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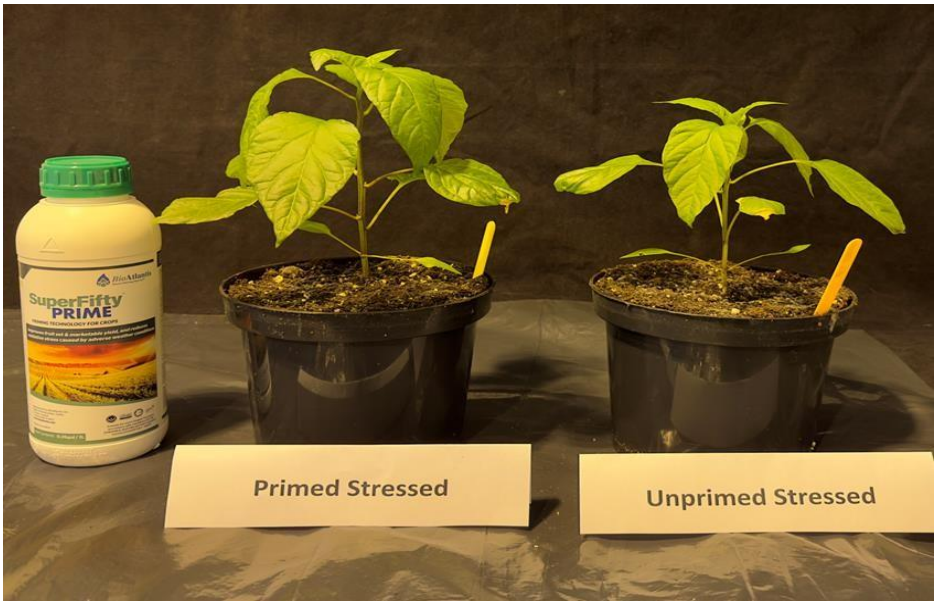
Title	Assessment of the effectiveness of SuperFifty® Prime in preventing cold stress in pepper (Capsicum annuum cv. Kurtovska Kapija)
Authors	Mahony, Cian
Publication date	2024
Original Citation	Mahony, C. 2024. Assessment of the effectiveness of SuperFifty® Prime in preventing cold stress in pepper (Capsicum annuum cv. Kurtovska Kapija). MRes Thesis, University College Cork.
Type of publication	Masters thesis (Research)
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Download date	2026-11-07 03:54:56
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Assessment of the effectiveness of SuperFifty® Prime in preventing cold stress in pepper (*Capsicum annuum* cv. Kurtovska Kapija).



Master of Research Thesis

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University College Cork 2024.

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Table of Abbreviations:

SFP	SuperFifty Prime	*PAL*	Phenylalanine ammonia lyase
SF	SuperFifty	*CHS*	Chalcone synthase
ROS	Reactive Oxygen Species	PQ	Paraquat
CAO	Cold Air Outbreak	PU	Primed Untreated
ICE	Inducer of CBF Expression	PT	Primed Treated
CBF	C-repeat (CRT)-binding factors	UU	Unprimed Untreated
COR	Cold Response gene	UT	Unprimed Treated
ml	Millilitre	BR	Biological Replicate
cm	Centimetre	L	Litre
hr	Hour	CHL	Chlorophyll
C	Celsius	mg	Milligram
v/v	Volume/volume	FW	Fresh Weight

1. Abstract:

The occurrence of abiotic stress is becoming increasingly common as climate change progresses, and this poses a major challenge for agriculture. Plant Biostimulants are a new form of abiotic stress management which induces the plants natural defence systems in a process known as "molecular priming". SuperFifty® Prime is a highly concentrated *A. nodosum* extract (500g/L) which has been proven to mitigate the detrimental effects of different abiotic stressors. Peppers were chosen for this experiment (*Capsicum annuum)* as they are a commercially important crop with an extremely low tolerance to cold conditions. In this project we aimed to investigate how a priming and recovery application of SuperFifty® Prime on a cold sensitive crop helped avoid and mitigate the damage caused by cold and uncover the molecular mechanisms behind this protection.

At the physiological level, the results showed that two applications of SuperFifty® Prime improved plant performance as demonstrated by monitoring the following parameters: fresh weight, electrolyte leakage, chlorophyll content, flavonoid content and the occurrence of necrotic tissue. The application of SuperFifty® Prime also improved plant recovery after the cold stress event. At final harvest, there was no significant difference in yield parameters between Primed Cold-Treated and Unprimed Untreated plants. At the molecular level, five genes of interest were selected. Three of these genes; *ICE 1, CBF 1* and *COR 314,* are part of most studied cold signalling pathway known as the *ICE-CBF-COR* transcriptional cascade. The two other selected genes; *PAL 1* and *CHS 1,* are important for flavonoid production. A similar pattern of expression was observed for all five genes with a clear difference between Primed and Unprimed Cold-Treated plants. Expression of *ICE 1* and *COR 314* are also upregulated in Primed Untreated plants, despite these plants not experiencing cold stress inducing conditions. Based on the evidence from this experiment, two applications of SuperFifty® Prime improved yield and productivity in Primed Cold- Treated plants and has the potential to improve grower incomes in areas affected by cold stress.

2. Introduction:

2.1 Climate change and Cold air outbreaks:

Climate change is one of the most significant challenges facing agriculture in modern times, with climate change leading to high variability in weather conditions and less predictable seasons (Easterling & Wehner, 2009). While a changing climate alone represents a major challenge, this is further exacerbated by global population growth, which is predicted to increase from 6.7 to 9.3 billion by 2050 and increasing atmospheric CO_2 concentrations which are set to rise from 380 ppm to 540-990 ppm by 2100 (Anwar *et al*, 2013). Compounding these problems, incidences of abiotic stress on crops will become more common due to the increased occurrence of adverse weather conditions associated with climate change. To meet the global food requirements of future human populations, farmers must increase agricultural output to meet the rising demands, eventually doubling current production. Food must also be produced in a more sustainable manner, in line with government policies and plans in relation to climate change and the environment.

While the term "Global Warming" suggests that heat and drought stress are the main problems associated with climate change, studies have shown that persistent cold snaps will also become more and more common as we progress through the 21st Century (Kodra *et al*, 2011). A study conducted on cold-air outbreaks (CAOs), using seven global climate models, lead to the prediction that, while the average winter temperatures will significantly increase by the conclusion of the 21st century, CAOs will not disappear, and in fact will increase in incidence in some regions (Vavrus *et al*, 2006). CAOs that occur in spring or early summer could have devastating effects on agriculture as crops are at their most vulnerable stage during early development. In the early stages of germination, plant meristematic tissue shows amplified sensitivity to stressors such as cold (Takahashi et al, 2021). As the shoot apical meristem is vital to active plant growth and development (Olas et al, 2021), damage can have long lasting effects throughout the plants maturity. This poses a major problem for farmers as sudden decreases in temperature can impair normal plant physiological, biochemical, metabolic, and molecular processes, and these changes will negatively impact on yields. Cold stress can be divided into two categories, chilling and freezing. Chilling stress can be defined as the negative effects plants face when exposed to low but not freezing temperatures,

typically between 0°C - 15°C. In contrast, freezing stress is caused by temperatures under 0°C resulting in ice crystallization in the plant tissue.

Cold stress can cause immense financial damage to farmers and the agricultural sector at large. In 2009, prevailing cold temperatures in Japan caused significant crop losses and resulted in a 58 billion Yen (€310,000,000) loss (Soualiou *et al*, 2022). In Greece, cold conditions such as frost/chilling account for 34.5% of weather-related crop losses (Papagiannaki *et al*, 2014). Many countries are dependent on a select number of staple crops such as cotton, soybean, maize and rice, all of which are cold sensitive to temperatures under 15°C and unable to survive in these conditions over prolonged periods (Larcher, 1995). This means that cold stress is a huge threat to national economies, and a bad cold spell can cause substantial harm to a country's food production.

2.2 Abiotic Stress and Plant Responses:

Abiotic stressors can be defined as non-living environmental factors, for example, drought, heat, cold and salinity, which can have a negative impact on living organisms in a specific environment. In the agricultural sector, abiotic stress is one of the main reasons for massive monetary and production losses (Moglia & Portis, 2016). The experiments carried out in this thesis are focused on cold stress and the negative impacts it has on pepper production, a species with an extremely low tolerance to cold conditions. The optimal temperature range for pepper growth is between 21°C and 27°C, with growth impaired once the temperature reaches below 15°C and above 30°C (Deng *et al*, 2009). Cold exposure can cause stress, including damage to the cell membrane system, and may eventually lead to cell death. Cold exposure may also cause injury symptoms to the plant which typically appear 48 to 72 hours following cessation of cold exposure. Short term phenotypic symptoms can include injuries such as reduced leaf expansion, wilting and chlorosis (yellowing of leaves) and eventually areas of necrotic tissue may form on the leaf. Long term stress effects on plants that survive cold exposure are poor germination, stunted seedlings, withered and reduced tillering and reduced yield (Jiang *et al*, 2022). A study on wheat, published in 2021, showed that plants that had undergone cold stress at the juvenile stage suffered from stunted growth, diminished root-shoot surface area, leaf chlorosis, and disturbed water and nutrient relations. These impairments may lead to a severe reduction in wheat yield and quality (Hassan *et al*, 2021).

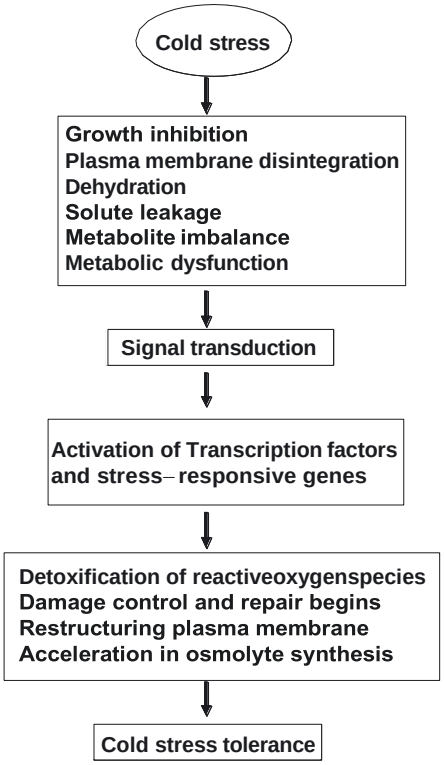


Figure 1: Fundamental responses of plants when subjected to cold. Cold may cause cold stress, and this is perceived by plants as a stress stimulus, which in turn leads to the activation of transcription factors and cold responsive genes. Diagram derived from (Kumar, 2009).

Unlike animals, plants are sessile and cannot avoid stressful environments, therefore they developed complex specialised mechanisms to survive. The most studied cold signalling pathway is the *ICE-CBF-COR* transcriptional cascade. This pathway responds to cold through C-repeat (CRT)-binding factors (CBFs), also known as dehydration-responsive element binding factors (DREBs), which are quickly induced by *ICE1* (*Inducer of CBF Expression*), which then bind to the promoter regions of *COR* genes to activate transcription (Lei *et al,* 2019). The transcripts of the *COR* gene activation are numerous, and most transcripts are thought to function in some capacity in cold acclimation and subsequent freezing tolerance. These transcripts include enzymes to biosynthesize osmo-protectants, transcription factors, protein kinases, proteins associated with lipid metabolism, cell wall modifiers and chloroplast proteins (Liu *et al*, 2019).

