive element in their promoter regions (Wang et al., 2018; Ritonga & Chen, 2020; Jan, 2024; Qian et al., 2024). It was reported that in DREB (Kargiotidou et al., 2008) and FtbHLH2, the transgenic plant promoter was induced by cold and that many cis-elements of the FtbHLH2 promoter collaborated in cold stress conditions (Yao et al., 2018). These findings further highlight the complexity of COR gene regulation, demonstrating that COR genes do not only support chilling tolerance but also play roles in other abiotic stress responses, such as ABA signalling and dehydration stress. This interplay between different stress pathways suggests that cold tolerance is part of a broader stress adaptation network, integrating multiple environmental signals. A deeper understanding of these mechanisms is essential for improving plant resilience to low temperatures and other stress factors.

2.2.4.2 A transgenic approach to attain cold adaptation and tolerance

As discussed earlier, the CBF/ DREB genes are very crucial when it comes to low temperature adaptation and signal transduction (Hsieh et al., 2002). The CBF1/DREB1b cDNA when introduced into tomato under the control of cauliflower mosaic virus 35S (CaMV35S) promoter improved tolerance to chilling, drought, and salt stress, although it resulted in a dwarf phenotype with reduced fruit set and seed number per fruit (Hsieh et al., 2002). Similar response was found in *Arabidopsis* (Gilmour et al., 2004; Welling & Palva, 2008), rapeseed (*Brassica napus*) (Jaglo et al., 2001), potato (Pino et al., 2007), and rice (Ito et al., 2006) in which constitutive overexpression of CBFs under the transcriptional control of the 35S

cauliflower mosaic virus promoter in transgenic plants resulted in severe growth retardation under normal growth conditions.

In arabidopsis and rice, the CBF/DREB1-dependent cold response pathway has been shown to play a predominant role in cold tolerance through the process of cold acclimation (Sanghera et al., 2011; Adhikari et al., 2022; Qian et al., 2024; Wang et al., 2024). These CBFs/DREBs are upstream transcription factors that bind to promotor cis element CRT/DRE and activate the expression of these COR genes (Sanghera et al., 2011; Wang et al., 2024). Overexpression of CBF1/DREB1b and CBF3/DREB1a was reported to enhance cold tolerance by inducing COR genes, such as COR78, COR47, COR15, rd29A, and COR6.6 in arabidopsis. These genes contribute to cold tolerance by producing hydrophilic peptides that protect cells from cold damage (Qian et al., 2024). In another study, the overexpression of the dehydrin gene *Saussurea involucrata* (SiDHN) led to increased cold tolerance in transgenic tomato plants by scavenging ROS and increasing membrane stability in plants kept at temperatures of 0°C and 4°C for 48 hours (Guo et al., 2019b). Moreover, over expression of CBF3 transcriptional activators was reported to cause many biochemical changes, such as the accumulation of sugar and proline (Gilmour et al., 2000). In consequence, the CBF/DREB1 genes are thought to be activators that integrate several components of the cold acclimation response by which plants increase their tolerance to low temperatures after exposure to chilling conditions (Qian et al., 2024; Wang et al., 2024). In overall, it can be concluded that the CBF/DREB1 pathway integrates multiple cold acclimation responses, improving plant survival under low temperatures. This knowledge could be beneficial for safflower production, particularly in phenological stages susceptible to cold stress. Introduction or over expression these genes could improve its ability to withstand chilling stress.

2.2.4.3 The role of messenger RNA in cold adaptation and tolerance

Transcriptional regulation at the stages of pre-mRNA processing, mRNA stability, and nuclear export plays a crucial role in cold acclimation and tolerance (Chinnusamy et al., 2007). During pre-mRNA processing, mRNA undergoes several modifications, including the addition of a 5' methyl cap, a poly (A) tail, and intron splicing. These processes are essential regulatory mechanisms of gene expression in eukaryotes. Splicing is particularly important for removing introns and generating functional mRNAs capable of translation (Chinnusamy et al., 2010). In genes containing multiple introns, alternative splicing can produce different protein variants from a single gene. Approximately 20% of plant genes undergo alternative splicing, which,

although not fully characterized, appears to influence processes such as photosynthesis, flowering, grain quality in cereals, and plant defence responses (Chinnusamy et al., 2010).

Several studies have linked intron splicing to abiotic stress responses. For example, in wheat, two early cold-regulated (e-cor) genes coding for ribokinase (7H8) and C3H2C3 RING finger protein (6G2) were found to undergo stress-dependent splicing under cold conditions (Mastrangelo et al., 2005). Similarly, Chinnusamy et al. (2010) reported that the barley homologs of these genes exhibited stress-dependent intron retention under cold stress. However, barley albino mutants, which have defective chloroplast development, failed to retain introns in these genes. In Arabidopsis, the Arabidopsis stabilized1 (sta1) mutant, which had impaired splicing of the cold-induced COR15A pre-mRNA, exhibited heightened sensitivity to chilling, ABA, and salt stress. STA1 encodes a nuclear pre-mRNA splicing factor that is upregulated by cold stress and facilitates COR15A splicing, a process essential for cold tolerance (Nakayama et al., 2007).

2.3 Cold signal perception and responsive pathways

2.3.1 Secondary messengers in cold signal transduction

Plants respond to low temperatures through complicated processes at different levels. It is reported that initially, plant cells recognize cold stress signals via PM rigidification, PM-bound G-protein associated receptors, or cold sensors, such as Ca^{2+} influx channels, two-component histidine kinases, protein and nucleic acid conformations, or metabolite concentrations (Chinnusamy et al., 2010; Wei et al., 2021; Adhikari et al., 2022; Qian et al., 2024; Wang et al., 2024). Thereafter, second messengers such as ROS, calcium ion, and inositol phosphates are produced (Wei et al., 2021; Chinnusamy et al., 2010; Adhikari et al., 2022; Wang et al., 2024). These second messengers further regulate the intracellular Ca^{2+} concentration. Disturbance in cytosolic Ca^{2+} concentration is recognized by calcium-binding protein (Ca^{2+} sensors), which interact with their target proteins, to change Ca signal in the cell. These proteins manage cold stress signal transduction, activate protein phosphorylation cascades, and adjust the expression of transcription factors and cold-regulated (COR) genes in plants (Wei et al., 2021; Chinnusamy et al., 2010; Sanyal et al., 2016; Adhikari et al., 2022; Wang et al., 2024). It is common that plant cells respond to different changes in their environment by releasing Ca^{2+} . These Ca^{2+} ions normally serve as secondary messengers that rapidly convert external signals inside the cell (Knight, 2002; Kudla et al., 2018; Adhikari et al., 2022; Wang et al., 2024). The same principle applies in cells of cold stress plants (Knight, 2002). The Ca^{2+}

will be introduced into the cell or obtained from intracellular stores. Accumulation of Ca^{2+} usually forms an influx, which is thought to be of the initial activities vital in monitoring thermal changes during cold acclimation (Monroy et al., 1997). The structural and transient patterns of Ca^{2+} influx are stimulus-specific and are known as Ca^{2+} signatures (DeFalco et al., 2010). Cytosolic Ca^{2+} signatures and cold-responsive gene expressions are induced during rigidification of the membrane, which is damaged by gadolinium, a mechanosensitive Ca^{2+} channel blocker. Hence this concept verifies that mechanosensitive Ca^{2+} channels are involved in cold hardening (Sangwan et al., 2001; Chinnusamy et al., 2010). The message carried by Ca^{2+} signatures can be interpreted by various pathways. Calcium signatures are detected by families of proteins, known as calcium-dependent protein kinases (CDPKs), calmodulins (CaMs), and calcineurin B-like proteins (CBLs) (Winfield et al., 2010; Ma et al., 2015; Zhu, 2016; Adhikari et al., 2022; Jan, 2024; Qian et al., 2024; Wang et al., 2024). These proteins, which contain a highly secured EF-hand motif that binds Ca^{2+} , have been shown to take part in the orchestration of calcium-directed signal transduction networks (Yang et al., 2004). Because of binding Ca^{2+} , CBPs undergo configuration modifications that enable them to interact and regulate the target proteins (DeFalco et al., 2010). In turn, these downstream effectors start a series of events that results in the large-scale reprogramming of gene expression that is exhibited in cold acclimation (Pareek et al., 2017; Adhikari et al., 2022; Jan, 2024; Wang et al., 2024; Qian et al., 2024). The specific cascades are not yet identified which initiates specific cold induced genes cascades as a clear identification cannot be made at transcription level (Winfield et al., 2010). Therefore, cold stress affects general hormonal downstream signaling pathways.

2.3.2 Rice

The sensing of cold occurrence and responsive pathway in rice was demonstrated by Guo et al. (2018). In their study, plants were reported to respond to low temperature stress by PM rigidification and cytoskeletal reorganization (Guo et al., 2018). The cold signal was perceived by the PM and temperature sensor (COLD1/RGA1) and other components, leading to an influx of Ca^{2+} , ROS production, abscisic acid (ABA) accumulation, and mitogen-activated kinase (MAPK) cascade (OsMKK6-OsMPK3) reactions (Guo et al., 2018; Qian et al., 2024). Among these, the main responsive pathway in the nucleus was the OsMAPK3-OsbHLH002/OsICE1-OsTPP1 pathway, which is regulated by ROS. In addition, Ca^{2+} signals were translated by a series of Ca^{2+} -binding proteins that induced transcription factors (OsMYB3R-2, OsMYBS3, and OsMADS57). Another pathway was the ABA signalling pathway, which was transduced

by ABF1/2 and the ABRE-contained OsNAC gene (Guo et al., 2018). The findings in rice support the general model of cold stress signalling by highlighting the essential roles of Ca^{2+} influx, ROS, and hormonal regulation. However, rice also demonstrates species-specific pathways, such as the OsMAPK3-OsbHLH002/OsICE1-OsTPP1 cascade, along with distinct ABA-driven transcription factors. These variations suggest that while the core components of cold stress signalling are conserved across various species, individual plants adjust their responses based on their genetic makeup and ecological adaptation strategies.

2.3.3 Arabidopsis

Detection of cold stress in arabidopsis plants, stimulates Ca^{2+} channels and the PM protein CaM-regulated receptor-like kinase (CRLK) 1/2, followed by rapid activation of Ca^{2+} signals and mitogen-activated protein kinase (MAPK) cascade pathways (Kleist and Luan, 2016; Guo et al., 2018; Wang et al., 2024). Ca^{2+} signals are translated by a sequence of Ca^{2+} binding proteins, causing downstream signal transduction. ICE1, which is a key regulator in the ICE1-CBF-COR pathway, is regulated by multiple post-translational modifications (Guo et al., 2018). One of the pathways is through phosphorylation of ICE1 by OST-1, which will trigger ICE1 transcriptional activity. There is a possibility of ICE1 to be ubiquitinated by HOS-1, leading to its degradation. Nevertheless, that is usually avoided by sumoylation of ICE1 by SIZ-1 and phosphorylation by OST-1. BTF3 and BTF3L can also be phosphorylated by OST-1 and their interactions with C-repeat binding factors (CBFs) are enhanced to stabilize CBFs under cold stress. During cold acclimatization, ICE1 can be phosphorylated by MPK3/6 in the MAPK cascade, leading to the degradation of ICE1 by an unknown E3 ligase. Meanwhile, 14-3-3 proteins, which are phosphorylated by Cold-Responsive Protein Kinase 1 (CRPK1), shuttle from the cytosol to the nucleus to promote the degradation of CBFs and thus, regulate the duration of the cold defence response (Liu et al., 2017; Guo et al., 2018; Wang et al., 2024). Generally, the cold stress response in arabidopsis shown to follow the universal cold signalling framework, involving Ca^{2+} influx, ROS accumulation, MAPK activation, and transcriptional regulation. However, its specificity lies in the ICE1-CBF-COR pathway, where ICE1 is tightly regulated by post-translational modifications (phosphorylation, sumoylation, and ubiquitination). These regulatory layers modify the CBF stability and degradation, ensuring a controlled and reversible cold response. This mechanism highlights a sophisticated balance between stress adaptation and energy conservation in arabidopsis.

2.3.4 General overview of cold signal perception and responsive pathway

Both rice and arabidopsis respond to cold stress through the rigidification of the PM, which triggers Ca^{2+} influx and activates MAPK signalling cascades. However, arabidopsis relies heavily on ICE1 as the master regulator of the CBF pathway, while rice integrates ABA signalling and specific transcription factors such as OsbHLH002, OsMYB3R-2, and OsNAC to regulate its cold stress response. This difference may be due their evolutionary adaptations: arabidopsis, a temperate model plant, has developed a more robust cold acclimation system, whereas rice, a tropical crop, has a weaker cold tolerance mechanism, making it more susceptible to chilling stress.

2.4 Summary of integrated plant responses to chilling stress

In summary, the interconnected morphological, physiological, biochemical and molecular responses of plants under chilling stress are illustrated in Figure 2.1 below. Briefly, the diagram highlights major activities involved from inception of cold stress and development of CI. The diagram further reveals the plant's ability to recover from sub-lethal chilling through the gradual restoration of normal metabolic processes. This visual representation provides a comprehensive overview of how plants respond and adapt to cold stress at multiple levels.

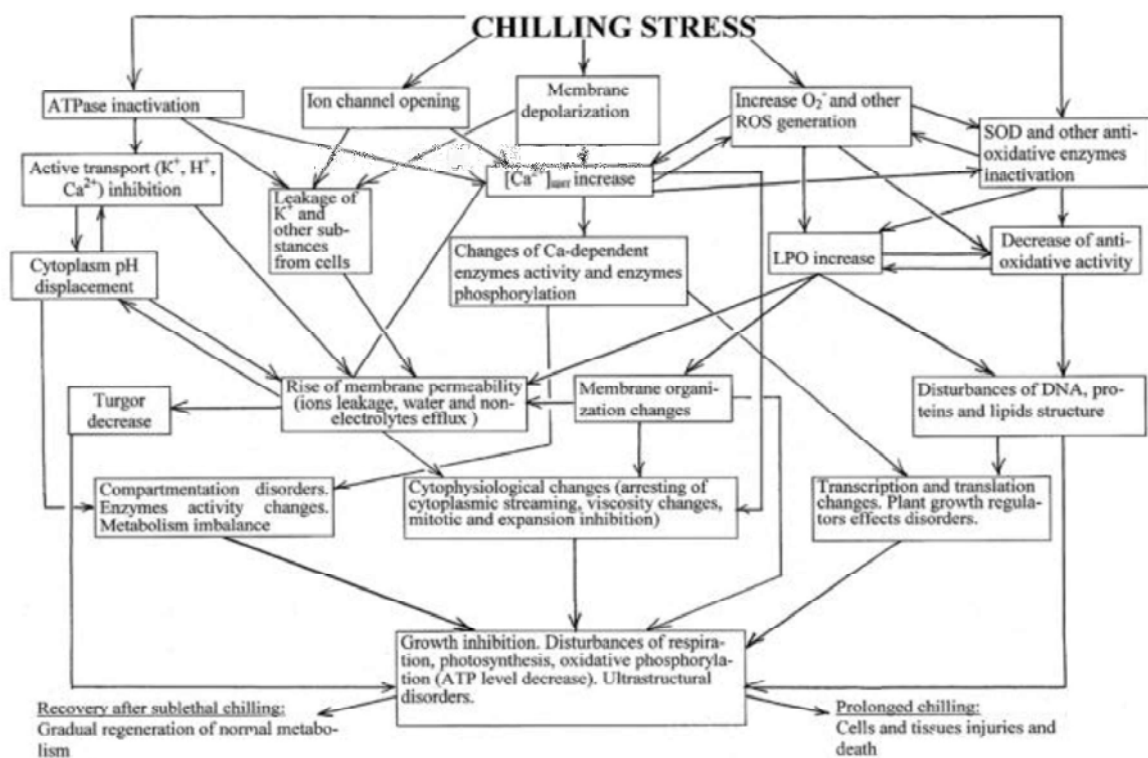


Figure 2.1. Schematic presentation of initiation and development of CI in cold- sensitive plant species. Adopted from Lukatkin et al. (2012).

2.5 Chilling injury acclimation in safflower

A chill injured safflower will exhibit damaged growing points and split stems, especially during the elongation phase of plant growth. This occurs when temperatures fall to -4°C in the winter season (GRDC, 2017; Kolanyane, 2022). Safflower accessions with a shorter elongation stage in winter are proposed to be better cultivars to be planted, since safflower is cold sensitive at that stage (Johnson et al., 2012).

Similarly, to other plants, a cold stressed safflower plant has been shown to undergo a series of biological changes, such as an increase in soluble sugars, cold stress proteins, and proline content (Thomashow, 1999; Cunningham et al., 2003), and an increase in unsaturated fatty acids and phospholipids of the plasma membrane (Arbaoui et al., 2007). The molecular mechanisms by which soluble sugars influence cold tolerance in plant cells are not yet clear (Zuther et al., 2004). But as of present, soluble sugars such as sucrose, fructose, and the raffinose family oligosaccharides (RFOs) role in cold acclimation, is to reduce cellular membrane damage by replacing cell water content with vitreous state that minimizes ice crystal formation and cold induced dehydration (Thomashow, 1999; Taji et al., 2002). The importance

of sugars is also to provide energy to maintain low temperature respiration in living cells, allowing cell metabolism to recover after cold stress, and mobilize resources for resumption of growth (Schrader & Sauter, 2002; Guy, 2003; Rife & Zeinali, 2003). Cold acclimation of safflower at 4°C in a photoperiod of 10 hours for four weeks showed an increase in soluble sugars (Landry et al., 2017). However, the increase in soluble sugars was not solely associated with cold tolerance in safflower. Other factors such as phenolics, hormones, dehydrin proteins, antioxidants, nitric oxide, proline, cell membrane fatty acid composition and amino acid content have been associated with cold tolerance of safflower plants (Zhu et al., 2007; Galiba et al., 2013). These factors affect water relations between the intra- and intercellular environments (Burchett et al., 2006), along with crop development (Johnson et al., 2012) play a role for successful cold tolerance and winter hardiness in safflower (Hekneby et al., 2006).

Furthermore, the length of exposure and specific cold acclimation conditions are key contributors in cold tolerance of winter hardy safflower (Bourion et al., 2003). Cold acclimation of winter hardy safflower genotype at 15°C/5°C day/night temperature, and 10-hour photoperiod resulted in more cold tolerant plants. Even when the plants were cold stressed at -15°C for 4 hours during their first, second and third week, survival rate increased from 28, 32 to 60%, respectively (Landry et al., 2017). Hence proving that longer exposure time can increase recovery rate depending on the acclimation environment. Additionally, the study showed that photoperiod was important in cold acclimation. In general, longer exposure (four weeks) of winter type safflower to low temperature resulted in increased low molecular weight carbohydrates as compared to spring genotypes.

2.6 Management strategies

Many adaptations strategies help crops to mitigate the negative impacts of low temperature stress. Agronomic practices such as selection of cold-tolerant cultivars, nutrient management, appropriate planting methods, time of planting, seed enhancements, external application of osmo-protectants, and irrigation management, may help improve cold tolerance in crops (Hassan et al., 2021). External application of organic compounds such as polyamines and their derivatives have been reported to mitigate the effects of extreme temperatures in plants (Liu et al., 2007).

2.6.1 Breeding for cold tolerance

Breeding for cold tolerance has led to the development of cultivars that can withstand low temperatures without significant damage. For instance, cold-tolerant wheat and barley varieties have been bred to thrive in cold temperate climates, ensuring crop viability and food security (Ferrante et al., 2021; Hassan et al., 2021; Sadok et al., 2022; Su et al., 2022; Ajayi et al., 2023). Some of the breeding methods previously utilized included participatory plant breeding (PPB) and marker-assisted selection (MAS) (Sadok et al., 2022; Su et al., 2022; Oyebamiji et al., 2024). The combination of these two breeding methods sought to merge the traditional and modern selection methods to achieve cold tolerance. The PPB technique involves farmers in the selection process to ensure that developed varieties meet local needs and environmental conditions, while MAS was used to identify genetic/Quantitative Trait Loci (QTLs) associated with cold tolerance (Ceccarelli and Grando, 2007; Ferrante et al., 2021; Raj and Nadarajah, 2022; Sadok et al., 2022; Su et al., 2022; Ajayi et al., 2023; La Borde et al., 2023). Several breeding methods have been identified that could be used to mitigate the effects of cold stress and other abiotic stresses. These methods include genetic engineering, genome editing and speed breeding (Ashraf et al., 2009; Hossain et al., 2021; Oyebamiji et al., 2024). In genetic engineering, resilient transgenic plants are created by directly inserting the desired specific genes into a plant cell. This process employs several techniques, such as agrobacterium-mediated and particle bombardment (Ashraf et al., 2009; Hossain et al., 2021; Oyebamiji et al., 2024). Genome editing methods entails modification of the plant trait by adding, removing, or altering genetic material at specific locations within the genome. The most prominent gene-editing method includes clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) (Zeng et al., 2020; Afzal et al., 2023; Oyebamiji et al., 2024). Meanwhile, speed breeding is an accelerated plant breeding technique that utilizes controlled environmental conditions like greenhouses, growth chambers and facilities equipped with light-emitting diode (LED) to control light, temperature and humidity. This method significantly shortens crop generation times (Haroon et al., 2022; Oyebamiji et al., 2024). It also allows researchers and breeders to grow multiple generations of plants within a single year, thereby expediting the development of improved crop varieties. The controlled environments are used to extend photoperiod up to 22 hours with artificial LED light in a wavelength of 400-700 nm (Kumari & Singh, 2022; Oyebamiji et al., 2024). This breeding method has been proven successful in rapidly developing various crops, including chickpea, groundnut, canola, barley, wheat, sunflower and peas (Watson et al., 2018; Pandey et al., 2022; Samantara et al., 2022).

With climate change causing erratic temperature fluctuations, cold-tolerant varieties act as a buffer against unexpected cold snaps, safeguarding food production and farmers' livelihoods. For example, cold-tolerant sorghum and millet varieties have been instrumental in improving cold resilience in semi-arid and cold-prone areas (Bettina et al., 2012; Chaturvedi et al., 2022; Mwamahonje et al., 2024). The release of varieties such as MP 472 in pearl millet and 'Macia' in sorghum represent successful outcomes of breeding for cold tolerance in regions with cool early-season temperatures like in India (Bettina et al., 2012; ICRISAT Annual Report, 2019; ICRISAT Annual Report, 2023). While in semi-arid regions, particularly Zimbabwe, climate smart sorghum hybrid varieties SH1 and SH2 have been bred (ICRISAT, 2024). Moreover, development of early-maturing millet hybrids like PMH 1 and PMH 2 suitable for short growing seasons have been released in semi-arid regions (Bettina et al., 2012; ICRISAT Annual Report, 2023; ICRISAT, 2024). This was achieved through multi-environment testing to identify genotypes with high resilience, the use of genomic and molecular breeding programs such MAS, and transgenic approach, which entails introducing cold-resilient genes from other species like antifreeze proteins. Additionally, farmers participation in genotype selection has been crucial (Bettina et al., 2012; Chaturvedi et al., 2022; Mwamahonje et al., 2024). The same breeding methods could be applied to enhance cold tolerance of safflower.

2.6.2 Nutrient management

Proper nutrient management is essential for optimizing crop growth, yield, and resilience to environmental stresses (cold, drought, or salinity) in most agricultural crops (Mündel et al., 2004; Dordas and Sioulas, 2008; Golzarfar et al., 2012; Taiz & Zeiger, 2013; Ahamed & Elseemat, 2019). In safflower production, nitrogen (N) and phosphorus (P) have been reported as vital nutrients required for crop growth and development (Golzarfar et al., 2012; Kolanyane, 2022). They also play a pivotal role in mitigating the effects of cold stress by enhancing plant physiological and biochemical responses (Geisseler, 2016). Generally, N enhances the synthesis of proteins, enzymes, chlorophylls and osmolytes that are crucial for cold tolerance (Marschner, 2005; Taiz & Zeiger, 2013). Adequate N levels improve photosynthetic efficiency and promote the production of antifreeze proteins, aiding plants in withstanding low temperatures (Dordas & Sioulas, 2008; Geisseler, 2016). To counteract cold stress, application of a slow-release or split doses of N is recommended to sustain availability during cold-prone periods without causing excess vegetative growth in most plants (Geisseler, 2016). In safflower, sufficient N ensures robust vegetative growth with a deep green colour, which in later growth stage is beneficial for seed and oil yield (Dordas & Sioulas, 2008; Ahamed &

Elseemat, 2019; Kolanyane, 2022). It also improves root growth, development, and uptake of other nutrients (Leghari et al., 2016). The application rate of N in safflower is influenced by many biotic and abiotic factors, such as genotype, climatic conditions, location, soil type and agronomic practices (Ahamed & Elseemat, 2019; Kolanyane, 2022), thus different recommendations have been made by different authors. Geisseler (2016) recommended a dose of 60-80 kg N/ha applied through split application, that is, 50% as a basal dose at sowing and 50% at the vegetative or pre-flowering stage. Dordas & Sioulas (2008) recommended application of 200 kg N/ha as it increased seed yield and yield components in their study. Seed yield, seed weight per plant, the number of heads per plant, number of seeds per plant increased by 19, 60, 32 and 41 (%), respectively compared with the control. Golzarfar et al. (2012) reported 150 kg N/ha as the adequate application rate as it resulted in the highest seed yield of 3227.50 kg/ha in their experiment. Al-Zubaidy & Al-Mohammad (2021) also reported the highest seed yield of 1762 kg/ha against 1074 kg/ha of control (0 kg N/ha) in safflower fertilized with 150 kg N/ha. Kolanyane (2022) recommended application rate of 40 kg N/ha for optimal safflower production in Botswana. Moreover, Singh & Singh (2013) reported high oil content of 33.5% in safflower fertilized with 80 kg N/ha compared to 32.4% from control plants.

As mentioned before, phosphorus is the second most essential element in safflower productivity (Dordas & Sioulas, 2008; Golzarfar et al., 2012; Kolanyane, 2022). It plays a crucial role in energy transfer and root development, which are very critical for most abiotic stress tolerance (Marschner, 2005; Hasanuzzaman et al., 2018; Kolanyane, 2022). Under cold stress, phosphorus facilitates membrane stability and energy metabolism, helping plants recover from cellular damage (Dordas & Sioulas, 2008; Geisseler, 2016). In safflower cultivation, phosphorus is also crucial for flower and seed formation (Dordas & Sioulas, 2008; Golzarfar et al., 2012). Typically, phosphorus is applied in the form of phosphorus pentoxide (P_2O_5) (Alivelu et al., 2014; Geisseler, 2016; Emongor & Oagile, 2017). Like N fertilization, different application rates have been proposed based on the various abiotic factors such as location, genotype, soil characteristics and climate. Ahamed & Elseemat (2019) suggested an application rate of 30-50 kg P_2O_5 /ha as optimal in their trials. Dordas & Sioulas (2008) recommended 60 kg P_2O_5 /ha. While Golzarfar et al. (2012) reported the highest seed yield and yield components after application of 100 kg P_2O_5 /ha. Singh & Singh (2013) reported that P application at 40 or 80 kg P_2O_5 /ha significantly increased safflower plant biomass and P uptake by 24.6-31.2% and 17.9-35.3%, respectively, compared to control plants. The same application

dose also increased the uptake of N and sulphur (S) by 17.0-24.1% and 11.5-23.8%, respectively, compared to control plants (Singh & Singh, 2013). Meanwhile, Kolanyane (2022) recommended a dose of 50 kg P/ha in combination with N. Generally, it was recommended to apply P_2O_5 as a basal dose before sowing to ensure early root development and uptake before cold stress onset (Dordas & Sioulas, 2008; Golzarfar et al., 2012; Ahamed & Elseemat, 2019). Lastly, although different application rates of N and P have been proposed, soil testing should be the key to advise the nutrients requirement of a particular soil before fertilizer application.

2.6.3 Planting time

The optimal planting time for safflower varies based on the climatic conditions of the region (Mündel et al., 1997; Emongor & Oagile, 2017; Kororoneo, 2023). In South Central India, safflower is typically sown in the autumn, around September to November, to take advantage of heavy soils that retain moisture from summer rains or irrigation (Alivelu et al., 2014). In regions with a Mediterranean climate, such as Australia, Mexico, Spain, certain countries in the Middle East, and California in the USA, safflower is usually grown during the winter season. Additionally, it can be cultivated as a spring crop in areas with climates like the Northern Great Plains of the USA and Canada (Emongor & Emongor, 2023). In semi-arid and arid regions, including the Republic of South Africa, Angola, Namibia, Botswana, and Eswatini, the best yields are often obtained during the winter under irrigation conditions. However, safflower can also be grown in the summer under rainfed conditions (Emongor & Oagile, 2017; Mosupiemang, 2024). It is recommended to plant early when sowing in the summer to prevent the timing of flowering from coinciding with the coldest months (Kolanyane, 2022; Kereilwe et al., 2023; Mosupiemang, 2024).

2.6.4 External application of osmo-protectants and plant growth regulators

The use of osmo-protectants and growth regulators is an effective strategy for reducing the impact of cold stress on plants (Sabagh et al., 2021; Raza et al., 2022a; Raza et al., 2023). Cold stress negatively affects plant growth and productivity by causing cellular damage, disrupting metabolic processes, and impairing photosynthesis. Applying osmo-protectants and growth regulators externally helps maintain cellular balance, enhances stress tolerance, and improves overall plant performance in cold conditions (Ding et al., 2019; Alotaibi, 2023; Oyebamiji et al., 2023).

Osmo-protectants are low-molecular-weight compounds that help stabilize cellular structures and maintain osmotic balance during stress conditions (Gupta et al., 2020; Oyebamiji et al., 2024). Examples of osmo-protectants include proline, glycine betaine and trehalose (Szabados & Savouré, 2010; Gupta et al., 2020; Raza et al., 2022b; Raza et al., 2023; Oyebamiji et al., 2024). The exogenous application of proline has been shown to enhance cold tolerance by stabilizing proteins, membranes, and enzymes, as well as scavenging ROS, thereby reducing oxidative damage (Szabados & Savouré, 2010). Additionally, proline functions as an osmolyte, protecting cells from osmotic stress induced by low temperatures. In chickpea (*Cicer arietinum* L.), Kaur et al. (2011) observed that exposure to 10°C led to flower and pod abortion, resulting in a notable reduction in yield. However, foliar application of 10 µM proline during the flowering stage enhanced sucrose and trehalose content in leaves, promoting flower retention and pod set, ultimately improving yield under chilling conditions. Similarly, Shin et al. (2018) demonstrated that pre-treatment of spinach seedlings with 10 mM proline enhanced cold tolerance following exposure to -1°C for two weeks. Proline-treated seedlings exhibited a 2.6-fold increase in endogenous proline content and maintained greater leaf and petiole turgidity compared to untreated seedlings, indicating improved cold stress resilience. Further supporting these findings, Mohammadrezakhani et al. (2021) examined the response of citrus (*C. reticulata* L., *C. sinensis* L. and *C. paradisi* L.) species to external application of proline and putrescine (0, 15, 20 mM) under cold stress (1, -1, -3°C) for 6 hours. Cold exposure significantly altered physio-biochemical processes, negatively impacting plant growth. However, exogenous application of proline and putrescine mitigated these effects by reducing ROS accumulation, improving osmotic balance, and increasing phenolic compound content, which are all crucial for cold stress tolerance. These findings reinforce the role of proline in enhancing physiological resilience and biochemical defence mechanisms against chilling stress across different plant species.

Conversely, glycine betaine alleviates oxidative stress by protecting the photosynthetic machinery and maintaining membrane integrity during cold stress (Ashraf & Foolad, 2007). Jarin et al. (2024) highlighted that accumulation of glycine betaine in transgenic tomato and rice exposed to cold stress, exhibited resistance by improving oxygen-evolving capacity of the PSII, greater stability of membrane integrity, reduced membrane lipid peroxidation and preservation of PSII electron transport. Meanwhile, trehalose boosts antioxidant enzyme activity, reducing oxidative damage and improving plant survival under low temperature conditions (Gupta et al., 2020; Raza et al., 2022b). Collectively, these osmo-protectants aid in

maintaining metabolic activities of plants undergoing cold stress, ultimately enhancing growth parameters such as germination rates, biomass accumulation, and yield.

Plant growth regulators play a crucial role in modulating the physiological and biochemical responses of plants, enabling them to adapt better to cold stress. These are small chemical molecules that help regulate plant growth and stress tolerance (Mubarik et al., 2021; Sabagh et al., 2021; Raza et al., 2022a). Example of plant growth regulators include abscisic acid (ABA), salicylic acid (SA), cytokinins (CKs), jasmonic acid (JA), indole-3-acetic acid (IAA), brassinosteroids (BRs), gibberellic acid (GA) and ethylene (ET) (Sabagh et al., 2021; Raza et al., 2022a; Raza et al., 2023). ABA has been shown to induce the expression of cold-responsive (COR) genes through the ABA-dependent signalling pathway, enhancing cold tolerance (Soualiou et al., 2022). Additionally, ABA helps maintain stomatal closure, which reduces water loss during stress (Chen & Murata, 2011; Finkelstein, 2013). Moreover, exogenous application of ABA mitigates the effect of cold stress by triggering ABA signalling to induce the transcription of antioxidant enzymes for ROS scavenging (Shi & Yang, 2014). Application of 1×10^{-5} mol L⁻¹ ABA was reported to increase CAT, SOD, POD, APX, and GR activities, thereby reducing H₂O₂ production in wheat plants cold stress at 0 and -24°C (Yu et al., 2020). Salicylic acid (SA) has been reported to improve cold tolerance by enhancing antioxidant defence and reducing the accumulation of ROS, which can damage cellular components (Khan et al., 2015). In another study, external application of 100 µM SA counteracted cold stress in wheat leaves at 4°C by increasing antioxidant enzyme activities and proline bioaccumulation, decreasing lipid peroxidation and enhancing the expression of marker genes such as TaMnSOD and TaFeSOD (Ignatenko et al., 2019). Exogenous application of 10 µM SA was reported to improve plant dry mass of watermelon cold stressed at 10/5°C (day/night) (Cheng et al., 2016). Cytokinins, when applied externally, have also been noted for their ability to mitigate the effects of cold stress by maintaining photosynthetic activity and delaying senescence, thereby contributing to overall plant growth (Zwack & Rashotte, 2015; Raza et al., 2023). Meanwhile, JA promotes the accumulation of secondary metabolites and proteins that are involved in cold stress tolerance, thereby strengthening the plant's defence mechanisms (Wasternack & Hause, 2013; Raza et al., 2023). Overall, growth regulators facilitate the activation of signalling pathways and physiological changes that prepare plants to cope with cold-induced stress. They also enhance stress recovery by improving cellular repair mechanisms and growth resumption.

Adoption of an integrated approach that combines breeding for cold-tolerant cultivars and proper agronomic practices can achieve sustainable and effective solutions to mitigate the

impact of cold stress on the productivity of safflower and other crops. This strategy will help ensure food security in the face of climate change while promoting environmental sustainability.

2.7 Conclusion

Several studies have demonstrated that plants undergo morphological, physiological, biochemical and molecular changes in efforts to mitigate the damaging effects of chilling stress on crop production. Extensive research on major crops such as rice, maize, beans, and arabidopsis have revealed key adaptive mechanisms, including changes in membrane fluidity, accumulation of osmo-protectants, activation of antioxidant defence systems, and expression of cold-responsive genes. However, despite safflower's potential as a cold stress-tolerant crop, research on its response to chilling stress remains limited both globally and within Africa. Few studies have investigated the morphological and bio-physiological effects of low temperatures on safflower at CI level, while information on its biochemical and molecular responses, including photosynthetic status, antioxidant activity and gene regulation (gene expression), is scarce. This lack of research hinders the development of chilling-tolerant safflower varieties, which are essential for expanding cultivation in cooler climates and ASALs experiencing mild winters. Therefore, in efforts to bridge this knowledge gap, this study investigated different aspects of CI of safflower such as the actual temperature and duration of exposure at which CI occurs at different phenological stages (rosette and bolting stages), physiological, biochemical and molecular responses of safflower to chilling stress.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Chilling injury incidence, severity, and plant recovery

3.1.1 Study site

The study was done at the Botswana University of Agriculture and Natural Resources (BUAN) Content Farm located at 59°24'S, 95°25'E and 993 m above the sea level. The soils are characterised as shallow and ferruginous tropical, mainly consisting of sandy loam soil (De-Wilt & Nightengale, 1996; Moatshe et al., 2020). The winter and summer temperature ranges from -1 to 30°C and 20 to 37°C, respectively, with average rainfall of 538 mm per annum (De-Wilt & Nightengale, 1996).

3.1.2 Experimental design

The study was conducted in two parts: one involving artificial stress induction and the other focusing on natural stress induction.

Artificial stress induction on safflower plants of the genotype Turkey was performed following a 2 x 4 x 4 factorial design. The treatments were growth stage (rosette & bolling), temperature (0, 4, 8, and 12°C), and duration of exposure (2, 4, 8, and 12 hours). The plants were put in refrigerators set at the above temperatures three times in one trial, but two trials were undertaken between March 2022 to August 2023. At each time and trial, different plants of the same genotype were put in the refrigerators.

Two field trials were undertaken in the winter of 2023 and 2024, respectively. The experimental design was a 2 x 4 factorial laid down in a completely randomized block design (RCBD) replicated three times. The treatments were growth stages (rosette and bolling stage) and four safflower genotypes (Turkey, Sina, Gila and PI 537636). All genotypes used in the study had been previously tested for adaptability under the semi-arid conditions of Botswana (Emongor et al., 2017). Trial 1 field study was done between March to October 2023. While trial 2 field study was conducted between May to November 2024.

3.1.3 Soil preparation and planting

For the artificial stress induction experiment, prior to planting, topsoil was collected and mixed with sandy soil in the ratio 3:1 (topsoil: sandy soil) for improved drainage. The soil mixture

was used to fill 10 L perforated plastic pots for each growth stage (rosette and bolling), and 5 g of NPK fertilizer in the ratio of 2:3:2 (22%) was applied/pot. The pots were watered to field capacity before planting. The next morning, two safflower seeds of uniform size were sown at a depth of 4 cm. Three weeks after emergence the plants were thinned to one plant per pot. The plantings were staggered (six to seven weeks apart) such that by the coldest months (June and July) some plants were in rosette stage while others in bolling stage.

For the field experiments, the site was cleared, ploughed and disc harrowed to a fine soil tilth. Soil was sampled to determine which fertilizers to apply prior to planting. Safflower seeds were sown directly at a seeding rate of two seed/hill and 2.5 cm depth. The plot size was 3 x 3 m with 1 m space between the plots and 45 x 20 cm plant spacing. Seedlings were thinned to one plant/hill 15 to 20 days after emergence. Similarly, the plants were planted in a staggered manner so that by winter season, some plants were in rosette stage while others in bolling stage.

3.1.4 Management practices

After sowing, plants were watered three times a week to keep the growth medium at field capacity. Weeds were also uprooted by hand continuously, as per when their appearance, so that they do not suppress plant growth.

