THE EFFECT OF CHILLING TEMPERATURES ON SOYBEAN SEEDLINGS

Research Report

Faculty of Science
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By

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Signed on the 10th of August 2018 in Johannesburg

Declaration

I declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

(Signature of candidate)

10th day of August 2018 in Johannesburg

Dedication

This research report is dedicated to my late dad, who taught me that there is nothing in this world I cannot accomplish if I am willing to put in the extra effort. I also dedicate it to my mum who has done everything in her power to see me complete my studies.

Acknowledgement

I would like to acknowledge the God of Prophet TB Joshua & Major 1, the author and finisher of my faith as well as the significant men of God whose prayers have made this possible.

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Abstract

Soybean is an economically important crop in South Africa and the South African Bureau for food and agricultural policy projected an increase in land assigned for commercial soybean. Environmental stresses can affect aspects of plant growth and development, consequently specific morphology, anatomically and physiological parameters. In this regard, the present study determined the responses of soybean seedlings to chilling stress. Results were a tool for evaluation of sensitivity of soybean seedlings to chilling stress.

Experiments were carried out to assess the possible effects of chilling temperatures (5°C and 15°C) with respect to control (25°C) on soybean leaves at seedling stage for 24 and 72 hours. The following were analysed- Leaf functional parameters (leaf vigour, shape, colour); Physical parameters dry weight (DW), fresh weight (FW) and relative water content (RWC); Microscopic analysis and Reactive Oxygen Species (ROS) investigation by assessing superoxide concentrations.

Full turgidity in leaves was evident in the control, leaf curling was observed at 15°C and wilting leaves were observed at 5°C temperature treatment. As samples were exposed to 5°C temperatures, FW and RWC declined during extended periods of exposure. Effects on growth indexes were observed as early as 24 hours. A significant decrease in RWC was observed between 15°C and 5°C with 9%±2 and 13%±2 respectively. When compared to the control, significant decrease occurred in the palisade mesophyll layer at 15°C (12±2µm) and adaxial layer at 5°C (12±2µm). This suggests that the upper epidermis was possibly the most affected part of the leaf and cell modification could have occurred. Changes in DW, leaf thickness, spongy mesophyll layer, abaxial layer and superoxide accumulation were not statistically significant and were not affected by chilling temperature stress. TEM studies illustrated changes in chloroplasts profiles, formation of vesicles, disappearance of starch grains, incipient plasmolysis could indicate that the low temperature treatments resulted in stresses which lead to more production of ROS and subsequently cellular damage, which was more pronounced on 5°C treatment. Therefore, environmental stresses can affect aspects of plant growth and development.

Our study illustrated that a decrease in RWC, curling of leaves and wilting of leaves as well as growth of microorganisms especially fungi are effects of chilling temperatures on soybean seedling leaves. It is important to determine how soybean phenology is influenced by short term chilling temperatures as these changes are directly associated with anatomical, morphological, physiological changes. This gives us information on developmental changes that can be used for predicting soybean phenology under field conditions where short term exposure to chilling temperatures is likely to occur during seedling development.

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Chapter 1

1.1. General Introduction

Soybean stands as one of the earliest farmed legumes that dates back prior to 2500 BC in China (DAFF, 2010). It was later introduced in other countries such as America and Canada in the 19th century. Momentum of the soybean market started picking up in 1990's and has significantly increased since the 2004/2005 according to a 2013 report done by the South African National Seed Organisation (SANSOR, 2013). Food health benefits of soybean are numerous and arise from the edible protein and oil constituents that contain vital fatty acids and vitamins. The estimated composition of soybean seeds on a dry weight basis include: Protein (33-40%); Carbohydrates (35%); Oil (19%), Minerals (5%); Saponins (2%) while Isoflavones and Lectins make up the reminder (Dixit, Antony, Sharma & Tiwari, 2011)

It has been established that one of the major abiotic stresses on subtropical important crops are low temperatures especially chilling stress. Yadav (2010) and Balestrasse, Tomato, Batlle and Noviega (2010) both state that chilling stress is injury that occurs to plant characteristics without ice crystals formation occurs between 0^{o}C and 15^{o}C, that are low enough to suppress plant growth and development. Soybeans (*Glycine max*) originating from warm climates have been established to exhibit symptoms of injury when exposed to chilling temperatures (Musser, Thomas, Wise, Peeler & Naylor, 1984; Chapman, 1985; Kratsch & Wise, 2000; Lukatkin, Brazaityte, Bobinas & Duchovskis, 2012; Borowski & Michalek, 2014)