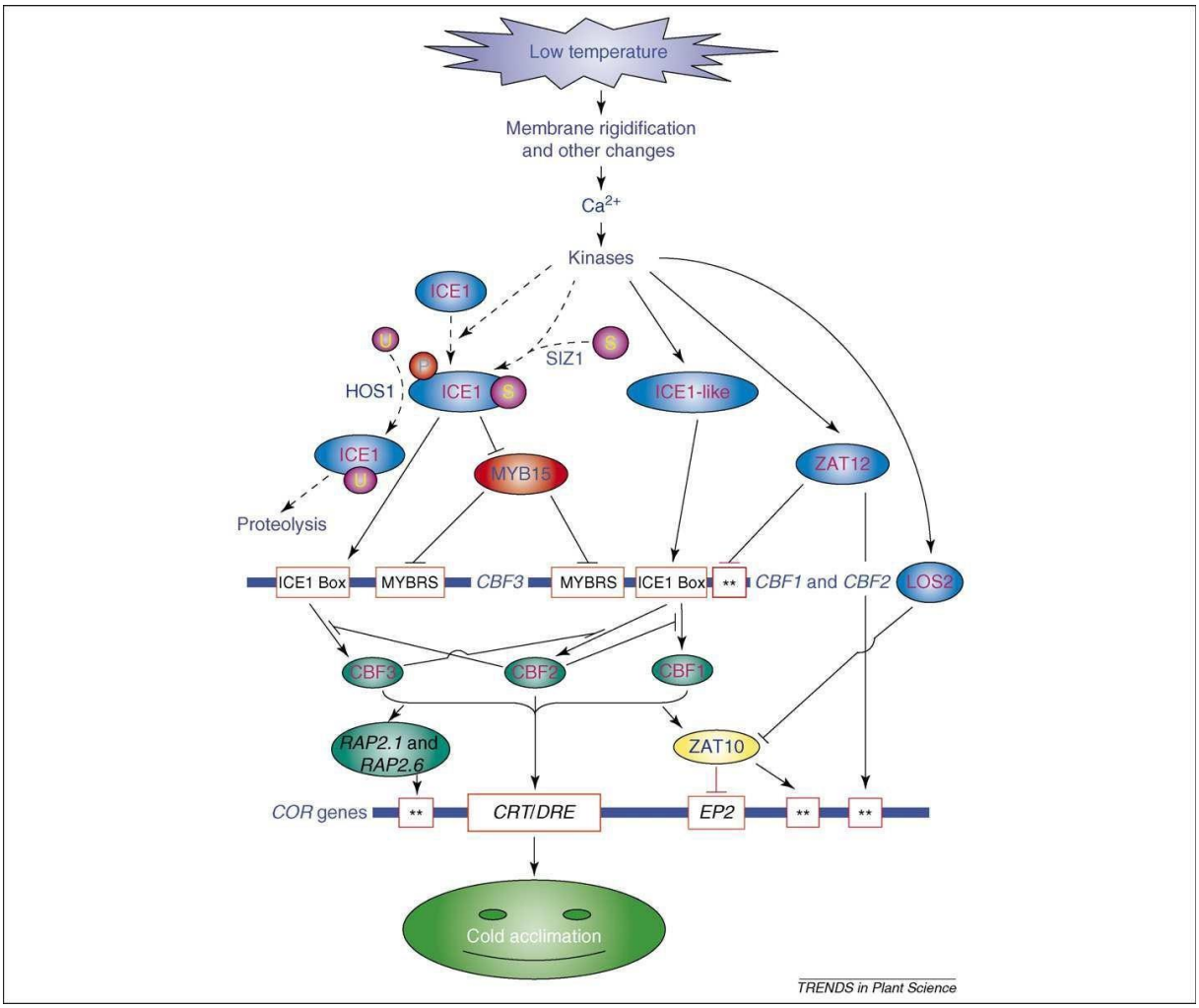


Figure 2: A diagram of the cold responsive network in *Arabidopsis* plants. *ICE1* is activated by cold stress through SUMOlyation and phosphorylation. Once stimulated through SUMOlyation, *ICE1* begins activation of *CBF* transcription. *CBF* genes then regulate the expression of *COR* genes that confer freezing tolerance. Diagram taken from (Chinnusamy *et al*, 2007).

Another method of plant protection against abiotic stress is through increased production of flavonoids. Flavonoids are a group of plant polyphonic secondary metabolites and comprise of four major classes: anthocyanins, flavonols, flananols and proanthogens. Secondary metabolites are the intermediate or product of metabolism and are not directly involved in the development and growth of the plant. They serve a number of different roles throughout the plant such as defence against other organisms, symbiosis, increased tolerance to abiotic stress and inducing flowering and abscission (Bano *et al*, 2023). Flavonoids are formed through two biosynthetic pathways, the phenylpropanoid pathway in which the enzyme phenylalanine ammonia lyase (PAL) is used and the polyketide pathway where the enzyme chalcone synthase (CHS) is used (Dias *et al* 2021). Plants subjected to cold can biosynthesize

more phenolic compounds than those growing under optimal conditions. The produced compounds are capable of scavenging free radicals decreasing the detrimental effects of oxidative stress. Enhanced expression of enzymes can be measured through the upregulation of the transcript levels of genes encoding key enzymes for phenolic production such as PAL and CHS (Sharma *et al*, 2019).

2.3 Biostimulants:

Plant Biostimulants enable a relatively new form of abiotic stress management. The European Biostimulant Industry Council (EBIC) has defined plant biostimulants as "substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality" (European Biostimulants Industry, 2012). One of the most efficient ways to apply plant biostimulants is through a mechanism known as "molecular priming". Molecular priming is an environmentally friendly strategy developed in recent years that has increased plants resistance to abiotic stressors, while also aiding traditional breeding and biotechnological procedures. Molecular priming is an approach of pre-treating a plant with a natural compound before a stress event, which induces the plants natural defence systems, increasing mineral uptake and antioxidant enzyme activity (Turhan *et al*, 2020).

A wide range of biostimulants are obtained from the marine macroalgae *Ascophyllum nodosum*, including the product used in this study, SuperFifty® Prime (SFP). SFP is a highly concentrated *A. nodosum* extract (500g/L) produced under conditions of high temperature and pressure (Omidbakhshfard *et al*, 2020). *A. nodosum* extracts are rich in polyphenols and unique polymers such as fucoidan and alginates, some of which are recognised for their antioxidant properties that could potentially mitigate harmful reactive oxygen species (ROS). While it is firmly established that *A. nodosum* extracts are effective on domesticated plants and can improve growth and resilience to abiotic stressors, a clearer understanding of the underlying molecular mechanisms is still required (Rasul *et al*, 2021).

Previous studies have proven that SuperFifty® Prime is efficient in mitigating the harmful effects of oxidative stress and drought (Rasul *et al,* 2021, Staykov *et al*, 2020 and Omidbakhshfard *et al,* 2020). The study undertaken by Rasul *et al* (2021) compared plants that were primed with SuperFifty® (a product similar to SuperFifty Prime) two days before the onset of drought (SF + drought), versus control plants sprayed with water (H_2O + drought) and

found that ROS levels in droughted plants were decreased when primed with SuperFifty®, whist relative water content remained higher. The study also found that electrolyte-leakage, a measure of cell damage, was diminished compared to control plants. This could be associated with downregulation of the dehydration and H_2O_2-induced NAC transcription factor *RD26*, which is involved in abiotic stress signaling, by priming with SuperFifty® (Rasul *et al*, 2021) .There have been numerous studies showing that the molecular responses underlying drought and cold stress are very similar as the majority of the genes that respond to drought at the transcriptional level also respond to cold (Shinozaki & Yamaguchi-Shinozaki, 1996). Therefore, it can be hypothesised that the compounds in SuperFifty® Prime that upregulated drought response genes in Rasul *et al.* (2021) may also be involved in the upregulation of key genes for cold response.

2.4 Reactive Oxygen Species (ROS):

Every plant accumulates Reactive Oxygen Species (ROS) as a by-product of metabolism. Whilst well-known for causing oxidative stress, ROS is also a secondary messenger in various biological processes, most importantly under stress conditions (Mittler, 2002). ROS accumulation can also be triggered by abiotic stressors such as high light, high/low temperature, nutrient deficiency and excess salinity. The impact of ROS rests on the equilibrium between the generation of ROS and its scavenging. (Ghosh and Majee, 2023). Plants have numerous antioxidants and anti-oxidative enzymes along with a variety of molecules to dissipate oxidant accumulation. However, when ROS production exceeds the plant antioxidizing capacity, this can lead to cell damage and eventual cell death by ROS toxicity.

A recent study (Omidbakhshfard *et al,* 2020) proved that an application of SuperFifty® (SF) leads to the induction of ROS marker genes, genes involved in ROS-induced programmed cell death and autophagy-related genes. Oxidative stress was induced by treating three-week-old *A. thaliana* Col-0 plants with 15µM of the superoxide producer paraquat (PQ). Treatment groups consisted of plants that were primed with SF two days before PQ application through foliar spraying (SF + PQ) while control plants were sprayed with water (H_2O + PQ). Severe electrolyte leakage was observed in paraquat treated control plants while plants primed with SF and then treated with paraquat had ion leakage levels similar to the wild type control (H_2O + H_2O). There was also a statistically significant difference in the occurrence of necrotic lesions

between both treatment blocks. Control plants (H_2O + PQ) showed significantly higher levels of necrotic tissue when compared with those primed with SF (SF + PQ).

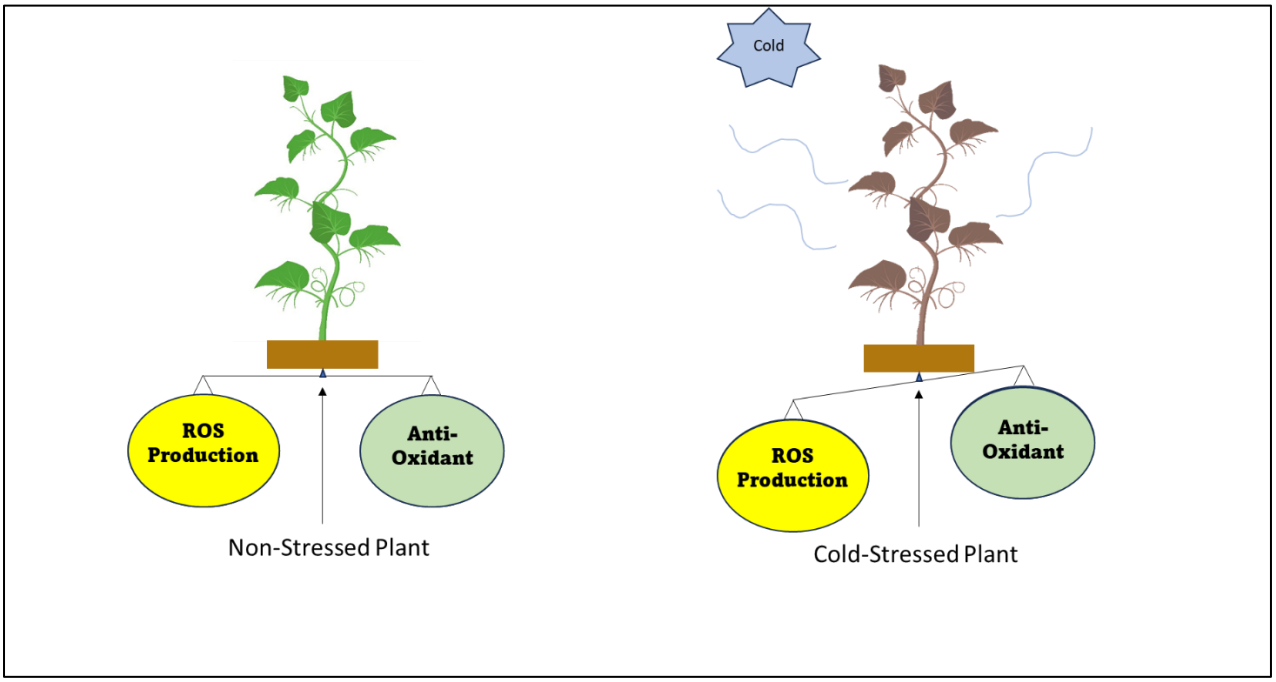


Figure 3: Diagram depicting ROS imbalance in a cold-treated plant compared to a non-cold-treated plant. The non-Cold-Treated plant is maintaining equilibrium as it is producing sufficient antioxidants to balance normal ROS production. The Cold-Treated plant is stressed, producing excessive levels of ROS which is over the threshold the plant can deal with naturally. With no intervention this will eventually result in cell death.

2.5 Objectives of thesis:

The aim of this research project was ro assess the efficiency of the plant biostimulant SuperFifty® Prime in mitigating the detrimental effects of cold on a cold sensitive crop variety. The author of this thesis was based in Bulgaria for the first two months of the research project, as he completed his allotted secondment time as part of the 'RESIST' project (EU HORIZON-MSCA, Grant ID: 823746). Therefore, the first biological replicate of this experiment was undertaken and accessed in the facilities of the Centre of Plant Systems Biology and Biotechnology (CPSBB) in Plovdiv, Bulgaria. Interestingly, it has been observed that variations in temperature due to climate change are increasing the impact that cold stress is having on agriculture in Bulgaria (Fadon *et al*, 2020). Cold temperatures that once occurred in January and early February are now occurring in March and April in Bulgaria. This is affecting early flowering species which typically have a low tolerance to cold stress. According to Mr. Nikolay

Kolev, fruit grower and chairman of the Union of Danube Fruit Growers, Bulgaria, "*Climate change is affecting the fruit sector. In recent years there has been a massive frost damage to early varieties and partial frost damage to medium-early varieties of fruit species.This year (2021) is no exception: January and February were extremely warm, and in March there were return frosts with temperatures up to -10 ° C, temperatures that are detrimental to apricots*" (Agrovar, 2021).

The first section of this project was focused on assessing the physiological responses associated with SuperFifty® Prime (SFP) and control (mock) treatments before and after plants were exposed to cold. The four treatment blocks were as follows: Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT). Each treatment group was sprayed twice (either with SFP – primed plants; or water – control plants), 48 hrs before and after cold treatment. Cold treatment consisted of 48 hrs of a constant 2^{o}C temperature. Separate to the period of cold treatment there was a gradual decrease and increase on either side of the treatment starting and finishing at 12^{o}C (±2^{o}C every hour). The following parameters were measured: fresh shoot weight, electrolyte leakage, chlorophyll content, flavonoid content, area of necrotic tissue and total fruit weight/number.

These analyses were then complemented with an assessment of gene expression levels associated with each of the tested conditions. Sample preparation and treatments were exactly the same as described above. Three genes were selected as candidates for analysis due to their importance in cold acclimation and frost tolerance: *Inducer of CBF Expression 1, C-repeat/DRE binding factor 1* & *Cold Regulated 314* (*ICE 1, CBF 1* & *COR 314*). A further two more genes were selected for their importance in flavonoid production: *Phenylalanine Ammonia Lyase* & *Chalcone synthase* (*PAL 1* & *CHS 1*). The expression of these three housekeeping genes (*UB3, BETA-TUB & GADPH*) was initially assessed to identify which of them would be most suitable to use as a normalisation standard in our qPCR analysis. This approach allowed me: 1) to establish a reproducible protocol for gene expression analysis in pepper including a working protocol for RNA extraction and cDNA synthesis; 2) to design and test specific set of primers that could be used to effectively measure the expression of the selected genes, and 3) to determine the expression levels of these genes of interest in pepper plants exposed to different SFP treatments and stress conditions.