3.1.5 Data collection

Data collection in the field was done in three consecutive days with a sample size of 10 plants per replication. The control data was collected at 9.7/24.6°C (first trial) and 9/23.3°C (second trial) average minimum and maximum temperatures before plants were naturally subjected to cold night temperatures (Appendix 1 & 2). However, in trial 2 of the field experiment the night minimum temperatures ranged between -2 and 12°C which did not induce CI in all four safflower genotypes because the plants were at the elongation stage. Therefore, only trial 1 field data was used.

3.1.5.1 Evaluation of chilling incidence and severity

After induction of cold stress, chilling injury incidence (CII) and severity were evaluated visually according to the method described by Pérez-Tello et al. (2001) for both the artificial and natural cold stress experiment. CII was evaluated on the number of leaves that showed CI symptoms (wilting, curling, and discoloured edges) out of the total leaves in a plant used per treatment expressed as a percentage. For artificial cold stress experiment, three plants were

used per treatment, while in the field 10 plants were observed for cold stress. While severity of CI was evaluated visually on a predetermined scale from 0 to 4, where 0 = no injury, 1 ≤ 10% leaf wilting, 2 ≤ 25% leaf wilting, 3 ≤ 50% leaf wilting, and 4 ≥ 51% leaf wilting, after which chilling injury severity index was calculated by the following equation:

$$\text{CI Index} = \sum_1^n \left(\frac{\text{Chilling score} \times \text{number of leaves at that chilling level}}{\text{Total number of leaves assessed}} \right) \text{.equation 1}$$

Where n represents the chilling score category/severity level.

3.1.5.2 Plant recovery

After chilling stress, the plants for the controlled experiment were kept at room temperature of 23°C, while those at the field were left intact at their natural growing environment. Visual observations were made to determine the duration of recovery in the chill-injured plants. The recovery of the plants was indicated by the return of plants to an upright and rigid posture, improved leaf firmness, and greenness in previously discoloured portions, and the development of new leaves with a normal green colour. Plant recovery was recorded as the number of plants that recovered (%) after a given duration (12, 24, 72 (hours) and 7 days) (Hu et al., 2016).

3.2 Screening for physiological, biochemical, and molecular changes

3.2.1 Experimental design

Based on the results from study 1 above (section 3.1.5.1), an experiment was conducted to determine the influence of temperature, duration of exposure, and safflower phenological stages on physiological, biochemical, and molecular changes on chilled safflower plants. For the artificial cold stress induction, the experimental design was 2 x 3 x 2 factorial, replicated three times. The treatments were safflower growth stages (rosette & bolting), temperature (0, 4 & 23°C) and duration of exposure (8 & 12 hours). Safflower plants were cold stressed in two refrigerators set at 0 and 4°C. Control plants were under room temperature which averaged 23°C during the study period. The study was repeated three times, and these were used as replications. While for the field trial the same experimental design used in section 3.1.2 was maintained.

3.2.2 Data collection

3.2.2.1 Determination of relative leaf electrolyte leakage

The relative electrolyte leakage (REL) was determined according to the method of Wu et al. (2014) with slight modifications. After stress induction, three leaves from each plant were harvested and discs were cut using a 10 mm diameter cork borer. The leaf discs were rinsed with deionized water to eliminate the electrolyte at the cut surface. Then 100 mg of leaf discs were weighed using an analytic balance (Adam Nimbus <http://adamequipment.com>). Then samples were incubated in 25 mL distilled water in a flask. The flask containing leaf discs was shaken with a gyratory shaker at 200 rpm at room temperature (25°C) for two hours. The initial conductivity reading was taken using a Bench top conductivity meter (CON 700) and recorded as conductivity 1 (C1). Thereafter, the samples were incubated for 10 minutes in boiling water bath (98°C) to stimulate the highest leaf leakage (Kovaleski & Gossman, 2021). The samples were allowed to cool to room temperature (25°C) prior to assay of REL (recorded as C2). Relative electrical conductivity (C%) was calculated using the formula $(C1/C2) \times 100$ (Liu et al., 2013).

3.2.2.2 Proline content determination

Proline was extracted by grinding 0.5 g of freshly harvested leaves in liquid nitrogen. The powdered leaf samples were homogenized in 10 mL of 3% aqueous sulphosalicylic acid in a test tube and centrifuged at 5000 rpm for five minutes. The supernatant was transferred into a 5 mL test tube and reacted with 2 mL of acidic ninhydrin and 2 mL of glacial acetic acid in a water bath at 95°C for one hour to develop the colours. Soon after removal from the water bath, the test tube was cooled in an ice bath for 30 minutes (Carillo & Gibon, 2011). To the reaction mixture, 4 mL of toluene was added and vortexed for a minute, then centrifuged at 1000 rpm for five minutes. The optical density of the samples was read at 520 nm using a spectrophotometer (SPECTROstar Nano, BMG Labtech microplate reader, Ortenberg, Germany) set to end point mode and the content of proline was calculated using standard curve (Zhang & Huang, 2013). Proline content was calculated as;

$$PC = [(\mu\text{g proline/mL}) \times \text{mL toluene}] / 115.5 \mu\text{g}/\mu\text{mole} / [(\text{g sample})/5]. \text{ equation 2}$$

Where, PC = Proline content, 115.5 is the molecular weight of Proline

3.2.2.3 Determination of leaf chlorophylls and carotenoid content

Prior to and after imposition of cold stress in safflower plants, five mature leaf samples were harvested from each plant and immediately wrapped in an aluminium foil to avoid chlorophyll breakdown. The samples were then labelled and placed in a re-sealable plastic bag and frozen at -80°C until the day of the analysis. During the analysis day, 1 g of safflower leaf sample from each treatment was finely crushed using a pestle and mortar and gently mixed. To this homogenized leaf material, 20 mL of 80% acetone and 0.5 mg MgCO₃ powder was added. The mixture was homogenized gently. Then the samples were put into a refrigerator at 4°C for four hours. Thereafter, the samples were centrifuged at 500 rpm for five minutes. The supernatant was transferred to 100 mL volumetric flask. The final volume was made up to 100 mL with addition of 80% acetone. The quantitative analysis of chlorophyll a, b, and a + b (total chlorophyll) was done. The colour absorbance of the solution was estimated spectrophotometrically using SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) set at end point mode and read at 645 and 663 nm wavelengths (Kamble et al., 2015) for chlorophyll, while 480 and 510 nm wavelengths were used to analyse carotenoids content against the solvent (Rajput and Patil, 2017). Acetone (80%) was used as a blank. The same procedure was followed for the controls. The following formulas were used to calculate chlorophyll and carotenoids contents.

$$\text{Chlorophyll a (mg/g tissue)} = \frac{[12.7 (A_{663}) - 2.69 (A_{645}) \times V]}{1000} \times W. \text{ equation 3}$$

$$\text{Chlorophyll b (mg/g tissue)} = \frac{[18.61 (A_{645}) - 4.68 (A_{663}) \times V]}{1000} \times W. \text{ equation 4}$$

$$\text{Total chlorophyll (mg/g tissue)} = \text{Chlorophyll a} + \text{Chlorophyll b. equation 5}$$

$$\text{Carotenoid content (mg/g tissue)} = \frac{[7.6 (A_{480}) - 1.49 (A_{510}) \times V]}{1000} \times W. \text{ equation 6}$$

Where, A= Absorbance at those specific wavelengths (480, 510, 645 and 663 nm), (7.6, 1.49, 11.75, 2.35, 18.61, 3.96) are coefficients at those wavelengths, V is volume of extraction solvent (mL), W is fresh weight of the sample extracted.

3.2.2.4 Measurement of chlorophyll fluorescence

For both the artificial and natural stress induction experiments, chlorophyll fluorescence (Chl) parameters from the adaxial of intact leaves were measured using a portable handheld MultiSpeQ field fluorimeter version 2.0 (PhotosynQ Inc, USA). Measurements for controls

were taken on a clear and sunny day (in May), between 10:00 and 13:00 hours for maximum light absorption by plants (Scharnagl & TerAvest, 2017). In the artificial stress induction experiment, cold stressed measurements were taken after the given duration (8 and 12 hours) of cold stress exposure. While for the field experiment (natural induction), measurements were taken after a cold night temperature at 6:00 am before the plants could recover from chilling stress (Scharnagl & TerAvest, 2017). The Chl fluorescence measurements taken included quantum yield of photosystem II (ϕ_{II}), non-photochemical quenching (ϕ_{NPQ}), quantum yield of non-regulated energy for PS II (ϕ_{NO}) and linear electron flow (LEF) (Kuhlgert et al., 2016; Scharnagl & TerAvest, 2017; Huang et al., 2018; Ratajczak et al., 2023).

3.2.2.5 Measurement of stomatal conductance

Stomatal conductance (gs) was measured using steady state SC-1 leaf porometer (Decagon Devices, Inc. USA) on intact leaves for both trials. For control plants, the youngest fully expanded and exposed to sunlight leaves were selected for measurement. Measurements were made by randomly placing the sensor head of the SC-1 porometer on the upper (adaxial) surface at the middle portion of the leaf for 30 seconds. The readings were taken on a clear day between 10:00 to 13:00 hours before winter season (May) (Ramya et al., 2016). For the artificial cold induced stress experiment, gs was taken immediately after the elapse of cold stress duration, that is, after 8 and 12 hours. Meanwhile, for the field experiment, measurements were taken in the early hours (0600 to 0800 hours after a cold night). For both experiments, readings were taken on three consecutive days to minimize variation (Ramya et al., 2016).

3.2.2.6 Determination of antioxidants enzyme activity

For determination of antioxidants enzyme activity (catalase, superoxide dismutase, and peroxidase), leaf samples were collected from each plant prior and post induction of cold stress. For extraction of superoxide dismutase and catalase, approximately 0.5 g of leaf sample were weighed and finely pulverized in liquid nitrogen with a cold mortar and pestle. The powdered samples were homogenized in a 50 mmol potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. The homogenate was centrifuged at 15, 000 rpm for 20 minutes at 4°C. The resultant supernatant was the crude enzyme extract (Miao et al., 2010).

3.2.2.6.1 Catalase enzyme activity

Catalase (CAT) enzyme activity was measured by monitoring the decrease in absorbance at 240 nm for one minute, after addition of H₂O₂ at 20°C (Miao et al., 2010). Reagents included

50 mM phosphate buffer (pH 7.0), 15 mM H₂O₂ and 0.1 ml enzyme extract. Safflower sample containing 0.1 ml enzyme solution and 1 ml H₂O₂ was read against a blank containing, 1 ml phosphate buffer and 0.1 ml enzyme solution using SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) spectrophotometer set to kinetic mode. The CAT activity was calculated from the extinction coefficient (40 mM⁻¹ cm⁻¹) for H₂O₂ (Miao et al., 2010).

3.2.2.6.2 Superoxide dismutase enzyme activity

The superoxide dismutase (SOD) enzyme activity was determined using a photochemical method that employs nitro-blue tetrazolium (NBT). A reaction mixture was prepared consisting of 0.003 mM riboflavin, 13 mM methionine, and 0.075 mM NBT in 50 mM phosphate buffer (pH 7.8). Next, 0.5 g of the safflower sample was added to the reaction mixture and exposed to light for 5 to 10 minutes. In this method, one unit of SOD activity was considered as the amount of enzyme required to give 50% inhibition of the rate of NBT reduction, measured at 560 nm using SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) spectrophotometer set at kinetic mode. The activities of SOD were quantified based on soluble protein bases (Miao et al., 2010).

3.2.2.6.3 Peroxidase enzyme activity

The peroxidase (POD) enzyme activity was assayed as per the method by Liang et al. (2013). In which 1 g of safflower leaf sample was weighed and finely ground in liquid nitrogen with a pre-cooled mortar and pestle. The powdered sample was homogenized in an ice bath of 5 mL 50 mM borate buffer (pH 8.7) consisting of 5 mM sodium hydrogen sulphite and 0.1 g polyvinylpyrrolidone (PVP). POD was extracted by centrifuging the homogenate at 10 000 rpm at 4°C for 25 minutes. A substrate mixture containing acetate buffer (0.1 mM, pH 5.4), ortho-dianisidine (0.25% in ethyl alcohol) and 0.1 mM 0.75% H₂O₂ was added to the enzyme extract (0.1 mL). POD activity was measured based on the change in absorbance of the brown guaiacol at 460 nm using SPECTROstar Nano (BMG Labtech microplate reader, Ortenberg, Germany) spectrophotometer set to kinetic mode.

3.2.2.7 Expression pattern of cold stress related genes during chilling injury of safflower

3.2.2.7.1 Sample collection

After induction of cold stress, three leaf samples were randomly cut from one plant in each pot to represent one biological replicate and snap frozen in liquid nitrogen and stored at -80°C until mRNA extraction (Hu et al., 2016).

3.2.2.7.2 RNA extraction and cDNA synthesis

Total RNA was extracted from the previously frozen leaf tissues using Quick-RNA™ Plant Miniprep kit (Zymo Research, Irvine, CA, USA) and following the manufacturer's instructions. The purity and concentration of RNA were determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the extracted RNA was confirmed using 0.8% agarose gel electrophoresis. Samples with good quality and quantity RNA were reverse transcribed to cDNA as a template for qPCR using ProtoScript® II First Strand cDNA Synthesis Kit (New England Biolabs Inc., Ipswich, MA, USA) with oligo (dT) primer. Success of cDNA synthesis was tested by amplification of the cDNA using translation initiation factor 5A (EIF 5A) and ubiquitin-conjugating enzyme E2 (UBCE2) (described in Table 3.1), the internal reference markers, to confirm the presence of amplification band. cDNAs that exhibited banding were used for quantification of the genes of interest, while those that did not show any banding had their synthesis repeated.

Table 3.1. The primer description of reference genes used for normalization of safflower target genes.

Unigene number	Gene symbol	Gene description	Sequence (5'–3')	Amplicon length (bp)	Tm of primer (°C)
comp409 96_c0	EIF-5A	Translation Initiation Factor 5A	F 5'-TGTCCTCATGTCAACCGTA-3'	120	59.96
			R 5'-GCATCATCAGTTGGGAGCTT-3'		60.23
comp318 83_c1	UBCE2	Ubiquitin-Conjugating Enzyme E2	F 5'-GAGATGGCACCGTGAGTTATG-3'	102	60.53
			R 5'-GCCCTTCATGTACAGAGTTGTG-3'		59.66

3.2.2.7.3 Gene of interest selection and design of primers

A collection of various cold responsive genes was identified from literature with the National Centre for Biotechnology (NCBI) Blastn procedures. However, the output of the BLAST did not match any sequence of safflower. Safflower pseudo-marker snap genes were extracted from the *Carthamus tinctorius* Genome Database (<https://www.safflower.scuec.edu>) following NCBI Blastn (<https://www.ncbi.nih.gov>) procedures to obtain mRNA sequences similar to that of safflower pseudo-maker snap genes. Confirmation of sequence similarities was done using Clustal omega Pairwise Sequence Alignment (EMBL-EBI-[www.ebi.ac.uk>msa](http://www.ebi.ac.uk/msa)). Genes with high percentage similarities were checked for cold responsiveness by studying their gene annotation; biological processes, functions and conserved domain sequences utilizing the PUBMED, Reverse Position Specific-BLAST (RPS-BLAST) and Conserved Domain Database (CDD) (Lu et al., 2020a, Wang et al., 2023a). Cold-responsive homologs were then used to design specific primers using Primer3Plus tool (<https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>). Details of the specific primers used are presented in Table 3.2.

Table 3.2. Primers used in qRT-PCR for amplification of safflower target genes.

Name	Sequence (5'–3')	Sequence length (bp)	Tm of the primer (°C)
CtAH1700–1	F 5'-ATGGTGAATTGGCATTGGTT-3'	20	47.68
	R 5'-AAGGTTCAACGGCTATGGTG-3'		51.78
CtAH1500–1	F 5'-CATGCTCCCTCCGAAAAATA-3'	20	49.73
	R 5'-AGCAATGCACGATACAGCAG-3'		51.78
CtAH500	F 5'-GTTAGGGTTGCAAGGGTGAA-3'	20	51.78
	R 5'-GGATGTCCTCCTCCTGACA-3'		53.83

bp-base pair, tm-melting temperature

3.2.2.7.4 mRNA quantification

The qRT-PCR was performed in a template containing 3 µl of cDNA, 10 µL of Luna qPCR master mix, 2 µM of the forward and reverse primers and topped up to 20 µL using DNase water. The reactions were performed on a Bio-Rad CFX 96 Connect real-time PCR detection system (BioRad Laboratories, Inc., Hercules, CA, USA) with each sample having three

independent biological replicates. Translation initiation factor 5A (EIF 5A) and ubiquitin-conjugating enzyme E2 (UBCE2) were used as reference genes (Table 3.1) and no template control (NTC) as a negative control. The qPCR test was run with the following thermal cycling conditions: initial denaturation at 95°C for three minutes, 40 cycles of 95°C denaturation for 15 seconds, annealing at the melting temperature of the primers for 15 seconds, and extension at 72°C for 30 seconds.

3.2.2.7.5 Analysis of the mRNA abundance

To determine relative fold differences for each sample, the CT value for each safflower gene was normalized to the CT value for the reference gene (EIF 5A and UBCE2). Relative expression level of the targeted was determined based on the delta CT method which utilizes the equation; $R = 2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001).

3.3 Statistical analysis

Data collected was subjected to analysis of variance using R-Programme version 4.2.3 (Doebioresearch package). Means were separated using the Least Significant Difference (LSD) at $P \leq 0.05$.

CHAPTER 4

4.0 RESULTS

4.1 Chilling injury incidence, severity and plant recovery

4.1.1 Chilling injury incidence

The results showed no signs of CII in safflower at the rosette stage in all temperatures and durations of cold exposure under artificial cold stress induction (Figure 4.1). Even after a prolonged period of 12 hours at 0 and 4°C, the exposed plants remained fresh, green, and healthy, like those kept at the warmer temperature of 23°C (Picture 1a, b). Meanwhile, CI incidence occurred in plants at the bolling stage exposed to 0 and 4°C for the duration of 8 and 12 hours, respectively (Figure 4.2, Picture 4.2). Still in bolling stage, plants exposed to 4°C exhibited CI symptoms when exposed for longer duration of 12 hours, but plants exposed to the same temperature for 8 hours or less did not develop CI symptoms. Whereas safflower plants exposed to 0°C for 8 and 12 hours suffered from CI (Figure 4.2, Picture 4.2). Notably, plants exposed to shorter durations (2 and 4 hours) showed no signs of cold stress damage at any of the tested temperatures. Similarly, safflower plants exposed to 8 and 12°C were unaffected as the plants were still intact and resembled those not cold-stressed (Figure 4.2, Picture 4.2).

On the other hand, cold stress at 2°C did not significantly ($P > 0.05$) influence the CII of safflower, irrespective of genotype, growth stage, and interaction of genotype and growth stage under the field conditions (Figure 4.3).

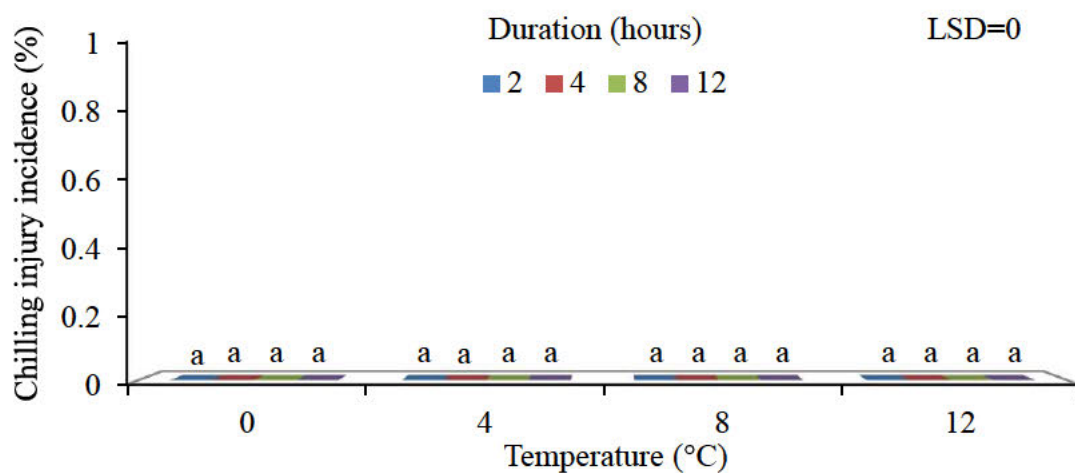
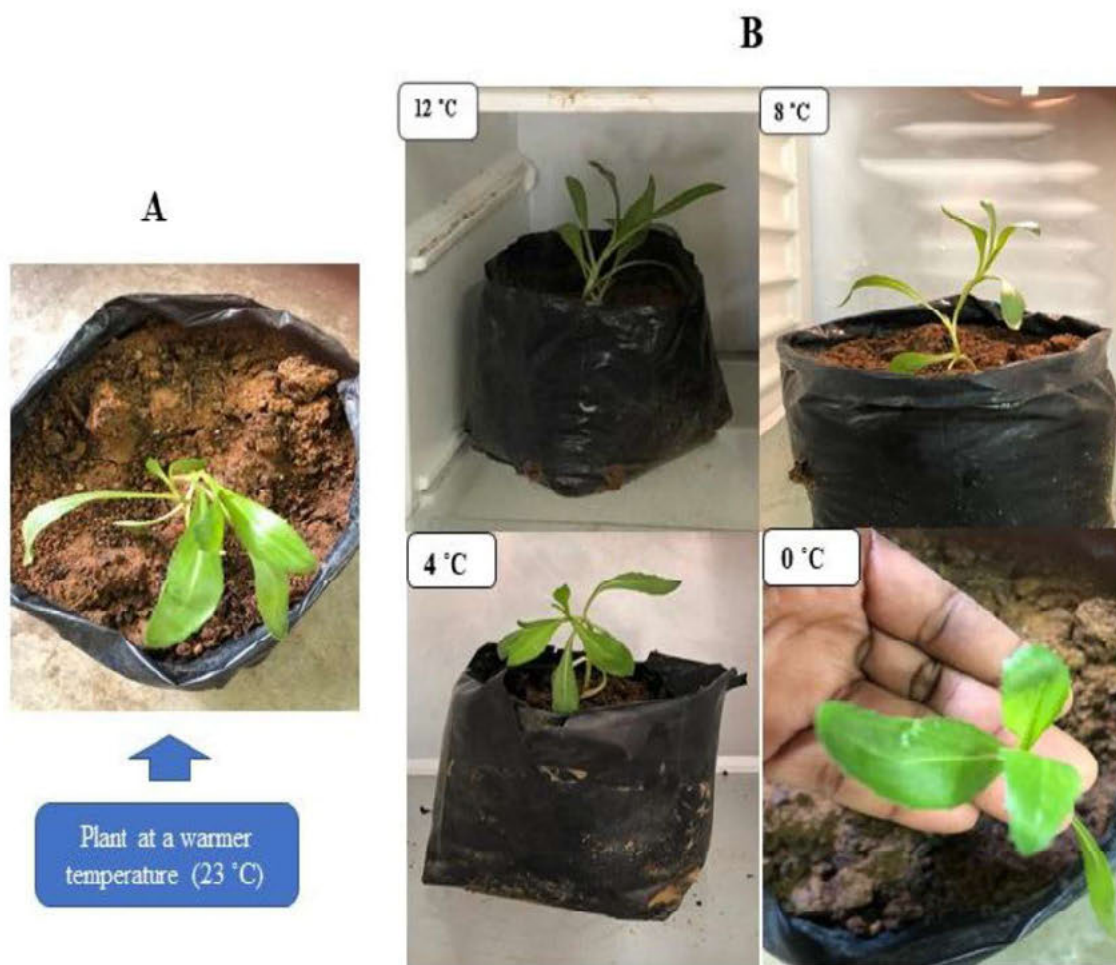


Figure 4.1. Influence of temperature and duration of exposure to CII of safflower at the rosette stage in artificial cold stress induction experiment. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.



Picture 4.1. The visual appearance of safflower plants after 12 hours of cold exposure at low temperatures of 0, 4, 8, and 12°C during the rosette stage.

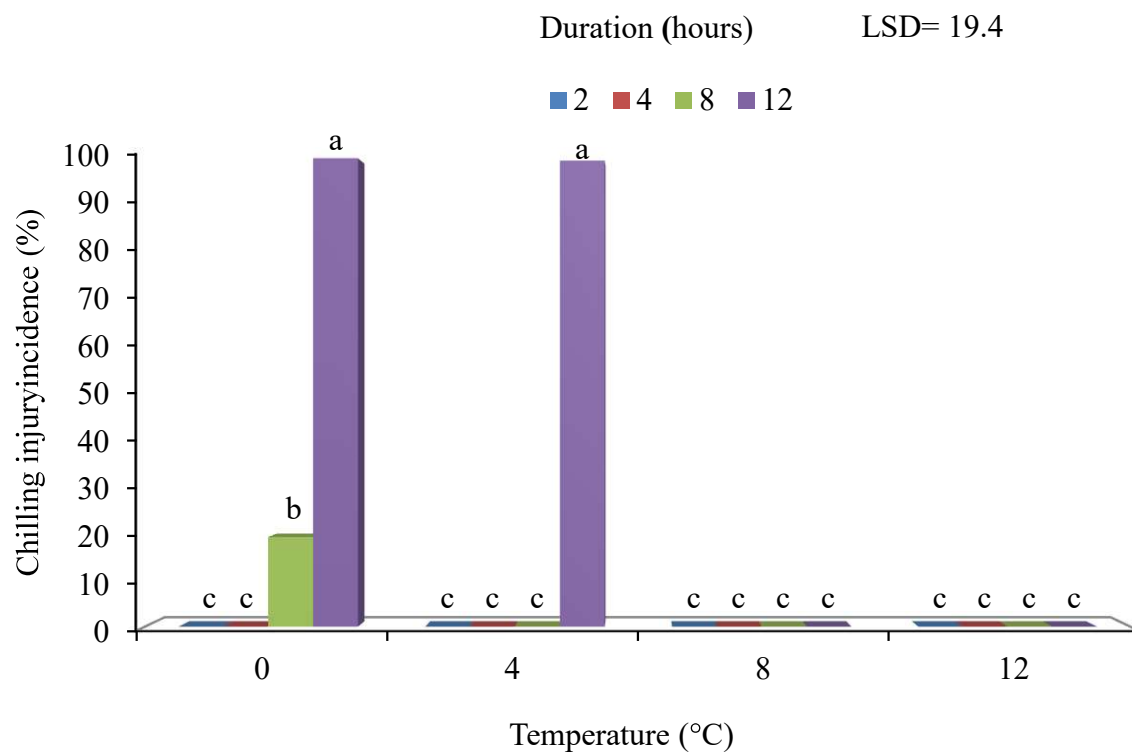


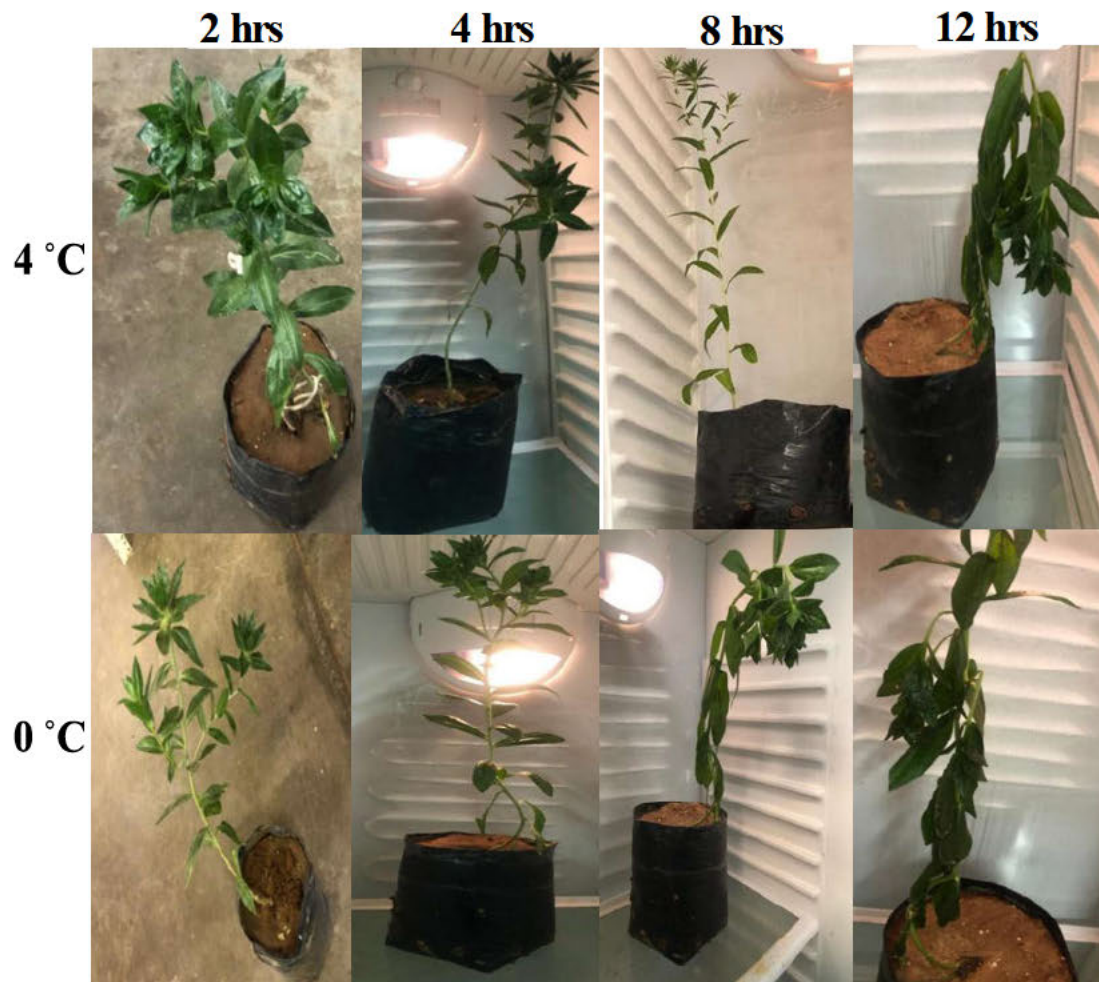
Figure 4.2. Influence of temperature and duration of exposure to CII of safflower at the boiling stage in artificial cold stress induction experiment. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

A



**Plant before
application of
cold stress**

B



Picture 4.2. The visual appearance of safflower plants after exposure to low temperatures of 0 and 4°C and durations of 2, 4, 8 & 12 hours at the bolling stage.

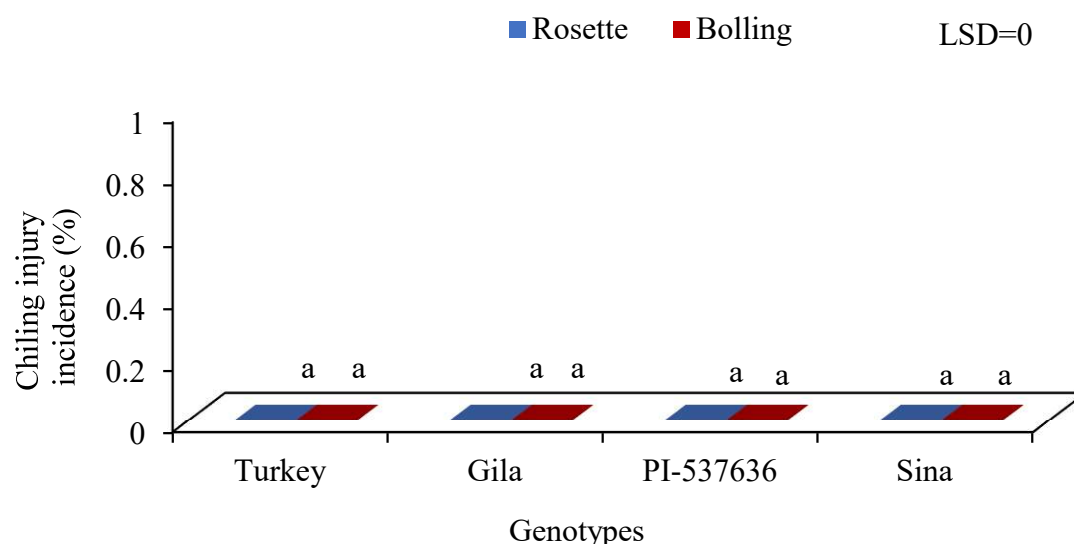


Figure 4.3. Influence of genotype and growth stage on the CII of safflower cold stressed at 2°C under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.1.2 Severity of chilling injury

Chilling severity was observable in plants exposed for 12 hours at 0 and 4°C at the bolling stage, as indicated by wilted, curled and discoloured leaf edges under artificial cold stress treatment (Picture 4.2b, Figure 4.4). Moreover, the potted soil in which plants were grown appeared patched and icy. In contrast, the severity was mild in plants exposed to 0°C for 8 hours at the same phenological stage. On the contrary, no significant differences were found on safflower plants exposed to 4°C for 2, 4, and 8 hours, and the soil in which the plants were grown remained moist (Picture 4.2, Figure 4.4). Since there was no evidence of CII on safflower plants during the rosette stage when exposed to all temperatures under study, there was no severity of CI at this developmental stage. Similarly, cold stress did not significantly ($P > 0.05$) influence severity of CI of safflower under field condition, irrespective of genotype and growth stage and their interactions (Figure 4.5).

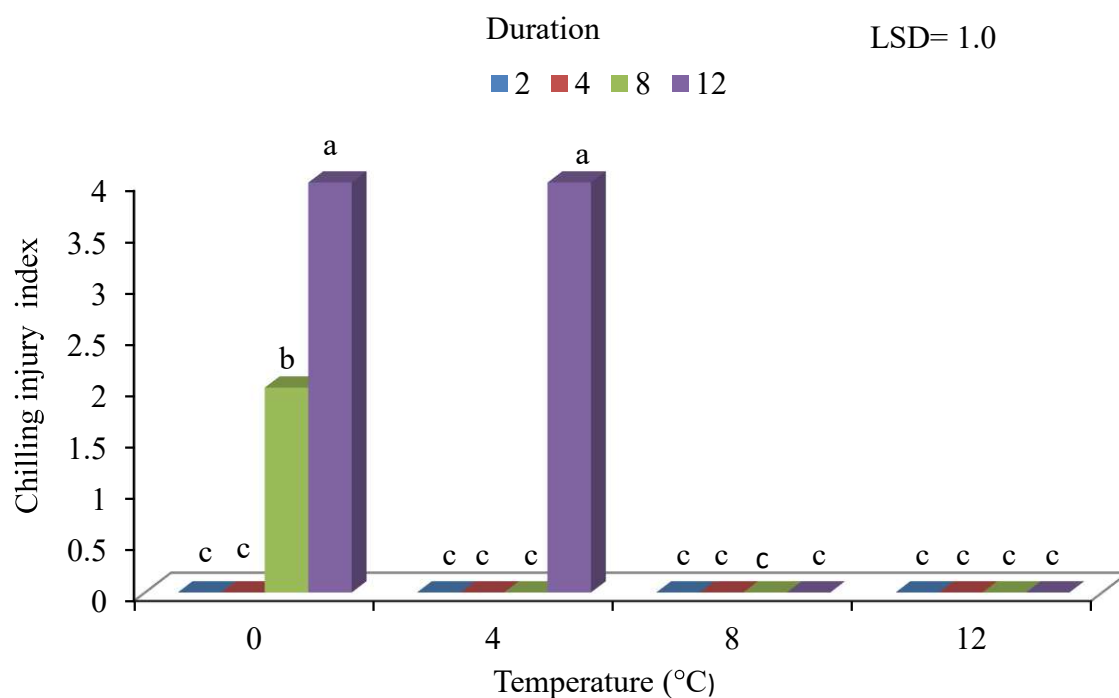


Figure 4.4. Influence of temperature and duration of exposure on severity of CI on safflower under artificial cold stress induction. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

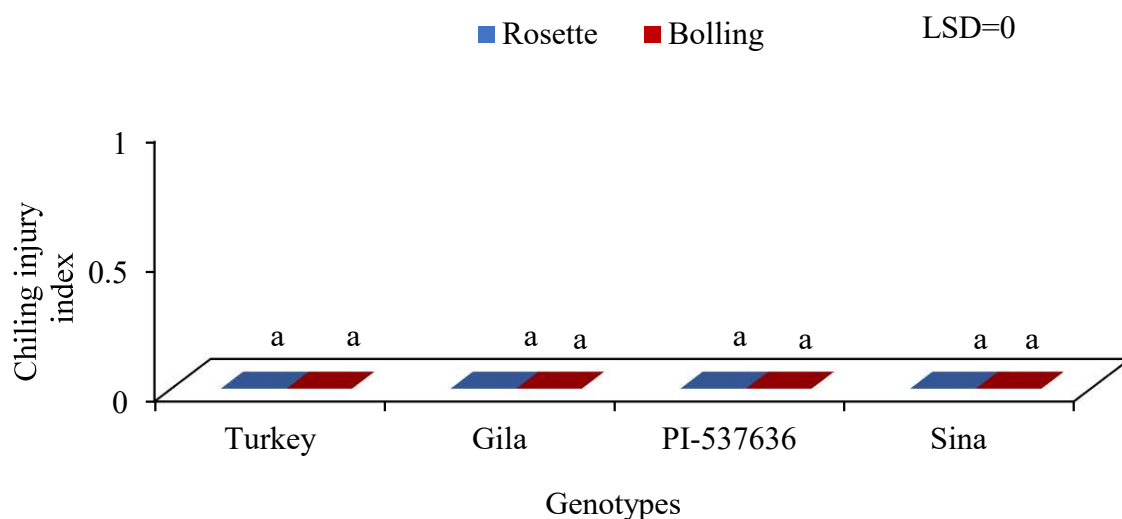


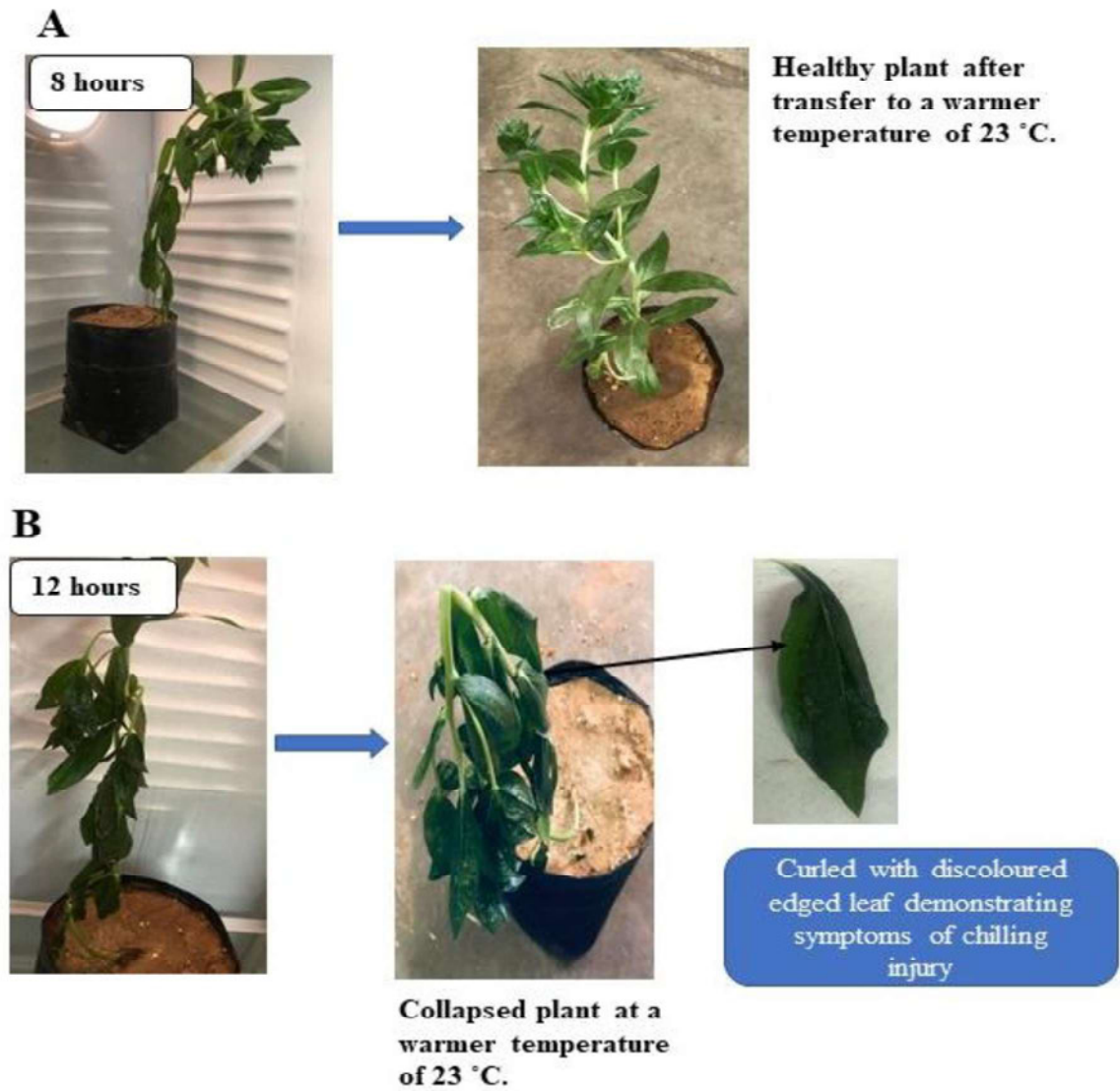
Figure 4.5. Influence of genotype and growth stage on CI severity of safflower cold stressed at 2°C under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.1.3 Plant recovery

Transferring plants previously cold stressed at 0°C and 8 hours to warmer temperatures of 23°C led to recovery of plants after 72 hours (Table 4.1, Picture 4.3a). Safflower plants exposed to low temperatures of 0°C and 4°C for a period of 12 hours did not recover (Table 4.1). The plants cold stressed at 0°C collapsed after 3 minutes of exposure to 23°C (Table 4.1, Picture 4.3 b). Whereas plants exposed to 4°C collapsed after 13 minutes (Table 4.1).