3. Methods and Materials:

3.1 Phenological:

3.1.1 Growth Conditions & Trial Layout:

The pepper cultivar, Kurtovska Kapija, was selected due to its importance in Bulgarian agriculture and its poor cold tolerance. Kurtovska Kapija plants typically grow between 50-60 cm high and produce 7-8 peppers per plant. There were three biological replicates (BR) in total (Figure 4); one undertaken and expressed in Centre of Plant Systems Biology and Biotechnology (CPSBB, Plovdiv, Bulgaria) and two in BioAtlantis (Tralee, Ireland). Plants were sown in seedling trays for two weeks post germination. Plants were then transplanted into growing pots (9 cm) and allowed to recover for a week to avoid stresses arising due to transplantation which could influence the experiment. A week after the conclusion of cold treatment, the remaining plants were transplanted into 1.5L pots. In the case of biological replicate 2, plants were transplanted into 5L pots to facilitate them growing to full maturity. Plants were sprayed with SFP at a concentration of 0.15% (*v/v*). Spraying was applied from the top, at a 15cm distance, until each leaf was covered in a fine mist.

In CPSBB, biological replicate 1 was assessed in an Aralab FitoClima PLH 12.000 growth chamber. Growth conditions were kept at 24°C ±2 under a 16-hr day / 8-hr night regime before cold treatment. Cold treatment was applied as 2°C under a 12-hr day / 12-hr night regime. Once the 48 hr of cold treatment had been completed, conditions reverted to pre-cold conditions. Light was supplied by Systion LED tubes with CCT of 4000 K and CRI of 80. The physiological components of the experiments biological replicates 2 & 3 were assessed in the growth chambers and laboratory at BioAtlantis. Plants were kept in the same light and temperature conditions as biological replicate 1. Cold-treatment was carried out in a display fridge that was customised with LED lights to ensure equal light distribution and the correct light timings outlined above were adhered to (https://www.nisbets.ie/nisbets-essentials-upright-display-cooler-black/dg280).

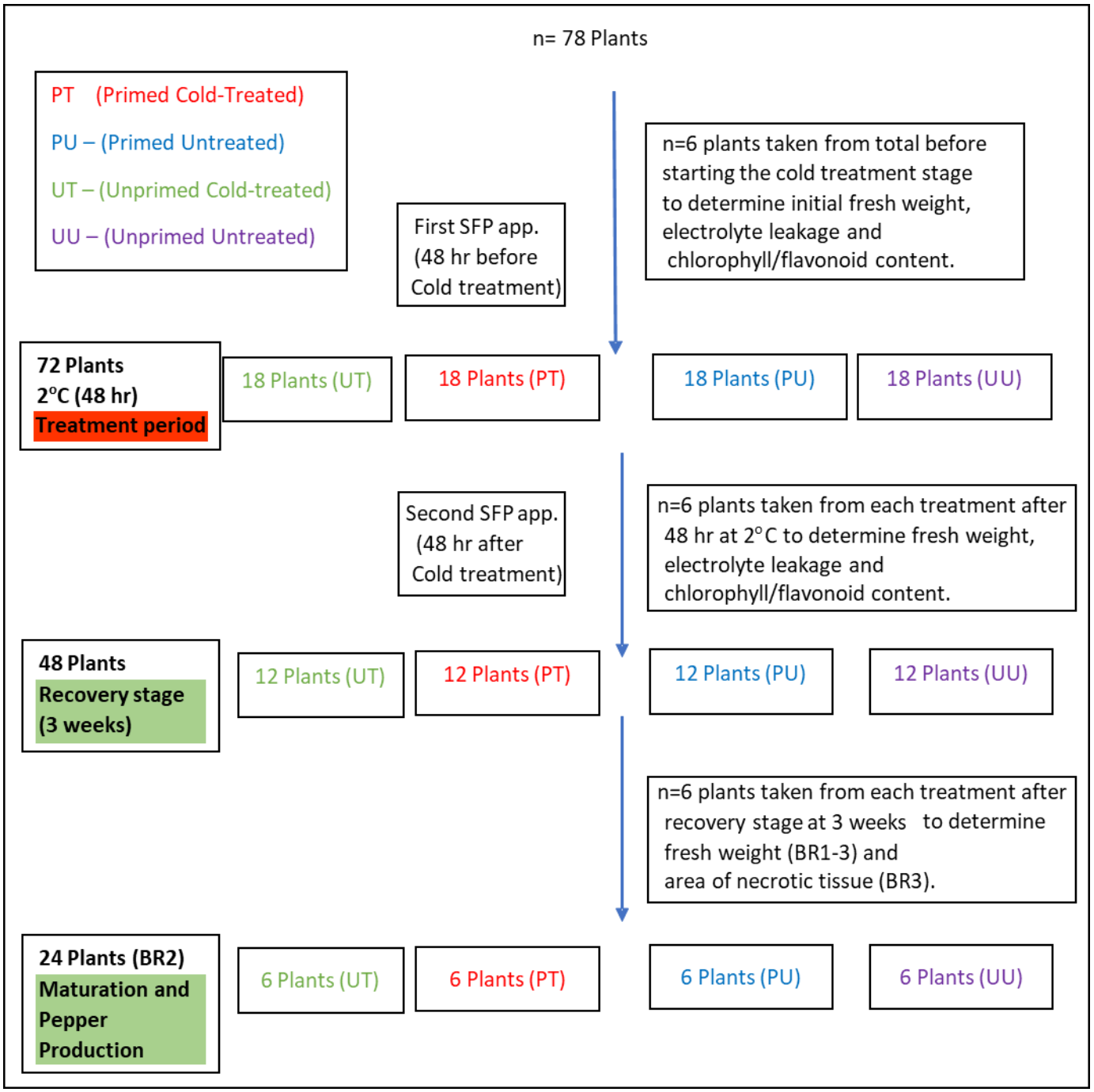


Figure 4: Diagram outlining the trial layout of the experiments. Plants were primed with SFP 48 hr before cold treatment. A second application of SFP was applied 48 hr into recovery stage. Physiological, phenotypic and biochemical parameters were measured at several timepoints for each biological replicate.

3.1.2 Fresh Shoot Weight:

Fresh shoot weight was taken at three points throughout each biological replicate. First measurements were taken before cold treatment commenced when the plants were a month old; six plants were taken from UU to get the initial fresh weight. Once cold treatment had concluded, six plants were taken from each condition (UU, UT PU & PT) to measure weight

after cold treatment had been applied. For biological replicate 1, a third fresh weight measurement was taken three weeks after cold treatment had been applied.

3.1.3 Electrolyte leakage:

Electrolyte leakage as a measure of membrane damage was measured and calculated using a protocol as described in (Gechev *et al,* 2004). Damaged leaves were first washed with distilled water to avoid any errors from surface impurities. Leaves were carefully removed from the main plant and immediately immersed in deionized water (30 ml) in a falcon tube (50 ml). Extra care was taken to avoid any damage to the leaf tissue during sampling. Sample tubes were shaken at 22^{o}C for 1 hr and an initial conductivity reading was made using a conductivity meter (HANNA, HI-8733). Readings were taken again after 24 hr, after which samples were boiled at 100^{o}C for 3 minutes and allowed to cool to room temperature. One final reading was taken on boiled samples to find the total conductivity. Electrolyte leakage was calculated as percentage of (initial conductivity – 24 hr conductivity) the total conductivity. The increase in electrolyte leakage (conductivity) was expressed as percent of the total.

3.1.4 Chlorophyll & Flavonoid content:

Two different methods were used to calculate chlorophyll and flavonoid levels. In biological replicate 1, which was conducted in Bulgaria, a spectrophotometer was used. In biological replicates 2 & 3, which were conducted in BioAtlantis, Tralee, the Dualex leaf sensor was used to calculate Chlorophyll & Flavonoid contents.

Biological Replicate 1:

Fresh leaf material (200 mg) was placed in a mortar with a small amount of liquid nitrogen (10 ml). Plant material was ground until the N had boiled off and the plant material had been crushed into a pale green powder. Once liquid nitrogen had evaporated, 80% (v/v) acetone (5 ml) was added to the mortar and the samples were grinded until a clear, green extractable liquid was produced. A pipette was used to extract 2 x 1.5 ml of the solution into labelled Eppendorf tubes. Tube 1 from each replicate was centrifuged at 900 x g for 5 minutes while the other Eppendorf tubes were kept as a back-up. Supernatant (1 ml) was then extracted from tube 1 from each replicate and placed in a cuvette for photometric analysis.

The spectrophotometer was zeroed before measuring the samples by placing ‘blank’ (80% v/v acetone (1ml) cuvettes in the two chambers. Once the baseline had been set, one cuvette

was removed, and a sample cuvette is placed in the measurement chamber. All samples had light absorbance measured at 663nm for chlorophyll a, then again at 645nm for chlorophyll b. The chlorophyll a & b contents for each sampling plot were calculated as a proportion of the original sample (Perez *et al*, 2018).

Chl a: $12.25*Abs._{663} - 2.79*Abs._{647}$ µg/mg/200mg Fresh Weight

Chl b: $21.5*Abs._{647} - 5.1*Abs._{663}$ µg/mg/200mg Fresh Weight

Chl a+b: $7.15*Abs._{663} + 18.71*Abs._{647}$ µg/mg/200mg Fresh Weight

Biological Replicate 2 & 3:

The Dualex leaf sensor was calibrated before measurements were taken. To calibrate, the leaf sensor was turned on and a reading was taken while empty. To make a measurement the clip was opened by pressing the handle and similarly aged leaves from each replicate were placed between the optical heads. Once the handle is released the measurement is automatically made. To ensure that the measurement taken is correct a long beep will emit from the sensor. Three short beeps meant that the measurement was wrong and needed to be redone.

3.1.5 Necrotic Tissue:

Given that there was not enough space or time to grow plants from biological replicate 3 (BR3) to maturity as was done in biological replicate 2 (BR2), the remaining plants in BR3 were selected for assessment of necrotic tissue damage. The largest true leaves from each plant from treatment blocks UT and PT were taken a week after cold treatment had concluded. Pictures were taken on a well-lit black background with a ruler in frame to illustrate the scale. Images were transferred to a computer where Image J (https://imagej.net/ij/) was used to calculate the total area of necrotic tissue on each leaf. Total area of necrotic tissue was then divided by total leaf area and multiplied by 100 to calculate the percentage of leaf covered in necrotic tissue.

3.1.6 Pepper Number and Weight:

Remaining pepper plants from biological replicate 2 were allowed to grow to maturity and produce harvestable fruit. Due to time and space constraints, the third biological replicate could not be grown to maturity, the time required between sowing to harvest was approximately 6 months. Plants were transplanted into 5 litre pots one month after the

conclusion of cold treatment and 50 g of Miracle Grow continuous release plant food was added. Plants were kept on an 18-hr day/ 6-hr night regime throughout the course of their maturity with a temperature of 26^{o}C during the day and 21^{o}C at night. Once plants had produced a full crop and peppers had ripened from green to red, a harvest was carried out and all peppers were carefully counted and weighted.

3.2 Gene Analysis:

3.2.1 RNA Extraction:

RNA extraction was done with precautions to protect RNA from degrading during the grinding and extraction processes. Leaves were cut from plants and immediately placed in a mortar with liquid nitrogen. Plant material was quickly ground down using a pestle until a fine powder was formed and liquid nitrogen had evaporated off. Samples were immediately placed in two labelled chilled Eppendorf tubes (1.5 ml) until approximately half full and moved into the -80^{o}C freezer. RNA was extracted from BR (2 & 3) using the Qiagen RNeasy Plant mini kit following the manufacturer's instructions.

Briefly, one half full Eppendorf was taken for each sample and filled with a buffer RLT (guanidium thiocyanate lysis buffer, Qiagen; 50 µl) (with β-ME added) and vortexed. The lysate was transferred to a QIAshredder spin column and centrifuged at full speed for two minutes. The remaining supernatant was then transferred to new Eppendorf (1.5 ml) and mixed with ethanol (96%-100%) at 0.5x of the total supernatant volume. Samples were then transferred to an Rneasy spin column and centrifuged for 15 s at 10,000 rpm. The flow through was discarded upon completion. The column was then washed with buffer RW1 (guanidine salt and ethanol) (350 µl) and this was centrifuged at 10,000 rpm for 15 s (the flow through was discarded). A mix (10 µl Dnase I stock solution & 70 µl Buffer RDD) was added to the spin column and allowed to sit for 15 minutes at room temperature. Upon completion, Buffer RW1 (350µl) was added and centrifuged at 10,000rpm for 15 s (the flow through was again discarded). This step was then repeated twice with Buffer RPE (ethanol; 500 µl), first at 15 s and secondly at 2 minutes. After centrifugation, the Rneasy spin column was removed from it's Eppendorf tube and placed in a new collection tube (1.5 ml). RNAse -free water (30 µl) was then added to the spin column. A final centrifuge was completed for 1 minute at

10,000 rpm to elute the RNA. A nanodrop machine was used after RNA extraction process to determine RNA concentration.