Table 4.1. Duration of recovery and plant death after transferring the chilled plant to 23°C.

Chilling temperatures (°C)	Duration of exposure (hours)	Duration of recovery after transfer to 23°C (hours)	Duration of plant death after transfer to 23°C (minutes)
0	2 & 4	Not affected	Not affected
0	8	72	Recovered
0	12	No recovery	3
4	2,4 & 8	Not affected	Not affected
4	12	No recovery	13
8	2, 4, 8 & 12	Not affected	Not affected
12	2, 4, 8 & 12	Not affected	Not affected



Picture 4.3. Illustration on the physical appearance of safflower plants after transfer from 0°C to a warmer temperature of 23°C at 8 (A) and 12 (B) hours of cold exposure at the bolting stage.

4.2 Physiological, biochemical, and molecular responses of chilled safflower plants

4.2.1 Relative leaf electrolyte leakage

Analysis of variance showed that safflower leaf relative electrolyte leakage (REL) was highly ($P < 0.001$) influenced by the interaction of growth stage, temperature and duration of exposure under artificial cold stress induction (Appendix 3). Exposure of safflower plants to 0 and 4°C at the bolling stage for a duration of 8 and 12 hours significantly ($P < 0.05$) increased REL compared to control plants at the same phenological stage and plants at the rosette stage (Figure 4.6). Safflower plants at the bolling stage and exposed to cold stress at 0°C for 12 hours had significantly ($P < 0.05$) the highest REL of 65.7% than plants at the same stage exposed to 0 and 4°C for 8 and 12 hours, respectively (Figure 4.6). When safflower plants are at the bolling stage were exposed to low chilling temperatures, lowering the temperature from 4 to 0°C and prolonging the exposure duration from 8 to 12 hours significantly ($P < 0.05$) increased REL from 14.47 to 65.67% (Figure 4.6). Even within the same exposure temperature increasing duration of exposure to chilling temperatures significantly ($P < 0.05$) increased REL of safflower plants in the bolling stage but not in the rosette stage (Figure 4.6). Safflower plants at the rosette stage exposed to chilling temperatures of 0 and 4°C for 8 and 12 hours did not significantly ($P > 0.05$) vary in their REL with control plants (Figure 4.6). Conversely, exposure of plants in bolling to 4°C significantly increased the leaf's REL from 9.9% to 14.5% and 17.1% in 8 and 12 hrs of exposures, respectively.

In the field experiment, REL exhibited significant ($P < 0.001$) variability depending on the interaction of growth stage and time of the day (Appendix 4). Natural exposure of safflower plants to 2°C (lowest temperature at night) remarkably ($P < 0.05$) increased REL from 7.74 (control) to 20.18% (morning), and 4.79 (control) to 6.81% (morning) in the bolling and rosette stages of development, respectively (Figure 4.7). As the day temperatures warms up the REL significantly ($P < 0.05$) decreased in both safflower plants in bolling and rosette phenological stages compared to plants in the morning. As a result, the REL value for plants in the rosette stage became almost comparable to that of the controls, measuring 4.79% against 4.93%, respectively (Figure 4.7).

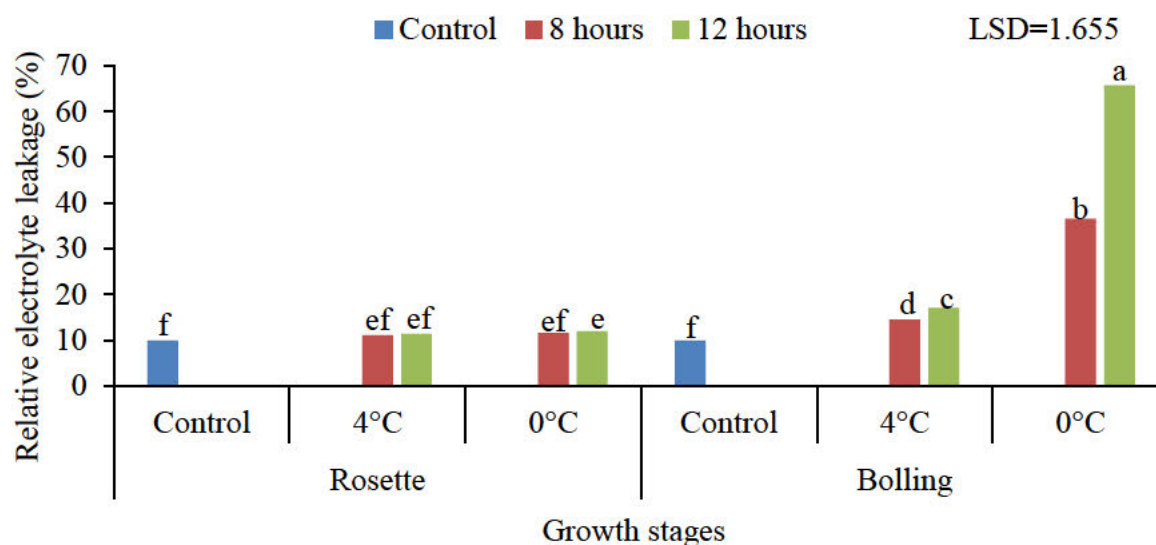


Figure 4.6. Influence of growth stage, temperature, and duration of exposure on relative electrolyte leakage (REL) of safflower under artificial cold stress induction. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

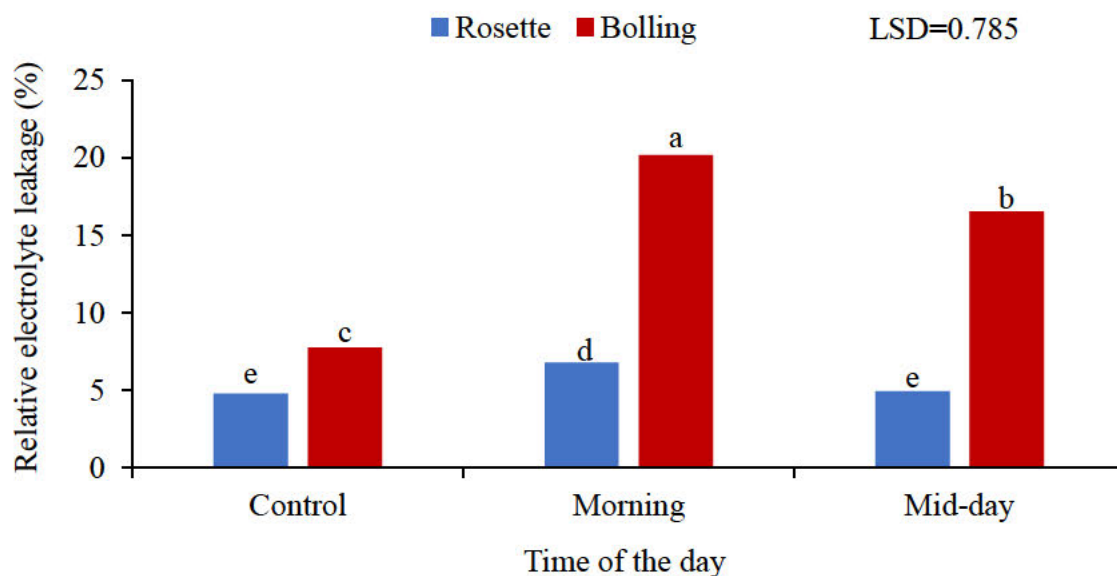


Figure 4.7 Influence of growth stage and time of the day on relative electrolyte leakage (REL) of safflower cold stressed at 2°C under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.2 Proline content

Under artificial cold stress induction, plants in both growth stages significantly ($P < 0.01$) produced higher proline content than control produced as influenced by the interaction of temperature, phenological stage and duration of exposure (Appendix 3). Safflower plants at the rosette stage exposed to chilling stress at 0°C for 12 hours significantly ($P < 0.05$) produced higher proline content of $8.84 \mu\text{mol/g FW}$ than plants in both development stages exposed to low temperatures of 0 and 4°C for 8 and 12 hours and control (Figure 4.8). Interestingly, safflowers at the rosette stage exposed to low temperatures of 0 and 4°C for a duration of either 8 or 12 hours produced remarkable ($P < 0.05$) higher proline content than plants at the bolting stage under the same condition (Figure 4.8). However, plants within the rosette stage exposed to low temperatures of 0°C for 8 hours, and 4°C for either 8 or 12 hours did not significantly ($P > 0.05$) vary in their proline contents but had higher proline content than control plants (Figure 4.8). Plants at the bolting stage exposed to chilling temperature of 0°C for either 8 or 12 hours did not significantly ($P > 0.05$) differ in their proline contents with plants exposed to 4°C for 12 hours but their proline contents were higher than control plants (Figure 4.8).

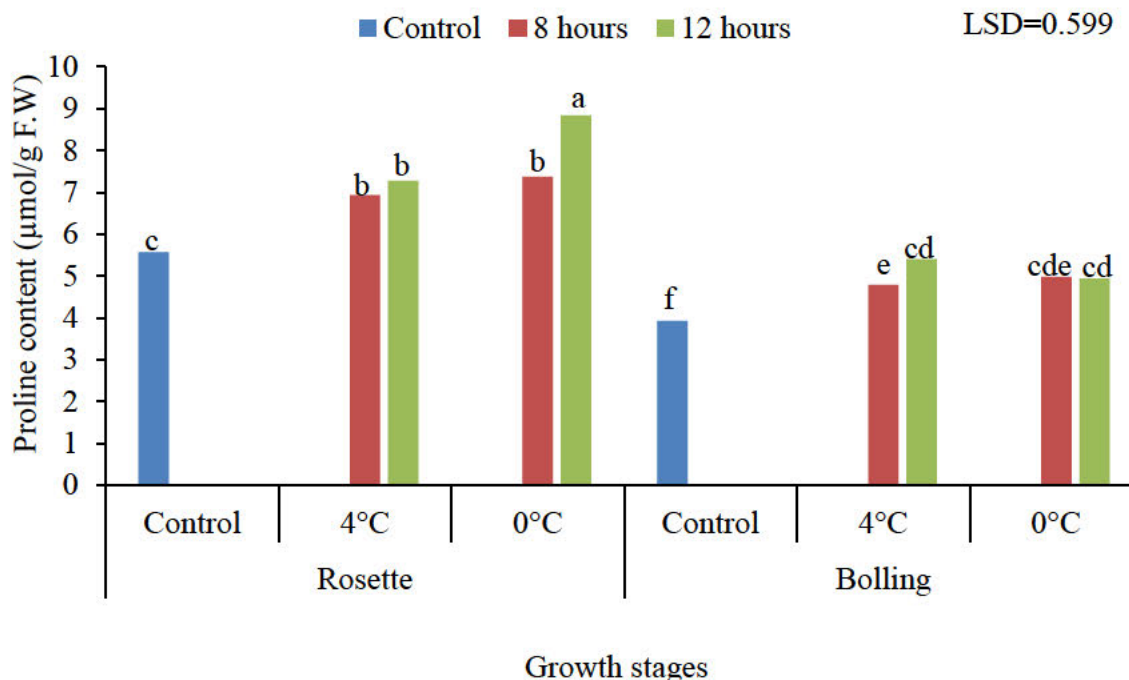


Figure 4.8. Influence of growth stage, temperature, and duration of exposure on proline content of safflower under artificial cold stress induction. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

In the field experiment, temperature, stage of development, and time had a statistical ($P < 0.001$) influence on leaf proline content (Appendix 4). Exposure of safflower plants to a chilling temperature of 2°C remarkably ($P < 0.05$) increased the leaf proline contents to $11.65 \mu\text{mol/g FW}$ and $10.70 \mu\text{mol/g FW}$ in the rosette and bolting stages, respectively, in the morning. The highest proline accumulation was recorded in the rosette stage in the morning. At mid-day, proline content did not differ significantly from the bolting stage morning values in either phenological stage (Figure 4.9).

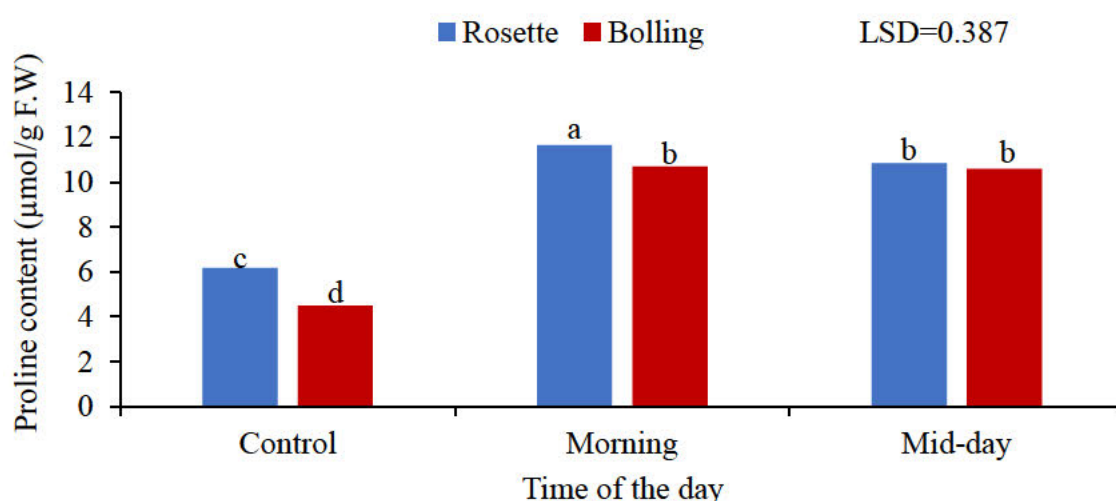


Figure 4.9. Influence of growth stage and time on proline content of safflower cold stressed at 2°C under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.3 Leaf chlorophylls and carotenoid content

The results of the study demonstrated that cold stress significantly ($P < 0.05$) caused variation in safflower chlorophyll a, chlorophyll b, total chlorophyll (a+b), and carotenoids contents depending on the interaction of growth stage, temperature, and duration of cold exposure (Figure 4.10, 4.11, 4.12, 4.13). Before imposition of cold stress, safflower plants at the rosette stage had significantly higher ($P < 0.05$) chlorophyll a (Figure 4.10), chlorophyll b (Figure 4.11) and total chlorophyll (a+b) (Figure 4.12) of 3.18 mg/g FW , 2.24 mg/g FW and 5.60 mg/g FW against 1.88 mg/g FW , 0.76 mg/g FW and 2.64 mg/g FW at the bolting stage, respectively (Figure 4.10, 4.11, 4.12). Cold stress resulted in a reduction in chlorophyll a, b, and a+b in all

the growth stages and durations of exposure (Figure 4.10, 4.11, 4.12). However, cold stress in all durations significantly ($P < 0.05$) reduced total chlorophyll, chlorophyll a, and chlorophyll b in the bolting compared to control plants but in the rosette stage the reduction was insignificant ($P > 0.05$). Within the rosette stage, chilling temperature and duration of exposure did not significantly ($P > 0.05$) reduce chlorophyll a content compared to control (Figure 4.10). In the bolting stage, leaf chlorophyll a content was significantly ($P < 0.05$) reduced by exposure of plants to 0°C for 12 hours (Figure 4.10). Meanwhile, chlorophyll b was notably reduced by 41.3% in chilling conditions of 4°C and 12 hours at the rosette stage. After which lowering the chilling conditions to 0°C for both 8 and 12 hours did not considerably ($P > 0.05$) reduce chlorophyll b content of the plants compared to plants exposure to 4°C for 12 hours during the rosette stage (Figure 4.11). Contrariwise, chlorophyll b was not significantly ($P > 0.05$) decreased by the interaction of temperature and duration of cold exposure at the bolting stage compared to control plants (Figure 4.11). Correspondingly, the total chlorophyll (a+b) content was statistically reduced in safflower cold stressed with 4°C for 12 hours and 0°C for 8 and 12 hours, respectively compared to control plants at the rosette stage (Figure 4.12). Whereas at the bolting stage the total chlorophyll (a+b) content was severely reduced from 2.64 to 1.19 mg/g FW in plants cold stressed at 0°C for 12 hours. However, plants exposed to 0°C for 8 hours, and 4°C for either 8 or 12 hours, did not statistically vary in their total chlorophyll contents at the bolting stage (Figure 4.12).

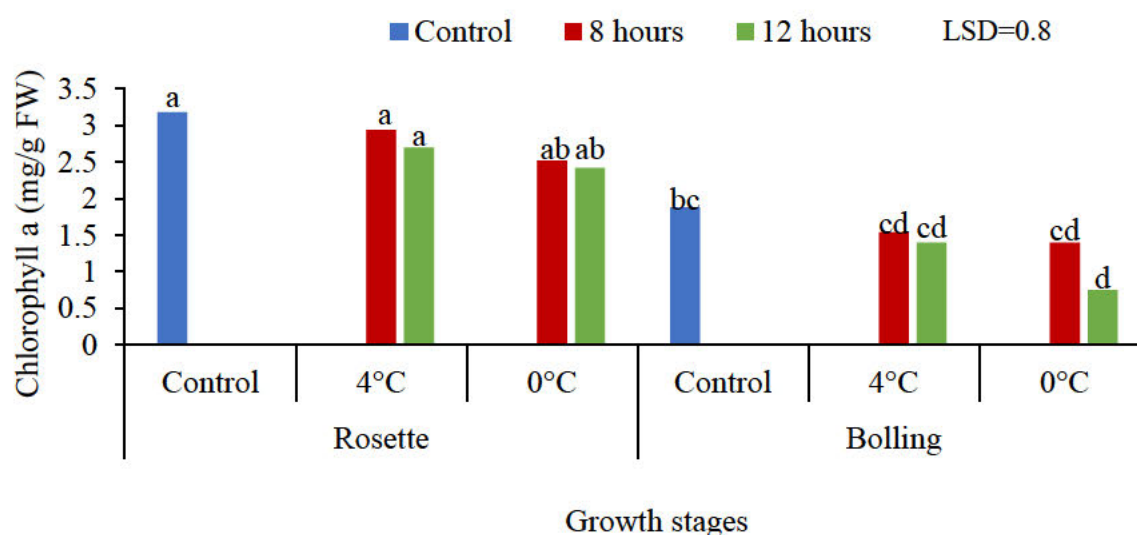


Figure 4.10. Influence of low temperature and duration on chlorophyll a of safflower at the rosette and bolting stage under artificial cold stress induction. Means with the same letters are not significantly different Means separated using the LSD at $P \leq 0.05$.

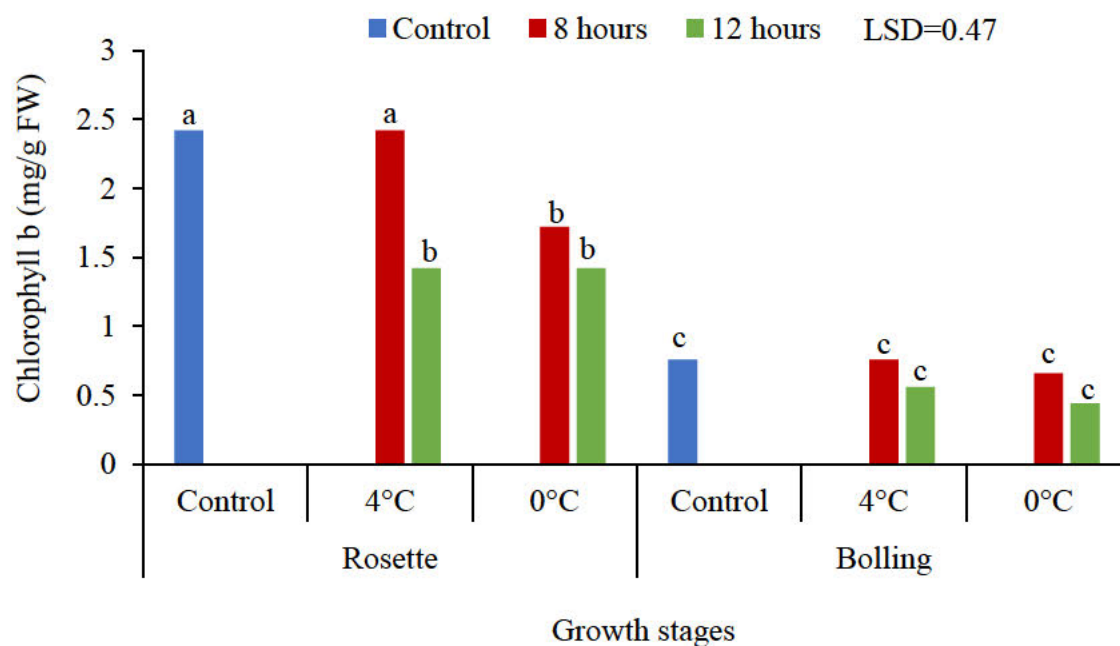


Figure 4.11. Influence of low temperature and duration on chlorophyll b of safflower at the rosette and bolting stage under artificial cold stress induction. Means with the same letters are not significantly different Means separated using the LSD at $P \leq 0.05$.

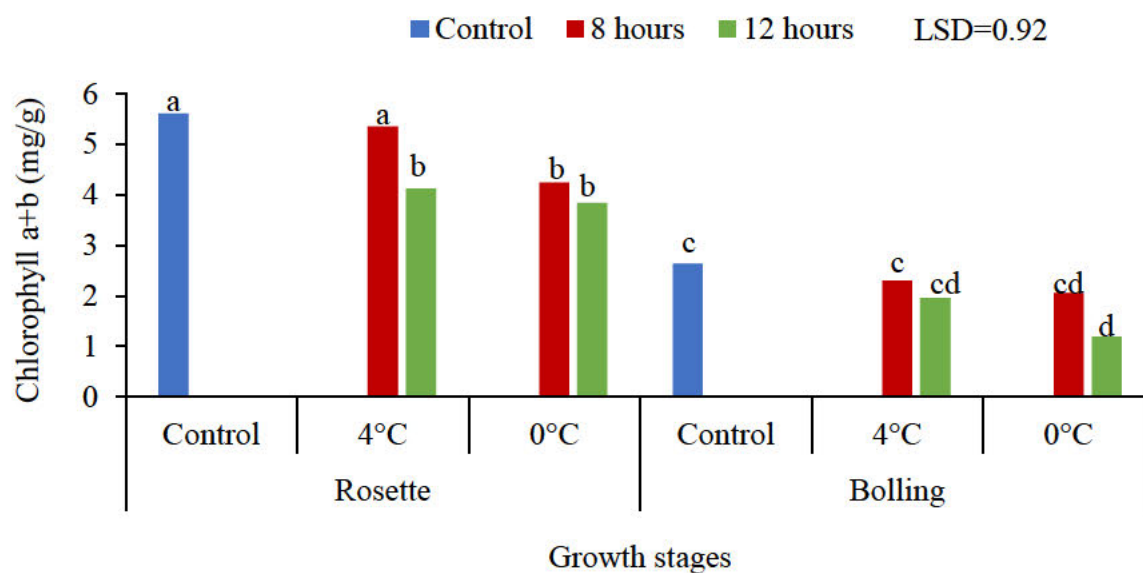


Figure 4.12. Influence of low temperature and duration on chlorophyll a + b of safflower at the rosette and bolting stage under artificial cold stress induction. Means with the same letters are not significantly different Means separated using the LSD at $P \leq 0.05$.

Contrary to the chlorophyll results, cold stress increased and decreased carotenoid content of plants in rosette and bolting stages, respectively (Figure 4.13). Safflower plants at the rosette stage exposed to 0°C for 12 hours had significantly ($P < 0.05$) higher leaf carotenoid content than control plants and plants exposed to cold stress at 4°C for 8 and 12 hours (Figure 4.13). In the bolting stage, safflower plants cold stressed at 4°C for 12 hours, and 0°C for either 8 or 12 hours had significant ($P < 0.05$) lower leaf carotenoid content than control plants but their carotenoid content did not vary significantly (Figure 4.13).

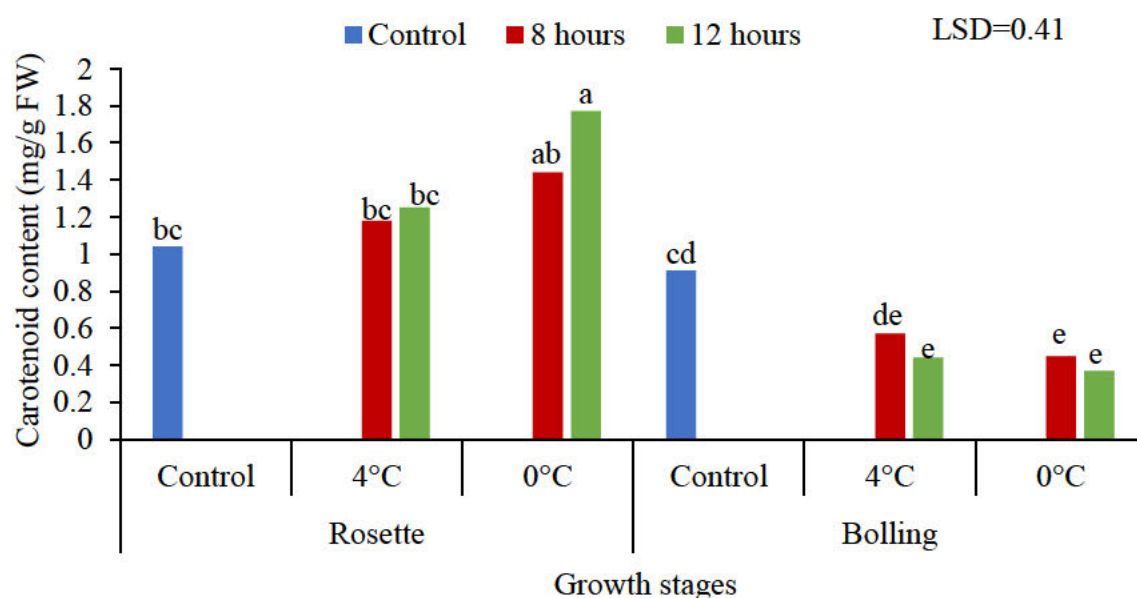


Figure 4.13. Influence of low temperature and duration on carotenoid content of safflower at the rosette and bolting stage under artificial cold stress induction. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

Under field conditions, safflower leaf chlorophyll content highly ($P < 0.001$) varied due to the interaction of genotype, growth stage and time of the day (Appendix 4). Generally, all genotypes exhibited high chlorophyll contents in both phenological stages prior to exposure to chilling temperatures. All safflower genotypes in both rosette and bolting stages exposed to chilling stress of 2°C at night had significantly ($P < 0.05$) lowered leaf chlorophyll content in the morning than control plants under field conditions (Figure 4.14). However, the reduction in leaf chlorophyll content in all genotypes was much higher in plants at the bolting stage compared to the rosette stage (Figure 4.14). In the morning, the genotype Sina had significantly ($P < 0.05$) higher chlorophyll contents of 73.87 and 61.97 (SPAD) at rosette and bolting stages,

respectively than other genotypes under field conditions (Figure 4.14). On the contrary, the genotype Turkey had the lowest leaf chlorophyll content in the morning compared to other genotypes at the same time in both phenological stages (Figure 4.14). As the temperature warms at midday, the leaf chlorophyll content increased in all genotypes and phenological stages compared to morning plants (Figure 4.14).

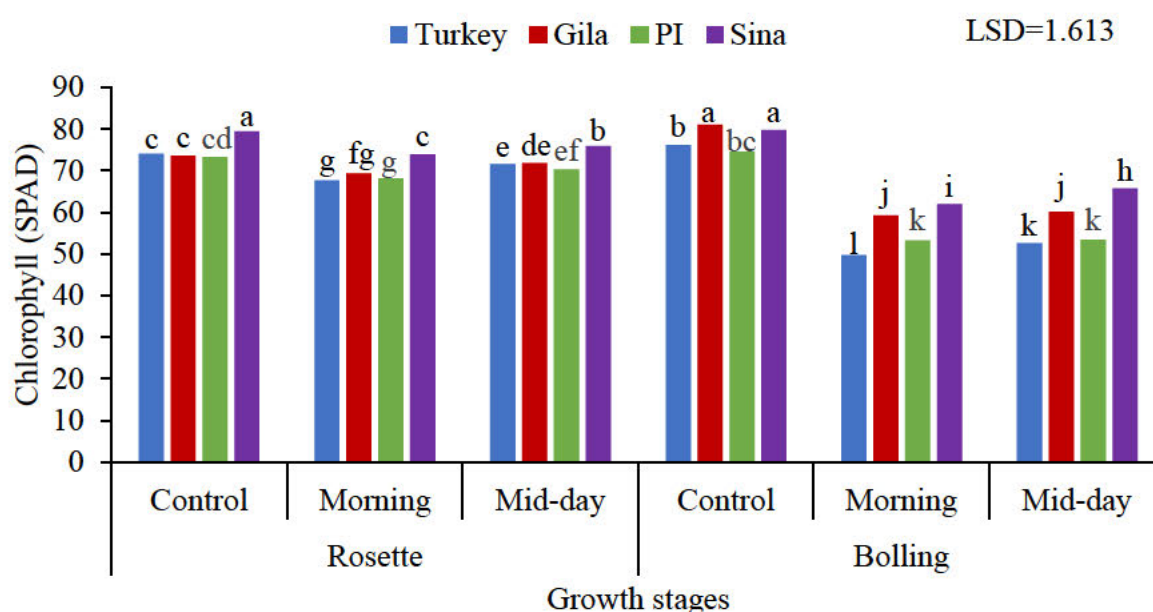


Figure 4.14. Influence of genotype, growth stage, and time of the day on chlorophyll content of safflower plants cold stressed at 2°C under field conditions. Means with the same letter are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.4 Chlorophyll fluorescence

In terms of chlorophyll fluorescence parameters, the quantum yield of photosystem 2 (Φ_{II}) under artificial cold stress significantly ($P < 0.05$) ranged between 0.35-0.71 and 0.19-0.40 at the rosette and bolling stages as influenced by the interaction of phenological stage, temperature, and duration of cold exposure (Figure 4.15, Appendix 5). Prior to cold stress induction, safflower plants at the bolling stage had notably higher Φ_{II} value of 0.40 versus 0.35 at the rosette stage. Exposure of plants to 4°C for 12 hours remarkably increased the Φ_{II} by 103.4% compared to control plants at the rosette stage and this value significantly ($P < 0.05$) the highest Φ_{II} in all phenological stages and durations of exposure to cold stress (Figure 4.15). The quantum yield of PSII (Φ_{II}) was significantly ($P < 0.05$) lower in cold stressed plants in the bolling stage than plants in the rosette stage in all temperatures and duration of exposure to cold stress (Figure 4.15). The lowest Φ_{II} (0.19 and 0.20) was observed in plants in the bolling

stage exposed to cold stress at 0 and 4°C for 12 hours (Figure 4.15). Extending the duration of exposure to 12 hours at 4°C further increased the Φ II value by 21.34% at the rosette stage, whereas at the bolting stage it decreased by 17.09%. Interestingly, reducing the chilling temperature to 0°C for 8 hours led to a 25.46% decrease in the Φ II value at the rosette stage, while at the bolting stage the value was increased by 57.68%. Moreover, prolongation of duration of exposure from 8 hours to 12 hours at 0°C increased Φ II value by 11.83% at the rosette stage, meanwhile a substantial decrease of 44.86% was observed at the bolting stage, resulting in value not significantly ($P \geq 0.05$) different from that of plants subjected to 4°C for 12 hours (Figure 4.15).

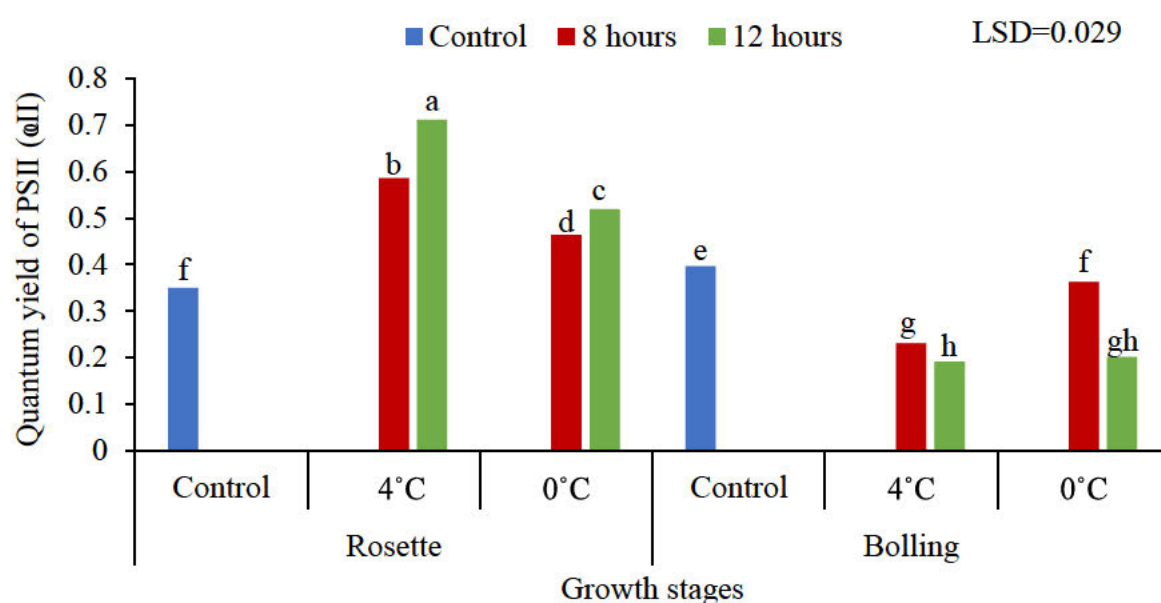


Figure 4.15. Influence of growth stage, temperature and duration of cold exposure on quantum yield of PSII (Φ II) of safflower under artificial cold stress induction. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

As for plants under field conditions, the Φ II was highly ($P < 0.001$) influenced by the interaction of growth stage and time of the day (Figure 16, Appendix 6). Before plants underwent cold stress, the plants had 0.46 and 0.57 Φ II value at the rosette and bolting stage, respectively (Figure 4.16a). Following chilling temperatures, a notable increase of 23.50% in Φ II was observed at the rosette stage plants during the morning, whereas a decrease of 9.87% was noted in plants at bolting stage during the same time (Figure 4.16a). The Φ II values measured at midday showed no statistical differences when compared to the morning values for both growth stages (Figure 4.16a). Additionally, the results demonstrated that the genotype Sina exhibited

the highest Φ_{II} value of 0.61 compared to other genotypes (Figure 4.16b). In contrast, the genotypes Gila and PI 537636 showed intermediate values of 0.53 and 0.54, respectively (Figure 4.16b). While Turkey recorded the lowest value of 0.50 compared to other genotypes (Figure 4.16b).