3.2.2 cDNA Synthesis:

The NZY first-strand cDNA synthesis kit was used to synthesize cDNA from the extracted RNA following the manufacturer's instructions. Two reaction mixes were prepared on ice before the reaction began. Mix 1 was made up of Oligo (dT; 1 µl) primer mix and 10x Annealing Buffer (1 µl) per sample. Mix 2 was made up of NZYRT 2x Master mix (10.3 µl) and NZYRT Enzyme Mix (2.6 µl) per sample. Lastly, 1 µg of total RNA was mixed with dH_2O up to a final volume of 6 µl. Initially 2 µl of Mix 1 and 6 µl of diluted RNA were added to a strip tube and this reaction was incubated at 65°C for 5 minutes in a thermocycler. Once this step was completed 12 µl of mix 2 was added to the strip tube and this mixture was incubated at 50°C for 30 minutes and then at 85°C for 5 minutes, consecutively. Lastly, RNase H (1 µl) was added and a final incubation was done at 37°C for 5 minutes.

Once cDNA synthesis was completed, expression of the housekeeping genes was assessed to ensure that the cDNA synthesis process was equally efficient and of sufficient quality to be used in a qPCR analysis. Three primer sequences for the housekeeping genes (*Beta Tubulin* (*B-TUB*), *Ubiquitin-conjugating protein* (*UB3*) and *Glyceraldehyde-3-phosphate dehydrogenase-GapCp* (*GAPDH*)) were obtained from a paper published by Wan *et al*, (2011).

3.2.3 Primer Design & Assessment:

Flavonoid biosynthesis genes were identified based on the two biosynthesis pathways referenced in the introduction section of this thesis. The cold stress response genes were identified from an extensive literature review. Three genes *ICE 1* (AT3G26744), *CBF 1* (AT4G25490) and *COR 314* (AT1G29390) were selected for their importance to the cold stress response. Two genes *PAL 1* (AT2G37040) and *CHS 1* (AT5G13930) were selected for their importance to flavonoid production. Full cDNA sequences were obtained on Tair (https://www.arabidopsis.org/index.jsp) and blasted to the Sol genomics website (https://solgenomics.net/) where the *Capsicum annuum* genome sequence can be found. Both cDNA strands were inserted into clustalw (https://www.genome.jp/tools-bin/clustalw) and conserved regions of the coding region were identified. Primers were designed around these regions.

Primers were first tested for amplification efficiency to optimise the final reaction concentrations. Amplification efficiency of a primer pair can be lower than 100% for a variety of reasons, the most common being poorly designed primers and non-optimal reagent concentrations. Secondary structures such as dimers and hairpins or inappropriate melting temperatures can also result in primer-template annealing which lowers amplification efficiency (Biosistemika, 2017). First, PCR was performed and the primer pairs specific for each gene were tested using the first strand cDNA. Amplicons of expected size were checked using an agarose electrophoresis gel (100 base pair ladder). Once the expected band had been found in the gel and no unspecific bands were present, two sets of primer pairs for each gene were tested in a qPCR reaction using the Applied Biosystems™ 7500 Real-Time PCR System SDS machine. A four-times dilution series was used so each gene had its primer pairs diluted to 1:4, 1:16 and 1:64. In a ten-fold dilution the Ct difference between two consecutive dilutions should be 3.3, assuming 100% amplification efficiency. After Ct values had been obtained, they were plotted on a logarithmic scale along with corresponding concentrations. A linear regression curve was generated through the data points and the slope of the trend line was calculated. Efficiency was calculated as $E=-1+10(^{-1/slope})$.

3.2.4 Quantitative real-time PCR (qPCR) Analysis:

Quantitative real time PCR was performed in 96 well plates using an Applied Biosystems™ 7500 Real-Time PCR System SDS machine. The protocol used was as follows: denature at 94^{o}C for 30 s followed by 40 cycles at 94^{o}C for 10 s, annealing and amplification at 60^{o}C for 30 s. Computation of a melting curve was undertaken by holding a temperature of 94^{o}C for 15 s, with a gradual increase in temperature from 60^{o}C to 94^{o}C in 0.5^{o}C increments for 15 s intervals. All reactions were performed in triplicates using SYBR Green-PCR Master Mix (Takara, Japan) using the most efficient of the gene specific primers. Expression data was normalised against *BETA-TUBILIN* as a reference gene using the $2^{-deltadeltaCt}$ method (Rao *et al*, 2013).

Results I: Physiological responses to cold treatment:

4.1.1 Fresh Shoot Weight:

The fresh weight measurements were taken from wild type control i.e., Unprimed Untreated (UU) plants before starting the cold treatment, the mean shoot weight was 1.92 grams (g) across all three biological replicates. Plants were then weighed immediately after the cold treatment period ended (Figure 5 (a)). Both Unprimed Cold-Treated (UT) and Primed Cold-Treated (PT) plants subjected to 48 h of cold treatment lost fresh biomass weight with UT weighting 1.57 g and PT weighting 1.74 g. The increase in biomass weight for both UU and PU was observed after 48 h of cold treatment with UU and PU weighting 2.51 g and 2.62 g respectively. This increase in fresh shoot weight in UU and PU was significantly higher compared to UT and PT after 48 h of cold treatment, however we failed to see a significant difference when comparing primed plants subjected to the cold treatment with those that were unprimed

However, this was not the case when fresh weight was assessed in 3 weeks post-cold treatment. At this time point there was a significant difference in fresh shoot weight between UT and PT (Figure 5 (b)), with UT weighting 9.04 g and PT weighting 11.43 g, a percentage increase of 26%. In addition, there was a slight difference (although non-significant) between UU (15.22 g) and PU (15.79 g) plants. However, both treatment blocks (UU, PU) weighed significantly more than UT and PT plants.

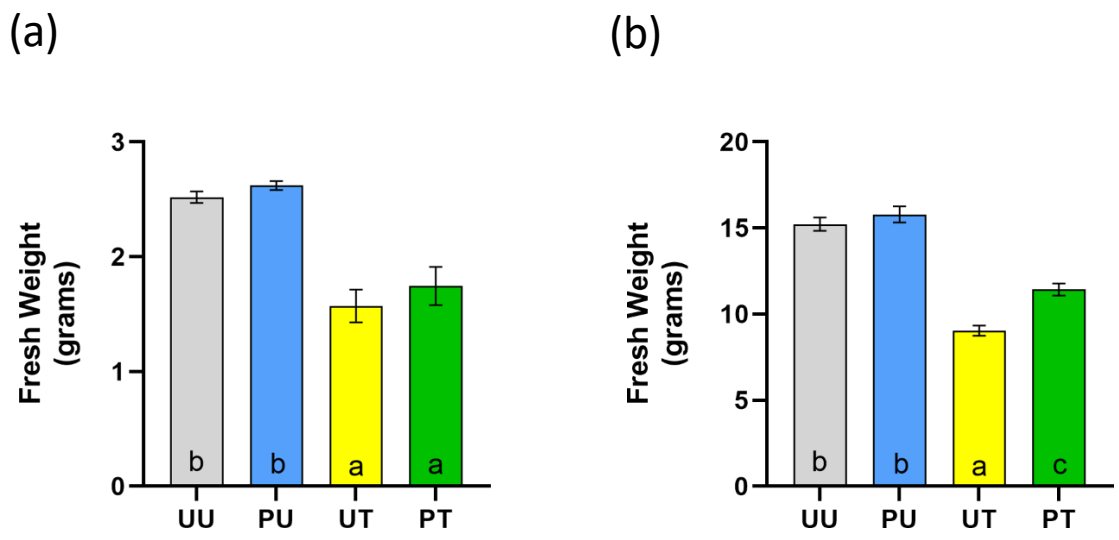


Figure 5. Mean fresh shoot weight from plants across all four treatment groups in three biological replicates. Panel a. Measurements of fresh shoot weight taken after 48 h of cold treatment. Panel b. Fresh shoot weights taken after three-week post-cold recovery period. Values are expressed as mean averages across three biological replicates (n = 18 (BR 1, 2 & 3); One-Way ANOVA followed by Tukey's multiple comparisons test was undertaken. p-value < 0.05 would indicate a significant difference. Different letters represent a significant difference between the treatment means. Sharing of letters denotes no significant difference between the means. Error bars denote standard error of the mean (SEM). Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

4.1.2 Electrolyte leakage:

Electrolyte leakage, as a measure of membrane damage under stress, was taken from all four treatment blocks immediately after cold treatment had concluded. As shown in Figure 6, leaves taken from Unprimed Cold-Treated (UT; 9.65% conductivity) plants showed significantly higher rates of electrolyte leakage than Unprimed Untreated (UU; 3.7% conductivity), Primed Untreated (PU; 3.69% conductivity) and Primed Cold- Treated plants (PT; 5.91% conductivity). Leaves in the UT (9.65 % conductivity) group had a 161% increase in electrolyte leakage over untreated plants (UU & PU) while having a 63.5% increase over PT (5.91 % conductivity). Primed Cold-Treated (PT) plants showed a significant reduction of 38.7% in electrolyte leakage when compared with Unprimed Cold-Treated plants (Figure 6).

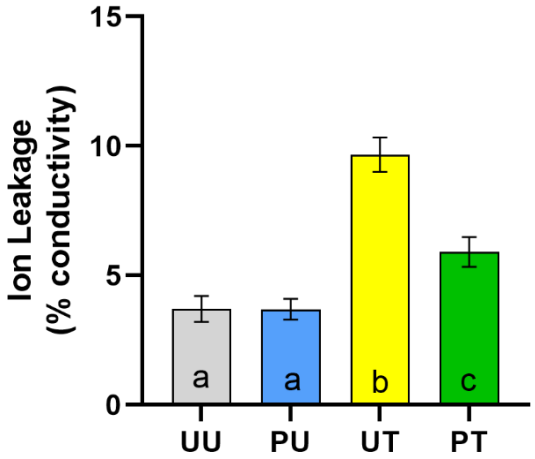


Figure 6. Electrolyte Leakage as a measure of membrane damage from plants across all four treatment blocks. Readings taken from all four treatment groups after conclusion of cold treatment. Values are expressed as mean averages across three biological replicates (n = 18

(BR 1,2&3); One-Way ANOVA followed by Tukey's multiple comparisons test was undertaken assuming a *p*-value < 0.05 as significance. Different letters represent a significant difference between the treatment means. Sharing of letters denotes no significant difference between the means. Error bars denote standard error of the mean (SEM). Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

4.1.3 Chlorophyll:

Chlorophyll levels (chlorophyll a, chlorophyll b and total chlorophyll) were assessed using standard protocols. No significance was found from these readings due to high variability and a low sample number, however a distinction can be observed between Unprimed Cold-Treated and the rest of the experimental groups. As shown in Figure 7 (a), chlorophyll a content in Unprimed Cold-Treated plants (UT; 21.76 µg/mg) was 25.7% lower than in Unprimed Untreated (UU; 29.32 µg/mg) plants. In Primed Cold-Treated (PT; 26.9 µg/mg) plants, chlorophyll a content was 8.1% lower than in Unprimed Untreated (UU; 29.32 µg/mg) biomass. Chlorophyll b levels displayed (Figure 7(b)) a similar trend since Unprimed Cold-Treated (UT; 6.4 µg/mg) levels were 32.6% lower than Unprimed Untreated (UU; 9.5 µg/mg). On the other hand, Primed Cold-Treated (PT; 10.5 µg/mg) had an increased chlorophyll b levels of 10.5%. Chlorophyll a+b is a combination of both chlorophyll pigments and gives a complete view of chlorophyll levels measured in the plant.