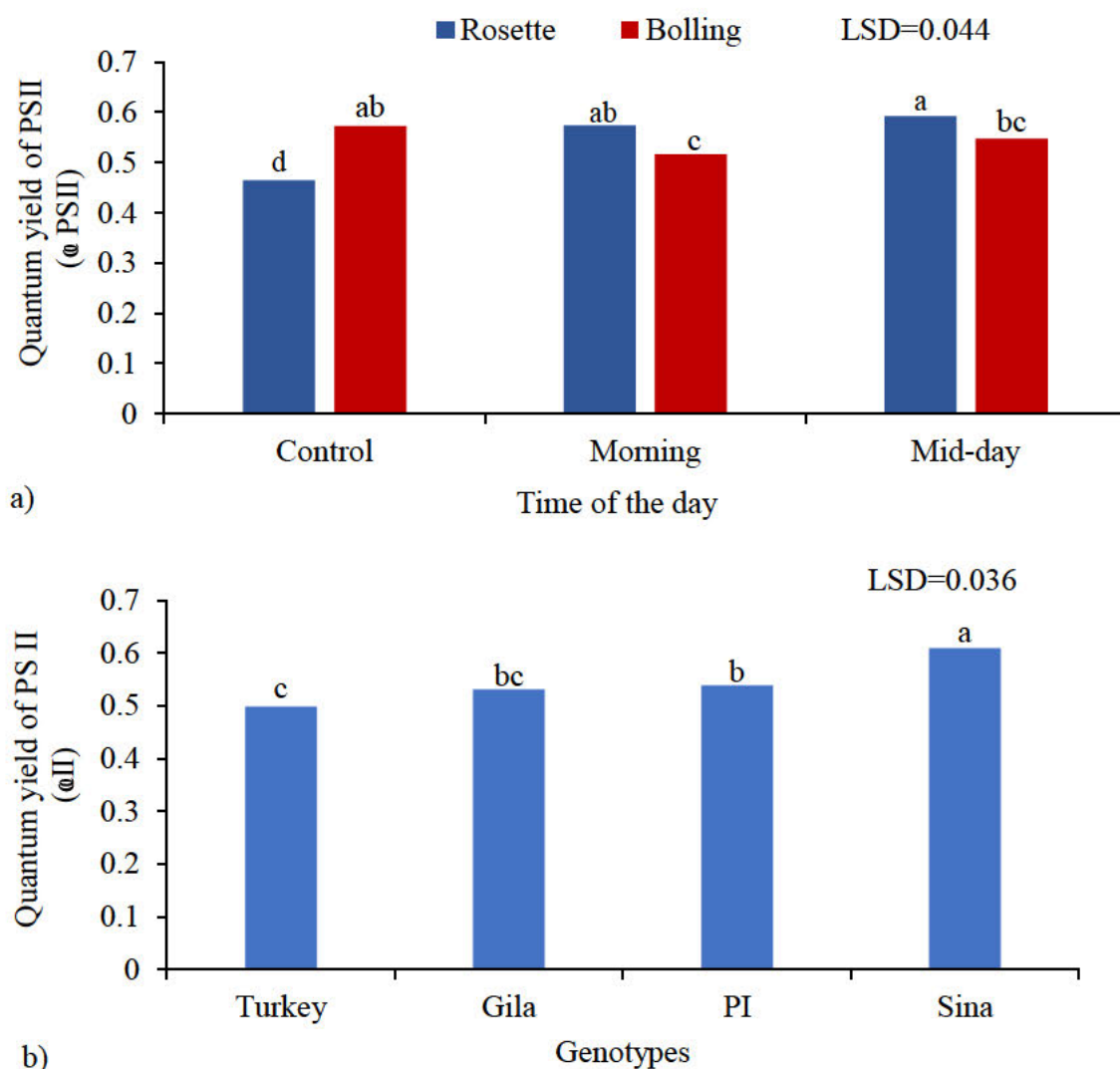


Figure 4.16. Influence of growth stage and time of the day (a), and genotype (b) on the quantum yield of PSII (Φ_{II}) of safflower under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

On the other hand, non-photochemical quenching (Φ_{NPQ}) was significantly ($P < 0.01$) influenced by the interaction of growth stage, temperature, and duration of cold exposure under artificial cold stress induction (Figure 17, Appendix 5). Prior to cold stress induction, Φ_{NPQ} values were comparable between the rosette and bolting stages, averaging 0.56. Exposure of

safflower plants to 4°C for 12 hours led to a notable increase in NPQ at the rosette stage, rising to 0.69 which was significantly ($P < 0.05$) the highest Φ NPQ value in all phenological stages, exposure temperatures and duration, and control plants (Figure 4.17). At the bolling stage, exposure of safflower plants to cold stress at 0 and 4°C for either 8 or 12 hours significantly ($P < 0.05$) reduced Φ NPQ compared to control plants and plants at the rosette stage (Figure 4.17). On average plants at the bolling stage experienced a pronounced reduction of 49.55% of Φ NPQ (Figure 4.17). The lowest Φ NPQ value of 0.12 was recorded in plants at the rosette stage exposed to 4°C for 12 hours (Figure 4.17).

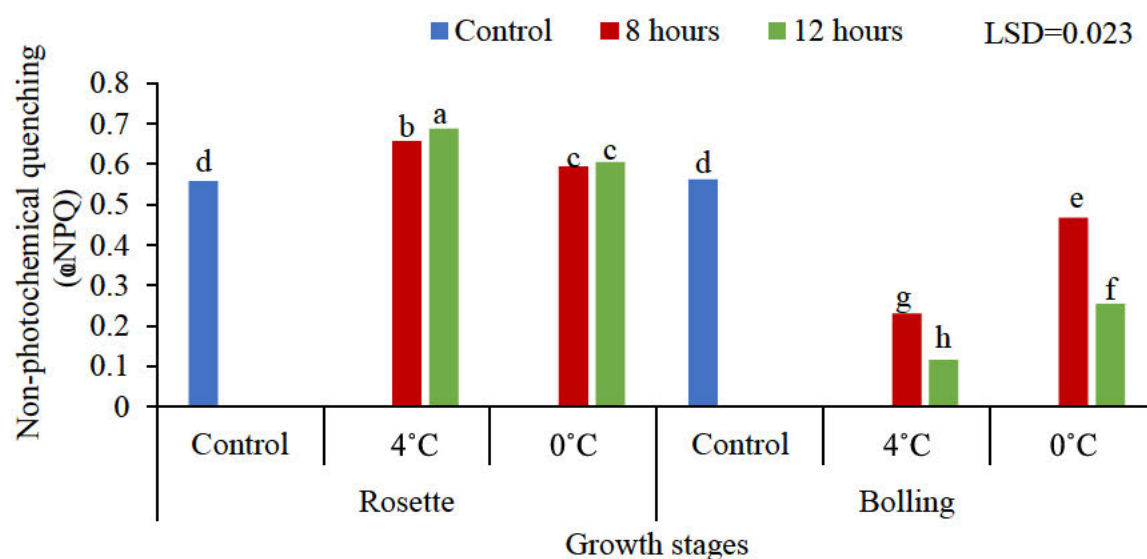


Figure 4.17. Influence of growth stage, temperature, and duration of cold exposure on non-photochemical quenching (Φ NPQ) of safflower under artificial cold stress induction. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

In contrast, safflower plants exposed to chilling conditions in the field were significantly ($P < 0.05$) influenced by the main effects of growth stage and time of the day but no interaction (Figure 18, Appendix 6). Plants at the bolling stage had a higher value of 0.64 compared to 0.62 of the rosette stage (Figure 4.18a). Additionally, mid-day measurements recorded the highest Φ NPQ value of 0.67, compared to the morning value of 0.64 (Figure 4.18b).

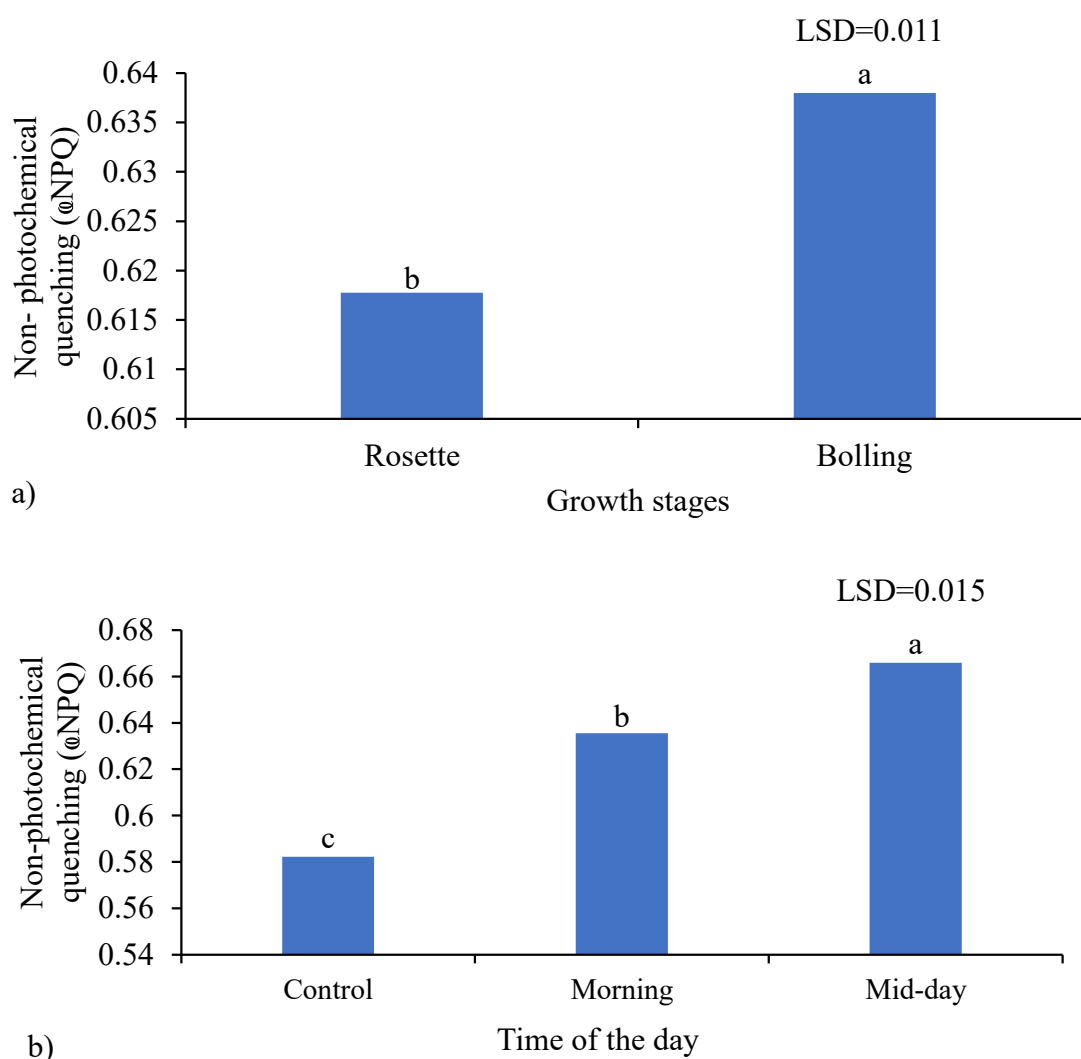


Figure 4.18. Influence of growth stage (a) and time of the day (b) on non-photochemical quenching (NPQ) of safflower under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

With regards to the quantum yield of non-regulated energy of PS II (Φ_{NO}), the interactions of growth stage and temperature and duration of exposure substantially ($P < 0.05$) influenced it (Figure 4.19, Appendix 5). Safflower plants at the rosette stage exhibited lower Φ_{NO} values than plants at the bolting stage under cold stress (Figure 4.19a). The highest and lowest Φ_{NO} value of 0.31 and 0.16 were observed in plants at the bolting and rosette stages exposed to 4°C (Figure 4.19a). Similarly, Figure 4.19b demonstrated that the duration of cold exposure and stage of safflower development influenced Φ_{NO} . Safflower plants at the rosette stage had significantly ($P < 0.05$) lower Φ_{NO} values than plants at the bolting stage stage irrespective of

duration to cold temperatures (Figure 4.19b). Safflower plants exposed to cold stress for 12 hours had the highest and lowest Φ_{NO} values of 0.32 and 0.16 at bolling and rosette phenological stages, respectively (Figure 4.19b).

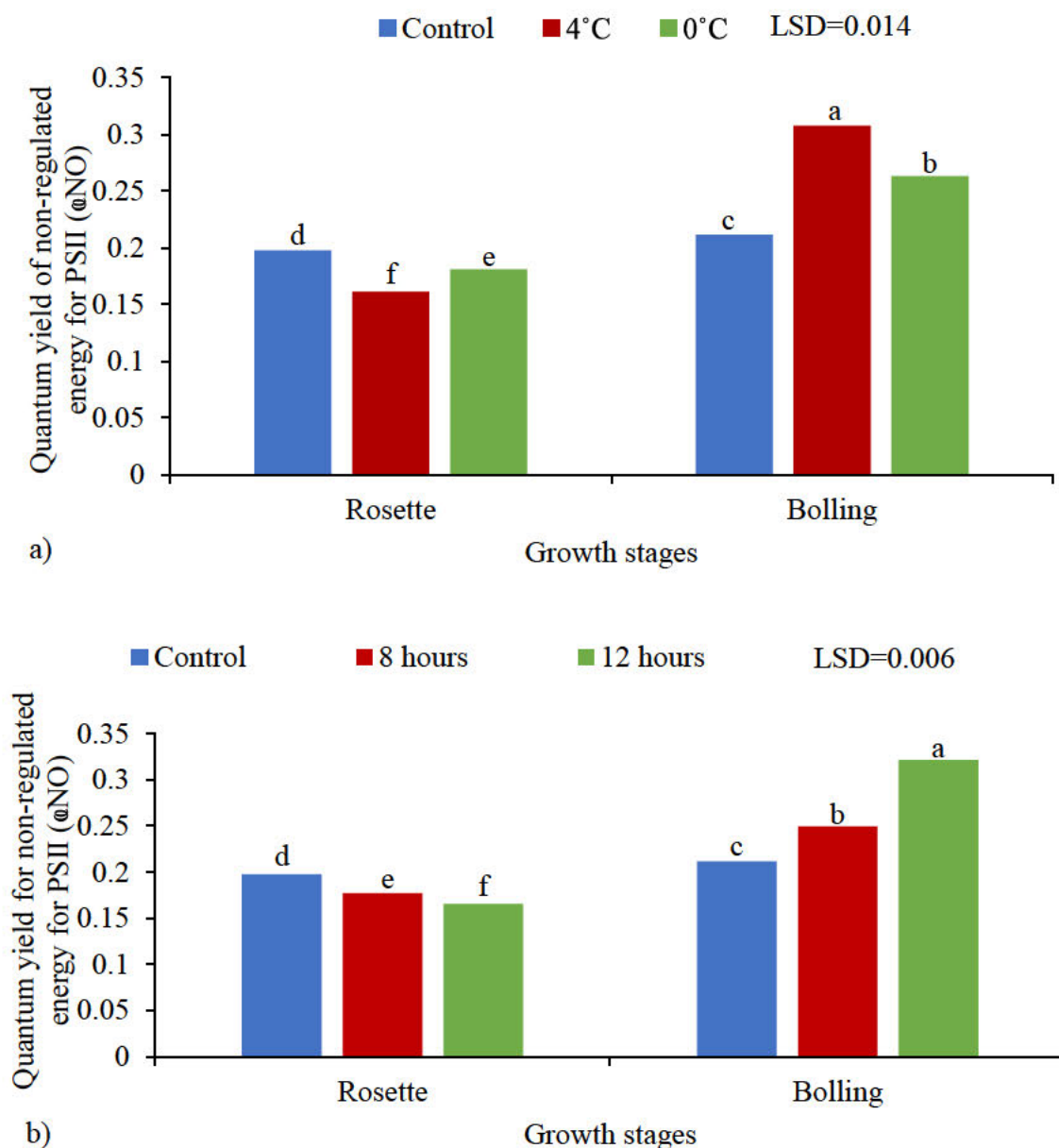


Figure 4.19. Influence of growth stage and temperature (a), and growth stage and duration of cold exposure (b) on quantum yield of non-regulated energy for PSII (Φ_{NO}) of safflower under artificial cold stress induction. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

Under the field trial, the Φ_{NO} of the safflower genotypes was notably ($P < 0.05$) influenced by the interaction of the phenological stage and time of the day (Figure 20, Appendix 6). Chilling temperatures of 2°C substantially ($P < 0.05$) increased the Φ_{NO} of safflower plants at the bolting stage (0.28) compared to plants at the rosette stage (0.2) (Figure 4.20). In general, plants at the bolting stage had significantly ($P < 0.05$) higher Φ_{NO} values than plants at the rosette stage under field conditions (Figure 4.20). The mid-day measurements were lower than the morning, with the Φ_{NO} value still higher at the bolting stage with 0.18 against 0.17 at the rosette stage. Notably, the mid-day Φ_{NO} at the rosette stage did not differ ($P > 0.05$) significantly from the control values observed at either growth stage (Figure 4.20).

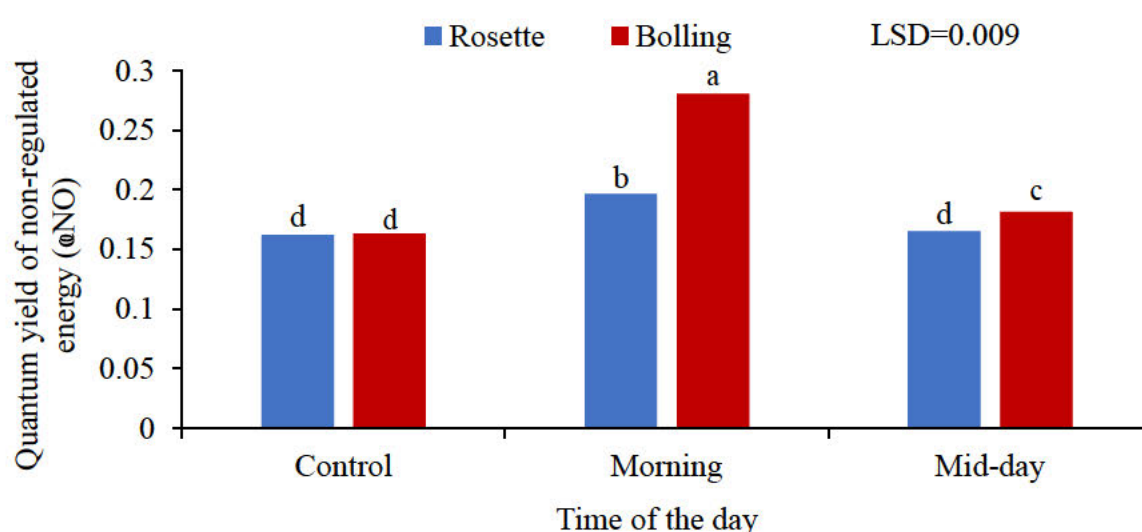


Figure 4.20. Influence of growth stage and time of the day on the quantum yield of non-regulated energy for PSII (Φ_{NO}) of safflower under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

The electron transport rate II (ETR II) was considerably ($P < 0.05$) influenced by main effects of growth stage and temperature under artificial cold stress conditions (Figure 21, Appendix 5). Safflower plants exposed to cold stress at the rosette stage had significantly higher ETR II value of 52.25 than plants at the bolting stage which had ETR II value of 36.17 (Figure 4.21a). Additionally, control plants exhibited an exceptionally elevated ETR II of 220.17, while plants subjected to cold stress at both 0 and 4°C showed relatively negligible values, approximately 0.22 (Figure 21b).

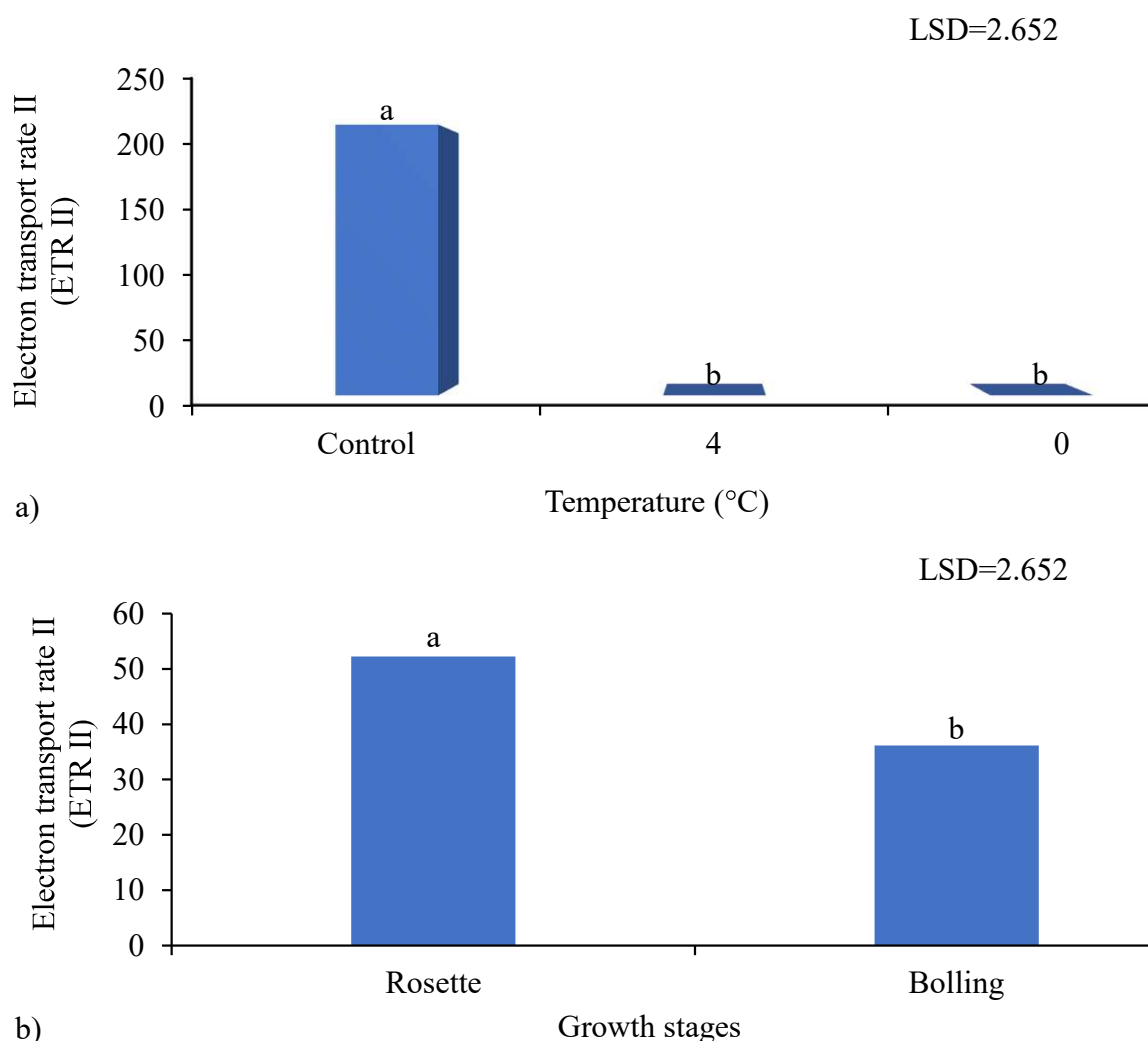


Figure 4.21. Influence of temperature (a) and growth stage (b) on electron transport rate II (ETR II) of safflower under artificial cold stress. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

The electron transport rate (ETR II) of naturally cold stressed plants substantially ($P < 0.01$) differed depending on the interaction of genotype x time of the day, and growth stage and time (Figure 22, Appendix 6). Prior to chilling stress, all the genotypes had relatively lower values, averaging approximately 0.08. Interestingly, the control plants displayed ETR II values comparable to those recorded in the morning. At mid-day, however, genotype-specific differences emerged: Sina recorded the highest ETR II value of 300.97, followed by Gila (272.02), PI 537636 (265.77), and Turkey (261.54) which were not significantly ($P > 0.01$) different from one another (Figure 4.22a). Furthermore, the interaction between growth stage and time of day revealed that plants at the rosette stage exhibited higher ($P < 0.05$) ETR II

(312.23) at mid-day. Overall, ETR II peaked at mid-day, with plants at the bolling stage also showing elevated levels amounting to 237.92 (Figure 4.22b).

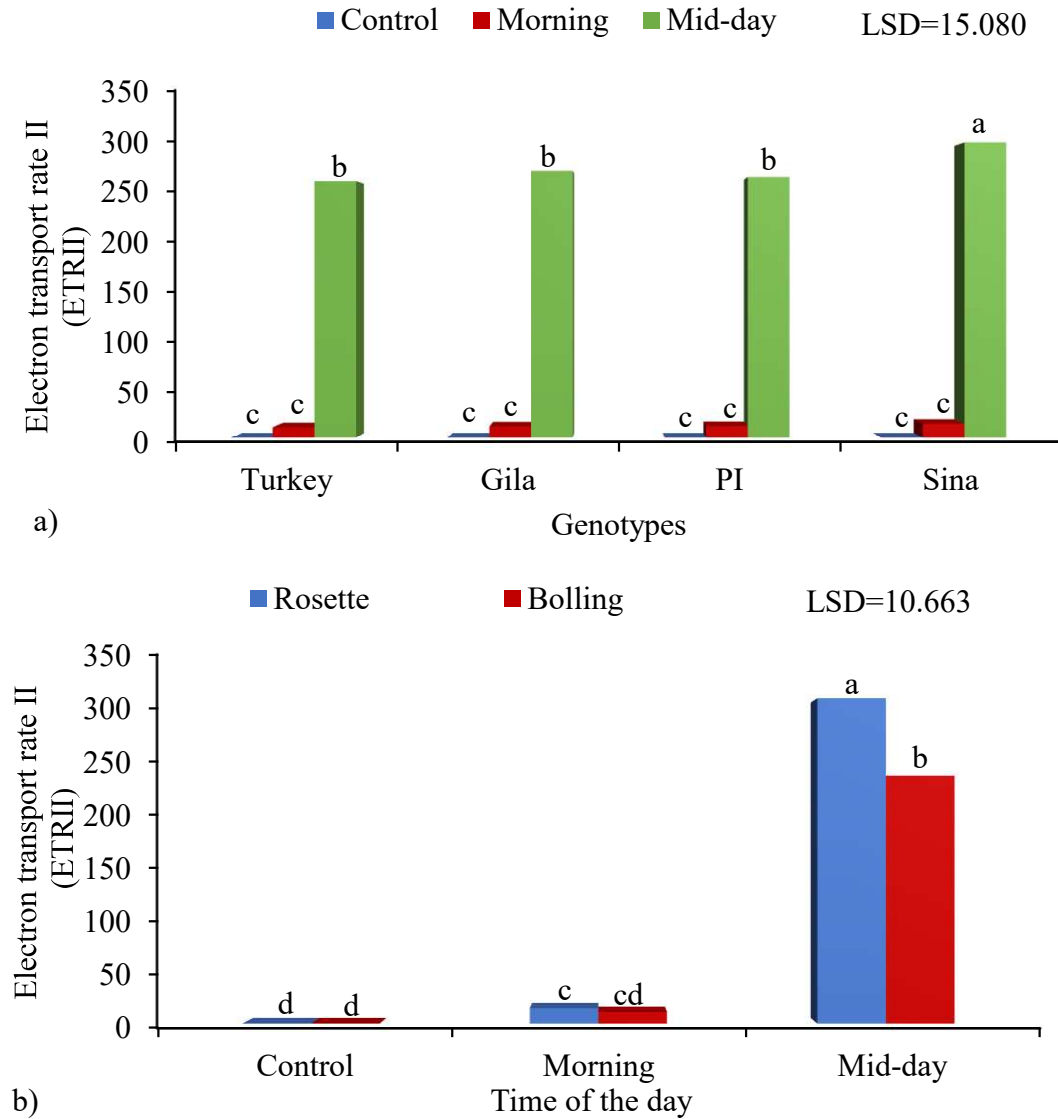


Figure 4.22. Influence of genotype and time of the day (a), and growth stage and time of the day (b) on ETR II of safflower under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.5 Stomatal conductance

The stomatal conductance (gs) of safflower plants under artificial cold stress induction was significantly ($P < 0.05$) influenced by the interaction of growth stage, temperature and duration of cold exposure (Figure 4.23, Appendix 3). Before imposition of cold stress, plants at the

rosette stage had higher g_s value of $630.31 \text{ mmol m}^{-2} \text{ s}^{-1}$ against $518.23 \text{ mmol m}^{-2} \text{ s}^{-1}$ at the bolting stage, respectively (Figure 4.23). Lowering the temperature to 4°C for 8 hours drastically reduced the g_s from 630.31 to 218.43 and from 518.23 to $131.76 \text{ mmol m}^{-2} \text{ s}^{-1}$ at the rosette and bolting stages, respectively. Increasing the duration of cold exposure to 12 hours at the same temperature further dropped the g_s to 199.07 and $17.87 \text{ mmol m}^{-2} \text{ s}^{-1}$ at the rosette and bolting stages, respectively. Further lowering the temperatures to 0°C for 8 hours did not significantly ($P > 0.05$) reduce the g_s of safflower at the rosette as compared to plants cold stressed at 4°C for 12 hours. While for the same chilling conditions (0°C and 8 hours) the g_s was remarkably reduced by 87.0 % at the bolting stage. Likewise, as in 4°C and 12 hours, prolongation of cold exposure to 12 hours severely reduced the safflower g_s to $9.20 \text{ mmol m}^{-2} \text{ s}^{-1}$ (Figure 4.23).

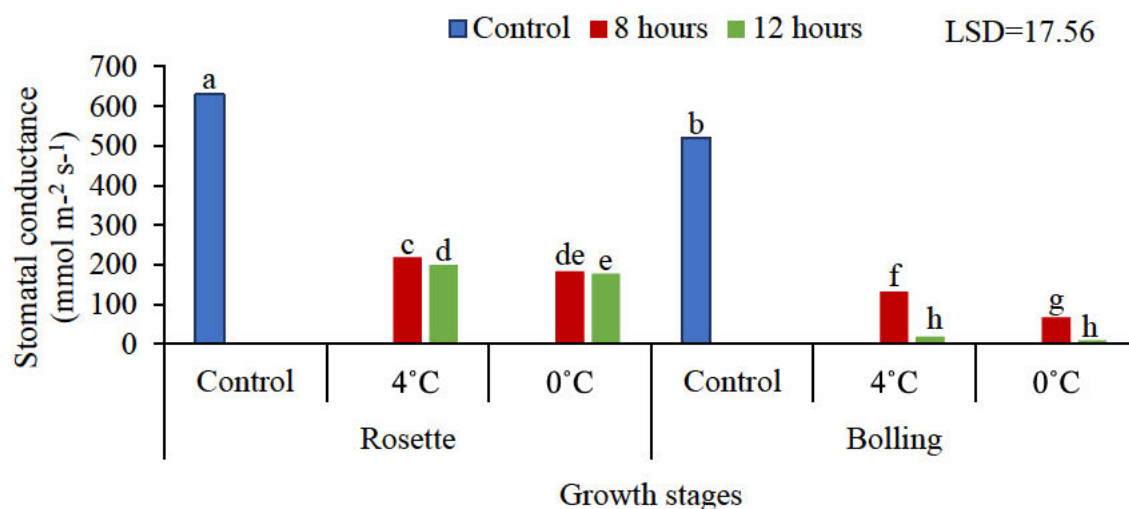


Figure 4.23. Influence of growth stage, temperature and duration of cold exposure on the stomatal conductance (g_s) of safflower under artificial cold stress experiment. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

Under field conditions, the g_s of safflower genotypes was statistically ($P < 0.05$) influenced by main effects of growth stage and time of the day of cold exposure (Figure 4.24, Appendix 4). Plants at the rosette stage had significantly ($P < 0.05$) higher g_s of $566.29 \text{ mmol m}^{-2} \text{ s}^{-1}$ than plants at the bolting stage which had g_s of $444.06 \text{ mmol m}^{-2} \text{ s}^{-1}$ (Figure 4.24a). Moreover, the genotypes had an average g_s of $691.52 \text{ mmol m}^{-2} \text{ s}^{-1}$ before stress induction. Cold stress of 2°C substantially reduced the genotypes g_s to an average of $311.35 \text{ mmol m}^{-2} \text{ s}^{-1}$ in the morning. However, in mid-day, the g_s of genotypes increased to an average of $512.67 \text{ mmol m}^{-2} \text{ s}^{-1}$ (Figure 4.24b).

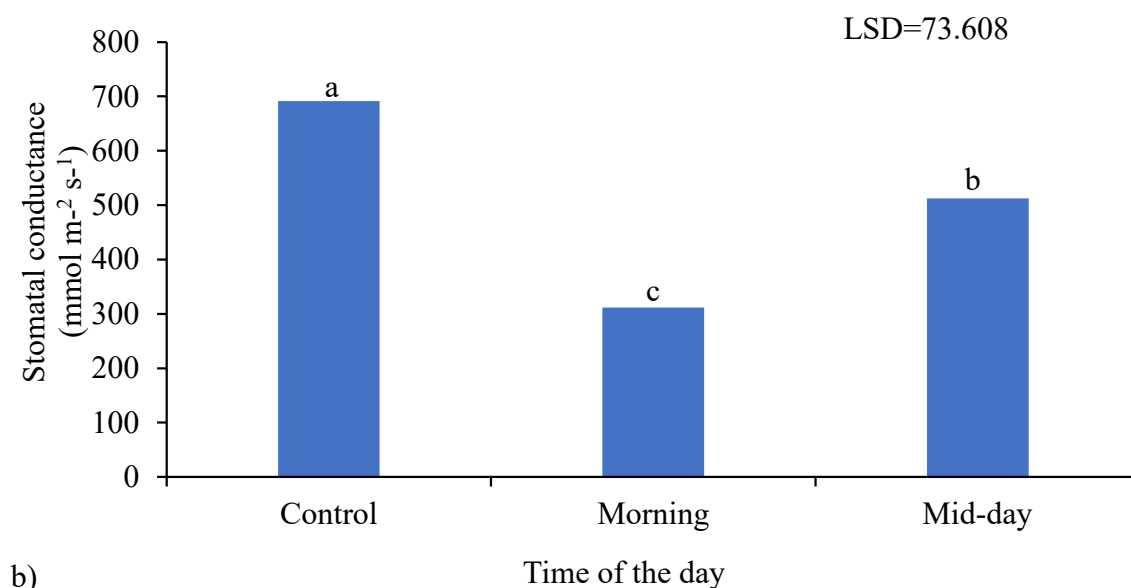
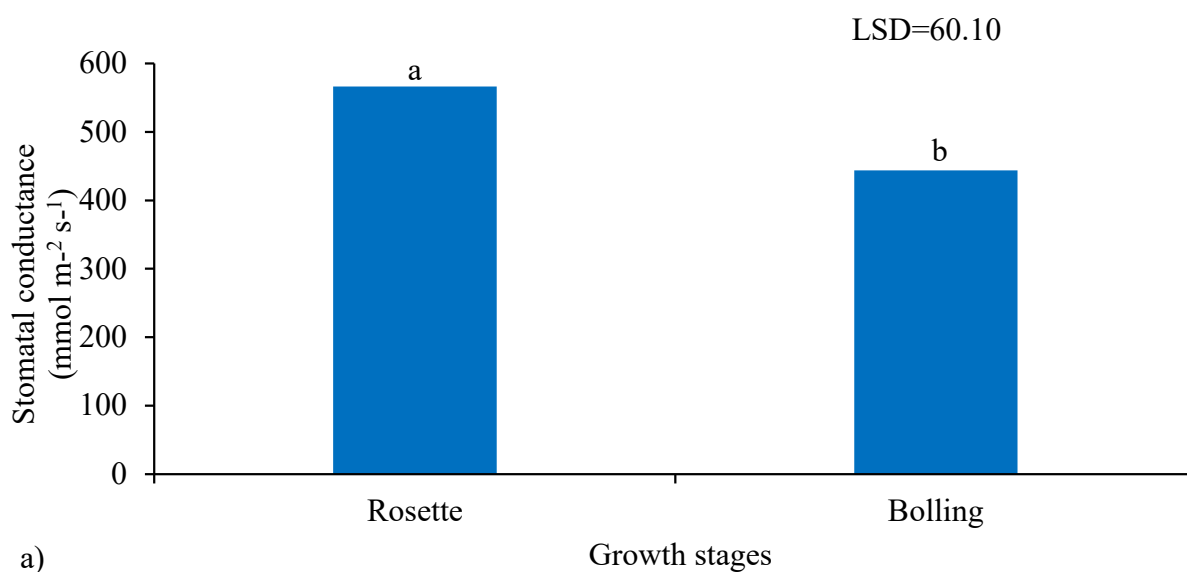


Figure 4.24. Influence of growth stage (a) and time of the day (b) on stomatal conductance (gs) of safflower cold stressed at 2°C under field condition. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.6 Antioxidants enzyme activity

4.2.6.1 Catalase

Catalase (CAT) activity highly ($P < 0.001$) varied with respect to the interaction of growth stage, temperature, and duration of cold exposure in safflower plants subjected to artificial cold stress induction (Figure 4.25, Appendix 7). At the rosette stage, the control plants exhibited

relatively low CAT activity ($1.09 \text{ U mL}^{-1} \text{ FW}$). Safflower plants exposed to chilling temperatures of 0 or 4°C for either 8 or 12 hours at either rosette or bolting developmental stages increased the activity of CAT compared to control plants except for plants exposed to 0 and 4°C for 12 hours at the bolting stage (Figure 4.25). Exposure of safflower plants to 4°C for 8 and 12 hours significantly ($P < 0.05$) increased CAT activity to $1.41 \text{ U mL}^{-1} \text{ FW}$ and $1.62 \text{ U mL}^{-1} \text{ FW}$, respectively at the rosette stage (Figure 4.25). The highest CAT activity of $1.80 \text{ U mL}^{-1} \text{ FW}$ was recorded in safflower plants exposed to 0°C for 12 hours in the rosette stage and it was significantly ($P < 0.05$) than any CAT activity of plants exposed to 0 and 4°C for either 8 or 12 in all phenological stages (Figure 4.25). In contrast, at the bolting stage, the control plants showed slightly higher CAT activity than those at the rosette stage, with a value of $1.16 \text{ U mL}^{-1} \text{ FW}$. When exposed to 4°C for 8 hours, CAT activity increased moderately to $1.27 \text{ U mL}^{-1} \text{ FW}$ but did not significantly change with prolonged exposure of 12 hours, which remained close to control levels ($1.14 \text{ U mL}^{-1} \text{ FW}$). Notably, under 0°C for 12 hours, CAT activity dropped sharply to $0.31 \text{ U mL}^{-1} \text{ FW}$, the lowest observed across all treatments (Figure 4.25). The CAT activity of safflower plants at the rosette stage exposed to chilling temperatures of 0 and 4°C for either 8 or 12 hours was significantly ($P < 0.05$) higher than that of plants at the bolting stage at the same temperatures and duration of exposure (Figure 4.25).

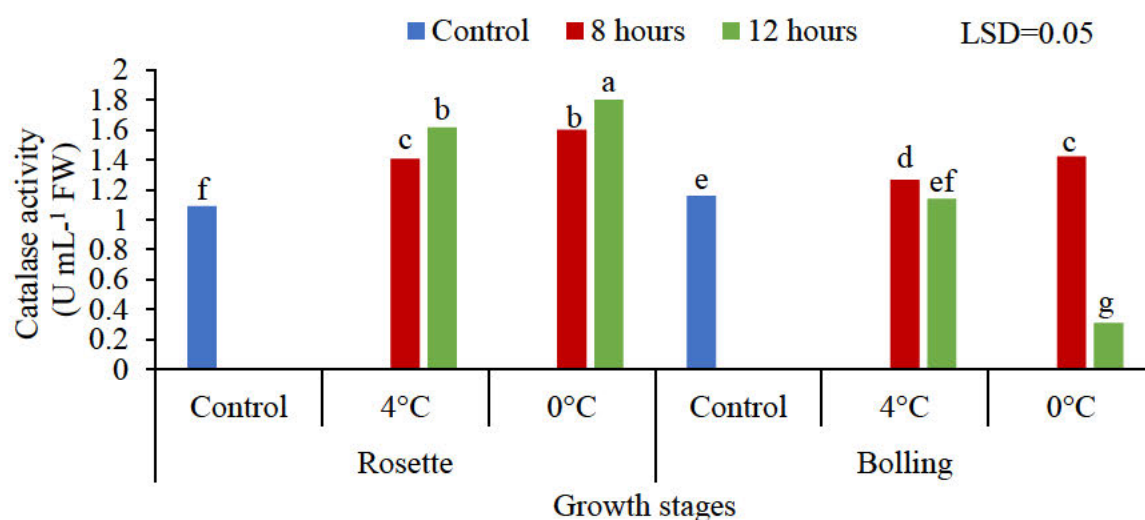


Figure 4.25. Influence of growth stage, temperature and duration of cold exposure on catalase (CAT) activity of safflower under artificial cold stress experiment. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

While on the other hand, safflower plants exposed to natural cold stress in the field at night exhibited significant ($P < 0.05$) differences in CAT activity based on the interaction of genotype and growth stage, and growth stage and time of the day (Figure 4.26, Appendix 8). The

genotype Gila recorded the highest CAT activity of $1.58 \text{ U mL}^{-1} \text{ FW}$ at the bolting stage than other genotypes at either rosette or bolting stage of development (Figure 4.26). The genotypes PI-537636, Sina and Turkey showed similar CAT activities each averaging $1.53 \text{ U mL}^{-1} \text{ FW}$ at the bolting stage but was significantly ($P < 0.05$) higher than CAT activity of all genotypes at the rosette stage except for genotype Sina (Figure 4.26). At the rosette stage, Sina ($1.52 \text{ U mL}^{-1} \text{ FW}$) maintained CAT activity closer to that of PI-537636 and Turkey recorded at the bolting stage. Meanwhile, the remaining genotypes consistently displayed lower CAT activity, averaging about $1.45 \text{ U mL}^{-1} \text{ FW}$ (Figure 4.26).

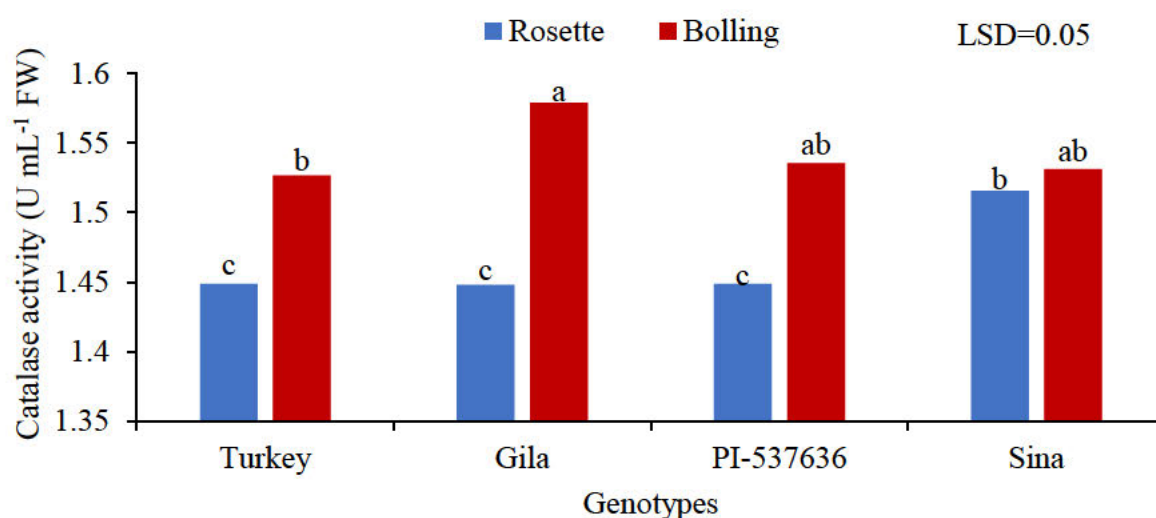


Figure 4.26. Influence of genotype and growth stage on catalase (CAT) activity of safflower cold stressed at 2°C under field condition. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

Moreover, CAT activity for safflower naturally cold stressed in the field was notably ($P < 0.001$) influenced by the interaction of growth stage and time of the day (Figure 4.27, Appendix 8). For both growth stages, cold stress increased the CAT activity. Initially before cold stress imposition, the CAT activity was about 1.27 and $1.46 \text{ U mL}^{-1} \text{ FW}$ at the rosette and bolting stage, respectively (Figure 4.27). During morning measurement, CAT activity increased to 1.66 and $1.57 \text{ U mL}^{-1} \text{ FW}$ at the rosette and bolting stages, respectively (Figure 4.27). However, by mid-day, CAT activity dropped to $1.47 \text{ U mL}^{-1} \text{ FW}$ at the rosette stage but CAT activity of plants at the bolting stage increased to $1.60 \text{ U mL}^{-1} \text{ FW}$ at mid-day (Figure 4.27).

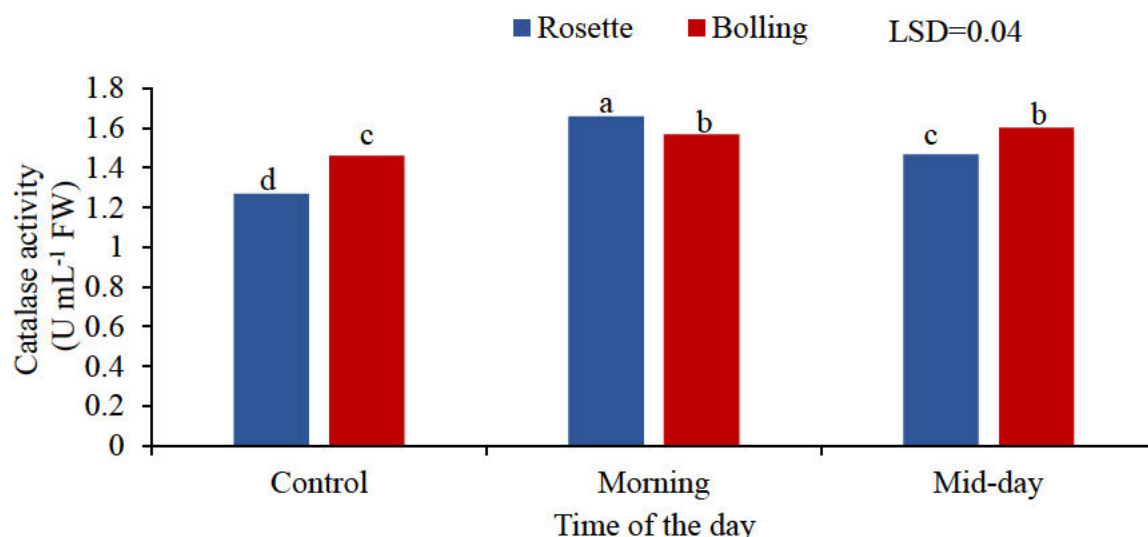


Figure 4.27. Influence of growth stage and time of the day on catalase (CAT) activity of safflower cold stressed at 2°C under field condition. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.6.2 Superoxide dismutase

Safflower plants highly ($P < 0.001$) differed in the activity of superoxide dismutase (SOD) depending on the interaction of growth stages, temperature, and durations of exposure to low temperature under artificial cold stress imposition (Figure 4.28, Appendix 7). Safflower plants cold stressed at 0 and 4°C for either 8 or 12 hours at the rosette stage had significantly ($P < 0.05$) higher SOD activity than plants at the bolling stage (Figure 4.28). The highest SOD activity of 4.45 U mL⁻¹ FW was observed on cold stressed plants at 0°C for 12 at the rosette stage (Figure 4.28). On the contrary, safflower plants at the bolling stage exposed to low temperature of 0°C for 12 hours had significantly ($P < 0.05$) the lowest SOD activity of 0.41 U mL⁻¹ FW (4.28). At the rosette stage, control plants had the lowest SOD activity of about 2.54 U mL⁻¹ FW. Cold exposure at 4°C for 8 and 12 hours significantly elevated SOD activity to 2.98 and 3.45 U mL⁻¹ FW, respectively. Further lowering the temperature to 0°C led to the highest SOD activity, that is, 4.00 U mL⁻¹ FW at 8 hours and 4.45 U mL⁻¹ FW at 12 hours. Meanwhile, at the bolling stage, the control plants had a slightly higher SOD activity of 2.68 U mL⁻¹ FW as compared to the rosette controls. Cold exposure at 4°C for 8 hours increased SOD activity to 3.06 U mL⁻¹ FW, but prolonged exposure of 12 hours resulted in a noticeable decline to 2.02 U mL⁻¹ FW. Under 0°C for 8 hours, SOD activity increased moderately to 3.32 U mL⁻¹ FW, but further exposure (12 hours) caused a dramatic decrease to 0.41 U mL⁻¹ FW (Figure 4.28).

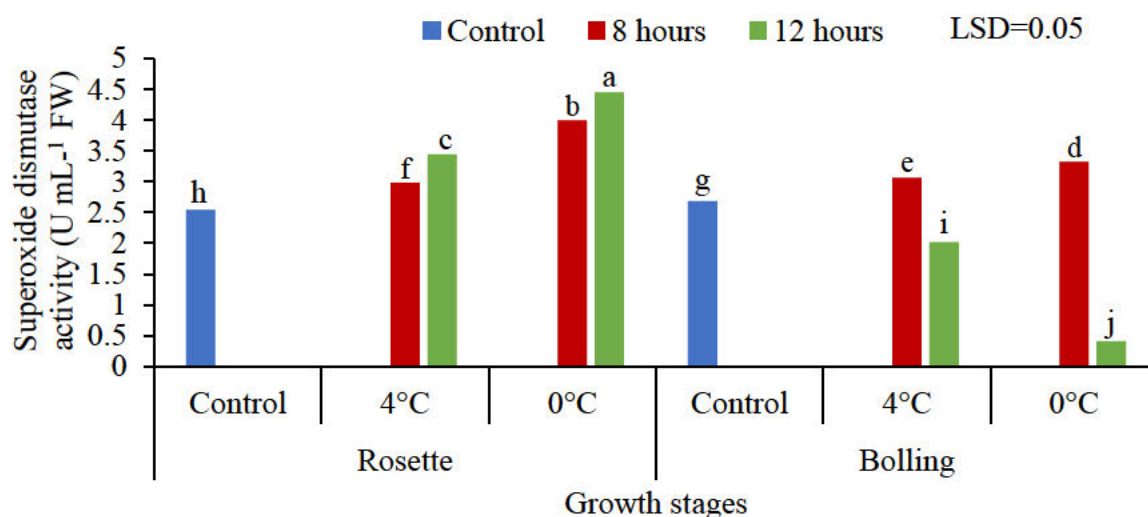


Figure 4.28. Influence of growth stage, temperature and duration of cold exposure on superoxide dismutase (SOD) activity of safflower under artificial cold stress experiment. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

Under field conditions, SOD activity was significantly ($P < 0.001$) influenced by the interaction of genotype and time of the day, and growth stage and time (Figure 4.29, Appendix 8). Prior to cold stress, all genotypes exhibited similar SOD activity ranging around $2.84\text{--}2.86 \text{ U mL}^{-1} \text{ FW}$. After cold stress, the genotype Sina at mid-day had notably ($P < 0.05$) high increase in SOD activity of $3.21 \text{ U mL}^{-1} \text{ FW}$ than any other genotype and time of the day (Figure 4.29). In the morning, the genotype Sina still had higher SOD activity of $3.14 \text{ U mL}^{-1} \text{ FW}$ than other genotypes and time of the day except of genotypes Gila, Turkey and PI-537636 at mid-day (Figure 4.29). Genotypes Turkey and PI-537636 showed relatively lower insignificant SOD activity of 3.07 and $3.08 \text{ U mL}^{-1} \text{ FW}$, respectively in the morning (Figure 4.29).

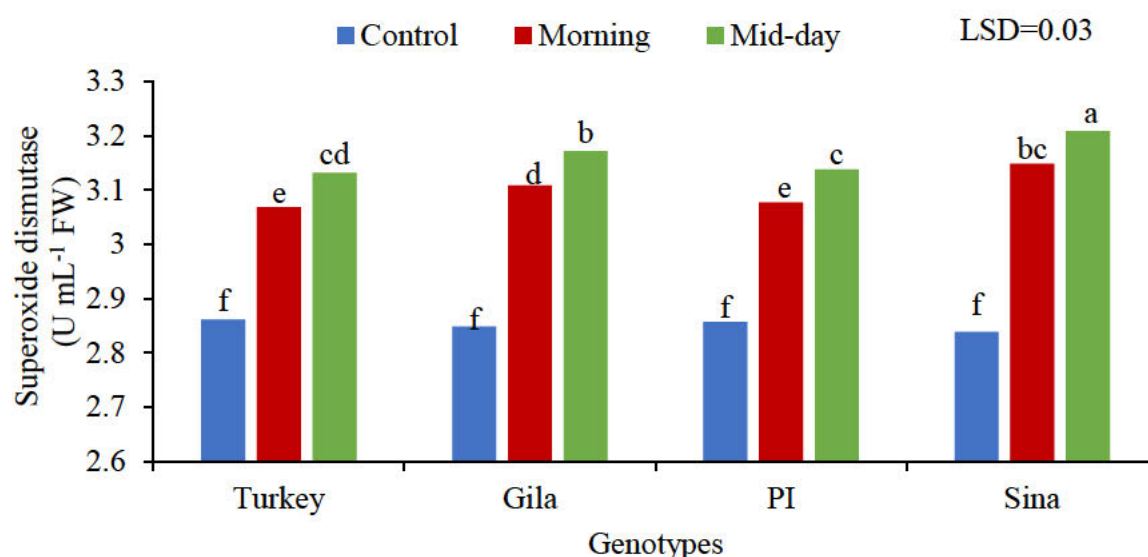


Figure 4.29. Influence of genotype and time of the day on superoxide dismutase (SOD) activity of safflower cold stressed at 2°C under field condition. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

In the interaction of growth stage and time, prior to cold stress imposition, SOD activity was about 2.75 and 2.95 U mL^{-1} FW at the rosette and bolting stages, respectively (Figure 4.30). In the morning, SOD activity increased to 3.16 and 3.04 U mL^{-1} FW for the rosette and bolting stages, respectively (Figure 4.30). By mid-day, the highest SOD activity of 3.19 U mL^{-1} FW was recorded at the rosette stage, while a slightly lower value of 3.14 U mL^{-1} FW was observed at the bolting stage (Figure 4.30).

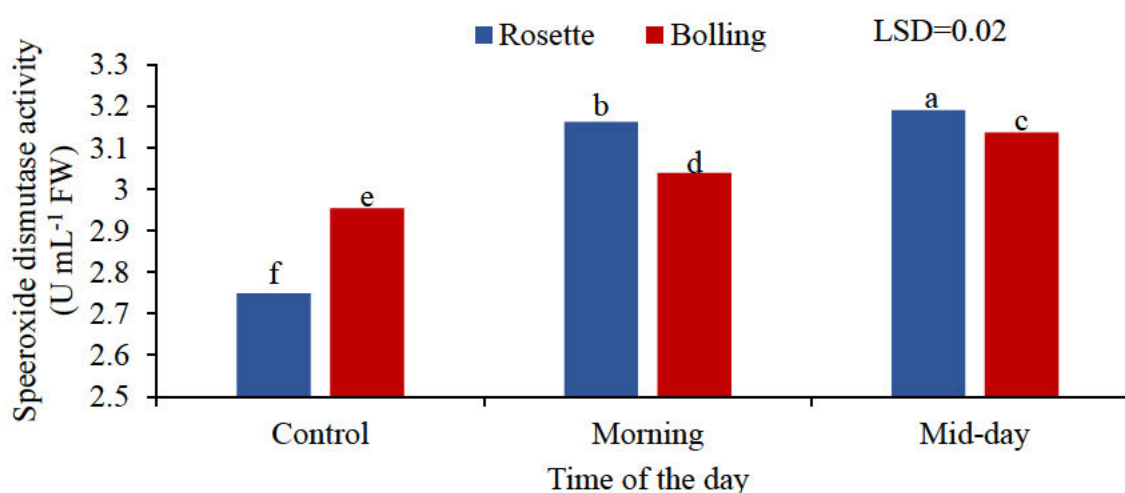


Figure 4.30. Influence of growth stage and time of the day on superoxide dismutase (SOD) activity of safflower cold stressed at 2°C under field condition. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.6.3 Peroxidase activity

Peroxidase (POD) activity was significantly ($P < 0.001$) influenced by the interaction of growth stage, temperature, and duration of cold exposure in safflower plants subjected to artificial cold stress (Figure 4.31, Appendix 7). At the rosette stage, the control plants exhibited relatively low POD activity of $0.81 \text{ U mL}^{-1} \text{ FW}$. Exposure of safflower to 0°C for 12 hours at the rosette stage ($2.29 \text{ U mL}^{-1} \text{ FW}$) significantly ($P < 0.05$) increased POD activity compared control plants and plants exposed to cold stress at different temperatures and exposure times (Figure 4.31). Plants at the bolting stage exposed to 0°C for 12 hours had significantly ($P < 0.05$) the lowest POD activity of $0.27 \text{ U mL}^{-1} \text{ FW}$ compared to any other treatment (Figure 4.31). Exposure of plants to 4°C for 8 and 12 hours increased POD activity to $0.97 \text{ U mL}^{-1} \text{ FW}$ and $1.37 \text{ U mL}^{-1} \text{ FW}$, respectively. While lowering of the temperature 0°C to remarkably increased POD activity to 1.70 and $2.29 \text{ U mL}^{-1} \text{ FW}$ at 0°C at 8 and 12 hours, respectively. In general, safflower plants exposed to 0 or 4°C for either 8 or 12 hours at the rosette stage had higher POD activity than plants under similar conditions but at the bolting stage (Figure 4.31).

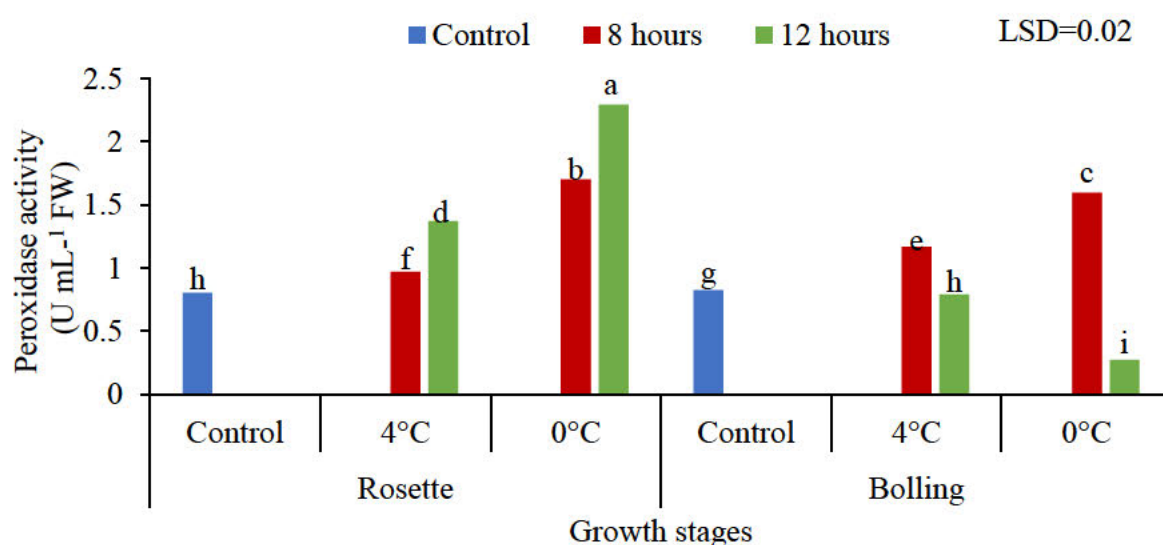


Figure 4.31. Influence of growth stage, temperature and duration of cold exposure on peroxidase activity of safflower under artificial cold stress experiment. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

As for safflower plants subjected to natural cold stress, POD activity was statistically ($P < 0.001$) influenced by the interaction of genotype and time of the day, and genotype (Figure 4.32, Appendix 8). Prior to cold stress, POD activity at the rosette and bolting stages averaged at 0.87 and $0.91 \text{ U mL}^{-1} \text{ FW}$, respectively (Figure 4.32a). In the morning, low POD activity of 0.87 and $0.82 \text{ U mL}^{-1} \text{ FW}$ in plants at the rosette and bolting stages, respectively was recorded

which were significantly ($P < 0.05$) different from each other (Figure 4.32a). As the air temperature increased, by mid-day the POD activity significantly ($P < 0.05$) increased to 0.97 and 0.94 U mL⁻¹ FW in plants at the rosette and bolting stages, respectively (Figure 4.32a).

Significant ($P < 0.01$) genotypic variation on POD activity was observed in the current study safflower plants chilled under field conditions (Figure 4.32b, Appendix 8). The genotype Sina exhibited significantly ($P < 0.05$) higher POD activity of 0.92 U mL⁻¹ FW than other genotypes (Figure 4.32b). The genotypes Gila, PI-537636 and Turkey did not remarkably vary in their POD activity (Figure 4.32b).

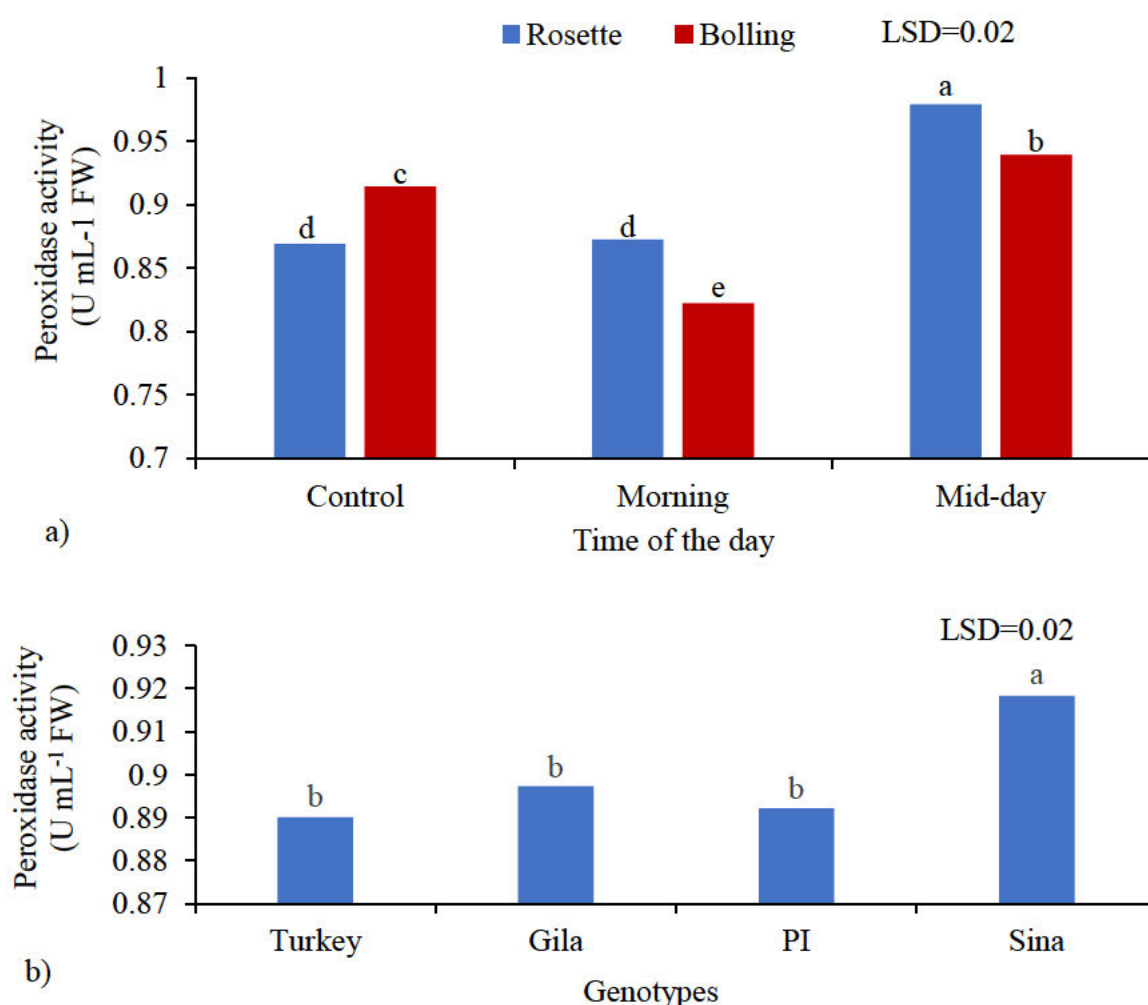


Figure 4.32. Influence of growth stage and time of the day (a), and genotype (b) on peroxidase (POD) activity of safflower cold stressed at 2°C under field conditions. Means with the same letters are not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.7 Expression pattern of cold stress related genes during chilling injury

4.2.7.1 Identification of homologous genes

The NCBI BLASTn analysis revealed that all the three safflower pseudo-marker snap genes showed similarities to genes of artichoke (*Cynara cardunculus* var. *scolymus*) organism (Table 4.2). The first gene, CtAH10T10001700.1, showed 84% similarity to an E3 ubiquitin-protein ligase CIP8-like protein, which is involved in protein ubiquitination and degradation. This process is crucial for regulating protein turnover and modulating cellular responses during cold stress. The second gene, CtAH10T10001500.1 was 94% identical to a RING finger and transmembrane domain-containing protein 2-like gene. This gene also functions in protein ubiquitination and degradation. Both genes play vital roles in maintaining cellular homeostasis under cold conditions, as ubiquitination marks damaged or misfolded proteins for degradation, thereby protecting cells from cold-induced stress. The third gene, CtAH10T10000500.1, showed 82% similarities to the protein Scarecrow, which is also associated with both biotic and abiotic stress resistance (Table 4.2).

Table 4.2. Description of homologs used in identify safflower cold responsive genes and how they confer cold tolerance

Safflower gene	Gene ID	Organism name	Gene description	Biological process	Percentage similarity (%)	Citation
CtAH10T10001700.1	112512063	<i>Cynara cardunculus</i> var. <i>scolymus</i> (Artichoke/globe artichoke)	E3 ubiquitin-protein ligase CIP8-like	- Protein ubiquitination and degradation -Cold stress resistance	84	Wang et al. (2022)
CtAH10T10001500.1	112512836	<i>Cynara cardunculus</i> var. <i>scolymus</i> (Artichoke/globe artichoke)	RING finger and transmembrane domain-containing protein 2-like	- Protein ubiquitination and degradation -Cold stress resistance	94	Han et al. (2022)
CtAH10T10000500.1	112513693	<i>Cynara cardunculus</i> var. <i>scolymus</i> (Artichoke/globe artichoke)	Protein Scarecrow	Biotic and abiotic stress resistance	82	Lu et al. (2020)

4.2.7.2 Sequence analyses of safflower and *Cynara cardunculus* var. *scolymus* pairwise alignment

The clustal omega pairwise sequence alignment results demonstrated regions of stronger and weaker similarities (Figure 4.33, 4.34, 4.35).

CtAH10T0001700.1	TATGGACATGGATGGCACCATAGCCGTTGAACCTTGGGACCAGCCACGGAGGTGTGTGCT	329
E3 Ubiquitin	TATGGACATGAATGGCACCATAACTGTGCAACCTTGGGATCAGCCACGGAGGTGTGTGCT	195

CtAH10T0001700.1	GTGCCATAGAATCATTTTCATCTGAGATCGAGTCCGATGGAGAGTTGAAGCTACGAGTAT	389
E3 Ubiquiti	GTGTCATAGAATCCTTTTCATCTGAGATCGA-----GGTTGAAGCTACAAGTAT	243

CtAH10T0001700.1	ATGTGGAGATTGCAAGTTTCTGTTTCTGGAAGATAACCATTCAGCATACCACAAGACA	449
E3 Ubiquitin	ATGTGGGAGTTGAAGTTTCTGTTTCTGGAAGATAGTGACATTCAGCATAACACAAGACA	303

CtAH10T0001700.1	AACAAGGTACAACAACCCGTTGCAATCTTTGGAATAATACAATTCGAGA-----	503
E3 Ubiquitin	AACAAGGTACAACAACA--ATTGCAATCTATTGAAAATATGCAATTCAGATATGAC	360

CtAH10T0001700.1	-----GTCTGAACACGGGAATCAATCAAGACGGTGGAGGAGAGTTTATC	548
E3 Ubiquitin	GAATCTGGTTACGGTCTCTGAGCATGGGAATCAATCAAGACGGTGGAGGAGAGTTTGTG	420

CtAH10T0001700.1	CGATACAGAAAGTGATGGGTTTGATTGATGTTCCGTTCAAGTTGATA-----	598
E3 Ubiquitin	TGATACAGAAAGTGATGGCTTTGATTGATGTTTCTGTTGAAGTTGATAGTGATGACC	480

CtAH10T0001700.1	-----CTCATCCAGACGATGCAAGTCTGAACCAAGTCAAATTGAGACAATGACGATGA	650
E3 Ubiquitin	TGCTCATCTCATTGATGATGCAAGTCTGAACCAAGTCAAATTGAGACAATGACGATGA	540

CtAH10T0001700.1	ATTGGAAGAAGTTGAAAACACAGTTGGAAGTTTGGTGGGAGAGTTGAGTGCATAGATC	710
E3 Ubiquitin	ATTGGAAGAAGTTGAAAACACAGTCGGAAGTTTGGTGGGAGAGTACAGCTACGATAGATC	600

CtAH10T0001700.1	GTTGGCAACTAATGGACGGAATCTCCAGCTGATTGGCTTAACGAGATTCTTCTCTGA	770
E3 Ubiquitin	GTTGGCATCCAATGGGCGGAATCTCCAAGTCGATTGGCTTAATGAGATTCTTCTCTGA	660

CtAH10T0001700.1	AGCTGAAAACCTAGAAAAGATCACATTATGTAGGGAACGCGGAGACTATCTTGATGCAAG	830
E3 Ubiquitin	AGCTGAAAACCTAGAAAAGATCACGTTATGTAGGGAATGCTGGAGACTATCTTGATGCAAG	720

CtAH10T0001700.1	TGGGTTTGAACAACCTGTTGGAGCGCTAGCGGAGACTGACGGTTACGGCTGGGAGCACC	890
E3 Ubiquitin	AGGGTTTGAACAACCTGCTGGAGCGTCTAGCGGAGAGCGGATGGTTACGGCGAGGAGCACC	780

CtAH10T0001700.1	GCCTGCCGCTGCTTCGTTTGTGAAGAGTTTGGAGCGGTTGGTGGCGGGGAGGATGATGG	950
E3 Ubiquitin	GCCTGCCGCTGTTTCGTTTGTGAAGAAATTTGGAGCGGTTGGTGGTGGATGAGGATGAG--	838

CtAH10T0001700.1	TGATTTGGTGTGTGTGATTGTGAAGGACAGTGTTGTGTTGGTAGTGTAGTGAACCGGCT	1010
E3 Ubiquitin	-GGTTTGGTGTGTGTGATATGAAGGATAGTGCTGTGTTGGTAGTGTAGTGAACCGGCT	897

CtAH10T0001700.1	TCCGTGTCTTCATGTGTACCATCCGTCATGCATCATGGCATGGTTAAATGCACGGAATAC	1070
E3 Ubiquitin	TCCTGTCTTCATGTGTACCATCCTTCATGCATCATGCCATGGTTAAATGCACGGAATAC	957

CtAH10T0001700.1	GTGTCGCTTTGTGTCGATACGAACTCCCAACAGATGACAAGGAGTGTGAGAGGAGGAGGA-	1129
E3 Ubiquitin	ATGTCGCTTTGTGTCGATACGAGCTCCCAACAGATGAAAGGGAGTGTGAGAGGAGGATGAG	1017

CtAH10T0001700.1	--GGAGAAGCGAAAGGC-----AATATGCATCATC	1157
E3 Ubiquitin	GAGGAGAAGCAGAAAGCCAGAGTGGAATGGAAGAGGAGTGGGTGAGGATGCATCATC	1077

CtAH10T0001700.1	GGGTGGCGGAGGAAGGTGGTGGGTTGTGGCGGTACCGCTAGCAAGCATGGTGGCTATTGG	1217
E3 Ubiquitin	AGGTGGCGGAGGAAGGTGGTGGGTTGTGGCGGCACCGTTAGCGACCATGATGGCTATTGG	1137

CtAH10T0001700.1	TGTTGTGTTGTGTTAGGGGAGGAGTTGATAGAAGCAGGAGATG--GTGTGCTTGTA	1274
E3 Ubiquitin	TTTTGTGGTGTGTTGGGGGAGGAGTCAACAGAAACAGGAGATGGTGGTGTGCTTGTA	1197

Figure 4.33. Pairwise sequence alignment of CtAH10T0001700.1 and E3 Ubiquitin ligase showing regions of similarities. ***** indicating stronger identity, * ** * indicating weaker similarities and ----- showing gaps.

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CtAH10T0001500.1    AAACAGGGTCGATCAGGCTACATCGTGGTTTGAGCAGGTCAATCCATTCGTTTTGCTACT 660
RING finger          AAACGGGGTTGATCAGGCTACCACGTGGATTGAGCAGGTTCTTCCATTCATTTTGCTACT 80
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    GTTGGCCGTCTTCATTGCGCAACATTTGCAAGGTATTCTTGTCACGATCCTGATTGCTGC 720
RING finger          GTTGGCCGTCTTCATTGCGCAACATCTGCAAGGTCTTCTTGTCACGATCTCGATTGCTGC 140
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    ATTCATGTTCAAATCAAATGACATTCTAAGGAAACAGACAGCTCTTAAGGGAGATAGGAA 780
RING finger          TTTCTTGTTCAAATCAAATGACATTCTAAGGAAACAGACAGCTCTTAAGGGAGATAGGAA 200
                      ***  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    GATATCAGTTTTAACGAGTGTCTCTCTTCTTCACACTTTATGTGGCTGGGGTTTATTG 840
RING finger          GATATCAGTTTTAACGAATGTTCTCTCTTCTTCACCTTTATGTGGCTGGGGTTTATTG 260
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    GTGGTATCGCAAGGATGGTCTCCTTTATCCATTGCTCATGCTCCCTCCGAAAAATATACC 900
RING finger          GTGGTATCGCAAGGATGATCTCCTTTATCCATTGCTCATGCTCCCTCCGAAAAATATACC 320
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    ACCTTTTGGCATGCAATTTTATAATAGTGGTGAATGATATTTGGTGAGGCAAGCAGC 960
RING finger          GCCTTTTGGCATGCAATTTTATAATAGTGGTGAATGATATTTGGTGAGGCAAGCAGC 380
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    CATGGTGGTAAAGTGTTTGCTCTTGATGTATTACAAGAATAGCAAAGGACTAAATTACAG 1020
RING finger          CATGGTGGTAAAGTGTTTGCTCTTGATGTATTACAAGAATAGCAAAGGACTAAATTACCG 440
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    AAAGCAGGGTCAAATGCTAAGATTGGTGGAGTATTATTACTGCTGTATCGTGCATTGCT 1080
RING finger          AAAGCAGGGTCAAATGCTAAGATTGGTGGAGTATTATTACTGCTATACCGTGCCTTGCT 500
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    TCCTGTACCTGTATGGTATCGGTTCTTTTGAACGAAGAATATGGCAGCCTGTTTTCTGC 1140
RING finger          GCCTGTTCTGTGTGGTATCGGTTCTTTTGAACGAAGAGTATGGCAGCCTATTTTCTGC 560
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

CtAH10T0001500.1    ACTAATAACGGGATTGTATCTCACATTCAAACACCTCTTTGGTGGATGAAGGTGGTGA 1200
RING finger          ACTAATAACTGGATTGTATCTGACATTCAAACACCTCTTTAGTTGAAAAG-GT----- 614
                      ****  ****  ****  ****  ****  ****  ****  ****  ****  ****

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Figure 4.34. Pairwise sequence alignment of CtAH10T0001500.1 and *Cynara cardunculus* var. *scolymus* RING finger and transmembrane domain-containing protein 2-like showing regions of similarities. **** indicating stronger identity, * ** * indicating weaker similarities and ----- showing gaps.

CtAH10T000500.1 SCARECROW	TCAGGAACCATAAACGTATTGGTGTCCGGTACCGTGACATCATAAAGAGAATGCTGCAACC TCAGGAACCATAAAGTATTGGTGTCCGAGCCGGTGACATCGTACAAAGAATGCTGCAACC *****	1021 1787
CtAH10T000500.1 SCARECROW	AGTGGACGGCAACGGCTCTCTTTTGTGACCTTCAACCTCTCCGGATTGAGATTCCCGA AGTGGACAGCAACAGCTTCTCTTTTGTAACTTCAATCTCTCCGGATTCAAATTCCCGA *****	1081 1727
CtAH10T000500.1 SCARECROW	CTTATCCGCCACAGCCGAGAACTCAAACGGGAGTCCAGTTTGTCTGCGAAATCCGACA CCTTATCAGCCACAGCCGAGAACTCAAACGGGAGTCCAGTTTATCTGCAAAATCAGACA * *****	1141 1667
CtAH10T000500.1 SCARECROW	ACCGTTTTCCGGTGGCTTCCAGGGCGTCCAACGAGTTCCCAATCCGGTGAGTCGGACGA ACCGTTTTCCGGTGGCTTCAAGGTATCCAACGAGTTGCCAATCCGGTGAGTCGTACAA *****	1201 1607
CtAH10T000500.1 SCARECROW	AAGGGGGTCCGCCTGGTCTGGATGCCAATATGTGAAAAGCCCCGGCCATTGCAACCCCT AAGCGGACCCGCTGGTCTGGATGCCAATATGTGAAAAGCCAGGCATTGCAAGGCTT **** *	1261 1547
CtAH10T000500.1 SCARECROW	GCATGATGTCCAGATCGATTATGTGACCCGGTCTTCCCTCTCGAAGGCTTCTGATCG GCATGATGTCCAGGTCGATGATGACCCCTATCTCTCTCTCGAATGCCTCTGTATCG *****	1321 1487
CtAH10T000500.1 SCARECROW	CCTGATTGGCGTGAAATGCGAGAACTTGACGAAAGGGCTGATGCCGTTGAACACTTGGA CTTGATTGGCTGTGAAATGTGAGAATTAACGAAAGGGCTGATGCCGTTGAACACTTGGA * *****	1381 1427
CtAH10T000500.1 SCARECROW	AGGCCGACGTCATCTTCTTGAGGGCTTGCGGAAGCGCTGC - - - -GGAAGGGCGGTGT AGGCTGACGTCATCTTCTTCAGCCCTTGCGGAACCGCTGCATTGGGAGGAAGGGTGTGT **** *	1435 1367
CtAH10T000500.1 SCARECROW	ATATCCCGAGGACGAGCTCATGAGCCGAGCCGACATGGCTTCCGAGAAGTAGGCTGCAA ATATCCCAAGACAAGAGCTCATGAGCCGTGCGGACATGGCTCTGAGAAATAGGCCGCAA *****	1495 1307
CtAH10T000500.1 SCARECROW	CTCGCTGAGCTGAAGTTCGTAAGGAGTTGAGAGCTCCGAGATCTCGAGTAGCATCTTGT CCCGCTGAGCAGAAGTCCCATATGGAGTTGAGAGTTCGGAGATCTCTAGTAGCATCTTGT * *****	1555 1247
CtAH10T000500.1 SCARECROW	TGGCCTCTTCAAAGTTATCGGCTGAAACCGCCTCCGCACATTGCAATAGCAAGGCCAGCA TGGCCTCTCAAAGTTGTGAGCTGAAACTGCCTCCGCACATTGCAAGGAGCAAAGCAAGCA *****	1615 1187
CtAH10T000500.1 SCARECROW	GGTGAGACCTTGTTCTGCTCTCGTTCTGCTGCTGATCTCCTCCCTTTTTTCTCTCTTA GATGTAGGCCTTGTTCTGCTCTTTTCTGCTGCCGTATCTTCTCTCTTCTCTCTTA * * * *	1675 1127
CtAH10T000500.1 SCARECROW	GCAACATAGCTGCTGCTTGTC - - - -C-G-TG-T-TA---TGGTGCGTTGCTGGTGGTG ACAACATTGCTGCTGCTTGCTGATGCTGCTGCTGTTGTTGGTGGTGGTGGTGGTGGTG *****	1723 1067
CtAH10T000500.1 SCARECROW	GTGGTGGTGGTGGAGACTCTT-C-G-TGGT--TG-TT-T-G-ATGAAGAAATA-G-G-A GTTGTGATGGTGGAGACTCTGAGCTGTTCTGGTTGTTGGATGAAGAAATAAGTGCAAG * * * *	1771 1007
CtAH10T000500.1 SCARECROW	GAGGAGGAGGTCCGGTGGTAGGGTTGTAATCGTCATTGACGAAGTGGTCATCCTGCTTTC CAGGAGGAGTCCAGTACTAGGGTTGTAATCGTCATTGAGGAAGTGGTCGTCCT---TTC *****	1831 950

Figure 4.35. Pairwise sequence alignment of CtAH10T000500.1 and *Cynara cardunculus* var. *scolymus* SCARECROW showing regions of similarities. ***** indicating stronger identity, * ** * indicating weaker similarities and ----- showing gaps.