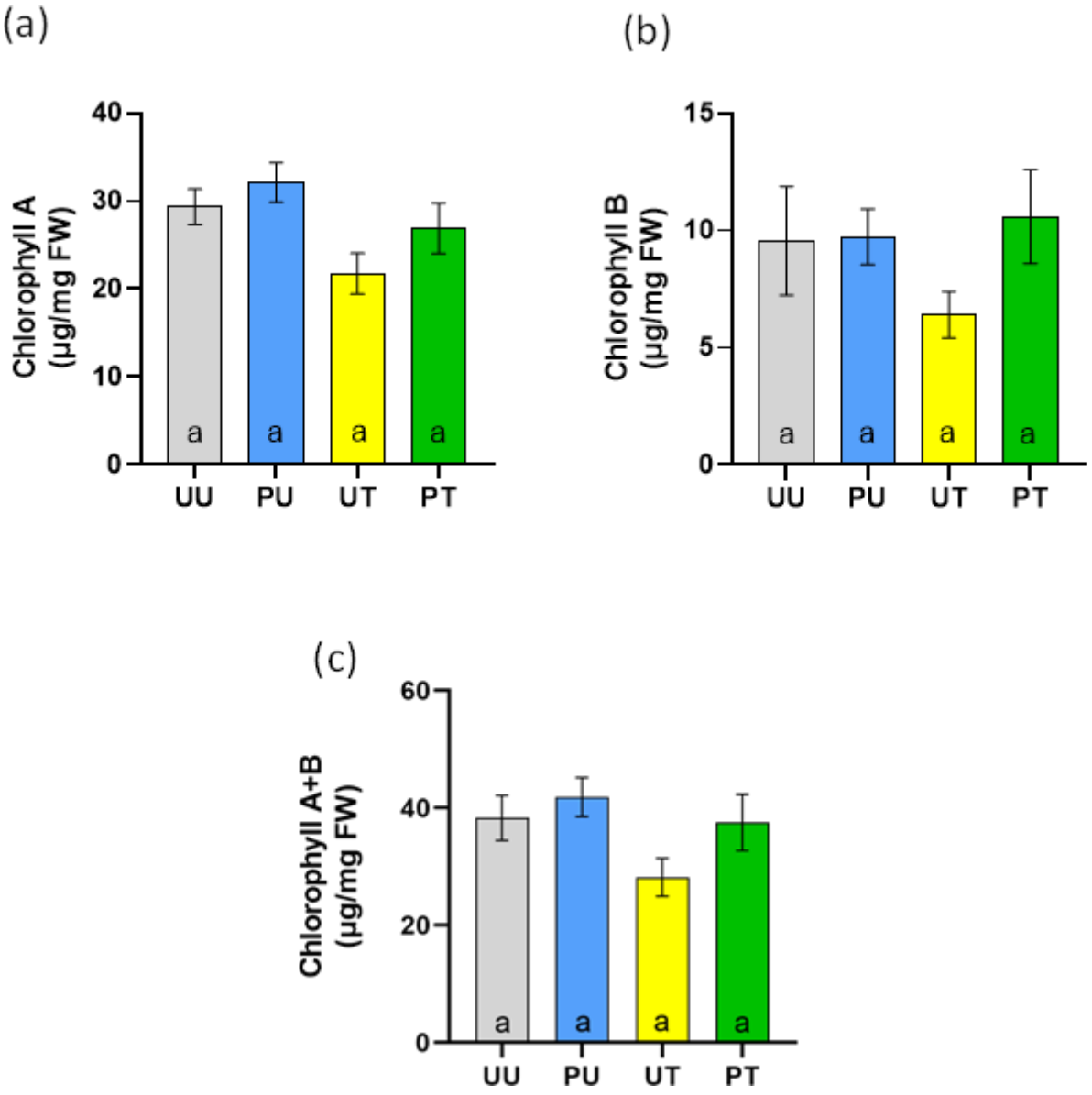


Figure 7. Chlorophyll levels from plants across all four treatment blocks from biological replicate 1. Readings taken from all four treatment groups after conclusion of cold treatment. Panel a. Chlorophyll a levels were measured as it is the primary pigment responsible for chlorophyll production. Panel b. Chlorophyll b levels were measured as it is an important accessory pigment in chlorophyll production. Panel c. Chlorophyll a+b is a combination of both pigments and gives an overview of total chlorophyll in each plant. Values are expressed as the mean average from the first biological replicate (n = 6 (BR 1); One-Way ANOVA followed by Tukey's multiple comparisons test was undertaken assuming a p-value < 0.05 as significance. Different letters represent a significant difference between the treatment means. Sharing of letters denotes no significant difference between the means. Error bars denote standard error of the mean (SEM). Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

Chlorophyll levels from biological replicate 2&3 were measured using a Dualex leaf sensor (unit = Chlorophyll Arbitrary Unit (CAU)) which allowed for a higher number of readings to be done which improved our statistical analysis. Nevertheless, these results corroborated those done with the spectrophotometer. Unprimed Cold-Treated plants (UT; 17.5 CAU) showed lower levels of total chlorophyll compared to the Unprimed Untreated (UU; 20.29 CAU) plants with a percentage decrease of 13.75%. Notably, there was no significant difference in chlorophyll levels between Primed Cold-Treated (PT; 19.47 CAU) and Unprimed Untreated plants (UU; 20.29 CAU) with Primed Cold-Treated plants having a small percentage decrease of 4%. Interestingly, the highest chlorophyll levels detected were from PU plants (PU; 22.1 CAU), which would suggest SFP promotes chlorophyll accumulation in healthy untreated plants.

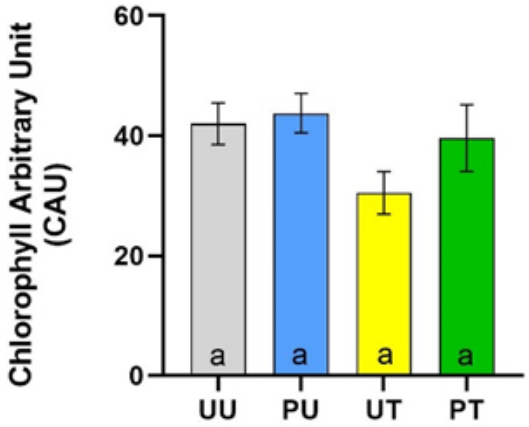


Figure 8. Chlorophyll levels taken from plants across all four treatment groups from biological replicate 2&3 using the Dualex leaf sensor. Readings taken from all four treatment groups after conclusion of cold treatment. Values are expressed as mean averages across two biological replicates (*n* = 12 (BR 2&3); One-Way ANOVA followed by Tukey's multiple comparisons test was undertaken assuming a *p*-value < 0.05 as significance. Different letters represent significant differences between the treatment means. Sharing of letters denotes no significant difference between the means. Error bars denote standard error of the mean (SEM). Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

4.1.4 Flavonoid:

Flavonoid levels from biological replicates 2&3 were recorded with the Dualex Leaf sensor (unit = Flavonoid Arbitrary Unit (FAU)) after cold treatment (Figure 8). Primed Cold-Treated

(PT; 0.58 FAU) had significantly higher levels of flavonoids than Unprimed Cold-Treated plants (UT; 0.52 FAU) with a percentage increase of 11.5%. Primed Cold-Treated plants also had higher levels of flavonoids than both Unprimed and Primed Untreated plants (UU; 0.48 FAU, PU; 0.48 FAU) and showed a 20.8% increase over the untreated controls. Unprimed Cold-Treated plants had higher levels of flavonoids than plants in the Untreated groups with a non-significant percentage increase of 8.3%.

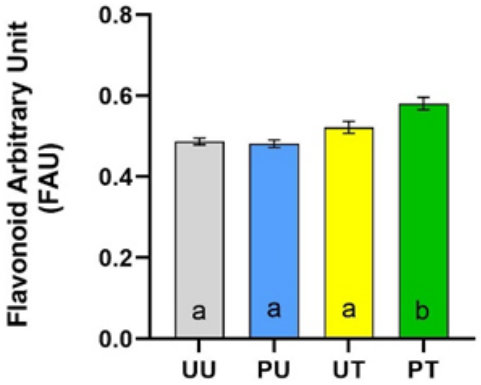
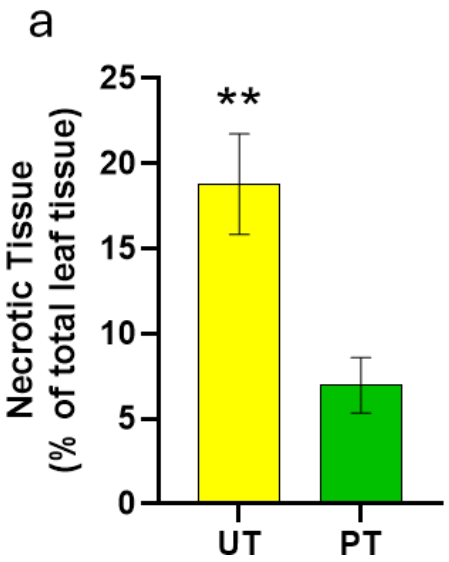


Figure 9. Flavonoid levels from plants across all four treatment groups from biological replicate 2&3 using the Dualex leaf sensor. Readings taken from all four treatment groups after conclusion of cold treatment. Values are expressed as mean averages across two biological replicates (*n* = 12 (BR 2&3); One-Way ANOVA followed by Tukey's multiple comparisons test was undertaken assuming a *p*-value < 0.05 as significance. Different letters represent significant differences between the treatment means. Sharing of letters denotes no significant difference between the means. Error bars denote standard error of the mean (SEM). Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

4.1.5 Necrotic Tissue:

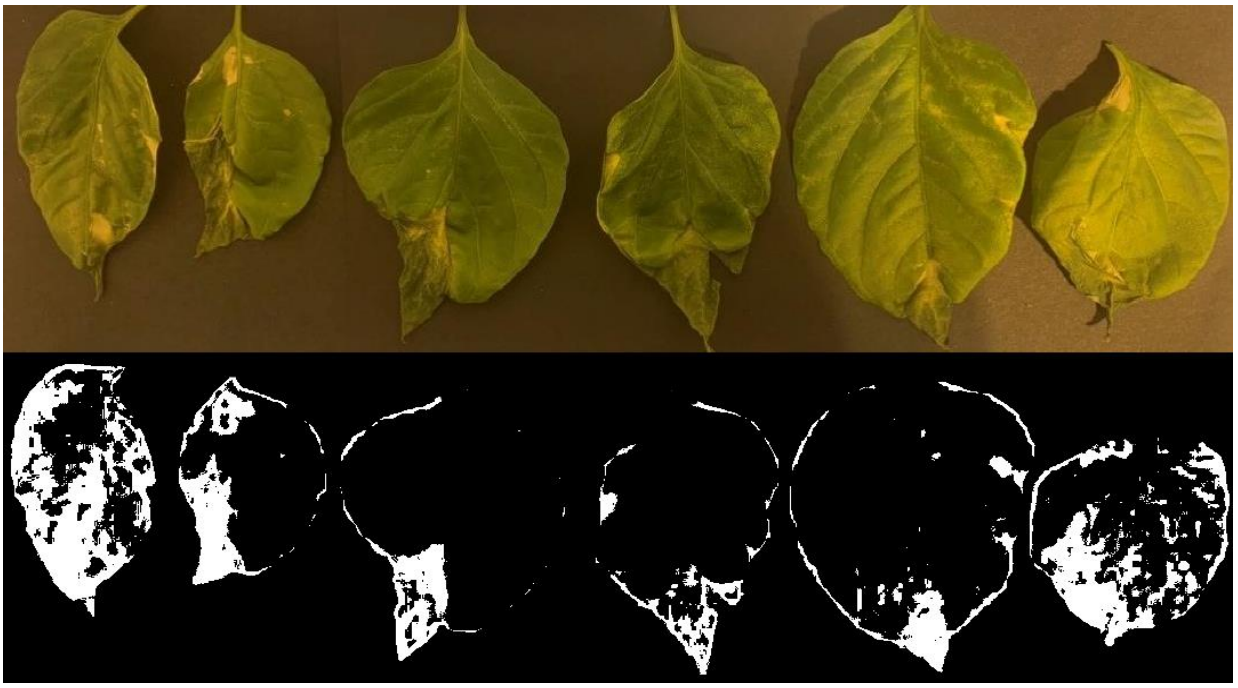
Necrotic tissue was measured as it is an important indicator of how badly the leaf tissue was damaged following cold treatment. Necrotic leaf tissue measurements were taken a week after cold treatment had concluded in BR 3. Only the two Cold-Treated treatment groups were measured for necrotic tissue as Untreated plants were kept in optimum conditions. Leaves

from plants not primed with SuperFifty® Prime (UT; 18.7%) had significantly larger areas of necrotic tissue than primed plants (PT; 6.9%) as can be seen in (Figure 9 (a)(b) & (c))



Unprimed Cold-Treated Plants:

(b)



Primed Cold-Treated Plants:

(c)



Figure 10. Panel a. Total area of necrotic tissue on leaves of plants a week after cold treatment had ended BR3. Panel b. Picture of leaves taken from plants in the Unprimed Cold-Treated group, includes areas of necrotic leaf tissue highlighted in Image J. Panel c. Picture of leaves taken from plants in the Unprimed Cold-Treated group, areas of necrotic leaf tissue highlighted in Image J. Values are expressed as the mean average from the third biological replicate (n = 6 (BR 3); Unpaired T-test was undertaken assuming a p-value < 0.05 as significance. Asterisk represents a significant difference between the treatment means. Error bars denote standard error of the mean (SEM). Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

4.1.6 Total weight and Number per plant:

Six plants from each treatment block in BR2 were kept to grow to full maturity. Plants were harvested, exactly 6 months after they were sown. Peppers from each plant were counted and weighed. Primed Untreated (PU; 118.6 g) had the largest fruit yield per plant, significantly higher than Unprimed Cold-Treated (UT; 92.3 g) and Primed Cold Treated (PT; 105.7 g) (Figure 10). There was no significant difference in yield between Unprimed Untreated plants (UU; 112 g) and Primed Cold-Treated plants. A similar trend was observed in pepper number per plant with the only significant difference between Primed Untreated (PU; 6) and Unprimed Cold-Treated (UT; 4.75). There was no significant difference between Unprimed Untreated (UU;

5.5) and Primed Cold-Treated (PT; 5.375), suggesting that SFP treatment has a protective effect on pepper yield and number under Cold-Treatment conditions.

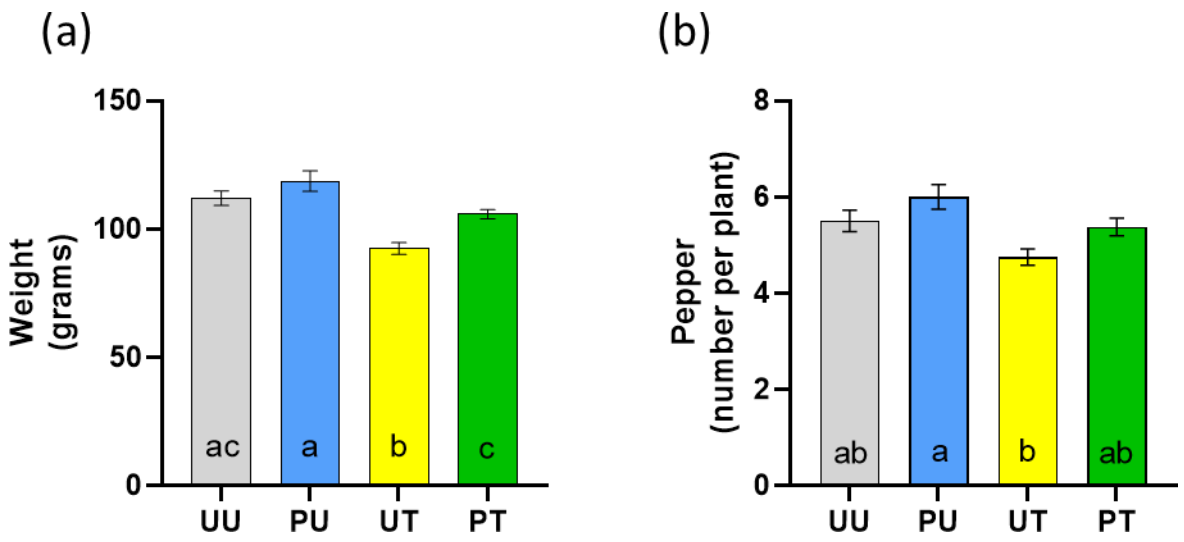


Figure 11: Panel a. Total pepper weight per plant from BR 2 taken after plants had grown to full maturity (180 days). Panel b. Total pepper fruit number harvested per plant. Values are expressed as mean averages from the second biological replicate (n = 6 (BR 2); One-Way ANOVA followed by Tukey's multiple comparisons test was undertaken assuming a p-value < 0.05 as significance. Different letters represent significant differences between the treatment means. Error bars denote standard error of the mean (SEM). Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

Results II: Gene Expression Analysis:

4.2.1 Selection of Housekeeping Genes:

Three housekeeping genes were selected as potential reference candidates as identified in Wan *et al*, (2011). Reference genes are those with constant expression levels regardless of biological differences and experimental conditions. Expression of these genes was used to normalise expression of targeted genes.

Table 1: A list of different housekeeping genes and their respective primers used in this study.

Gene Name	Description/Function	Forward Primer	Reverse Primer
b-TUB	*Beta Tubulin*	GAGGGTGAGTGAGCAGTTC	CTTCATCGTCATCTGCTGTC

UBI-3	*Ubiquitin-conjugating protein*	TGTCCATCTGCTCTCTGTTG	CACCCCAAGCACAATAAGAC
GAPDH	*Glyceraldehyde-3-phosphate dehydrogenase*	ATGATGATGTGAAAGCAGCG	TTTCAACTGGTGGCTGCTAC

Several PCR reactions were performed using the primer pairs specific to the housekeeping genes outlined in Table 1. The objective was to identify a housekeeping gene with the highest amplification efficiency for later gene expression studies using RT-qPCR. Each gene was tested on a sample from each treatment block (UU, PU, UT and PT). As can be seen in Figure 12, *b-TUB* showed the best amplification efficiency out of all the candidates tested and was selected as a reference gene for all the later qPCR studies.

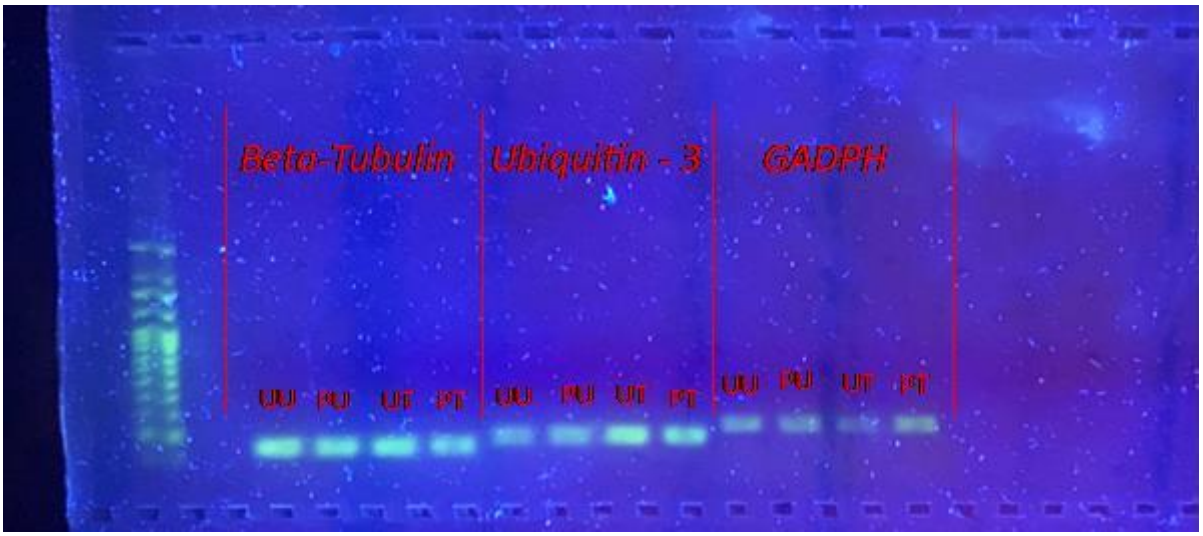


Figure 12: Gel picture showing bands of amplified products of different housekeeping gene candidates. Brighter band represents higher amplification of the respective gene. Note the *B-TUB* amplified product as the brightest bands representing efficient amplification across all four treatment groups. Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

4.2.2 Primer Design and Efficiency Testing:

Primers for our shortlisted genes were designed by identifying and selecting conserved coding regions in the genome of *Arabidopsis thaliana* and the Mexican Chile pepper (*C. annuum* line, CM334) (found on Sol Genomics website). Upon identifying the conserved coding regions, primers were designed pairing regions of cDNA approximately 18-24 base pairs in length.

Table 2: Table listing the different key marker genes used in this trial and their forward and reverse primers. Each shortlisted gene had two primer pairs designed to be assessed for amplification efficiency.

Gene Symbol	Gene	Forward Primer	Reverse Primer
ICE-1	*Inducer of CBF Expression 1*	GAAGAAAGGACTTCCTGC	CTTACTAATCTTTGGAAC
ICE-1 (2)	*Inducer of CBF Expression 1 (2)*	AGAGCAGTGAACATACAC	GTTGGATATCTTCCGAGCT
CBF-1	*C-repeat/DRE binding factor 1*	AAGAAGTTTCGAGAAACTCG	GTACCAAGCCAAATTCTTGA
CBF-1 (2)	*C-repeat/DRE binding factor 1*	GCTGAAATGGCTGCTAGAGC	CCGCCTTTTGAATATCTT
COR-314	*Cold Regulated Genes 314*	GGTTTGTTATGCTGCTGC	GGGACACAAGAAAAGACTTG
COR-314 (2)	*Cold Regulated Genes 314*	CAAAGGAAGGTGTTGTTC	GGCTAGGGTGGCAATAATTG
PAL-1	*Phenylalanine Ammonia Lyase*	CAGCTCAGAAGCTTCACG	CCGTTGTTGTSGTAATCATTG
CHS-1	*Chalcone synthase*	GGGGCCAGCCCAAATCGAAG	AGCAACCTTGTTGGTACATC

Once primers had been designed, PCR reactions were performed to check primer amplification efficiency and specificity (Table 3). Selection of the best primer pair used for each gene in qPCR depended on successful amplification. The first pair of primers was used for each gene as they showed the most promising bands across all gene candidates (Figure 13) (Figure 12).

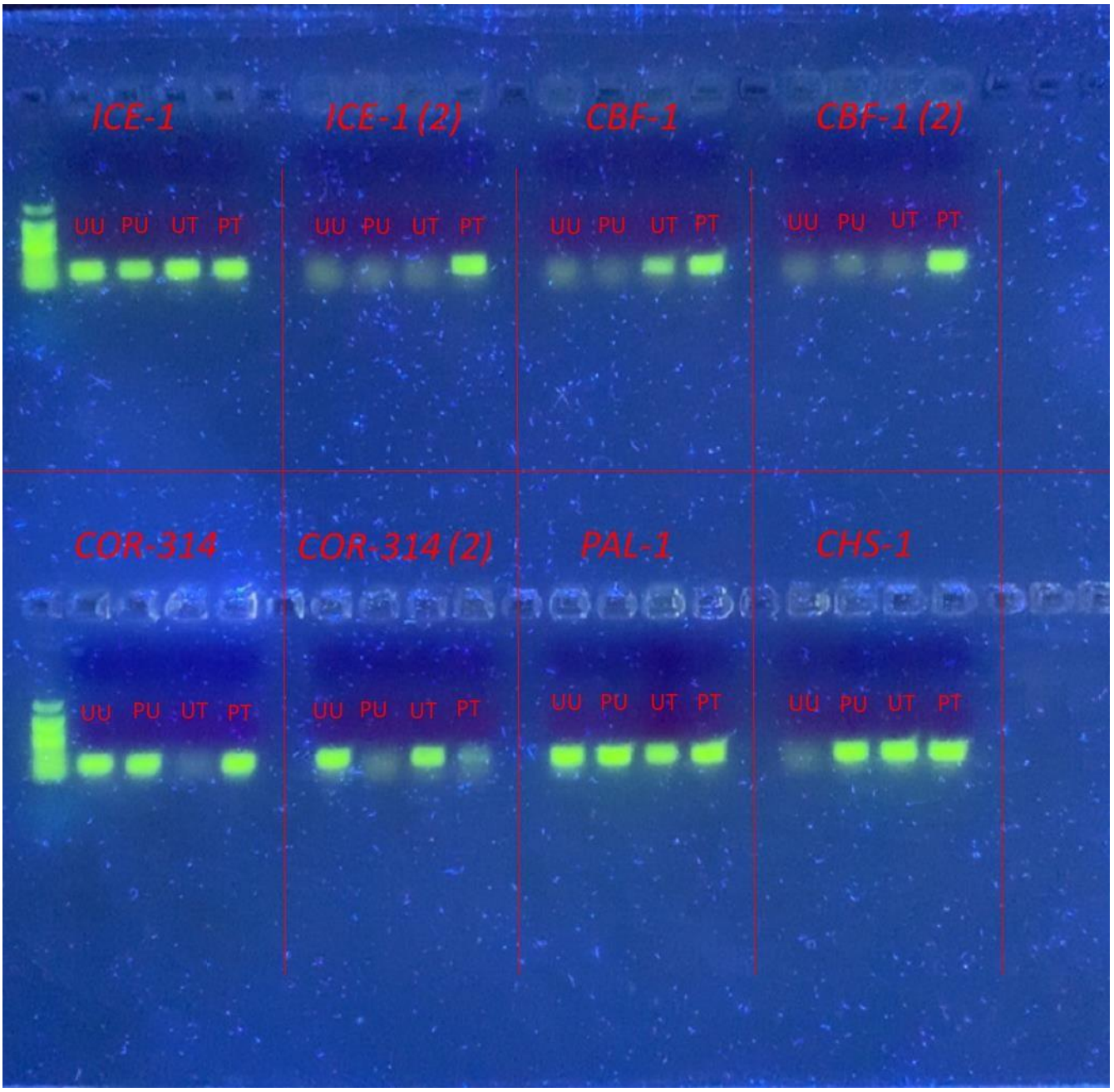


Figure 13: Picture showing the gene candidates for qPCR and their bands in the agarose gel. Brighter band represents higher amplification of the gene. The first primer pair from each gene showed the highest amplification across all four treatment groups. Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

Table 3: The primer pairs of these housekeeping and marker genes were accessed for amplification efficiency. Only the first *COR* primer pair was sampled as only 8 primer pairs could fit in the 96 well plate.

Gene Symbol	Gene	Efficiency Value
ICE-1	*Inducer of CBF Expression 1*	1.096734341%

ICE-1(2)	*Inducer of CBF Expression 1 (2)*	1.416249418%
CBF-1	*C-repeat/DRE binding factor 1*	1.218302462%
CBF-1(2)	*C-repeat/DRE binding factor 1*	1.24963819%
COR-314	*Cold Regulated Gene 314*	1.165561241%
b-TUB	*Beta-Tubulin*	1.171202532%
PAL-1	*Phenylalanine Ammonia Lyase*	1.23691947%
CHS-1	*Chalcone synthase*	1.127496491%

4.2.3 qPCR analysis:

To provide more insights into how SuperFifty® Prime mitigates the damage caused by cold treatment, the expression levels of the key marker genes for cold tolerance and flavonoid production outlined in the Materials and Methods section were investigated. The gene expression values for Unprimed Untreated (UU) plants were used as a threshold for the subsequent analysis and their expression level was given the arbitrary value of 1. As shown in Figure 14, Unprimed Cold-Treated plants (UT) had the highest expression for the three marker genes (*ICE 1, CBF 1* & *COR 314*) responsible for cold acclimation and freezing tolerance. When compared with the Unprimed Untreated plants, UT exhibited a 22-fold increase in *ICE 1* expression and a 19.3-fold increase in *CBF 1* expression. Notably, Unprimed Cold-Treated plants exhibited a 120-fold increase in *COR 314* expression when compared with the control (UU) condition. Interestingly, the expression values for Primed Cold-Treated plants (PT) were the most similar to those of the Unprimed Untreated (control) plants with only a 12.3-fold increase in *ICE 1* expression, a 3.6-fold increase in *CBF 1* expression and a 15.8-fold increase in *COR 314* expression.