4.2.7.3 Structural analyses of Conserved domain of *Cynara cardunculus* var. *Scolymus*

The RPS blast and CDD analyses revealed that E3 ubiquitin protein ligase CIP8-like and RING finger and transmembrane containing protein 2-like genes encode RING proteins domain and belong to RING_U Box super family. The E3 ubiquitin protein ligase CIP8-like are identified by the cross-brace motif (RING-H2) while the RING finger and transmembrane containing protein 2-like are characterized by a unique cross-brace motif RING-HC_RNFT1-like with zinc binding site (Figure 4.36a, b). SCARECROW, a member of the GRAS family, is characterized by a lengthy domain (Figure 4.36c).

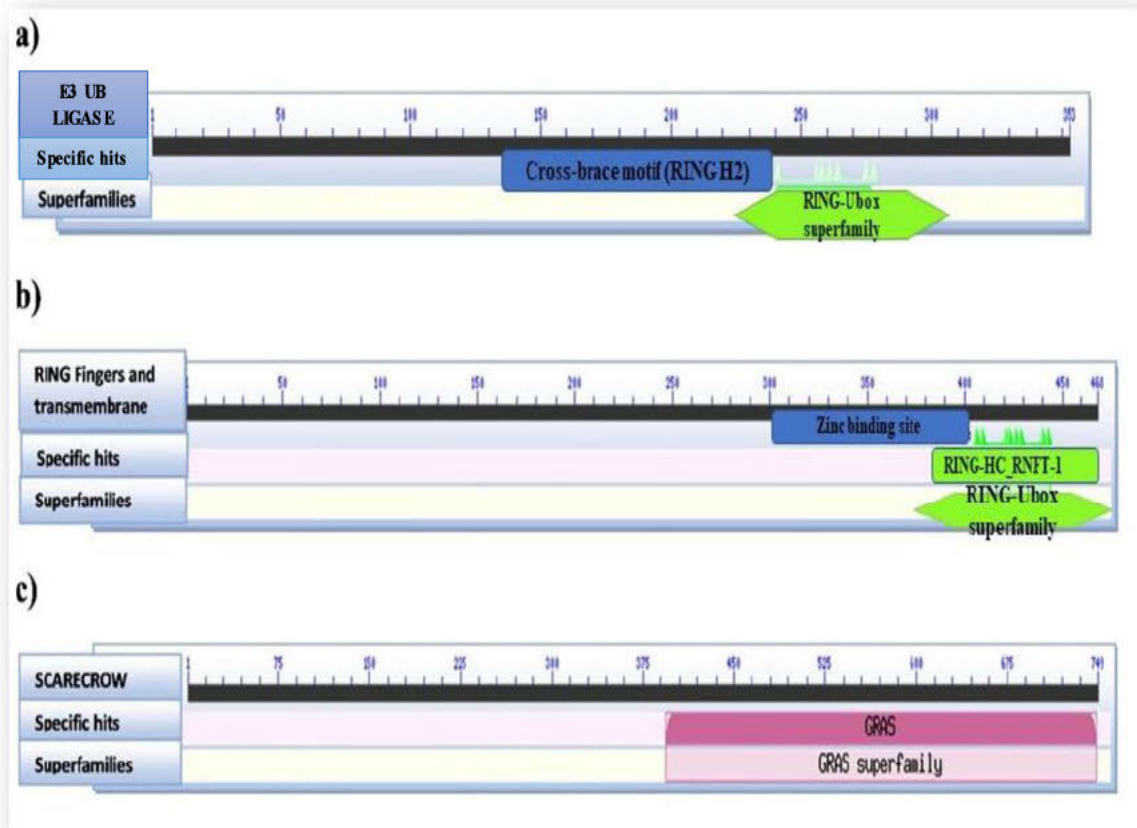


Figure 4.36. Simplified graphical representation of the conserved domain architecture showing specific hit and superfamilies of cold responsive *Cynara cardunculus* var. *scolymus* genes.

4.2.7.4 Relative gene expression

The expression pattern of all three safflower genes showed variability depending on either main treatment effects or interactions of genotype and growth stage, and genotype and duration of exposure (Figure 4.37, Appendix 9). Upregulation of CtAH0001700.1 gene was highly ($P < 0.01$) influenced by growth stage and temperature (Figure 4.37a and b, Appendix 9). Higher expression level was observed at bolting stage as compared to rosette and notably increased with decreasing temperature (Figure 4.37a and b). Control plants (at 23°C) had the least gene expression. Exposing the plants to cold stress of 4°C tripled (from 1 to 3 FC) the gene expression, while lower temperature of 0°C resulted in 2.5- fold increase in gene expression against the control (Figure 4.37b).

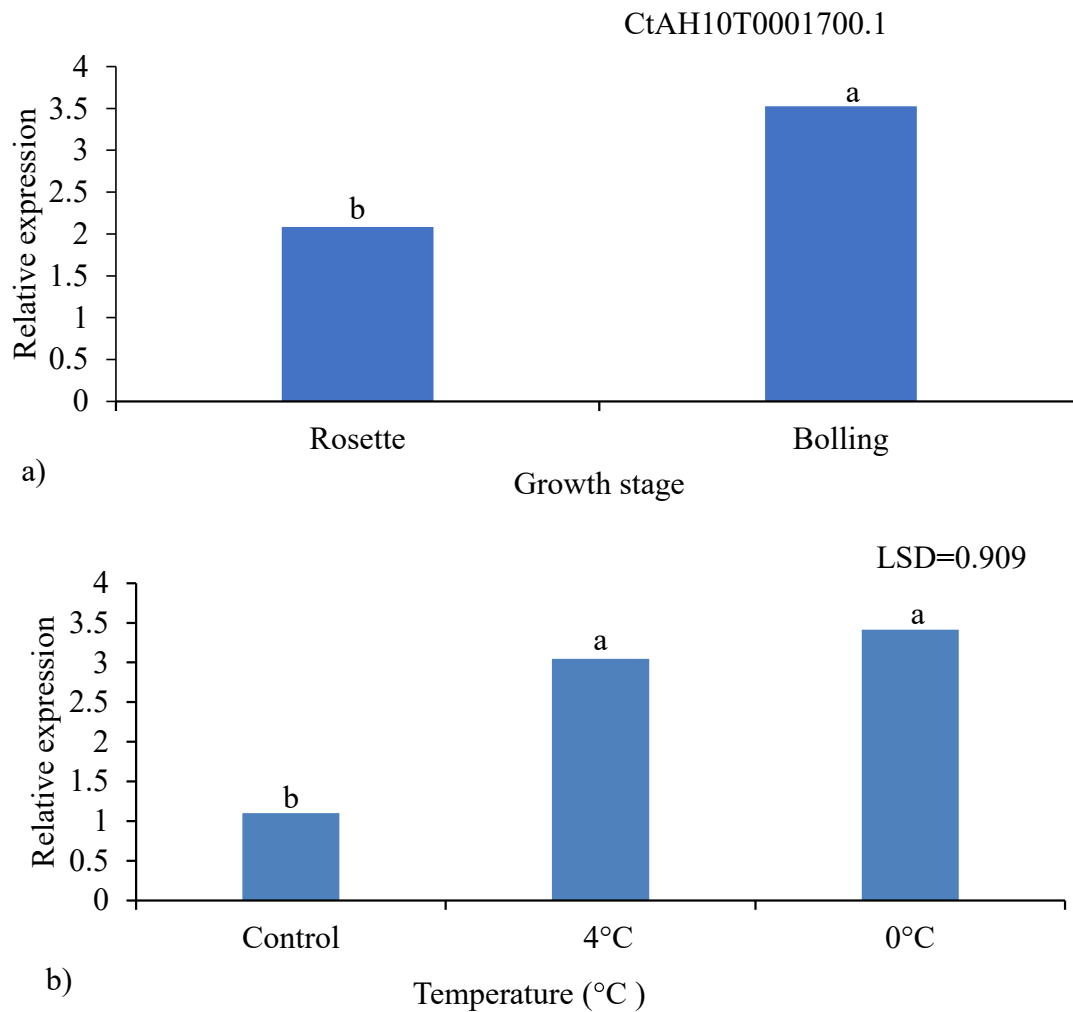


Figure 4.37. Relative expression level of CtAH10T0001700.1 safflower gene under artificial cold stress treatment. The relative expression level results were presented as (a) influenced by safflower growth stages, and (b) temperature. Means with the same letters were not significantly different. Means separated using the LSD at $P \leq 0.05$.

Similarly, CtAH10T0001500.1 was notably ($P < 0.1$) expressed at the bolling stage (Figure 4.38, Appendix 9). However, unlike CtAH0001700.1, the gene was highly expressed in plants cold stressed at 4°C for 8 hours. For both growth stages, plants in warmer temperatures (23°C) exhibited similar gene expression (1.3 FC bolling against 1.1 FC rosette) (Figure 4.38). Exposure of plants to lower temperature of 4°C for 8 hours significantly ($P < 0.05$) reduced gene expression by 46.2 % in bolling stage, while it doubled (from 1.1 to 2.2 FC) at the rosette

stage (Figure 4.38). Prolongation of cold stress to 12 hours resulted in insignificant decrease (from 1.3 to 0.97 FC) in bolting stage and almost similar gene expression in rosette stage (1.11 against 1.27 FC) relative to the controls. Meanwhile, cold exposure of 0°C did not significantly ($P > 0.05$) influence CtAH10T0001500.1 expression in both growth stages at 8 and 12 hrs of cold exposure (Figure 4.38).

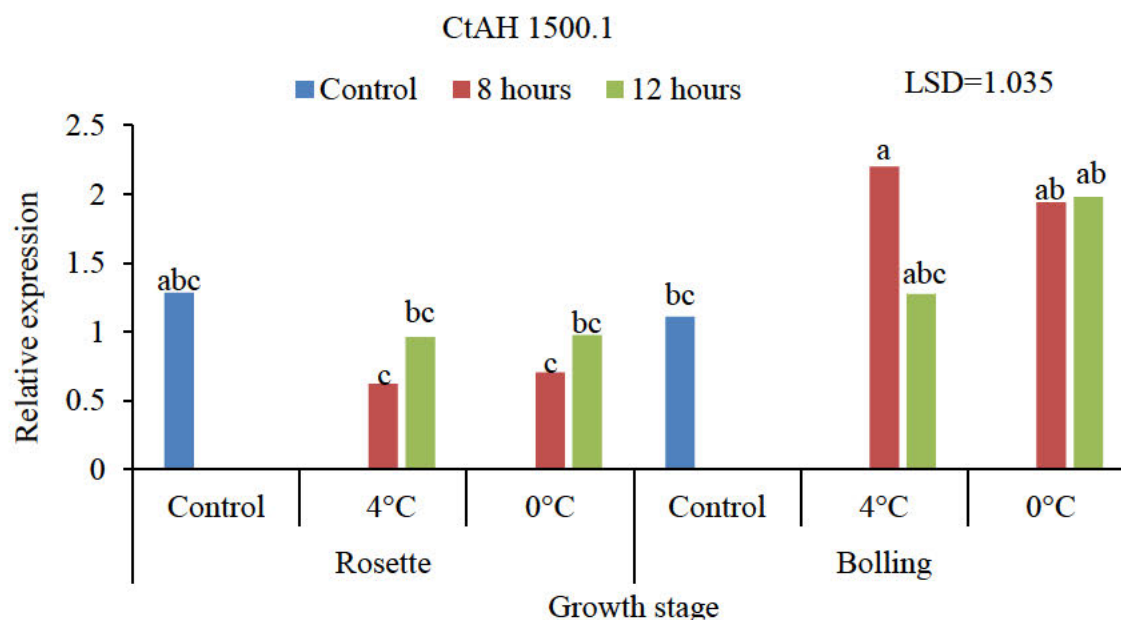


Figure 4.38. Relative expression level of CtAH10T0001500.1 safflower gene under artificial cold stress treatment. Means with the same letters were not significantly different. Means separated using the LSD at $P \leq 0.05$.

Contrary to the other genes, CtAH10T0000500.1 gene expression gradually increased with lower temperature and increment in duration of exposure at the rosette stage (Figure 4.39a). Plants at 23 °C had a relative expression of 1, which increased 2-fold and 4-fold at 4 and 0°C, respectively (Figure 4.39a). A similar trend was observed in duration of exposure as relative gene expression increased from 1, 3 and 4-fold with prolongation of cold from 0, 8, and 12 hrs, respectively (Figure 4.39a). In comparison to the rosette stage, the gene was significantly downregulated in all cold treatment, regardless of the chilling temperature and duration of exposure at the bolting stage (Figure 4.39b).

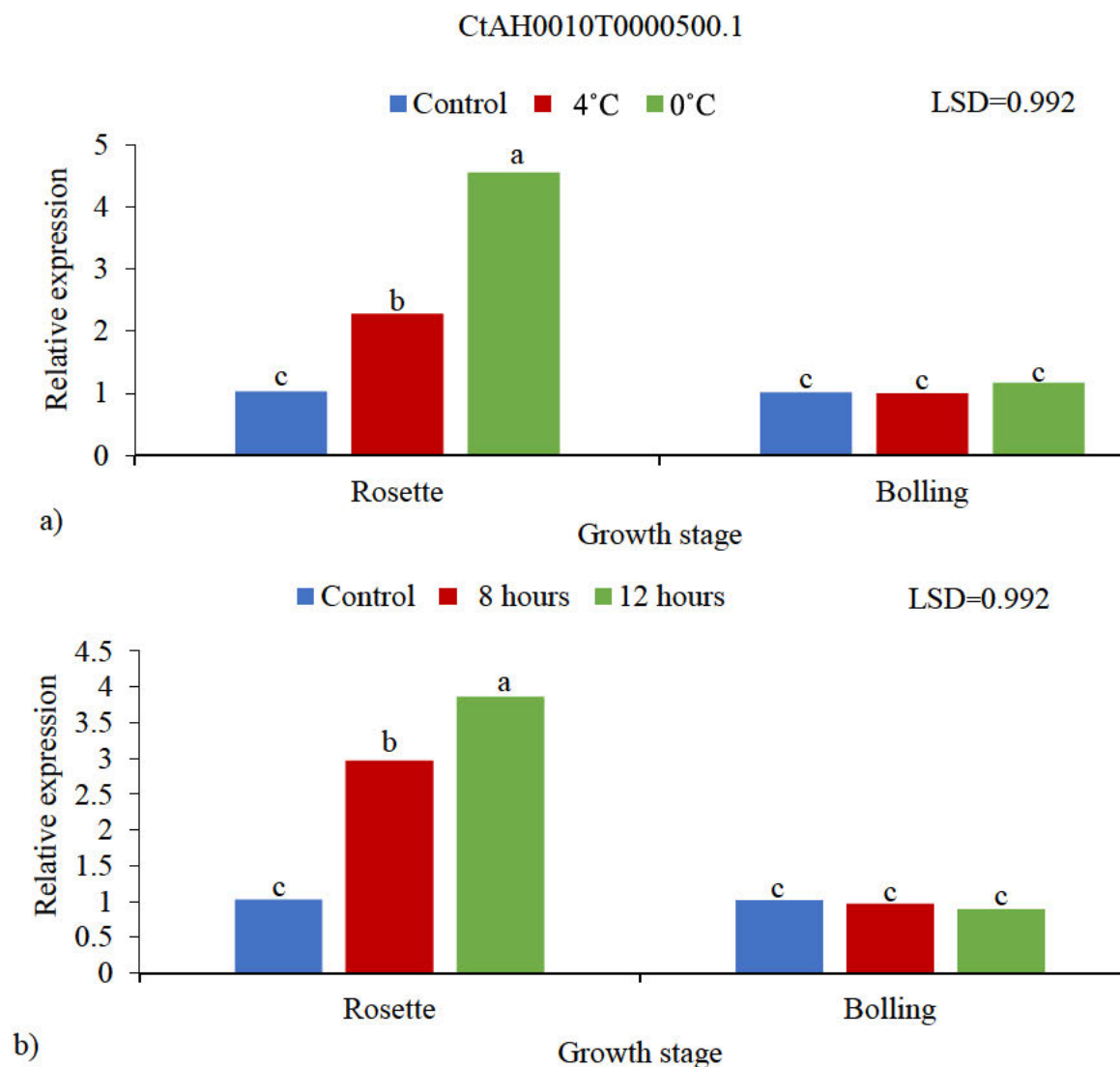


Figure 4.39. Relative expression level of CtAH10T0000500.1 safflower gene under cold artificial cold stress treatment. The relative expression level results were presented as (a) influenced by interaction of safflower growth stages and temperature, and (b) interaction of growth stage and duration of cold exposure. Means with the same letters were not significantly different. Means separated using the LSD at $P \leq 0.05$.

4.2.7.5 Correlation analysis of safflower genes with REL and proline content from artificial cold stress treatment

Correlation analysis between safflower cold responsive genes and relative electrolyte leakage (REL) showed a significant positive correlation at the rosette stage (0.48: $P < 0.05$ for CtAH10T0001700.1 and 0.43: $P < 0.05$ for CtAH10T0000500.1), except for CtAH10T0001500.1 (-0.47: $P < 0.05$) gene with negative correlation (Table 4.3). At the bolling stage, a non-significant inverse relationship was noted between CtAH10T0001500.1 (-0.17: $P < 0.05$) and CtAH10T0000500.1 (-0.92: $P < 0.05$) with relative electrolyte leakage (REL), while CtAH10T0001700.1 (0.49: $P < 0.05$) had non-significant positive correlation (Table 4.3). A similar trend of relationship was observed between safflower cold responsive genes and proline content as of relative electrolyte leakage at the rosette stage. CtAH10T0001700.1 and CtAH10T0000500.1 still had positive significant correlations of 0.41 and 0.32 ($P < 0.05$), respectively. Whereas CtAH10T0000500.1 (-0.41: $P < 0.05$) negatively correlated with proline content in the rosette stage. Contrary to correlation results between relative electrolyte leakage (REL) and cold responsive safflower genes, the genes had a significant positive relationship with proline content (0.96: $P < 0.05$ for CtAH10T0001700.1 and 0.68: $P < 0.05$ for CtAH10T0001500.1), excluding CtAH10T0000500.1 (-0.19: $P < 0.05$) with a significant inverse correlation (Table 4.3).

Table 4.3. Pearson correlation analysis of cold responsive safflower genes with relative electrolyte leakage and proline content.

Parameter	Growth stage	CtAH10T0001700.1	CtAH10T0001500.1	CtAH10T0000500.1
Relative electrolyte leakage (REL)	Rosette	0.48***	-0.47***	0.43***
	Bolling	0.49ns	-0.17ns	-0.92ns
Proline	Rosette	0.41***	-0.41***	0.32**
	Bolling	0.96*	0.68***	-0.19***

Values are significant at $P < 0.05$; *0.05, **0.01 & ***0.001.

CHAPTER 5

5.0 DISCUSSION

5.1 Chilling injury incidence, severity, and plant recovery

The present study revealed an occurrence of CI in safflower plants at the bolting stage, particularly in those exposed to prolonged cold stress (0 and 4°C) for 12 hours or more under artificial cold stress induction. Plants at the rosette stage, however, did not exhibit any CI in any of the treatments studied under artificial cold stress induction. These results implied that plants in the bolting stage under artificial cold stress induction were cold-susceptible, while those in the rosette were cold tolerant. Landry et al. (2017) associated the cold tolerance in safflower at the rosette stage with the ability of the meristems to regenerate new leaves rapidly, forming a compact cluster of leaves that quickly replace older ones. The regrowth mechanism is a key trait essential for continued photosynthesis and nutrient uptake hence survival of plants at the rosette stage under cold stress (Rapacz, 1999; Rys et al., 2020). Dajue and Mundel (1996) attributed cold tolerance in safflower to its growth habit of forming a bulky close-to-ground rosette leaves which protects the leaves from CI. Studies on cold tolerance of rosette-forming plant species like arabidopsis, cabbage, and lettuce have demonstrated that the dense rosette arrangement near the soil surface provides a favourable microclimate that acts as a natural barrier to minimize heat loss and offers retention of soil warmth, conditions that could exacerbate CI. Moreover, the dense forming ground-hugging rosette leaves have been reported to reduce exposure of plants from chilly air, thereby protecting the meristems from cold damage (Agrawal et al., 2012; Zuther et al., 2012). Unlike at the bolting stage, leaves are oblong and alternate on the erect stem, exposing the plants to environmental stresses (OECD, 2022). Therefore, it can be concluded that cold tolerance in safflower at the rosette stage is primarily due to its growth habit and the protective role of its close-to-ground rosette leaves, while the CI at the bolting stage could be due to lack of those protective adaptations.

The visible symptoms of CI in safflower were indicated by wilted, curled, and discoloured leaf edges. Several factors have been associated with the wilting of cold-stressed leaves (Farooqi et al., 2018). One major factor is the disruption of cell membrane function, which compromises membrane integrity and turgor pressure, leading to water leakage from the cells. Moreover, chilling temperatures can slow down or impede the transport of water and nutrients through the plant's vascular system, thereby reducing water availability to the leaves and triggering wilting (Farooqi et al., 2018; Ritonga & Chen, 2020). Additionally, cold-induced dehydration may

occur due to impaired water uptake by the roots, either from excessively cold soil that inhibits root activity or from reduced water availability in frozen soil (Bracale & Coraggio, 2003; Farooqi et al., 2018). Notably, the soil used to cultivate safflower in the current study appeared extremely dry and icy, particularly in severely affected plants cold stressed at 0 and 4°C for 12 hours. These findings therefore suggested that cold induced dehydration in safflower could have been due to damaged cell membranes and reduced water uptake, both of which limited the plant's capability to maintain turgor pressure, hence wilting. However, further investigation of other physiochemical and molecular changes is necessary for a more comprehensive conclusion of factors that caused CI in safflower at the bolting stage.

In tropical and subtropical plants, visible manifestation of chilling symptoms has been observable between 0-15°C (Purvis, 2004; Wang et al., 2016a). The results of the current study under artificial cold stress fell within the range, as chilling in safflower was observed between 0 and 4°C. This finding, however, contrasts with the report from GRDC (2017), which noted the development of chilling symptoms in safflower at frost temperatures of -4°C. Nonetheless, other studies have confirmed that CI can occur in various crops when temperatures fall below 15°C. Moreover, it is essential to note that sensitivity to chilling varies significantly among species and is influenced by factors such as temperature, duration of exposure, and growing conditions (Chen et al., 2020; Sari et al., 2020). Lipton et al. (2011) observed chilling incidence in asparagus plants (*Asparagus officinalis* L.) after 10 days of cold exposure at 0°C, leading to loss of sheen, glossiness, and graying of the plant tips. Chen et al. (2000) reported incidence of CI in Peace lily plants (*Spathiphyllum wallisii* L.) exposed to temperatures of 3 and 7°C. The visible symptoms varied depending on the variety of the plant. In the mini variety, symptoms appeared within 24 hours of exposure to 3°C, while for the 5598 variety, symptoms emerged after 5 days of cold exposure at the same temperature. Mesa et al. (2022) mentioned that CI in tomato plants grown in the field occurred between 10-12°C. The Ontario Ministry of Agriculture, Food and Rural Affairs (2022) reported CI in tomato plants at temperatures between 0-5°C, while in bell pepper (*Capsicum annuum* L.) plants it was observed at temperature between 0-10°C. The variability in CI thresholds among plant species highlights the complexity of cold stress responses, which are influenced by factors such as temperature, exposure duration, and plant variety. While safflower exhibited chilling symptoms primarily between 0-4°C, other crops, including tomato and pepper, display chilling injuries at temperatures as high as 12°C. These differences emphasize the importance of understanding

species-specific chilling sensitivity to develop effective cold management strategies in horticultural production.

Furthermore, the results of this study demonstrated that prolonged exposure of 12 hours at 0 and 4°C severely affected safflower plants at the bolling stage, leading to total loss of the plants. This finding underscored the significance of the duration of cold exposure in sensitive species. Similar results have been reported in other studies, where prolonged exposure to low temperatures resulted in severe damage or total loss of cold-sensitive plant species. For instance, Sari et al. (2020) reported incidence of CI in bottle gourd (*Lagenaria siceraria*) seedlings cold stressed at 4°C for 36 hours. Increasing the duration to 60 hours increased the extent of CI. Damage on growing points, true leaves and cotyledons rose from 2.3, 5.1 and 5.0 to 3.5, 6.4 and 6.4 scale ratings, respectively when comparing the 36- and -60 hours chilling durations (Sari et al., 2020). Kozik and Wehner (2014) reported total loss of water melon seedlings when exposed to chilling temperature of 2°C for 36 hours against 12 and 24 hours which did not cause any damage to the seedlings at both 2 and 4°C. Lainé et al. (2023) documented intense chilling damage of 50% reduction in shoot dry weight and leaf area in maize cold stressed at 4°C for prolonged duration of 7 days. Similarly, in the current study, low temperatures of 0 and 4°C coupled with 12 hours exposure duration severely affected safflower plants, with remarkable plant death at the bolling stage. These findings highlighted the critical importance of managing the duration of chilling exposure to mitigate damage in safflower and other cold-sensitive crops.

On the other hand, Ercoli et al. (2004) pinpointed that visibility of chilling symptoms do not occur only during exposure time in chilling temperatures but also after removal from low temperatures to warmer conditions. In their study, sorghum plants kept at 2, 5, and 8°C for eight days exhibited visual CI damage after removal from chilling temperatures. In agreement to this study, Petruccelli et al. (2022) reported that chilling temperatures of 7.5 to 12.5°C do not show chilling injury symptoms in olive (*Olea europaea* L.). Nonetheless, metabolic processes like respiration and photosynthesis are significantly reduced, thus resulting in decreased production. According to Fiorino and Mancuso (2000), visual symptoms of C in olive become noticeable when the temperature dropped to 0/-3°C, with slight damage to younger leaves and one-year shoots. Further drop of temperature to below -10°C resulted in irreversible damaged of plants (Sanzani et al., 2012). Thus, the above studies revealed that the manifestation of chilling symptoms during exposure time is not the only indicator of CI.

Clark et al. (2000) reported that rubber plant (*Hevea brasiliensis* Muell. Arg.) exhibited photosynthetic injury after exposure to chilling temperatures of 10°C for four hours. However, the plants returned to their previous values of photosynthetic rates when transferred to temperature of 28°C. Landry et al. (2017) reported that cold acclimation of safflower at 15/5°C day/night temperature for a period exceeding four weeks reduced the survival rate of plants, when later transferred to -15°C. Sari et al. (2020) reported that bottle gourd previously cold stressed at 4°C for 36, 48 and 60 hours recovered after two weeks of removal from chilling treatment. Plants in a shorter duration (36 and 48 hours) recovered first while plants for 60 hours still had traces of chilling symptoms after two weeks of recovery (Sari et al., 2020). On the other hand, Ercoli et al. (2004) reported that sorghum (*Sorghum bicolor*) plants exposed to 2 and 5°C for eight days did not recover any growth after 10 days of recovery, which was indicated by zero values of relative growth rate (RGR). While the plants at 8°C were able to re-gain some growth after four and eight days of cold exposure. Hence, demonstrating that the ability of plants to adapt to low temperatures decreases with decreasing temperature and longer exposure time. Consequently, low temperature and long duration of cold treatment increase the severity of CI.

Contrary, to this investigation, plants naturally exposed to 2°C in the field did not exhibit any sign of CI at both rosette and bolling stages. Several factors could explain the difference in safflower's response to chilling stress at similar temperatures. One factor could be the gradual cooling and acclimatization of plants in the field versus the abrupt dropping of temperatures in the refrigerator. As evidenced by meteorological data (Appendix 1 & 2), field temperatures typically decreased progressively, allowing safflower plants to acclimate to cooler conditions over time. Unlike in the refrigerator, plants that were grown outdoors were suddenly introduced to chilling temperatures of 4 and 0°C, not giving the plants time to adjust and activate their cold tolerance mechanisms, hence manifesting CI symptoms in cold susceptible plants. The results of the current study agree with the findings of Zeng et al. (2014), who reported that gradual cooling of Cassava (*Manihot esculenta*) from 24°C to 14°C and subsequently subjecting them to cold stress at 4°C, enhanced cold tolerance. In contrast, plants subjected to direct cold stress at 4 from 24°C showed low resilience to cold stress. Their argument was that gradual cooling enabled plants to undergo physiological adjustments, such as hardening, which enhanced their tolerance to lower temperatures. Moreover, when plants are cooled slowly, ice formation tends to occur outside the cells (extracellular freezing), which is less damaging. Whereas rapid cooling can lead to ice forming inside the cells (intracellular freezing), causing

significant cellular damage. Another factor that may contribute to the observed difference is the variable microclimatic conditions in the field. Unlike the uniform, static, and artificially maintained low temperatures in the refrigerator, field conditions include soil insulation, air circulation, and diurnal light exposure, which can mitigate the effects of chilling temperatures. Braem et al. (2023) elucidated that vegetation structures, such as grasses and tree canopies, influence soil insulation by increasing relative humidity (RH) and stabilizing temperatures near the ground. Their study demonstrated that dense deciduous tree canopies, variable canopy cover, and meadows with sparse tree cover had 17.69, 10–15, and 5–10% higher RH, respectively, compared to flat and no vegetation sites. This buffering effect significantly reduces temperature extremes, potentially mitigating chilling stress in plants. Similarly, under field conditions, the presence of natural vegetation, residual plant cover, or adjacent canopy structures (in the rosette stage) may have contributed to increased soil RH and reduced temperature variability, thus protecting safflower plants from CI. Lu et al. (2020b) reported that soil insulation increased soil temperature to a depth of 5–15 cm, and this increase enhanced root and shoot growth of maize in their study. Additionally, Song et al. (2021) explained that wind speeds above 2 m/s significantly affected frost development by enhancing air mixing, which disrupted temperature inversions and hindered frost accumulation. While on the other hand, constant air like that observed in refrigerated environments leads to air stratification, which increases the likelihood of CI. Similarly, it is possible that natural wind flow in safflower grown in the field maintained adequate air circulation, thereby preventing the formation of temperature inversions near the ground. This air movement likely reduced the occurrence of frost/ CI during susceptible bolting growth stage, providing a natural buffer against CI. Furthermore, exposure to natural light during the day maintained some level of photosynthetic activity, thereby preventing photoinhibition and oxidative damage. In contrast, the refrigerator environment, characterized by low light or complete darkness, could have disrupted photosynthetic processes, which led to the accumulation of ROS and increased susceptibility to CI (Liu et al., 2028). In summary, the difference in CI responses between field-acclimated safflower and refrigerator-exposed plants were likely due to the contrasting temperature dynamics and environmental conditions, highlighting the significance of gradual acclimation and microclimatic variation in cold stress tolerance.

5.2 Physiological, biochemical and molecular responses of chilled safflower plants

5.2.1 Relative leaf electrolyte leakage

Electrolyte leakage is an important indicator of injury that occurs in the cell plasma membranes after exposure to either biotic or abiotic stresses. When a cell is stressed, electrolytes such as K^+ ions are leaked out, resulting in loss of cell membrane integrity. The number of electrolytes lost is usually used as a measure of cell viability and death after stress exposure (Demidchik et al., 2014; Hatsugai & Katagiri, 2018). In the present study, both safflower plants at the rosette and bolting stages exhibited relatively low and comparable levels of solute leakage (9.9%) under artificial cold stress treatment, indicating that cell membranes were intact and not significantly stressed. This observation aligned with the findings of Wu et al. (2022), who reported that cold-tolerant tomato seedlings exposed to cold stress at 4°C for 12 hours exhibited unaltered membrane structure, demonstrating stable membrane integrity despite cold exposure. Nonetheless, a significant number of solutes were lost at the bolting stage relative to rosette stage at all the chilling conditions. However, lower temperature of 0°C resulted in higher solutes leakage with severe damage at 12 hours of exposure (65.7% REL). High electrolyte leakage values at bolting stage suggested high solutes leakage and susceptibility of the stage to cold stress. These results aligned with the notion that reproductive stages, such as bolting, are inherently more susceptible to CI due to increased metabolic and physiological demands (Wang et al., 2018). In comparison, Amala et al. (2022) reported that a cold-susceptible African marigold (*Tagetes erecta*) genotype exhibited even higher electrolyte leakage (86.08%) after prolonged exposure to winter conditions (three months). In contrast, a cold-tolerant marigold genotype displayed a relatively lower REL of 62.45% under the same conditions. These findings suggested that while safflower at the bolting stage showed a high degree of cold sensitivity, it is less vulnerable compared to the cold-susceptible marigold genotype. However, it is important to note that the duration of exposure differed between these studies: safflower experienced acute chilling stress (12 hours), while marigold was subjected to prolonged cold (three months). Despite this difference, the relatively high REL in safflower at bolting due to cold stress indicated that this stage is prone to cold stress-induced membrane damage, like cold-sensitive marigold genotypes. Thus, the vulnerability of safflower during the reproductive phase remains a critical limitation, necessitating the development of cold-tolerant varieties to mitigate yield losses. Longevity in cold stress was also found to play a significant role in loss of electrolytes. At the bolting stage, plants chilled for 12 hours in both 4 and 0°C had higher

REL than plants exposed to the same temperatures but shorter duration of eight hours. These findings agree with the results reported by Liu et al. (2013) and Rani et al. (2021).

Although plants under natural stress induction in the field did not show any visible symptoms of CI, a remarkable increase in REL was observed after exposure of plants to 2°C. A sharp rise was noted in plants at the bolting stage compared to rosette plants in the morning. This stage-specific sensitivity further emphasized the concept that reproductive stages are more vulnerable to chilling stress than vegetative stages (Wang et al., 2018). Notably, REL values decreased by mid-day, with rosette plants REL comparable to control level, while the bolting stage showed a significant but reduced REL. Diurnal variations in REL can result from temperature acclimation and the plant's ability to restore membrane integrity during the warm mid-day period (Li et al., 2019), as it was observed that during mid-day the temperature rose to 17.3°C in the current study (Appendix 1). Moreover, Rai et al. (2017) elucidated that increased daytime temperatures may enhance the activation of protective mechanisms such as the accumulation of compatible solutes like proline and increased activity of membrane-stabilizing proteins. Overall, the rosette stage probably had a better membrane repair mechanisms than the bolting stage, contributing to the observed tolerance both in the artificial and natural stress induction treatment.

5.2.2 Proline

Proline is regarded as the most important biomarker and signalling molecule associated with stress tolerance (Ashraf et al., 2018; Hosseinifard et al., 2022). The results of the current study demonstrated that prior to cold stress exposure, safflower plants at the rosette stage exhibited higher proline content than those at the bolting stage under artificial cold stress experiment. This implies that rosette plants had a more robust proline baseline accumulation, associated with better inherent stress preparedness (Rai et al., 2017). The findings of the current study are consistent with observations made by De-Freitas et al. (2019), who identified elevated protein concentrations in cold-tolerant rice varieties, measuring between 0.14–0.18 $\mu\text{mol/g}$ FW, compared to cold-sensitive varieties that exhibited low protein levels ranging from 0.09–0.10 $\mu\text{mol/g}$ FW prior to the application of cold stress. Moreover, Ji et al. (2020) also reported a cold-tolerant variety of sweet pepper to contain approximately 1.17 $\mu\text{mol/g}$ FW relative to 1.09 $\mu\text{mol/g}$ FW of a susceptible variety before any cold stress.

Exposure of safflower plants to low temperatures resulted in a high accumulation of proline in plants at the rosette stage, peaking at 0°C after 12 hours chilling temperature exposure. High

amounts of proline are to accumulate in plants to avoid dehydration induced by chilling temperatures, as a result maintaining the osmotic potential equilibrium and stabilizing proteins and membrane structures in plants (Verbruggen & Hermans, 2008; Rai et al., 2017). De-Freitas et al. (2019) reported higher proline content in cold-tolerant rice varieties (0.44–0.53 $\mu\text{mol/g}$ FW) after 72 hours at 10°C than cold-sensitive varieties (0.38–0.40 $\mu\text{mol/g}$ FW) under the same conditions. While both varieties showed elevated proline content, the elevation was more pronounced in the cold-tolerant ones. Tatar et al. (2013) also reported that cold-tolerant chickpea (*Cicer arietinum* L.) accumulated approximately 300 $\mu\text{g/g}$ (0.006 $\mu\text{mol/g}$) proline after cold exposure at 7°C, representing a 247% increase over control plants. In contrast, the cold-sensitive chickpea variety accumulated about 127.4 $\mu\text{g/g}$ (0.003 $\mu\text{mol/g}$). Additionally, Ji et al. (2020) reported progressively elevated levels of proline content in sweet pepper subjected to cold stress at 4°C for 7 days. Before the onset of cold stress, plants exhibited a proline content of approximately 1.17 $\mu\text{mol/g}$ FW, which increased to about 1.48 $\mu\text{mol/g}$ FW by the end of the exposure. In contrast, the cold-sensitive cultivar showed inconsistent accumulation of proline throughout the 7 days of cold stress. While the proline content initially rose from 1.09 to 1.17 $\mu\text{mol/g}$ FW after the first day, it subsequently decreased to 1.13 $\mu\text{mol/g}$ FW by day 5. However, by day 7 of cold exposure, the proline content had risen again to 1.22 $\mu\text{mol/g}$ FW. The fluctuating proline levels in the cold-sensitive cultivar indicated a less stable or less effective stress response, possibly reflecting a reduced ability to adapt to sustained low temperatures (Ji et al., 2020). While a consistent and progressive accumulation of proline under cold stress, as seen in the cold-tolerant sweet pepper cultivar, was likely a protective response contributing to cold tolerance (Ji et al., 2020). Hence, this argumentation could be used to justify that safflower plants at the rosette stage were more stable, while plants at the bolting stage responded slowly to counteract against the effect of cold stress.