When UT and PT responses are compared, a striking difference between expression levels can be observed. Plants that were Cold-Treated and primed with SuperFifty® Prime showed significantly lower levels of gene expression across all three genes (*ICE 1, CBF 1* & *COR314*) than those that were unprimed before Cold-Treatment. For *ICE 1,* expression in Unprimed Cold-Treated plants (UT; 21.9) was almost double that of Primed Cold-Treated plants (PT; 12.3). For *CBF 1,* the difference is even larger with Unprimed Cold-Treated plants (UT; 18.08) having an expression five times higher than that of Primed Cold-Treated plants (PT; 3.6). Lastly

the most significant difference is observed in *COR 314* expression with a six-fold increase in expression when comparing the Unprimed Cold-Treated (UT; 111.8) with the Primed Cold-Treated (PT; 15.86).

It is also noteworthy to mention that even in Primed Untreated plants (PU) there was a substantial increase in the expression values of two of the cold response genes (*ICE 1* & *COR 314*) despite never experiencing cold stress conditions. Expression values of *ICE 1* and *COR 314* were 7.2-fold and 29-fold respectively higher in PU plants when compared with Unprimed Untreated plants. On the other hand, *CBF 1* expression levels were only 0.5 times higher in PU than in Unprimed Untreated plants. Moreover, it was found that in Primed Untreated plants *COR 314* expression levels were even higher than those of the Primed Cold-Treated plants, suggesting that the treatment with SFP promotes the expression of this gene regardless of exposure to cold conditions (PT *versus* PU).

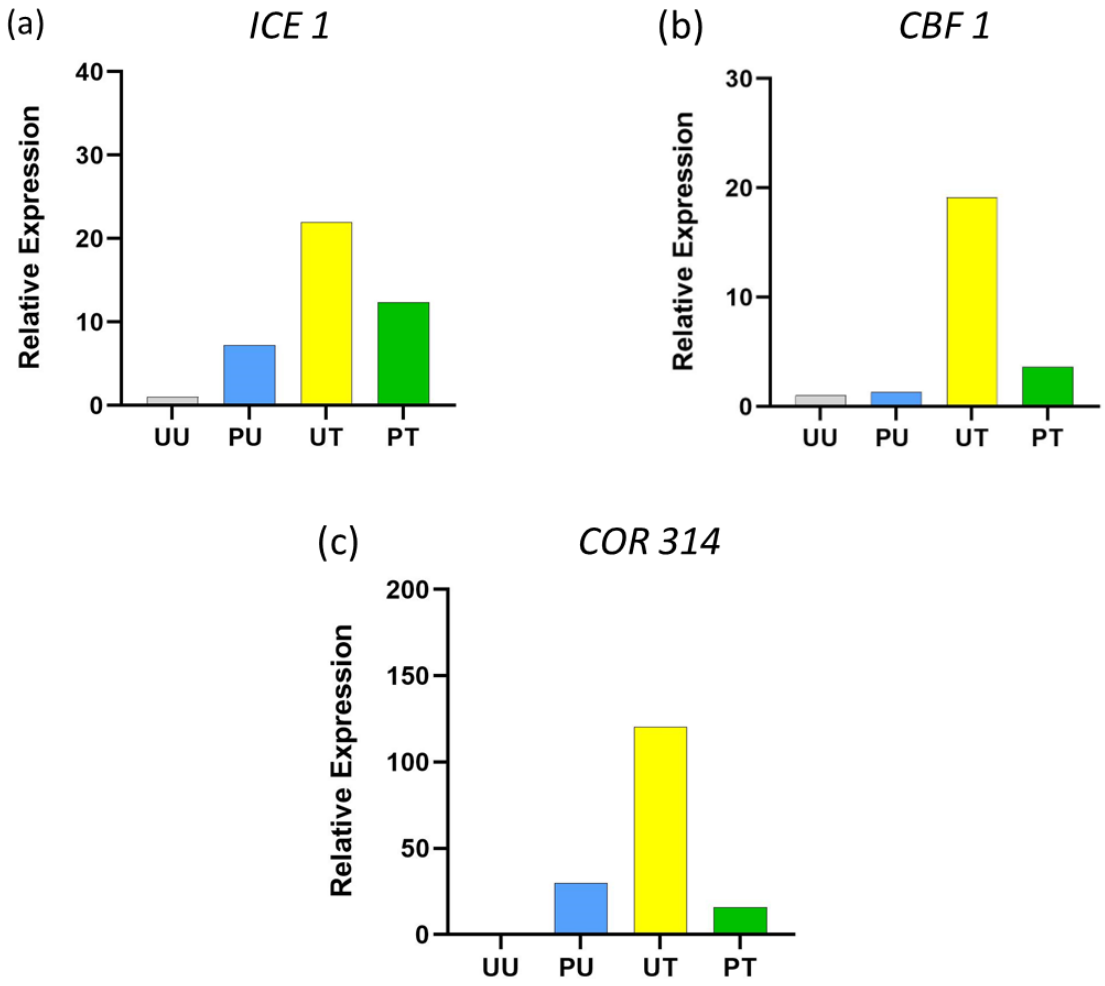


Figure 14: Relative Expression of three cold stress response genes. Six plants were sampled from every treatment block in BR (2&3) and one pooled sample per BR was used to measure gene expression. The expression value of Unprimed Untreated (UU) was used as the threshold for each gene and has a value of 1 in every graph. Panel a. Relative expression of *ICE 1* gene. Panel b. Relative expression of *CBF 1* gene. Panel c. Relative expression of *COR 314* gene. Error bars were omitted due to the small sample size. Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold-Treated (UT).

After the analysis of cold responsive genes, a similar approach was used to determine the expression levels of two genes responsible for encoding key enzymes in each pathway of flavonoid production (*PAL 1 & CHS 1*). It was found that both exhibited a similar expression pattern as observed previously for the cold responsive genes. Relative expression of *PAL 1* was higher in Unprimed Cold-Treated plants compared to other treatment blocks, there was a 344-fold increase in its expression when compared with that of Unprimed Untreated (UU) plants. Primed Untreated plants had the second highest level of *PAL 1* expression with a 127-

fold increase. Lastly the difference in relative expression values between Primed Cold-Treated and Unprimed Untreated plants was smaller with only a 23.5-fold increase in *PAL 1* expression. Interestingly, relative expression for *CHS 1* followed the same pattern, exhibiting a 201.8-fold increase in relative expression in Unprimed Cold-Treated (UT) and a 69.3-fold increase in Primed Untreated (PU) plants. Lastly, the relative expression value of *CHS* 1 was similar between Primed Cold-Treated and Unprimed Untreated plants with the Primed Cold-Treated plants having only a 1.97-fold increase over the negative control.

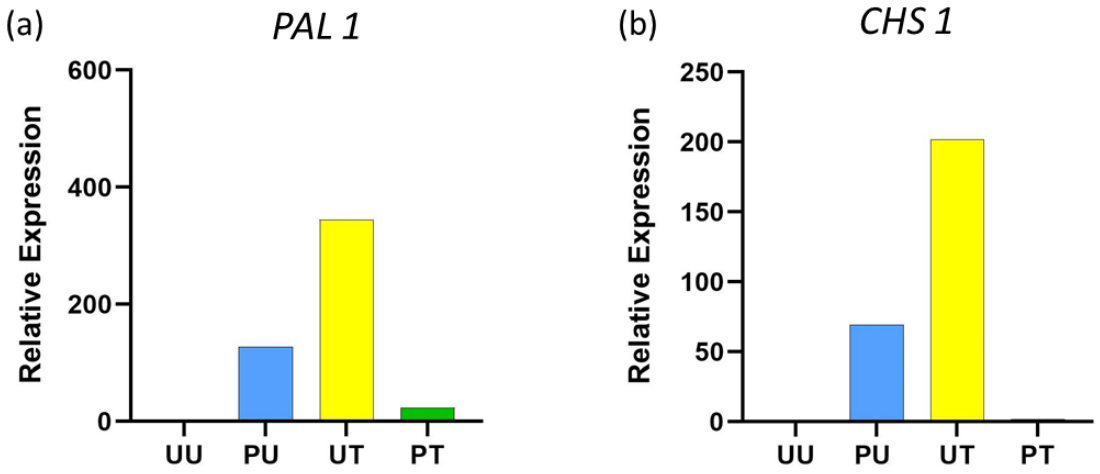


Figure 15: Relative Expression of two genes involved in flavonoid production. Six plants were sampled from every treatment group per BR and one pooled sample per BR was used to measure gene expression. Unprimed Untreated (UU) was used as the threshold and has a value of 1 in every graph. Panel a. Relative expression of *PAL 1* gene. Panel b. Relative expression of *CHS 1* gene. Error bars are omitted due to small sample size. Primed Untreated (PU), Unprimed Untreated (UU), Primed Cold-Treated (PT) and Unprimed Cold- Treated (UT).

Given the time constraints, only two samples could be tested for real time qPCR analysis over the course of the experiment which led to large error bars in some of the graphs. This could be resolved by increasing the number of qPCR analyses conducted in future research which would provide a more concrete set of datapoints. However, importantly the data followed the same pattern in both biological replicates as the values of expression for Unprimed Cold-Treatment plants were substantially larger across all five genes tested.

5. Discussion:

5.1 Effect of cold stress on early plant development in pepper:

Incidents of cold stress occurrence can cause significant damage to a plant or crop at any stage of development, but particularly at the early growth stages when they are at their most vulnerable. Damage from cold stress can cause a variety of detrimental effects such as lowering of metabolism, drought stress symptoms, mechanical damage, and eventual cell, and even plant, death (Adhikari *et al,* 2022). Exposure to cold can also cause long term stress damage to the plant and can result in poor seed development, stunted growth and a subsequent reduction in yield parameters.

Pepper plants (*Capsicum annum*) are widely grown in areas with high temperatures and ideally require temperatures within the range of 25^{o}C – 30^{o}C to grow optimally. This optimal temperature requirement renders pepper plants especially susceptible to cold stress. Any temperature below 15^{o}C can cause stress and damage to the plant (Manasa *et al,* 2022). Previous studies have shown that cell membranes are the first part of plant cells to be affected by cold stress, leading to an imbalance of membrane fluid content. This subsequently leads to leakage of electrolytes and amino acids from the damaged cell (Seo *et al*, 2010). Electrolyte leakage was assessed in this study, and the data demonstrated that Unprimed Cold-Treated plants have a significant increase in electrolyte leakage when compared to Unprimed Untreated plants (Figure 6). This indicates that the cells in plants that underwent Cold-Treatment were negatively affected and high levels of leakage occurred.

Another activity of the plant that is highly vulnerable to cold stress is photosynthetic activity and chlorophyll synthesis, possibly contributing to the lowering of metabolism mentioned above. An extensive study on stress responses in pepper plants during cold exposure (Nandagopal & Shanmugam, 2022) found that all twelve pepper genotypes tested in their experiment had significantly lower levels of chlorophyll due to a cold stress induced by exposure to a temperature of 10^{o}C. Our findings are in agreement with this study as Unprimed Cold-Treated plants had significantly lower levels of chlorophyll than Unprimed Untreated plants. A similar trend of reduced chlorophyll due to cold stress can be observed in a wide variety of cold sensitive crops such as eggplants (Wu *et al,* 2014), sugarcane (Zhang *et al,* 2015) and grapes (Aazami *et al,* 2021). Chlorophyll content is an important stress marker used

in the assessment of the severity of abiotic stress in plants. A significant reduction indicates high stress in Unprimed Cold-Treated plants compared to Unprimed Untreated plants.

Given that an increase in electrolyte leakage is an early sign of stress caused by cold exposure, and that chlorosis of plants in this study had begun, it can be hypothesised that production of reactive oxygen species (ROS) in Unprimed Cold-Stressed plants may have increased significantly. Given that the end result of increased ROS production is cell damage or death, occurrence of necrotic leaf tissue should be identifiable on the leaves of highly stressed plants. This indeed is the case in Unprimed Cold-Treated plants as 18% of the plant leaf tissue area consists of necrotic tissue one week after the occurrence of cold stress (Figure 10b). Lastly, yield parameters were lower in Unprimed Cold-Treated than Unprimed Untreated plants, indicating that the applied cold-treatment was severe enough at the seedling stage and led to long term effects four months later at the reproductive stage in plants. Therefore, it can be concluded that the trial parameters in this experiment were successful in sufficiently stressing the plant whilst also allowing it to recover and progress through its full life cycle.

5.2 SuperFifty® Prime mitigates damage caused by cold treatment:

Previous studies have comprehensively proven that pre-treatment with SuperFifty® Prime (SFP) can successfully prime and prevent much of the detrimental effects of exposure to abiotic stressors (e.g. drought) on plant growth and yield in crops such as tomatoes (see Introduction, section 2.3) (Rasul *et al*, 2021). The findings in this experiment show that SuperFifty® Prime successfully mitigated much of the cold stress damage outlined in the previous section as well as improving fresh weight recovery and flavonoid levels. Firstly, while electrolyte leakage in Primed Cold-Treated plants was significantly lower than in Unprimed Cold-Treated plants, it was still significantly higher than in Unprimed Untreated plants. Previous SuperFifty® Prime studies on abiotic stressors (drought & herbicide induced stress) by Rasul *et al.* (2021) and Omidbakhshfard *et al.* (2020) also measured electrolyte leakage, the results of which are consistent with the findings in this study with one discrepancy. Both Rasul and Omidbakhshfard found that there was no significant difference in electrolyte leakage between their Unprimed Untreated and Primed Stress-Treated plants. A possible explanation is that the severity of the stress used in this experiment was too intense leading to higher membrane damage when compared with the previous studies.

Another study by Staykov *et al.* (2020) established that a priming application of SFP significantly reduces the loss of chlorophyll fluorescence from oxidative stress induced through herbicide (Paraquat) exposure. Staykov *et al.* (2020) also used the same cultivar of pepper plants as one of three model crops (*Arabidopsis thaliana*, pepper and tomato) and found that while SFP did not significantly improve chlorophyll levels in Primed Untreated plants, it did significantly decrease chlorophyll loss in Primed Paraquat-Treated plants and improved recovery of chlorophyll levels after stress. Our results show that while a priming application of SFP did decrease chlorophyll loss in Primed Cold-Treatment plants, it also significantly increased chlorophyll content in Primed *versus* Unprimed Untreated plants (Figure 7&8). It is worth noting that the environmental set-up was slightly different in both trials which may have contributed to the lack of an increase in chlorophyll in Primed Untreated plants observed by Staykov et al (2020).

Given that both electrolyte leakage and chlorophyll loss was not as severe in Primed Cold-Treated plants versus Unprimed Cold-Treated plants, this may explain, in part, the reduction in occurrence of necrotic tissue in the leaves in Primed Cold-Treated plants. There are several other possibilities that may also explain the decrease of necrotic tissue. Our findings show that there were significantly higher levels of flavonoids in Primed Cold-Treated plants which may have also played a role in the reduction of the occurrence of necrotic tissue. Substantial research has been undertaken in the last decade indicating that flavonoids and other polyphenols play a key role in the scavenging of ROS. In fact, a recent study (Genzel *et al,* 2021) found that flavonoid production contributed to cold tolerance in pepper plants and that cold stress induces the production of flavonoids. A reduction in the occurrence of necrotic tissue as a result of a decrease in oxidative stress was documented by Omidbakhshfard *et al.* (2020), who found that a priming application of SF induces expression of *ascorbate peroxidase 4* (*APX 4*) which assists in the detoxification of superoxides and peroxides.

Finally, the data collected post-cold treatment in this study is proof that a second application of SuperFifty® Prime aids in the recovery of the plant. Fresh weight measurements before and after cold exposure showed that primed plants retained more of their fresh weight during cold-treatment and grew quicker in the weeks thereafter (Figure 5). Yield data show that there was no significant difference between Primed Cold-Treatment and Unprimed Untreated plants both in terms of weight and number of fruits (Figure 11). Given the difference in results

between Primed Cold-Treated and Unprimed Untreated plants, it is clear that two applications of SuperFifty® Prime primed plants to better withstand cold and recover quicker. Based on the evidence from this experiment, two applications of SuperFifty® Prime improved yield and productivity in Primed Cold-Treated plants and has the potential to improve grower incomes in areas affected by cold stress.

5.3 Uncovering the molecular mechanisms underlying SuperFifty® Prime mode of action:

Molecular Priming is a method of inducing a plant's natural defence system through applying various organic or inorganic molecules and biostimulants, with the aim of protecting plants against upcoming abiotic stressors. Upon the onset of severe cold conditions, plants can initiate an active response to prevent stress (i.e., cold shock) and this involves enhanced or altered expression of certain genes to induce cold tolerance. The three main cold response genes selected for our study were *Inducer of CBF Expression 1* (*ICE 1), C-repeat/DRE binding factor 1* (*CBF 1*) & *Cold Regulated Gene 314* (*COR 314*) found in the *ICE-CBF-COR* transcriptional cascade (Ritonga *et al,* 2020). Overall gene expression patterns for all three genes were similar in this study (Figure 14). *ICE 1* encodes a MYC-like basic helix-loop-helix (BHLH) transcription activation factor that binds to a specific region (MYC recognition sequence) in the promoters of *CBF* genes during cold stress (Chinnusamy *et al*, 2003; Tang *et al*, 2020). *ICE 1* was highly upregulated in Unprimed Cold-Treated plants with a 22-fold increase in expression over Unprimed Untreated plants. This significant increase in *ICE 1* expression after a period of 48 h at 2^{o}C demonstrates that plants (Unprimed) were severally affected by cold stress. *ICE 1* is recognised as a master regulator, responsible for the induction of several *CBF* genes and a variety of other cold responsive transcription factors and regulatory genes and early upregulation will promote cold tolerance throughout the plant (Chinnusamy *et al*, 2003). Primed Untreated plants had a 7-fold increase in *ICE 1* expression over the basal level expressed in Unprimed Untreated plants. This is a significant increase, especially considering that 48 h of severe stress at 2^{o}C only increased expression in Unprimed Cold-Treated plants by 22-fold. Notably, *ICE 1* expression was only increased up to 12.3-fold in Primed cold-treated plants (versus Unprimed Untreated), demonstrating a clear effect of priming on the expression level of this gene.

CBF 1, a transcriptional activator which typically induces cold-regulated gene expression to enhance plant tolerance to freezing (Yang *et al*., 2005), was also highly expressed in Unprimed & Primed Cold-Treated plants but was only marginally upregulated in Primed Untreated. Interestingly, *ICE 1* does not control the expression of *CBF 1* (*CBF 1* is instead induced by *ICE 2* upregulation in a different pathway) therefore, the lack of upregulation of *CBF 1* in Primed Untreated plant suggests that SuperFifty® Prime may not activate this specific pathway but could probably just act to upregulate the *ICE 1 – CBF 2/CBF 3* pathway to induce cold acclimation. Expression levels of *CBF 1* in Primed Cold-Treated plants were substantially lower than Unprimed Cold-Treated plants despite the data indicating that pre-treatment with SuperFifty® Prime had very little effect on *CBF 1* expression. This indicates that *CBF 1* regulation might be more complex and relies on different pathways.

The *COLD REGULATED 314* (*COR 314*) gene's product is an integral component of the inner membrane of a plant's chloroplast and has been proven to function in cellular responses to cold and water deprivation. The gene-product has been linked to cold acclimation and abscisic acid accumulation (Owaka *et al,* 2008). The expression of *COR 314* in Primed Untreated plants was 29-fold higher than expression in Unprimed Untreated and almost twice as high as Primed Cold-Treated plants. Unprimed Cold-Treated plants had an expression increase of 120-fold although it is worth noting that Unprimed Cold-Treated plants had lower chlorophyll and fresh weight than Primed Cold-Treated which would indicate severe stress occurring in the chloroplast.

In this experiment, clear evidence is seen of molecular priming as both *ICE 1* and *COR 314* are upregulated in Primed Untreated plants, despite not experiencing cold stress inducing conditions. The combination of the physiological and the gene expression data provides strong evidence that a priming application of SuperFifty® Prime could help in mitigating much of the issues caused by cold stress through inducing vital cold tolerance genes before a stress event has occurred. The clear difference in gene expression levels of *ICE 1*, *CBF 1* and *COR 314* between Primed and Unprimed Cold-Treated plants provides clear evidence that SuperFifty® Prime is influencing the plant at the molecular level.

Given that flavonoid levels in Primed Cold-Treated plants were significantly higher than in plants of all other treatment groups (Figure 9), the expected result would be that both *PAL 1* and *CHS 1* would be upregulated in Primed Cold-Treated plants. This is not the case however

as both Primed Cold-Treated, and Unprimed Untreated control plants have almost the same expression levels for *PAL 1* and *CHS 1.* The expression of *PAL 1* and *CHS 1* in Unprimed Cold-Treated plants is highly upregulated yet there is only a non-significant increase in flavonoid levels. There are several possible explanations for this observation. Firstly, although both genes have a role in encoding the proteins responsible for flavonoid production (described in detail in Introduction, section 2.2), the flavonoid production pathway is not solely dependent on the expression of *PAL 1* and *CHS 1* genes. A study on Red Sage plants found that a group of 108 genes are involved in flavonoid biosynthesis (Yang *et al,* 2022). This indicates that the flavonoid biosynthesis pathway is complex and involves an array of genes and regulatory factors. Therefore, without a complete picture of all the genes and regulatory components such as transcription factors, it is difficult to conclude why flavonoid levels are significantly higher in Primed Cold-Treated plants, while expression of *PAL 1* and *CHS 1* is not. Additionally, while the expression levels of *PAL 1* and *CHS 1* were measured, there was no data collected on the breakdown of flavonoids in the plant system. The physiological data collected in this study showed that Unprimed Cold-Treated plants were more severely damaged by the cold treatment than Primed Cold-Treated plants. Therefore, it is possible that more flavonoids are being consumed in Unprimed Cold-Treated plants as a survival/ stress adaptation mechanism of plants to reduce ROS levels (Agati *et al,* 2020).

5.4 Plant Biostimulants: an effective method of mitigating cold stress in the future of agriculture:

There are several alternative methods to engineer cold tolerance in plants including, transforming plant species through the introduction of cold tolerance-enhancing genes, breeding to develop a new cold tolerant variety and the molecular tailoring of genomes for enhanced stress adaption. In the last decade, revolutionary new genome editing techniques involving site-specific zinc-finger endonucleases and RNA-guided nucleases such as Cas9 allow scientists to precisely edit a desired genome sequence and improve crops species to better withstand the stressors they will encounter throughout the growing season.

While there is undeniably huge potential in the fields of genetic manipulation and breeding, there are also a number of drawbacks to these methods. Breeding a new cold tolerant cultivar of pepper through traditional cross breeding will take a large amount of time and resources. With the average pepper cultivar taking 5-6 months to reach full maturity, even using modern

techniques such as speed breeding, it is a multiple year project. Using new genetic engineering techniques to create a new cold tolerant pepper cultivars would substantially reduce the time needed to get the same outcome using traditional methods. Also, despite extensive efforts, these solutions remain greatly restricted due to the complex nature of cold stress tolerance and the molecular pathways involved. It would also require extensive legislation to register the genetically modified cultivar in some regions such as the European Union where registration must be completed on a case-by-case basis. Also introducing a GMO plant into a country's agriculture would mean encountering resistance from groups against the use of GMOs in agriculture, in addition to significant regulatory challenges.

Findings from the current study will contribute to efforts aimed at defining the role of seaweeds and their extracts in agriculture and industry to meet the EU drive towards secure, sustainable and safe means of food production (REGULATION (EU) 2019/1009 and REGULATION (EC) No 1069/2009). E.U. legislation outlines the role of plant biostimulants and recognises that biostimulants can be used alongside fertilisers to optimise their efficiency and reduce their use. Plant biostimulants, through offering enhanced abiotic stress tolerance and optimising fertiliser output, can play a key role in achieving the aims of the Green Deal 2020 (European Commission, 2020), which aims to make the E.U. carbon neutral by 2050. E.U. policy and regulations also allow organically certified biostimulants to contribute towards the Farm to Fork strategy, which is an essential part of the European Green Deal. This strategy aims to create a sustainable food system maximising food production while adapting to climate change.

Treatment methods using plant biostimulants, including SuperFifty® Prime, have several advantages besides improving cold stress tolerance. They can be applied in any country, regardless of species variety or weather conditions. Also, products such as SuperFifty® Prime can act at a molecular level to moderate abiotic stress response pathways and can be used as a sustainable means to improve yield under environmental stress-inducing conditions. Biostimulants are cost effective as increased yield will cover cost of application and provide a broad protection over a variety of different stresses (e.g. drought & cold).

6. Acknowledgements:

I would like to thank a number of people who have helped me throughout the course of my masters. Firstly, thank you to both Dr. Rossana Henriques and Prof. Marcel Jansen for all their help throughout the last year. They were extremely generous with their time and knowledge, and it was fantastic to get to learn so much from them both. I would also like to thank all the lab staff in U.C.C who were always on hand to help whenever I needed it. Also, I wish to extend my gratitude to Prof. Gerard Killeen and Dr. Barbara Doyle Prestwich who accommodated me after my car accident and allowed me to complete their modules.

I am grateful to BioAtlantis and John T. O'Sullivan for sponsoring my Master of Research Project. I would also like to thank Dr. Sujeeth Neerakkal and Dr. Kieran Guinan for their advice and for all the corrections I put them through. I am grateful to Gavin, Daniel, Lauren and Rachel for helping me through a very difficult year, especially for their help after my accident. Lastly, thanks to Dr. Fiaz Rasul for his patience, both in putting up with me for the last three years and for teaching me most of what I know.

Finally, thank you to my parents and to Sandy for their constant support and encouragement.

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