Tatar et al. (2013) associated chickpea genotypes which produced significantly low levels of proline with being cold sensitive. Their argument was that cold sensitive plants respond slowly when it comes to proline accumulation, hence yielding relatively low levels of proline which was inadequate to provide a strong defence mechanism. Consequently, leading to osmotic pressure imbalance in the plant cells and ultimately occurrence of CI in plants as evidenced in safflower plants at the bolting.

Similarly, control safflower plants under field conditions exhibited higher proline content of 6.17 $\mu\text{mol/g}$ FW at the rosette stage compared to plants at the bolting stage (4.49 $\mu\text{mol/g}$ FW). However, cold exposure of 2°C in both stages exhibited a marked increase in proline content,

reaching 11.65 $\mu\text{mol/g}$ FW and 10.70 $\mu\text{mol/g}$ FW in the rosette and bolling stages, respectively, in the morning. This surge highlighted safflower plants adaptive response to chilling, consistent with the results reported by Ashraf and Foolad (2007) who found that cold stress triggered proline biosynthesis as a protective mechanism in safflower (Ashraf & Foolad, 2007). While at mid-day, the proline content in both phenological stages did not significantly differ from the morning values of the bolling stage. This suggested that proline accumulation might have reached a threshold level, stabilizing despite temperature fluctuations.

5.2.3 Leaf chlorophylls and carotenoid content

5.2.3.1 Leaf chlorophylls

In the current study, the photosynthetic pigments of safflower plants significantly varied depending on the interaction of growth stage, temperature, and duration of exposure in controlled cold stress trial. Control safflower plants at the rosette stage exhibited significantly higher contents of chlorophyll a, chlorophyll b and total chlorophyll (a+b) in comparison with plants at the bolling stage. These results suggested that safflower plants at the rosette stage inherently possessed high chlorophyll concentration, which is essential for efficient photosynthetic activity, hence enhanced vegetative growth. Emongor et al. (2017) and Moatshe (2019) reported that safflower plants at the rosette and elongation stages had high total leaf chlorophyll content though influenced by genotype. Leaf chlorophyll is essential in sunlight capture and production of energy for manufacturing plant photosynthates (Srichaikul et al., 2011; Mirza et al., 2013; Chen et al., 2024). In support of the current results, Reddy et al. (2004) reported that earlier growth stages in cold-sensitive crops tended to have higher chlorophyll levels compared to reproductive stages due to their leaf area and active photosynthetic activity. Moreover, Kayess et al. (2020) reported that cold tolerant rice variety contained 3.957 mg/g Chl a, 2.118 mg/g Chl b and 5.051 mg/g total chlorophyll (Chl a+b), values in the same range as safflower at the rosette in control plants. In the current study, cold stress generally led to an insignificant reduction in chlorophyll a across most growth stages under controlled cold stress induction. An exception occurred at the bolling stage, where chilling at 0°C for 12 hours drastically reduced chlorophyll a by 60.1%. This reduction of chlorophyll induced by cold stress of 0°C for 12 hours demonstrated that longer duration at lower temperatures remarkably influenced chlorophyll a content of safflower plants at the bolling stage and increased the chances of CI development. On the other hand, chlorophyll b content decreased by 41.3% after exposure to 4°C for 12 hours at the rosette stage. Surprisingly, lowering the temperature to 0°C

for 8 and 12 hours did not significantly further reduce chlorophyll b. This suggested a potential cold acclimation response, where initial chilling may have triggered protective mechanisms that stabilized pigment content during more severe cold exposure. In contrast, chlorophyll b at the bolting stage was not significantly affected by the combination of temperature and exposure duration, indicating probably that chlorophyll b may be less sensitive to cold at this stage. This pattern aligned with the findings of Hussain et al. (2018) and Zhao et al. (2020), who accentuated that chlorophyll b degradation was more prominent in vegetative tissues than in reproductive parts during cold stress. Lastly, the total chlorophyll content was significantly reduced at 4°C for 12 hours (by 26.4%) and at 0°C for 12 hours during the rosette and bolting stages (by 54.92%), respectively. These results further emphasized that cold stress at 0°C and longer duration of 12 hours was particularly detrimental to safflower plants at the bolting stage. The reduction in chlorophyll a, b and total chlorophyll in the current study could also be attributed to the loss of RBP genes which resulted in safflower leaf chlorophyll degradation (Wang et al., 2016b; Wu et al., 2016; Zhang et al., 2016; Cui et al., 2019). RNA-binding proteins (RBPs) like pentatricopeptide repeat protein (PPR) and ribonucleoproteins (RNPs), are needed for maintaining chloroplast development and photosynthetic electron transfer, regulating the transcript levels of genes involved in chloroplast transcription/translation and photosynthesis without interfering with RNA processing (Gu et al., 2014; Wang et al., 2016b; Morita et al., 2017). Low temperature stress can also negatively influence the structure and development of chloroplasts, leading to abnormal thylakoid membranes and disrupted chloroplast morphology and physiology (Gu et al., 2014; Wang et al., 2016b; Morita et al., 2017). Low temperature stress has been reported to negatively impact safflower growth and development leading to a reduction in leaf chlorophyll content, photosynthetic capacity, seed yield, and potentially plant death (Shahrokhnia & Sepaskhah, 2017; Kereilwe et al., 2023; Eslam et al., 2024).

The reduction in chlorophyll content of cold stressed plants has been reported in previous studies. For instance, Partelli et al. (2009) reported 30, 27 and 29 (%) reduction in Chl a, Chl b, total chlorophyll (Chl a+b) contents, respectively in coffee plants (*Coffea canephora* and *Coffea arabica*) experiencing 13/8°C day/night temperatures for two weeks. Aghaee et al. (2011) reported 50% reduction in total chlorophyll content of rice seedlings exposed to cold treatment of 15/10°C day/night temperatures for two weeks. Tatar et al. (2013) reported reduction in total chlorophyll content, from 2.54 to 1.74 mg/g, in chickpea leaves exposed to chilling temperatures of 7°C. Li et al. (2021) also reported a significant decrease in this

photosynthetic pigment in pepper seedlings exposed to 15/5°C (day/night) cold treatment for 15 days, with a more severely sensitive cultivar decreasing by 54.0, 72.1 and 69.3 (%) Chl a, Chl b and Chl a+b, respectively, relative to their controls kept at 28/18°C (day/night). Xu et al. (2022) reported reduction in chlorophyll content in two *Camellia japonica* species cold stressed at 4 and -4°C for 24 hours, of course with severe damages in freezing temperature. Bhandari et al. (2018) also reported a decline in Chl contents of paprika (*Capsicum annuum* L.) exposed to 10°C. The recorded Chl a was 10.7 mg/g at day 1 and decreased to 2.4 mg/g after day 32 of the experiment, while Chl b decreased from 3.8 to 1.0 mg/g. The amount of chlorophyll in leaves can serve as an important indicator of stress level induced by low temperature stress in plants (Zhao et al., 2020).

Moreover, the results of the present study demonstrated that shorter duration of less than 8 hours in low temperatures of 0 or 4°C did not affect the chlorophyll content of safflower under artificial cold stress trial. Similar results have been observed by Orzechowski et al. (2021) in potatoes who reported that shorter durations in cold treatment did not influence the chlorophyll breakdown; hence chlorophyll can either be maintained or increased while the opposite is true for longer durations. In their study, Orzechowski et al. (2021) observed a significant reduction in chlorophyll content of potato plants after 12 hours of cold treatment at 2°C.

Similarly, under field condition stress induction in the current study, the results demonstrated that cold exposure of 2°C reduced chlorophyll content across safflower genotypes, with more severe impacts at the bolting stage, particularly in the morning. The morning decrease in chlorophyll content was associated with the rapid onset of chilling stress, causing stomatal closure to reduce water loss, thereby limiting CO₂ uptake and decreasing photosynthetic efficiency (Allen & Ort, 2001). Moreover, the genotype Sina (61.97 SPAD) showed a greater resilience by maintaining relatively high chlorophyll levels indicating that it had better pigment retention under cold stress than other genotypes suggesting a genetic advantage in stress resilience (Reddy et al., 2004). Decrease in chlorophyll content due to cold stress has been attributed to destabilization of chloroplast membranes resulting from the inefficient photosynthetic capacity of the leaves under low temperature stress (Tatar et al., 2013; Gadallah et al., 2020; Zhao et al., 2020). Additionally, chlorophyll content reduction due to cold stress was associated with suppression of chlorophyll biosynthesis resulting from inhibition of activity of the chlorophyll biosynthesis enzymes such as chlorophyllase and divinyl reductase (DVR) (Tatar et al., 2013; Gadallah et al., 2020; Zhao et al., 2020). In line with these findings, Nagata et al. (2007) reported that the enzyme activity of DVR was optimal at 30°C and reduced

at 10°C, with extremely high temperatures of 50°C resulting in no enzyme activity. According to Ashraf and Harris (2013) and Hajihashemi et al. (2018), the suppression in chlorophyll biosynthesis was attributed to the inhibition in δ -aminolevulinic acid (ALA) biosynthesis under temperature stresses (7 and 42°C). The decrease in chlorophyll content was attributed to the closure of the stomata in efforts to minimize water losses associated with chilling temperatures, simultaneously elevating the resistance of atmospheric CO₂ entry, which is essential for photosynthesis (Enneb et al., 2020). During chilling stress, plants often close their stomata to minimize water loss and prevent dehydration (Enneb et al., 2020). This stomatal closure reduces transpiration, but it also limits the entry of atmospheric CO₂ into the leaf (Enneb et al., 2020). Since CO₂ is essential for photosynthesis, reduced availability can lead to a decrease in photosynthetic efficiency and, consequently, a decline in chlorophyll content over prolonged stress. The rate of photosynthesis reduced is a clear indication of reduced chlorophyll in the leaves (Enneb et al., 2020).

Zhao et al. (2020) reported that the inhibition of ALA biosynthesis led to reductions of 19.4% and 46.8% in rice seedlings at temperatures of 18°C and 12°C, respectively, compared to those grown at 28°C. They explained that this inhibition hampers the conversion of Pchlide into chlorophylls. Pchlide is a precursor molecule essential for chlorophyll biosynthesis, playing a crucial role in the formation of chlorophyll a and b. Any disruption in Pchlide production hampers chlorophyll biosynthesis, particularly under stress conditions, leading to decreased chlorophyll content and impaired photosynthetic efficiency (Zhao et al., 2020).

Wu et al. (2020) also mentioned that low chlorophyll content could be due to photoinhibition and downregulation of light harvesting complex a and b (Lhca and Lhcb) associated with low light reception under low temperatures. In conclusion, results highlighted that safflower's chlorophyll stability is growth-stage dependent, with the rosette stage showing better pigment retention under chilling conditions compared to the flowering stage both under the artificial and natural cold stress conditions. The sharp reduction in chlorophyll during the bolting stage of safflower underscores the vulnerability of reproductive tissues to cold stress. Breeding strategies should focus on enhancing chlorophyll stability and stress resilience during bolting and flowering stages to mitigate cold-induced yield losses in safflower.

5.2.3.2 Carotenoids

Carotenoids are also essential photosynthetic pigments that play a major role in photosystem assembly, light harvesting and photoprotection (Ashraf & Harris, 2013; Yang et al., 2021). In

addition, they play an important role as a precursor in signalling during plant development under any abiotic stress (Ashraf & Harris, 2013). The results of the current study showed that cold stress increased carotenoid content of plants at the rosette stage compared to control plants. The increase in leaf carotenoids with low temperature stress and duration of exposure exceeding eight hours at the rosette stage was explained by a reduction in leaf chlorophyll content observed in the current study, which unmasked pre-existing carotenoids. Low temperature can trigger an increase in ethylene biosynthesis in plants by upregulation of the enzymes ACC synthase and ACC oxidase, which are important enzymes in ethylene production (Huang et al., 2023; Nergi & Arndt, 2023). Ethylene can promote chlorophyll degradation by increasing the activities of chlorophyllase and Mg-dechelataase, and regulating gene expression related to chlorophyll biosynthesis and degradation (Yin et al., 2016; Tipu & Sherif, 2024). However, safflower plants at the bolting stage in the current study had low levels of carotenoids. This suggested that the carotenoid system may have been damaged by low temperatures, implying that safflower plants at the bolting stage were more susceptible to cold stress compared to plants at the rosette stage under controlled conditions of cold stress induction. The reduction in carotenoids content in the current study could also be associated with the down regulation of genes responsible for carotenoids synthesis at low temperature (Li et al., 2021; Yang et al., 2021). The other contributing factor could be because as safflower approaches physiological maturity, the entire plant and the leaves lose their green colour and other plant pigments due to senescence (Emongor & Emongor, 2023). Reduction in photosynthetic pigments means low carotenoids and chlorophyll contents. Many studies have correlated low carotenoids content with low temperature to plants cold susceptibility (Li et al., 2021; Yang et al., 2021; Šamec et al., 2022). Furthermore, Suneel-Kumar et al. (2016) reported that phenological stages of safflower immensely influenced total carotenoids content. In their study, safflower analysed for carotenoids at the 30th day after sowing had the highest content ranging between 9558 to 14893 µg/100g (equivalence of 0.09558–0.14893 mg/g), and plants analysed after the 70th day had the lowest content ranging between 1476.00 to 4066.40 µg/100g (equivalence of 0.01474–0.040664 mg/g). The results of Suneel-Kumar et al. (2016) affirm that safflower plants at the rosette stage contained high carotenoid content compared to the latter stages, as evidenced by the results of controls in the present study, 1.04 mg/g FW versus 0.91 mg/g FW at the rosette and bolting, respectively. The results of the current study are in agreement with those reported by Šamec et al. (2022), who associated the cold susceptibility to low carotenoids content in kale sprouts grown at 8°C (0.51 ± 0.004 mg/g dry weight) as compared to those grown at 21°C (0.64 ± 0.06 mg/g dry weight). Li et al. (2021) also found a

significant decrease of 43.3% carotenoids content in cold sensitive pepper seedlings cold stressed at 15/5°C (day/night) for 15 days. Whereas Kayess et al. (2020) reported a colder tolerant rice variety to contain 1.213 mg/g carotenoids content.

5.2.3.3 Effect of photosynthetic pigments on photosystems

Like chlorophyll, low content of carotenoids reduces the photosynthetic efficiency of plants. Consequently, leading to reduced growth or total plant death as observed in plants grown at 0°C. When photosynthetic pigments (chlorophyll and carotenoids) are exposed to any stress factors (low temperature, drought or salinity stress), they are disrupted, thereby reducing the light-absorbing efficiency of both photosystems (PSI and PSII) (Zhang et al. 2011), which can be followed by a reduction in the level of excess excitation energy, as an alternative to increasing the level of energy dissipation, hence a lowered photosynthetic efficiency (Hajihashemi et al., 2018).

5.2.4 Chlorophyll fluorescence

Chlorophyll (Chl) fluorescence is a rapid and harmless method intended to investigate the efficiency of the photosystem II (PSII) of plants (Fernández-Calleja et al., 2020). Particularly, if the fluorescence originates from the de-excitation of the first excited singlet-state of Chl a molecule. Chl b molecules do not emit fluorescence because excited energy from it is instantly converted to Chl a (Roháček et al., 2008). Chl fluorescence is related to the photosynthesis pigments in the sense that it utilises the light energy trapped by leaf chlorophylls and carotenoids (Roháček et al., 2008; Fernández-Calleja et al., 2020). It is crucial to note that the light energy trapped by chlorophylls and carotenoids is not only used for Chl fluorescence, but it can also be used in photochemistry or dissipated as heat energy. These three pathways are always in competition, when fluorescence is maximized then the two others are minimised (Roháček et al., 2008).

5.2.4.1 Quantum yield of photosystem II

This is the portion of light energy absorbed by photosystem II, which is directed towards photochemistry to make ATP and NADPH and ultimately sucrose for photosynthesis (Scharnagl & TerAvest, 2017). Generally, under stress conditions, plants with cold-tolerant characteristics typically exhibit a higher quantum yield of photosystem II (ϕ_{II}) compared to their controls (Mishra et al., 2014). This indicates that cold susceptibility is associated with a lower ϕ_{II} .

The results of the current study demonstrated that safflower plants at the rosette stage displayed cold-tolerant characteristics as plants acquired a higher ϕ_{II} after stress imposition in the controlled cold stress induction experiment. These results are consistent with those of Mishra et al. (2014), who reported that cold-stressing arabidopsis plants at 4°C for two weeks increased the ϕ_{II} as compared to plants grown in room temperature (22°C). The ϕ_{II} ranged from 0.6–0.64 in cold-stressed plants, while for the room temperature ranged between 0.56–0.58. Mishra et al. (2014) associated a higher ϕ_{II} with the capability of the plants to gain their photosynthetic efficiency during the period of cold treatment. Therefore, it could be concluded that safflower in the rosette stage did not suffer from CI because it was able to continue photosynthesizing under low temperatures.

Moreover, the control plants in the current study showed that ϕ_{II} increased with decreasing temperature and an increasing exposure duration. Safflower plants at the rosette stage initially had a low mean value (0.35) of ϕ_{II} before stress imposition. Cold- stressing safflower plants at 4°C led to a gradual increase in ϕ_{II} ; with higher mean values of 0.59 and 0.71 after cold exposure of 8 and 12 hours, respectively. These results agree with those of Tesa et al. (2018) who reported an increase in ϕ_{II} from 1% at 27°C to 5% at a lower temperature of -196°C in perennial plant leaves. Contrary to the results of the current study, Huang et al. (2011) reported a much lower ϕ_{II} ranging from 0.2–0.5 in evergreen tropical (*Erythrophleum guineense*) trees exposed to 4°C versus the 0.6–0.7 of trees kept at 25°C, a pattern like that exhibited by safflower plants in the bolling stage. Huang et al. (2011) attributed the lower ϕ_{II} to the slowed delivery of electrons from the PSII to P700+ due to low temperature. The other contributing factor could be inhibition of oxidation of plastoquinol (PQH₂) at the cytochrome b/f complex due to low temperature, or limited diffusion of plastoquinone (PQ) and plastocyanin (PC) to convey electrons to PSI, which is helpful in getting rid of excess excited energy (Hendrickson et al., 2003; Huang et al., 2011). Therefore, the above explanation could apply to safflower plants at the bolling stage which suffered from CI.

In addition, Prinzenberg et al. (2020) argued that shorter durations of cold exposure can bring temporary reduction of ϕ_{II} . In their study, cold stressing arabidopsis accessions at 5°C for two days decreased the ϕ_{II} from 0.69 ± 0.024 (value of controls at 21°C) to 0.528 ± 0.07 . Nevertheless, prolonging the duration of exposure to seven days led to 0.56 ± 0.057 increment in ϕ_{II} . Prinzenberg et al. (2020) obtained similar results of the current study when safflower plants are at the bolling stage exposed to either 0 or 4°C for eight or 12 hours which resulted in significantly low quantum yield of PSII (ϕ_{II}) than plants in the rosette stage. The lowest ϕ_{II}

of values of 0.19 and 0.20 were observed in plants in the bolling stage exposed to cold stress at 0 and 4°C for 12 hours. Although low temperatures initially decreased the plants ϕ II relative to control results in the current study from 0.40 to 0.23 and 0.36 at 4 and 0 °C, respectively when exposed to eight hours, safflower plants managed to survive, which meant their photosynthetic apparatus were not totally damaged by low temperature. Reduction in ϕ II enlightened that photosynthesis efficiency can respond immediately to low temperature stress causing disruption to light-use efficiency, consequently causing photo-inhibition (Baker & Rosenqvist, 2004). At low temperature, light harvesting genes like proteins Lhca2*1 and Lhcb4*2 are likely to be suppressed (Hannah et al., 2005; Zhao et al., 2020), hence resulting in the leaves not receiving enough light for efficient photosynthesis (Mishra et al., 2014). This situation is unfavourable to plants, which do not have the capability to develop new leaves at low temperature, because leaves developed at low temperatures are reported to regain full photosynthetic activity. That is because plants must maintain a high need for assimilates due to active growth (Savitch et al., 2002). Since safflower is actively growing at the rosette stage that could be a contributing factor assisting it to maintain its photosynthetic efficiency under cold stress conditions. While photo-inhibition at low temperatures severely impaired the ϕ II of safflower plants at the bolling stage. With the unfortunate case of plants cold stressed for 12 hours, failing to regain their photosynthetic efficiency, resulting in total plant death in the controlled stress induction experiment.

With regards to natural exposure of safflower to cold stress, plants at the rosette stage showed a lower ϕ II efficiency of 0.46 against 0.57 of the bolling stage. This difference could be attributed to the physiological adaptations associated with developmental changes. During the rosette stage, plants primarily focus on leaf area expansion and light capture, whereas during the bolling stage, they shift toward reproductive growth and might exhibit enhanced photosynthetic efficiency to meet the increased energy demand (Olvera-González et al., 2013). Cold stress of 2°C generally increased ϕ II by 23.50% at the rosette stage, while at the bolling stage it was decreased by 9.87% in the morning. At the rosette stage ϕ II value was high in the morning because of the interaction of low irradiance and temperature. Kao et al. (2004) highlighted that the combination of low irradiance and temperature resulted in high ϕ II in mangrove species. In their study, ϕ II was at peak (0.5–0.8) at 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux (PPF) and low (0.08–0.38) at 1 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF at both 15 and 25°C. That argumentation was in line with the findings by Hogewoning et al. (2012), who reported that at low light intensities, natural photosynthesis has a very high ϕ II electron transfer of about 0.83.

stability. Additionally, genotypes with superior PSII efficiency may have enhanced protective enzyme activity, which helps reduce oxidative damage by maintaining higher levels of antioxidant enzymes.

5.2.4.2 Non-photochemical quenching

Prior to cold stress, results from the current study demonstrated comparable non-photochemical quenching (ϕ NPQ) values at both the rosette and bolling stages, averaged 0.56 under artificial cold stress conditions. This similarity suggested that both developmental stages maintained similar energy dissipation efficiencies under optimal conditions. Upon exposure to chilling temperatures of 4°C for eight hours, ϕ NPQ at the rosette stage significantly increased by 17.86%, indicating enhanced photoprotective activity. This aligns with the findings of Flexas and Medrano (2002), who reported that non-stressed grapevines thermally dissipated up to 65% of absorbed light, which increased to 75-92% under chilling conditions. Similarly, Hendrickson et al. (2004) observed an increase in ϕ NPQ from $64 \pm 1\%$ to $75 \pm 3\%$ of total absorbed radiation in grapevines subjected to a drop in temperature from 25°C to 10°C for 20 minutes. Huang et al. (2011) also reported significantly higher ϕ NPQ values (0.2-0.6) at 4°C compared to 0.1-0.2 in control plants grown at 25°C. Tezara et al. (2008) attributed such increases in thermal dissipation to PSII down-regulation, emphasizing ϕ NPQ's critical role in photoprotection. Hence, the increased ϕ NPQ in the rosette stage of safflower likely prevented photoinhibition under cold stress. Non-photochemical quenching is an energy partitioning parameter that quantifies the amount of light energy captured by PSII and dissipated as heat in the leaf, thereby protecting the photosynthetic machinery from photo-damage (Kramer et al., 2004; Parida et al., 2007; Ashraf and Harris, 2013). Typical ϕ NPQ values range from 0 to 0.85 (PhotosynQ MultispeQ Device User Guide, 2017). Under temperature stress, lower ϕ NPQ values indicate a weakened protective mechanism of PSII and, consequently, increased plant stress (Hendrickson et al., 2003; Schreiber & Klughammer, 2008).

In contrast, safflower plants at the bolling stage showed a significant 58.93% decrease in ϕ NPQ, indicating a compromised ability to activate heat dissipation pathways and a higher vulnerability to cold-induced photoinhibition. When the cold exposure at 4°C was extended to 12 hours, ϕ NPQ at the rosette stage increased modestly by 4.61%, suggesting possible acclimation to prolonged chilling. However, at the bolling stage, ϕ NPQ decreased drastically by 49.55%, indicating a pronounced breakdown in photoprotective capacity. This decline may be attributed to impaired activation of ϕ NPQ mechanisms or a reduction in photoprotective

pigment synthesis. These findings mirror those of Rácz et al. (2023), who reported significant ϕ NPQ reductions in cold-stressed bell peppers, with values dropping to 0.09 and 0.13 from control levels of 0.22 and 0.31 after 5 weeks of chilling (15/10°C). Similarly, Chen et al. (2024) observed reduced ϕ NPQ in tea seedlings subjected to 0°C, with Dali and Siqui varieties decreasing from 0.36 ± 0.02 to 0.08 ± 0.01 and from 0.27 ± 0.01 to 0.11 ± 0.01 , respectively, after 15 days. Further reduction of temperature to 0°C for eight hours in safflower plants caused a slight 13.53% decrease in ϕ NPQ at the rosette stage, suggesting a temporary suppression of photoprotection possibly due to impaired thylakoid membrane fluidity or damage to ϕ NPQ-related proteins. Interestingly, the bolting stage exhibited a dramatic 302.60% increase in ϕ NPQ, possibly indicating an overcompensation mechanism likely via rapid activation of heat dissipation pathways or increased synthesis of xanthophyll cycle pigments, as noted by Rácz et al. (2023) and Chen et al. (2024).

However, extending the 0°C exposure to 12 hours did not significantly alter ϕ NPQ at the rosette stage of safflower plants, indicating that the initial response was sustained. Oppositely, at the bolting stage experienced a marked 45.42% reduction in ϕ NPQ, suggesting that prolonged extreme cold stress overwhelmed its photo-protective mechanisms, leading to a collapse in ϕ NPQ efficiency. This may point to photo-inhibition or structural damage to the photosynthetic apparatus. These contrasting responses highlight a clear developmental stage-specific cold stress tolerance in safflower. The rosette stage exhibited consistent and moderate ϕ NPQ activation under cold stress, indicating a robust and adaptive photoprotective strategy. Conversely, the bolting stage displayed heightened sensitivity, with either suppressed or unsustainable ϕ NPQ responses under prolonged or extreme chilling conditions.

Under field conditions, ϕ NPQ at the bolting stage was slightly higher than at the rosette stage which suggested that maturing plants activated greater photo-protective mechanisms to dissipate excess light energy than plants at the rosette stage possibly due to elevated metabolic demands or increased light exposure. As observed in the artificial cold stress experiment, the enhanced ϕ NPQ at the bolting stage under field conditions reflected an adaptive strategy to safeguard PSII from photodamage (Tezara et al., 2008).

Additionally, diurnal fluctuations were observed, with ϕ NPQ values higher at mid-day than in the morning. This trend corresponds with increased mid-day light intensity ($1069.24 \mu\text{mol m}^{-2} \text{s}^{-1}$) and temperature (17.3°C), as shown in Appendix 1 and 10, which likely triggered an upregulation of thermal dissipation. Cooler morning conditions (2°C and $78.58 \mu\text{mol m}^{-2} \text{s}^{-1}$)

increased Φ_{NO} to overproduction of ROS under cold conditions, which impaired light-harvesting complexes and reduced the efficiency of energy transfer between LHCII and PSII. Similarly, Bhandari et al. (2018) reported an increase in Φ_{NO} from 0.20 to 0.44 in cold-stressed paprika seedlings, and Huang et al. (2011) observed similar elevations in evergreen tropical trees under 4°C conditions. The results of the current study align with the observed irreversible photo-inhibition of PSII at the bolting stage of safflower under sustained artificial cold, ultimately leading to plant mortality.

Under natural cold stress conditions, all genotypes had similar pre-stress values of 0.16 at both stages, indicating comparable PSII efficiency and minimal baseline stress. However, morning exposure to naturally low temperatures notably increased bolting-stage values to 0.28, reflecting cold-induced PSII photoinhibition and/or impaired protective capacity. Interestingly, mid-day values declined to 0.18 (bolting) and 0.17 (rosette), with the latter not significantly different from controls, could be probably due to partial recovery with rising temperatures (2–17.3°C) and light intensity (Appendix 1 & 10). These patterns differ with Lazzarin et al. (2024), who observed increasing Φ_{NO} in tomato with rising light intensity, reporting morning values of 0.29 (75 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and noon values of 0.38 (1300 $\mu\text{mol m}^{-2} \text{s}^{-1}$). In the current study, it appears that temperature, not light, was the main driver of morning Φ_{NO} increase. Moreover, the mid-day decrease in Φ_{NO} observed in the current study supports the role of reversible, temperature-sensitive photoinhibition and recovery dynamics, distinct from Lazzarin's light-driven patterns. Collectively, findings from both artificial and natural of cold induced stress experiments in the present study demonstrated that the bolting stage of safflower was more susceptible to chilling-induced increases in Φ_{NO} . However, the diurnal fluctuations in the natural setting allow partial recovery, highlighting how environmental variability may offer resilience advantages that are not captured under static, controlled conditions.

5.2.4.4 Electron transport rate II

The electron transport rate (ETR) is a light-adapted parameter which reflects the relative rate of energy conversion in PSII (Kao et al., 2004). It is directly related to Φ_{II} by the equation, $\text{ETR} = \Phi_{II} \times \text{PAR} \times 0.84 \times 0.5$ (Kao et al., 2004; Olvera-González et al., 2013).

The electron transport rate of photosystem II (ETR II) was markedly influenced by both growth stage and temperature, highlighting the sensitivity of the photosynthetic machinery to developmental cues and environmental stress in safflower in this study. Under artificial cold stress conditions, plants at the rosette stage demonstrated a significantly higher ETR II (52.25)

than those at the bolting stage (36.17). This could probably suggest that safflower plants at the rosette stage possessed a greater capacity to maintain photosynthetic electron flow during cold stress, possibly due to the presence of younger, more resilient leaves with higher physiological plasticity. The temperature effect results in the current study revealed that control plants had an exceptionally high ETR of 220.17, underscoring optimal photosynthetic efficiency under favourable conditions. These results are in line with findings by Kao et al. (2004), who reported a high ETR value in the range 150–200 in mangrove leaves exposed to 25°C, relative to 0–50 of plants exposed to 15°C. However, under cold stress at 0°C and 4°C, ETR II dropped drastically to approximately 0.22, regardless of growth stage. This dramatic decline indicated severe inhibitory effect of low temperatures on PSII activity in safflower plants. Zhou et al. (2004) attributed the decrease in the ETR to both the decrease in the number of open PSII reaction centres and the efficiency of energy capture by these open centres. Meanwhile, Kao et al. (2004) associated the decrease in ETR with photo-inhibition of PSII. Ratajczak et al. (2023) clarified that when photosynthetic electron transport in PSII was reduced, phosphorylation was curbed hence leading to production and accumulation of active O₂. Under severe chilling conditions, there might be blockage of electron transport which favours the conversion of O₂ produced into a highly active form referred to as singlet oxygen (¹O₂). This form of O₂ has the capacity to oxidize the chlorophyll in the reaction centre of PSII. Usually in that state, the activities of enzymes involved in carbon dioxide (CO₂) fixation are hindered robustly, thus increasing the amount of energy captured which eventually surpasses the one required for photosynthesis. Over time, the amount of excess energy dissipated as heat in the antennae of PSII increases while the absorbing efficiency of PSII is reduced, hence occurrence of photo-inhibition (Marocco et al., 2005). However, the author of the current study could not straight away assume that was the case since most previous studies have attributed photo-inhibition as an effect of chilling damage of PSI (Tjus et al., 1998; Sonoike, 1998; Kudoh and Sonoike, 2002). Since the interest of the current research was not in the activity of PSI, it was not identified which of these two mechanisms might be responsible for the reductions in ETR looking to the fact that safflower plants at the rosette stage did not suffer chilling at either 4 or 0°C. Hence more research could still be done to find the actual cause of reduction in ETR in safflower.

In contrast, ETR II response under natural cold stress was significantly influenced by the genotype and time of day interaction. Prior to exposure to chilling, ETR II values across genotypes were uniformly low (~0.08), indicating a basal photosynthetic state likely due to low

morning temperatures or light intensity as data was collected in low morning temperature of 2°C and low light intensity of 78.58 $\mu\text{mol m}^{-2} \text{s}^{-1}$. However, by mid-day, there was substantial genotype-specific differences, with the genotype Sina recording the highest ETR II, while the latter three had almost similar values. These findings could suggest genotypic variability in photochemical efficiency and potential differential tolerance to cold stress during natural diurnal cycles in safflower.

Moreover, the growth stage $\times$ time of day interaction revealed that rosette stage plants had significantly higher ETR II at mid-day, again confirming earlier observations from the artificial stress experiment where the rosette stage showed greater cold tolerance. Bolling stage plants, while showing lower values, still demonstrated improved ETR II at mid-day, emphasizing the reversible nature of cold inhibition under natural conditions when light and temperature improve. In support of the above results, Campbell et al. (2019) reported a clear diurnal progression in both PAR and ETR in maize, with ETR peaking at midday across all developmental stages. For instance, at vegetative stage, PAR increased from 572 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the morning to 812 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at noon, concurrently with an ETR rise from 80.2 to 97.7. A similar pattern was observed during early senescence (reproductive stage), where PAR rose remarkably from 490 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the morning to 1,275 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at midday, accompanied by a substantial increase in ETR from 15.2 to 28.9. This mid-day rise could be due to the electron transport machinery retaining some functional capacity and remaining responsive to increased light availability, although at a diminished rate compared to the vegetative stages. These findings confirm that midday conditions, characterized by peak light intensity stimulate the highest rates of electron transport, thereby explaining the ETR II peaks observed in the current study. Furthermore, the lower morning values can be attributed to suboptimal light levels and delayed activation of the photosynthetic apparatus. Altogether, these results underscore the importance of considering diurnal variation in photosynthetic measurements, particularly under stress conditions that may further modulate ETR II responses.

In comparison of the methods of cold induction, artificial cold stress (0 and 4°C) caused a severe and sustained suppression of ETR II relative to the natural setting, where mid-day recovery of ETR II was evident. Additionally, the maximum ETR II under control conditions in the refrigerator experiment was lower than the peak mid-day values recorded in naturally cold-stressed plants. The results of the present study suggested that artificial cold stress imposed in a static, dark environment which may be more physiologically extreme and

damaging than fluctuating field conditions, where plants can adjust to gradual changes and capitalize on periods of favourable microclimate.

5.2.5 Stomatal Conductance

The results of the current study demonstrated a clear relationship between temperature and stomatal conductance (gs), for as temperature increased, gs also increased in the controlled refrigerator cold induced experiment. At both the rosette and bolling growth stages, safflower plants not exposed to cold stress exhibited significantly higher gs values than those subjected to cold stress for both the artificial and natural stress induction conditions. These results were further confirmed by the field results, in which safflower genotypes displayed a lower gs in the morning average of $311.35 \text{ mmol m}^{-2} \text{ s}^{-1}$, while that value improved to average of $512.67 \text{ mmol m}^{-2} \text{ s}^{-1}$ during mid-day. Since gs measurements were taken at midday, the findings confirmed that stomatal conductance peaks during the day when sunlight and temperature are highest. These observations align with those of Hills et al. (2014), who reported a positive correlation between maximum stomatal conductance (ranging from 0.9 to $2.8 \text{ mmol m}^{-2} \text{ s}^{-1}$) and the mean maximum temperatures of the hottest month. Huang et al. (2016) also reported a gradually increase in gs of alpine evergreen oak tree (*Quercus guyavifolia*), which was recorded to be about $0.05 \text{ mol m}^{-2} \text{ s}^{-1}$ at early hours of the morning (0900 hours) and peaked to a value about $0.36 \text{ mol m}^{-2} \text{ s}^{-1}$ at 11:00 hours, although the experiment was conducted in summer.

Furthermore, the results of the current study indicated that gs decreased across both developmental stages in response to cold stress, reinforcing the hypothesis that safflower is more cold-tolerant during the early vegetative phase. This pattern was consistent with previous studies, which showed that plants with higher gs under stress conditions tend to be more cold-tolerant, while lower gs values are indicative of cold sensitivity (Kao et al., 2004; Aghaee et al., 2011). For example, Kao et al. (2004) found that a cold-tolerant mangrove species exhibited a gs of $159 \pm 16 \text{ mmol m}^{-2} \text{ s}^{-1}$ under chilling conditions (15°C for 1 hour), whereas the cold-sensitive species recorded a significantly lower value of $107 \pm 12 \text{ mmol m}^{-2} \text{ s}^{-1}$. Similarly, Aghaee et al. (2011) reported that cold-tolerant rice seedlings maintained a gs of $180 \text{ mmol m}^{-2} \text{ s}^{-1}$ under cold stress ($15/10^{\circ}\text{C}$ day/night) for two weeks, while cold-sensitive genotypes dropped to $43 \text{ mmol m}^{-2} \text{ s}^{-1}$ under the same conditions. In agreement with the results of the current study, Ramya et al. (2016) reported that in general gs was high in plants during their peak vegetative stage and decreased along with maturity due to senescence of leaves. In the current study, the rosette stage of safflower has vigorous vegetative growth compared to the

bolting stage as plants approach maturity or senescence, which could be a contributing factor to the g_s difference in the two developmental stages.

Moreover, the controlled experiment highlighted that prolongation of plants at lower temperatures resulted in safflower plants having low g_s . Initially, plants at both stages exhibited high g_s , but these values declined significantly with continued exposure to low temperatures. Consequently, safflower plants at 4°C and eight hours had higher g_s compared to plants cold stressed at 0°C and 12 hours. Cold stress decreased stomatal conductance of safflower plants through stomatal closure as a mechanism to conserve water and reduce water loss under cold stress conditions and reduce dehydration and damage. Georgieva & Vassileva (2023) and Yang & Guo (2018) reported that stress (cold, heat, salt, and water) decreases stomatal conductance of plants mainly through stomatal closure as a mechanism to conserve water and reduce water loss under stress conditions and reduce dehydration (wilting) and damage. Ku et al. (2018) and Haque et al. (2024) reported that both biotic and abiotic stresses have a significant influence on g_s regulation. Chilling temperatures have been reported to delay stomatal opening in cold-sensitive plants (Ku et al., 2018; Hague et al., 2024; Guo & Cao, 2015; Joshi et al., 2020). Additionally, low temperature stress has been reported to regulate phytohormones that induce stomatal closure, such as abscisic acid (ABA), ethylene, salicylic acid, and brassinosteroids (Agurla et al., 2018; Miura & Tada, 2014; Lang et al., 1994). Kao et al. (2004) reported that a drop in g_s from $191 \pm 7 \text{ mmol m}^{-2} \text{ s}^{-1}$ at 25°C to $107 \pm 12 \text{ mmol m}^{-2} \text{ s}^{-1}$ after just 1 hour of cold exposure at 15°C, and further down to $0.1 \text{ mmol m}^{-2} \text{ s}^{-1}$ after 10 days of cold treatment in mangroves. Aghaee et al. (2011) also documented a significant reduction in rice seedling g_s from $442 \text{ mmol m}^{-2} \text{ s}^{-1}$ (under 29/22°C) to $43 \text{ mmol m}^{-2} \text{ s}^{-1}$ after cold exposure at 15/10°C. Meanwhile, Ikkonen et al. (2012) also reported that prolonged continuous exposure of cucumber (*Cucumis sativus* L. cv. Zozu Iya) to 12°C for 6 days significantly reduced the plants g_s by 50% relative to controls. However, in their study, they found that short term exposure of cucumber plants to low temperature can be used to harden plants (Ikkonen et al., 2012).

Numerous studies have attributed reduced g_s under cold stress to stomatal closure triggered by cold-induced water stress (Kao et al., 2004; Aghaee et al., 2011; Jurczyk et al., 2019; Han et al., 2022). Li et al. (2017) emphasized that water stress leads to reduced stomatal density, aperture size, and area, morphological traits that directly influenced stomatal conductance. Under such conditions, reductions in g_s and transpiration helped to improve overall water use efficiency (WUE). In this context, it is likely that safflower plants at the rosette stage were able to activate these mechanisms to limit dehydration, whereas plants at the bolting stage failed to

do so, resulting in excessive water loss and wilting. Wilkinson et al. (2001) further observed that cold-sensitive plants often cannot effectively close their stomata, which leads to uncontrolled water loss. This was evidenced by open stomata in cold-sensitive tobacco plants after 3.5 hours at 7°C, in contrast to the cold-tolerant *Commelina communis*, which closed its stomata within one hour at the same temperature, thereby maintaining leaf relative water content even after several hours. Allen and Ort (2001) linked stomatal closure in cold-tolerant species to chilling-induced loss of Rubisco activity, leading to an accumulation of internal CO₂ (Ci). In contrast, cold-sensitive species may be unable to initiate stomatal closure due to limited Ca²⁺ uptake by guard cells during low temperatures. The role of Ca²⁺ in stomatal regulation is to activate anion channels, leading to depolarization and subsequent opening of K⁺ channels. The resultant ion efflux reduces turgor pressure in guard cells, prompting stomatal closure (Kollist et al., 2014).

Additionally, Driesen et al. (2020) emphasized the role of stress duration in modifying stomatal traits. Their findings indicated that prolonged exposure to water stress can alter stomatal density in newly formed leaves, thus adjusting CO₂ uptake and transpiration rates. This may explain the better cold tolerance observed at the rosette stage of safflower plants, where new leaf growth was active and may have helped in maintaining physiological functions under cold stress. Finally, the extremely low *g_s* values recorded after 12 hours of cold exposure of safflower plants highlighted that safflower at the bolting stage had limited capacity to cope with stress. In this stage, stomatal closure appeared to occur only after the onset of severe wilting. Similar results were observed in cold-sensitive tobacco plants by Wilkinson et al. (2001). The very low *g_s* values recorded in the current study were indicative of complete stomatal closure and suboptimal photosynthesis because CO₂ uptake was effectively halted. Kao et al. (2004) and Aghaee et al. (2011) reported *g_s* values of 0.1 mmol m⁻² s⁻¹ and 43 mmol m⁻² s⁻¹, respectively, as thresholds indicating full stomatal closure. These findings collectively underscore the critical influence of temperature and developmental stage on *g_s*, highlighting safflower's greater cold tolerance during early vegetative growth, a trend exhibited by other parameters discussed before.

5.2.6 Antioxidants enzyme activity

5.2.6.1 Catalase activity

The responses of catalase (CAT) activity under artificial and natural cold stress conditions in safflower underscored the dynamic, developmental stage, temperature and duration of

exposure to low temperature-dependant nature of antioxidant defence mechanisms in plants. Both treatments triggered alterations in CAT activity, but with distinct patterns based on the developmental stage, temperature, duration of exposure, and genotype. In both artificial and natural conditions, CAT activity displayed an initial increase at the rosette stage, signifying the plant's rapid response to cold-induced oxidative stress. Under artificial cold stress, CAT activity in safflower plants at the rosette stage plants increased from a basal level of 1.09 U mL⁻¹ FW (control) to 1.41 U mL⁻¹ FW at 4°C (eight hours), 1.62 U mL⁻¹ FW at 4°C (12 hours), and peaked at 1.80 U mL⁻¹ FW at 0°C (12 hours), reflecting robust activation of H₂O₂-scavenging pathways. Similarly, in the natural cold stress scenario, CAT activity measured in the morning rose to 1.66 U mL⁻¹ FW in safflower plants at the rosette stage and 1.57 U mL⁻¹ FW at the bolting stage, again indicating an early defence response against cold-induced ROS accumulation. This trend supported the notion that catalase plays a crucial role in mitigating oxidative stress during early plant development (Kusvuran et al., 2016; Tayu et al., 2025). CAT plays a vital role in neutralizing ROS especially hydrogen peroxide (H₂O₂) which builds up under different abiotic stress conditions, such as cold stress in plants. The enzyme CAT is primarily located in glyoxysomes of lipid-storing tissues in plants and has a tetrameric structure that facilitates the conversion of H₂O₂, generated from the β -oxidation of fatty acids, into water (H₂O) and molecular oxygen (O₂), thereby contributing to the antioxidant defence system in plants (Kusvuran et al., 2016; Tayu et al., 2025).

Comparable findings on CAT activity have been reported in other crops. De-Freitas et al. (2019) reported that CAT activity increased from 0.07–0.13 U mg⁻¹ to 0.28–0.35 U mg⁻¹ in cold-tolerant rice seedlings exposed to cold stress at 10°C for 72 hours. However, it decreased in cold-sensitive varieties, from 0.46–0.21 U mg⁻¹ under the same conditions. Similarly, Massardo et al. (2000) observed that in a cold-tolerant oat (*Avena sativa* L.) variety, CAT activity increased significantly from 149.5 ± 1.1 to 298.6 ± 2.1 U mg⁻¹ when comparing plants grown at 17 °C (control) and 3°C (cold stress). Conversely, the susceptible variety showed a slight decrease from 115.5 ± 2.1 to 110.0 ± 2.8 U mg⁻¹ under the same temperature treatments. Ji et al. (2020) also reported an increase from 0.075 to 0.011 U mg⁻¹ in CAT activity of belly pepper cold stressed at 4°C for seven days. While for the cold sensitive cultivar it decreased from 0.07 to 0.068 U mg⁻¹. The findings of the current study and those reported in literature (De-Freitas et al., 2019; Ji et al., 2020; Kusvuran et al., 2016; Tayu et al., 2025; Massardo et al., 2000) highlighted that elevated CAT activity was strongly associated with cold tolerance

and underscores the enzyme's role in mitigating oxidative stress during the early stages of plant growth.

While initial activation of CAT occurred under both artificial and natural cold stress inductions, the patterns of sustained response varied significantly. Under artificial cold stress, particularly at the bolling stage, CAT activity showed limited or impaired induction. Although a mild increase was observed at 4°C for eight hours (1.27 U mL⁻¹ FW), CAT activity declined to near control levels (1.14 U mL⁻¹ FW) after 12 hours and drastically dropped to 0.31 U mL⁻¹ FW at 0°C for 12 hours. This marked reduction may imply that safflower plants at the bolling stage may exhibit greater vulnerability to severe cold stress, resulting in an inability to effectively scavenge H₂O₂. Several factors could contribute to this issue, including sensitivity at specific developmental stages, irreversible damage, or exhaustion of defence mechanisms. Plants experience varying degrees of tolerance to abiotic stresses depending on their developmental stage (Kusvuran et al., 2016; Tayu et al., 2025). For instance, during the bolling stage, which involves significant resource allocation for reproductive development, there may be a trade-off in the plant's ability to tolerate stress (Tayu et al., 2025). Additionally, prolonged exposure to sub-zero temperatures could result in irreversible damage to enzyme structures or the machinery responsible for enzyme synthesis. On the other hand, extended and severe cold stress may exceed the plant's capacity to maintain high levels of CAT activity, ultimately leading to a decline (Tayu et al., 2025).

Conversely, during natural cold stress, CAT activity at the bolling stage remained relatively stable, with no significant decline between morning and mid-day measurements. This contrast may reflect the less abrupt and diurnally modulated nature of natural stress, allowing partial recovery or less physiological shock. Lastly, genotypic wise, Gila showed the strongest antioxidant response at the bolling stage, while PI-537636, Sina, and Turkey exhibited moderate but comparable activity. Notably, Sina maintained relatively high CAT activity in both phenological stages, indicating consistent defence potential. In contrast, the remaining genotypes displayed lower activity, pointing to weaker oxidative stress responses. These patterns highlighted that genotypes Gila and Sina were colder tolerant than PI-537636 and Turkey based on CAT activity.

5.2.6.2 Superoxide dismutase activity

Safflower plants cold stressed at 0 and 4°C for either eight or 12 hours at the rosette stage had significantly higher superoxide dismutase (SOD) activity than plants at the bolling stage. The

highest SOD activity of 4.45 U mL⁻¹ FW was observed on cold stressed plants at 0°C for 12 at the rosette stage. On the contrary, safflower plants at the bolling stage exposed to low temperature of 0°C for 12 hours had significantly the lowest SOD activity of 0.41 U mL⁻¹ FW. The high SOD activity in safflower plants at the rosette stage under cold stress of 0 and 4°C for either 8- or 12-hours exposure indicated a strong oxidative stress response. While the low SOD activity in safflower plants at the bolling stage suggested reduced enzymatic stability or adaptive capacity. Cold exposure at 4°C for eight and 12 hours significantly elevated SOD activity to 2.98 and 3.45 U mL⁻¹ FW, respectively. Further lowering the temperature to 0°C led to the highest SOD activity, that is, 4.00 U mL⁻¹ FW at eight hours and 4.45 U mL⁻¹ FW at 12 hours. SOD is important in protecting plants from CI by scavenging superoxide radicals that are harmful to cellular metabolism during stressful conditions such as exposure of plants to low temperatures (Clare et al., 1984; Thomas et al., 1999; De-Freitas et al., 2019; Ji et al., 2020). SOD catalyses the conversion of superoxide into H₂O₂ and O₂ leading to reduction of the amount of ROS and reducing cellular damage (Clare et al., 1984; Thomas et al., 1999; De-Freitas et al., 2019; Ji et al., 2020). Increased SOD activity has been reported in literature during stressful conditions such chilling temperatures demonstrating a protective mechanism against oxidative damage, therefore enhancing tolerance to CI (Thomas et al., 1999; De-Freitas et al., 2019; Ji et al., 2020). In line with the results of the current study, De-Freitas et al. (2019) reported that the cold tolerant rice varieties had SOD activity in the range 0.28-0.32 U mg⁻¹ after exposure to chilling temperature of 10°C for 72 hours, while sensitive varieties had 0.21–0.28 U mg⁻¹ SOD activity. Furthermore, Ji et al. (2020) reported that SOD activity in cold-tolerant bell pepper seedlings increased from 0.038 to 0.050 µmol mg⁻¹ following exposure to 4°C for seven days. In contrast, cold-sensitive cultivars showed a slight decline in SOD activity, from 0.036 to 0.035 µmol mg⁻¹, suggesting a limited or compromised ROS-scavenging capacity under chilling conditions which could be compared to the results of safflower at the bolling stage. Furthermore, Guo et al. (2012) demonstrated that SOD activity in bell pepper leaves responded dynamically to chilling stress (10/6°C). Activity initially declined slightly, from 5.2 U/g FW at 3 h to 4.6 U/g FW at 12 hours, relative to control values (5.0 and 4.5 U/g FW, respectively). However, a compensatory increase followed, with SOD activity reaching 6.2 U/g FW by 120 hours, exceeding control levels. This pattern underscores the enzyme's involvement in long-term cold stress mitigation and supports the notion that enhanced or sustained SOD activity correlates with greater cold resilience, particularly during earlier developmental stages of safflower.

Under field conditions, SOD activity was significantly influenced by the interplay of genotype and time of the day, and growth stage and time. The genotype Sina at mid-day had notably higher increase in SOD activity of $3.21 \text{ U mL}^{-1} \text{ FW}$ than any other genotype and time of the day. The genotypic variation in the activity of SOD in response to cold stress and time of the day indicated genotype and environment interaction, and varying levels of chilling tolerance. Safflower has been reported to show varying levels of chilling tolerance depending on the developmental stage and genotype (Emongor & Emongor, 2023; Kereilwe et al., 2023; Landry et al., 2017; Johnson et al., 2006; Mündel et al., 1992; Yazdi-Samadi & Zali, 1979). Safflower is considered cold-tolerant at the rosette stage, however certain phenological stages such as elongation and bolting are more susceptible to CI (Emongor & Emongor, 2023; Kereilwe et al., 2023; Landry et al., 2017; Johnson et al., 2006; Mündel et al., 1992; Yazdi-Samadi & Zali, 1979). The high SOD activity in the genotype Sina followed by Gila showed that Sina was most cold tolerant genotype followed by Gila. The high activity of SOD under cold stress indicated that it plays a critical role in safflower's cold stress defence mechanisms suggesting a potential for therapeutic applications in mitigating oxidative stress.

5.2.6.3 Peroxidase activity

The peroxidase (POD) activity of safflowers in the current study responded strongly to cold stress, with significant interactions between growth stage, temperature, and exposure duration under artificial cold stress experiment. At the rosette stage, the control plants exhibited relatively low POD activity of $0.81 \text{ U mL}^{-1} \text{ FW}$. Exposure of safflower to 0°C for 12 hours at the rosette stage ($2.29 \text{ U mL}^{-1} \text{ FW}$) significantly increased POD activity compared control plants and plants exposed to cold stress at different temperatures and exposure times. Plants at the bolting stage exposed to 0°C for 12 hours had significantly ($P < 0.05$) the lowest POD activity of $0.27 \text{ U mL}^{-1} \text{ FW}$ compared to any other treatment. The high POD activity at the rosette stage showed a robust enzymatic response of safflower plants to oxidative stress helping plants to tolerate cold stress. Whereas at the bolting stage POD activity was low which suggested compromised defence capacity at this developmental phase. Peroxidases are important in aiding plants to tolerate cold stress by scavenging ROS that are biosynthesized and accumulate during cold exposure (Pandey et al., 2017; Kapoor, 2015; Das & Roychoudhury, 2014). Peroxidases aid in maintaining cellular homeostasis and avoid oxidative damage caused by ROS, thus contributing to plants capability to withstand and recover from low chilling temperatures (Pandey et al., 2017; Kapoor, 2015; Das & Roychoudhury, 2014). De-Freitas et al. (2019) reported that POD activity in rice seedlings increased from 0.18–0.35

U mg⁻¹ to 0.35–0.56 U mg⁻¹ after 72 hours of exposure to cold stress at 10°C compared to controls. Additionally, Ji et al. (2020) reported that POD activity in cold-tolerant bell pepper seedlings increased from 0.14 to 0.20 $\mu\text{mol mg}^{-1}$ following exposure to 4°C for seven days. In contrast, cold-sensitive cultivars showed a slight decline in SOD activity, from 0.13 to 0.14 $\mu\text{mol mg}^{-1}$. Similarly, Guo et al. (2012) reported that in bell pepper leaves, POD activity under chilling treatment (10 °C/6 °C) rose markedly compared to the control. At three hours, POD activity under chilling was approximately 8.7 $\mu\text{mol g}^{-1}$ FW, compared to 7.0 $\mu\text{mol g}^{-1}$ FW in the control. Activity peaked at six hours, with an approximate of 10.8 $\mu\text{mol g}^{-1}$ FW activity under chilling stress, while control values remained around 7.2 $\mu\text{mol g}^{-1}$ FW. From 12 to 120 hours, chilling-stressed plants maintained higher POD activity (ranging between 9.2 and 10.0 $\mu\text{mol g}^{-1}$ FW), consistently exceeding control levels, which plateaued around 6.5 to 7.5 $\mu\text{mol g}^{-1}$ FW. The results of Guo et al. (2012) demonstrated that chilling stress upregulates POD activity in plants. The sustained elevation of POD in chilling-treated plants underscores its critical role in H₂O₂ detoxification and cold tolerance mechanisms, reinforcing the observed developmental and stress-duration sensitivity of antioxidant responses in safflower.

Under field conditions, plants at the rosette stage showed a delayed increase in POD activity by mid-day, implying a gradual activation of defence mechanisms. Conversely, at the bolting stage plants initially showed reduced activity, which later recovered, indicating a slower but adaptive response. Meanwhile, genotypically, Sina displayed the strongest POD response, while Gila, PI-537636, and Turkey showed comparable but slightly lower activity. These results suggested that POD activity may contribute to genotype- and stage-specific cold tolerance, with Sina demonstrating the most consistent enzymatic resilience and cold tolerant genotype. Genotypic variation in POD activity has been reported in safflower caused by abiotic stresses such as cold and water deficit (Yeloojeh et al., 2019; Önder et al., 2020). This implies that different safflower germplasm show variation in POD activity. The genotypic variation of POD activity in safflower was reported to be influenced by genetic and environmental interaction (Yeloojeh et al., 2019; Önder et al., 2020). Some safflower genotypes such as Sina in the current study showed higher POD activity under cold stress conditions, suggesting the genotype was cold stress tolerant. This further suggested that some safflower genotypes are better equipped to handle cold stress due to their inherent genetic constitution.

5.2.7 Expression pattern of cold stress related genes during chilling injury

The genomic data in the current study demonstrated that most of the homologs selected to identify cold responsive safflower genes came from *Cynara cardunculus* var. *scolymus*, commonly known as Artichoke/globe artichoke (López-Anido & Martin, 2021). Artichoke is a flowering perennial plant that belongs to the family Asteraceae (López-Anido & Martin, 2021), the same family as of safflower (Li et al., 2015). Cannon et al. (2004) reported that during evolution, plants of the same family form genes with similar functions or structures through the process called whole genome duplication. This information suggested that the two plants could have evolved from a common ancestor hence high structural similarity, sequence homology and similar response to cold stress.

To associate safflower genes with cold responsiveness, the biological processes, functions, conserved domains and structure of artichoke obtained from nucleotides blasting were studied (Han et al., 2022; Wang et al., 2022; Wang et al., 2023b). Genome annotation data, RPS-BLAST and CDD analyses revealed that E3 ubiquitin protein ligases CIP8-like and RING finger transmembrane domain-containing protein 2-like are RING finger proteins of RING/U-box superfamily (Han et al., 2022; Wang et al., 2022; Wang et al., 2023b). Under cold stress, the RING fingers act as E3 ubiquitin protein ligases and interact with E2 ubiquitin conjugating enzymes, thereby promoting ubiquitination of specific target transcription factors (Han et al., 2022).

In rice, the ubiquitination activity and expression pattern of the RING-H2 finger protein COLDINDUCIBLE (OsCOIN) was positively induced by low temperature (Liu et al., 2007). Relating to the current study, CtAH10T0001700.1 with a similar structure as E3 ubiquitin ligase (RING-H2) was highly expressed in the bolting than rosette stages of safflower. However, following the outcome of the current study results (Kereilwe et al., 2023), safflower plants in the rosette stage were cold tolerant while those in the bolting stage were susceptible, contradicting to the expression pattern of RING-H2 finger protein. Overexpression of CtAH10T0001700.1 in the bolting stage could be attributed to its CIP8-like character. CIP8 RING finger proteins are known to be repressor of light-responsive genes critical at plants flowering stage. These RING finger proteins also possess the ubiquitin ligase activity and directly interact with bzip transcription factor hy 5 (HY5), a positive regulator of photomorphogenesis, for degradation in darkness to reduce or inhibit flowering. Under cold stress and darkness, the dual role of E3 ligase gene prompts its production in large quantities, hence overexpression of CtAH10T0001700.1 in the bolting than rosette stage (Sun et al., 2019). The

CtAH10T0001500.1 which was like RING finger transmembrane domain-containing protein 2-like (HC) was downregulated in the rosette and upregulated in the bolting stage. Based on RING-HC finger protein High Expression of Osmotically Responsive Genes 1 (HOS1), these genes are downregulated under cold stress (Cui et al., 2018; Lourenço et al., 2013; Al-Saharin et al., 2022; Han et al., 2022). In Arabidopsis and rice, the RING-HC finger protein interacted with Inducer of CBF Expression 1 (ICE1) to mediate ICE1 ubiquitination and degradation through the 26S protease pathway, hence inhibiting expression of C-repeat binding factors (CBF) transcription factors which are essential for expression of Dehydration responsive element (DRE) required to increase osmoregulation substances in order to attain cold tolerance (Lourenço et al., 2013; Al-Saharin et al., 2022; Han et al., 2022). The results implied that CtAH10T0001500.1 in the rosette stage followed the expression pattern of RING-HC proteins to attain cold tolerance. While cold susceptibility in the bolting stage could be attributed to overexpression of HOS1.

The gene expression results further showed that CtAH10T0000500.1 safflower gene like *Cynara cardunculus* var. *scolymus* Scarecrow was significantly upregulated in the rosette stage while down regulated at the bolting stage. The gene expression level increased with decreasing temperature and increasing duration of exposure and was at peak at cold treatment of 0°C for 12 hours. The expression pattern of CtAH10T0000500.1 followed a similar trend as of GRAS genes. GRAS genes are a family of plant-specific transcriptional factors formed by three functional proteins named; GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR of GAI (RGA), and SCARECROW (SCR) (Lu et al., 2020c; Tong et al., 2023). In most plant species, cold stress significantly induces upregulation of the GRAS genes relative to controls (Peng et al., 2013; Li et al., 2017; Ye, 2013). Slightly differing in banana transplants, GRAS gene expression exhibited multidirectional regulation. Out of the 7 genes studies, four showed positive expression while three were negatively regulated (Tong et al., 2023). However, in overall, the increase in GRAS gene expression was positively correlated with plant resistance to cold stress (Li et al., 2017; Ye, 2013).

Furthermore, the correlation analysis demonstrated that E3 ligase genes associated with relative electrolyte leakage (REL) differently. CtAH10T0001700.1 was positively correlated to REL while the opposite was noted for CtAH10T0001500.1 in both phenological stages. These two genes confer cold tolerance by upregulation of CtAH10T0001700.1 and downregulation of CtAH10T0001500.1. Overexpression of the genes in the bolting stage rendered safflower plants more sensitive to cold stress hence high solutes leakage and membrane damage. High

electrolyte leakage has been associated with overexpression of E3 ligase genes such as AtCHIP and OsHOS1 in Arabidopsis and rice (Yan et al., 2013; Fang et al., 2016). On the other hand, the suppression of RING finger gene OsSRFP1 has been reported to increased free proline content of cold stressed rice leaves (Fang et al., 2016). Therefore, it could be concluded that both CtAH10T0001700.1 and CtAH10T0001500.1 with lower expression at the rosette stage of safflower plants has higher proline content as evidenced by the results of the present study. Meanwhile, the GRAS gene CtAH10T0000500.1 positively correlated with REL and proline content in the rosette stage and inversely associated with the two physiological variables at the bolting stage of safflower plants. Li et al. (2019) reported a significant positive correlation between electrolyte leakage and proline content. Thus, as cold stress triggers electrolyte leakage, upregulation of the GRAS gene triggers proline synthesis and accumulation hence conferring cold tolerance to safflower plants at the rosette stage. While suppression of the gene decreases proline synthesis and increases electrolyte leakage, leading to greater susceptibility of safflower plants to cold stress at the bolting stage. Together, these gene expression patterns highlight the molecular basis of safflower's enhanced cold tolerance at the rosette stage and increased susceptibility at the bolting stage.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

The study provided a comprehensive understanding of safflower's cold stress response, unequivocally establishing its developmental stage-dependent cold tolerance, with the rosette stage consistently exhibiting greater resilience than the more vulnerable bolting (reproductive) stage. This variation in susceptibility was apparent in both controlled artificial cold stress and natural field conditions, although symptoms like leaf wilting, curling, and discoloration at 0-4°C after 12 hours of exposure were confined to artificial settings. The absence of such visible injury under natural field conditions, despite similar low temperatures, was attributed to environmental modulating factors such as gradual cooling, enhanced acclimation, soil insulation, natural light exposure, and air circulation, all of which mitigated acute cold stress under field conditions.

At the physiological level, the rosette stage's superior tolerance was underpinned by its maintained cell membrane integrity, evidenced by low REL, and a robust osmotic adjustment mechanism characterized by high baseline proline content and a more consistent, proactive accumulation of this osmo-protectant during cold stress. In contrast, the bolting stage consistently showed greater electrolyte leakage and lower, less stable proline accumulation, indicative of compromised cellular stability. The photosynthetic machinery also demonstrated stage-specific responses; the rosette stage maintained significantly higher levels of chlorophylls and carotenoids, which are crucial for light harvesting and photo-protection, while the bolting stage experienced severe reductions in these pigments, particularly under intense and prolonged cold. This was further reflected in chlorophyll fluorescence variables: the rosette stage exhibited more effective photoprotective heat dissipation (increased ϕNPQ) and better regulation of excitation energy, whereas the bolting stage showed a greater decline in PSII efficiency (ϕII) and increased energy dissipation as heat (ϕNO), signalling higher photoinhibition and poor energy regulation. Variations due to time of the day were also critical, with morning chilling intensifying stress responses across most variables, though partial recovery was often observed by mid-day under natural conditions as temperatures and light intensity increased.

Beyond photosynthetic health, gs clearly delineated the stage-specific responses, with the rosette stage effectively regulating gas exchange and water loss under cold, while the bolling stage exhibited drastic reductions in gs, indicative of prolonged stomatal closure and increased dehydration risk. This physiological divergence was further supported by antioxidant enzyme activity, where consistently higher and more sustained levels of CAT, SOD, and POD at the rosette stage underscored its enhanced capacity to combat cold-induced oxidative stress. In comparison, the bolling stage displayed reduced or unstable enzyme activity, suggesting its limited ability to manage the ROS. Notably, comparisons between artificial and natural cold stress revealed a more gradual and stable enzyme response under natural conditions, emphasizing the importance of environmental context in shaping biochemical defences.

At the molecular level, the differential cold stress tolerance was linked to the expression patterns of specific genes. At the cold-tolerant rosette stage, low expression of E3 ligase-like genes (CtAH10T0001700.1 and CtAH10T0001500.1) appeared to support cold resilience by minimizing protein degradation pathways, while high expression of a GRAS gene (CtAH10T0000500.1) correlated with increased proline accumulation. Conversely, during the cold-sensitive bolling stage, overexpression of E3 ligase genes and suppression of the GRAS gene may have contributed to higher electrolyte leakage and reduced proline biosynthesis, exacerbating cold-induced damage. Furthermore, genetic variation among genotypes, such as the superior chlorophyll retention in Sina and stronger antioxidant responses in Gila and Sina, highlighted inherent genetic capacities for cold tolerance.

Safflower's cold tolerance was profoundly influenced by its developmental stage, with the rosette stage possessing a robust array of physiological, biochemical, and molecular mechanisms to withstand chilling stress, a capacity significantly diminished at the bolling stage. These findings highlighted the critical role of developmental stage, exposure duration, and environmental context in shaping safflower's cold stress response and underscore the urgent need for targeted breeding strategies aimed at enhancing cold tolerance, particularly during the vulnerable reproductive phase, to mitigate potential yield losses. Addressing the cold susceptibility of the bolling stage was paramount, as safflower's inherent adaptability makes it a valuable candidate for sustainable agriculture in a changing climate.

6.2 Recommendations

This study recommends future research to focus on enhancing safflower's cold tolerance during the bolling (reproductive) stage, which has been identified as the most susceptible to chilling

stress. Breeding programs should aim to develop genotypes that retain physiological and biochemical stability under low temperatures by incorporating traits associated with the rosette stage, such as efficient osmotic adjustment, strong antioxidant capacity, and stable photosynthetic pigment composition. Molecular studies should further investigate the regulatory roles of key genes, including E3 ligase and GRAS family members, to understand their contribution to cold stress signalling and tolerance. Physiological indicators such as chlorophyll retention, proline accumulation, and antioxidant enzyme activity could serve as effective screening tools for identifying cold-tolerant lines. Moreover, field-based investigations are needed to evaluate the influence of environmental factors, including gradual temperature decline, light intensity, and soil insulation, on cold acclimation and recovery. Integrating molecular, physiological, and agronomic approaches will be essential for developing safflower cultivars capable of maintaining productivity under fluctuating and low-temperature conditions, thereby improving its suitability for cultivation in cooler climates.

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APPENDICES

Appendix 1. Temperature (°C) data for the field experiment (first trial 2023). Sourced from Botswana Meteorological Services (2023) database.

MONTHS OF DATA COLLECTION	APRIL		MAY		JUNE		JULY	
DAY	Min	Max	Min	Max	Min	Max	Min	Max
1	18	31	14	27	6	20	3	22
2	16	29	14	27	3	26	5	22
3	17	30	15	29	3	22	4	22
4	16	30	15	29	8	30	4	22
5	14	27	13	29	6	26	3	22
6	15	27	13	27	6	26	3	22
7	14	29	14	29	9	26	3	22
8	16	28	13	29	11	26	4	23
9	17	30	12	23	10	27	2	18
10	16	26	10	24	1	15	2	15
11	14	26	8	26	3	22	2	16
12	11	27	8	26	2	25	2	18
13	12	29	7	28	7	19	2	18
14	13	30	9	25	2	23	2	28
15	12	28	9	25	3	24	4	20
16	11	25	10	23	2	22	7	22
17	12	25	10	23	2	22	5	24
18	11	27	11	22	3	25	5	24
19	10	27	11	23	6	25	4	23
20	9	27	7	22	4	22	4	21
21	9	28	6	24	2	22	7	15
22	12	30	3	23	0	23	10	21
23	14	29	3	26	3	21	7	25
24	11	30	7	30	7	22	4	28
25	11	29	7	30	5	23	7	22
26	15	30	8	24	3	23	7	22
27	12	31	9	26	9	26	8	25
28	16	31	9	24	8	23	9	27
29	14	30	10	26	1	20	9	29
30	14	32	10	24	1	20	6	17
31			10	22			2	20

Appendix 2. Temperature (°C) data for the field experiment (second trial 2024). Sourced from Botswana Meteorological Services (2024) database.

MONTHS OF DATA COLLECTION	MAY		JUNE		JULY		AUGUST	
DAY	Min	Max	Min	Max	Min	Max	Min	Max
1	11	30	10	27	9	23	8	24
2	10	29	7	17	10	25	13	27
3	10	29	3	19	8	22	7	30
4	10	29	3	22	8	26	5	32
5	10	27	1	25	1	24	6	24
6	8	27	4	19	1	22	5	23
7	8	29	4	18	0	18	7	26
8	9	31	3	19	-2	22	7	30
9	12	28	3	20	0	16	10	29
10	11	28	5	19	0	17	10	29
11	8	29	4	19	-1	26	5	32
12	8	29	4	20	0	23	5	28
13	10	26	4	20	2	26	8	30
14	14	23	3	23	2	26	8	27
15	12	26	6	21	5	26	8	27
16	11	28	2	22	2	22	4	27
17	10	28	4	22	3	26	3	28
18	9	28	7	22	2	26	6	29
19	10	27	7	24	4	26	6	29
20	9	26	4	26	4	26	6	27
21	10	26	7	26	5	26	10	28
22	6	24	7	22	3	23	7	30
23	10	24	7	22	4	26	10	24
24	10	26	5	22	6	29	10	26
25	8	26	8	21	5	26	8	29
26	7	26	10	23	3	24	10	31
27	7	26	8	21	2	26	10	33
28	3	25	8	21	2	23	7	31
29	7	24	9	21	1	25	9	28
30	6	25	9	21	2	19	10	23
31	6	26			3	26	12	23

Appendix 3. ANOVA summary table for REL, proline, chlorophyll and stomatal conductance of safflower under artificial cold stressed conditions.

Treatment	REL	Proline	Chlorophyll	Stomatal conductance
Growth stage	<2.2e-16***	4.16e-14***	1.36e-07***	<2.2e-16***
Temperature	<2.2e-16 ***	2.06e-08***	0.03*	<2.2e-16***
Duration of exposure	<2.2e-16 ***	5.0e-4***	0.16ns	2.06e-10 ***
Growth stage x temperature	<2.2e-16 ***	7.0e-4***	0.92ns	0.35ns
Growth stage x duration	<2.2e-16 ***	0.04*	0.56ns	3.10e-08***
Temperature x duration	<2.2e-16 ***	0.42ns	0.64ns	5.0e-4***
Growth stage x temperature x duration	<2.2e-16 ***	0.01**	0.40ns	0.02*

Significance: '****' $P \leq 0.001$; '***' $P \leq 0.01$; '**' $P \leq 0.05$; $P \leq 1.00$ -ns (not significant). 'e' is the exponential notation which informs to multiply the base number by 10 to the nth power (nth power given as the preceding number after e). Values in the columns are probability of a greater F ($P > F$).

Appendix 4. ANOVA summary table for REL, proline, chlorophyll and stomatal conductance of safflower naturally cold stress under field conditions.

Treatment	REL	Proline	Chlorophyll	Stomatal conductance
Genotype	0.98ns	1.00ns	< 2.2e-16***	0.28ns
Growth stage	<2e-16***	2.10e-11***	< 2.2e-16***	0.0002 ***
Time	<2e-16***	<2.2e-16***	< 2.2e-16***	5.216e-13 ***
Genotype x growth stage	0.98ns	1.00ns	9.52e-14***	0.94ns
Genotype x time	1.00ns	1.00ns	1.98e-06***	0.82ns
Growth stage x time	<2e-16***	2.03e-05***	< 2.2e-16***	0.91ns
Genotype x growth stage x time	1.00ns	1.00ns	1.04e-06***	0.99ns

Significance: ‘****’ $P \leq 0.001$; $P \leq 1.00$ -ns (not significant). ‘e’ is the exponential notation which informs to multiply the base number by 10 to the nth power (nth power given as the preceding number after e). Values in the columns are probability of a greater F ($P > F$).

Appendix 5. ANOVA summary table for quantum yield of PS II (@PS II), non-photochemical quenching (@NPQ), quantum yield of non-regulated energy for PSII (@NO) and electron transport rate II (ETR II) of safflower under artificial cold stressed conditions.

Treatment	Quantum yield of PS II (@PSII)	Non-photochemical quenching (@NPQ)	Quantum yield of non-regulated energy for PSII (@NO)	Electron transport rate II (ETR II)
Growth stage	<2.2e-16***	<2.2e-16***	<2.2e-16***	5.3e-11***
Temperature	3.86e-15***	<2.2e-16***	3.08e-11***	<2.2e-16***
Duration of exposure	0.46ns	7.38e-11***	1.42e-08***	0.99ns
Growth stage x temperature	5.89e-13***	9.43e-16***	5.53e-09 ***	0.95ns
Growth stage x duration	1.67e-11***	7.32e-13 ***	6.03e-11 ***	0.96ns
Temperature x duration	1.23e-06***	5.16e-05***	0.47ns	0.99ns
Growth stage x temperature x duration	0.08.	0.003**	0.10ns	1.00ns

Significance: ‘****’ $P \leq 0.001$; ‘***’ $P \leq 0.01$; ‘.’ $P \leq 0.1$; $P \leq 1.00$ -ns (not significant). ‘e’ is the exponential notation which informs to multiply the base number by 10 to the nth power (nth power given as the preceding number after e). Values in the columns are probability of a greater F ($P > F$).

Appendix 6. ANOVA summary table for quantum yield of PS II (@PS II), non-photochemical quenching (@NPQ), quantum yield of non-regulated energy for PSII (@NO) and electron transport rate II (ETR II) of safflower naturally cold stress under field conditions.

Treatment	Quantum yield of PS II (@PSII)	Non-photochemical quenching (@NPQ)	Quantum yield of non-regulated energy for PSII (@NO)	Electron transport rate II (ETR II)
Genotype	1.64e-06***	0.14ns	0.69ns	0.01**
Growth stage	0.91ns	0.001**	< 2.2e-16***	4.29e11***
Time	0.01**	7.76e-15***	< 2.2e-16***	< 2.2e-16***
Genotype x growth stage	0.06.	0.90ns	0.99ns	0.20ns
Genotype x time	0.64ns	0.88ns	0.95ns	0.01**
Growth stage x time	2.10e-06***	0.53ns	< 2.2e-16***	4.35e-14***
Genotype x growth stage x time	0.73ns	0.99ns	1.00ns	0.26ns

Significance: ‘****’ $P \leq 0.001$; ‘***’ $P \leq 0.01$; ‘.’ $P \leq 0.1$; $P \leq 1.00$ -ns (not significant). ‘e’ is the exponential notation which informs to multiply the base number by 10 to the nth power (nth power given as the preceding number after e). Values in the columns are probability of a greater F ($P > F$).

Appendix 7. ANOVA summary table for catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) of safflower under artificial cold stressed conditions.

Treatment	CAT	SOD	POD
Growth stage	<2.2e-16***	<2.2e-16***	<2.2e-16***
Temperature	<2.2e-16***	<2.2e-16***	<2.2e-16***
Duration of exposure	<2.2e-16***	<2.2e-16***	<2.2e-16***
Growth stage x temperature	<2.2e-16***	<2.2e-16***	<2.2e-16***
Growth stage x duration	<2.2e-16***	<2.2e-16***	<2.2e-16***
Temperature x duration	<2.2e-16***	<2.2e-16***	<2.2e-16***
Growth stage x temperature x duration	<2.2e-16***	<2.2e-16***	<2.2e-16***

Significance: ‘****’ $P \leq 0.001$; $P \leq 1.00$ -ns (not significant). ‘e’ is the exponential notation which informs to multiply the base number by 10 to the nth power (nth power given as the preceding number after e). Values in the columns are probability of a greater F ($P > F$).

Appendix 8. ANOVA summary table for catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) activity of safflower under field conditions.

Treatment	CAT	SOD	POD
Genotype	0.13ns	3.29e-07***	<1.5e-03**
Growth stage	<4.28e-08***	0.07.	< 6.7e-03**
Time	<2.2e-16***	<2.2e-16***	<2.2e-16***
Genotype x growth stage	0.01*	0.09.	0.76ns
Genotype x time	0.43ns	1.95e-06***	0.88ns
Growth stage x time	<7.91e-13***	2.2e-15***	1.23e-09***
Genotype x growth stage x time	0.81ns	0.98ns	0.40ns

Significance: ‘****’ $P \leq 0.001$; ‘***’ $P \leq 0.01$; ‘**’ $P \leq 0.05$; ‘.’ $P \leq 0.1$; $P \leq 1.00$ -ns (not significant). ‘e’ is the exponential notation which informs to multiply the base number by 10 to the nth power (nth power given as the preceding number after e). Values in the columns are probability of a greater F ($P > F$).

Appendix 9. ANOVA summary table for safflower genes under artificial cold stressed conditions.

Treatment	CtAH10T0001700.1	CtAH10T0001500.1	CtAH10T000500.1
Genotype	0.002**	0.013*	1.45e-12***
Growth stage	0.002**	0.134ns	7.41e-10***
Time	0.211ns	0.220ns	0.207ns
Genotype x growth stage	0.152ns	0.530ns	0.002**
Genotype x time	0.810ns	0.630ns	0.021**
Growth stage x time	0.379ns	0.917ns	0.794ns
Genotype x growth stage x time	0.358ns	0.054.	0.936ns

Significance: '****' $P \leq 0.001$; '***' $P \leq 0.01$; '**' $P \leq 0.05$; '.' $P \leq 0.1$; $P \leq 1.00$ -ns (not significant). 'e' is the exponential notation which informs to multiply the base number by 10 to the nth power (nth power given as the preceding number after e). Values in the columns are probability of a greater F ($P > F$).

Appendix 10. Light intensity measured under field conditions during the morning (from 0800-1000am), mid-day (from 1100-1200 noon) and late afternoon (1500-1600) times.