To obtain a good plant yield, seedling vigour is one important characteristic of plant growth because the processes are highly dependent on temperature. Jannink, Orf, Jordan and Shaw (2000) states that the advantage of seedling vigour is to heighten plant survival and develop the leaf surface area. Different plant studies have illustrated that exceeding phenotypic elasticity of plants influences the physiology, morphology, viability and crop yield especially in agriculture (Watanabe & Kume, 2009; Shah, Huang, Cui, Nie, Shah, Chen & Wang, 2011). Therefore, agricultural sector becomes vulnerable at a monetary and corporeal level such as costs of agricultural produce that can result in resource reallocation that might alter agronomic and international trade-offs among countries (Deke, Hoos, Kasten, Klepper, & Springer, 2001). Gbetibuo and Hassan (2005) have illustrated how major food crops in South Africa are marginally sensitive to temperature than precipitation by analysing agriculture, climate and edaphic data around the country. Van Jaarsveld and Chown (2001) have reported

that South-Western regions of the country are likely to experience increased early winter frontal.

According to a report submitted by a farmer to Grain South Africa in July 2015, the 2014/2015 soybean production season was extremely stressful for farmers. Reason being that the planting periods experienced anomalies in climate behaviour. It was stated that after germination, hot and cool days occurred together with very cold nights during early and mid-November resulting in mixed yields. Seedling emergence and vigour were affected in the first five weeks which is a critical stage of soybean growth (Grainsa, 2015). This can be correlated to the notion that chilling temperatures affect subtropical plants such as soybean which can lead to reduction or complete crop failure. Therefore, this research attempts to address effects of chilling temperatures on soybean seedling leaves.

1.2. Literature Review

Physical and economic parameters of the agricultural sector are susceptible to changes in the environment because plants have optimum limits for growth and metabolism which are subject to abiotic stresses such as chilling temperatures. Agronomic alterations and development of temperature tolerant genotypes through breeding programs are promising; however, an effective approach will be incorporating adaptive forms of research including plant physiology (Wahid, Gelani, Ashraf & Foolad, 2007).

In South Africa, the main soybean production areas include Mpumalanga [43%], Free State [32%] and KwaZulu-Natal [10%] (Dlamini, Tshabalala & Mutengwa, 2013). The climate in these areas is suitable for soybean cultivation and production is related to soil type, temperature, rainfall, topography, indigenous vegetation and seasons (Gbetibuo & Hassan, 2005). Planting periods start from end of October to end of December with mean maximum and minimum temperatures ranging from 21^{o}C to 25^{o}C and 11^{o}C to 15^{o}C respectively. The latter is usually experienced at night.

Soybeans grow in subtropical regions hence are susceptible to low temperature stress. The Ideal climate conditions for growth are 20^{o}C to 30^{o}C with temperatures less than 20^{o}C impeding growth (Balestrasse *et al.*, 2010). In South Africa, major crop production has been projected to move towards the Western and Northern Cape areas where there are frequent lower temperatures than Mpumalanga and KwaZulu-Natal. Following the spreading out of soybean production towards colder climates, studies on the physiological responses and acclimation of soybeans to these conditions are essential.

1.2.1. Importance of Soybean Plant

1.2.1.1. Crop Utilisation

Soybean production dominates the international market for oilseed and protein meal according to the Department of Environmental Affairs [DEA] (NAMC, 2011). Assessments previously done by Department of Agriculture, Fisheries and Forestry (DAFF) show that the average household in South Africa consumes 15% of soybean products in the form of soups, sauces, breakfast cereals and soymilk (Dlamini *et al*., 2013). The largest consumer is the livestock industry. It uses 60% of soybean produced as animal feed because of the high demand of local quality protein. The vegetative part of the plant is used in fodder or made into organic manure (DAFF, 2010). Twenty five percent of consumption in the form of oil and protein is mainly used in industry as an additive to manufacture products such as soap, printing inks, oilcloth, linoleum and paints (Erickson, 2015). By-products of oil/protein extraction such as lecithin phospholipids are used as stabilising and wetting agents in pharmaceutical industries (DAFF, 2010)

1.2.1.2. Economics of Soybean Production in South Africa

The economic value of soybeans lies in the oil and protein content (Brumm and Hurburgh, 1990). According to DAFF, the input of soybean in agricultural production has increased from a meagre R84 000 to R3 684 252 between 1947 and 2013 (SAGL, 2013). This illustrates that the market values and production have improved favourably, and it has been proposed that the gross value of soybean is now the third from maize and wheat. In 2013 approximately 748 318 tonnes of the soybean produced, and future projections lie between 1 759 000 to 3 290 000 tonnes per annum (SAGL, 2013; NAMC, 2011). An amount of 15 406 tonnes of the soybean was exported with increased demands for full fat soya and products. However, despite the increase in production, 94% of soybean oil consumed locally are exports predominantly from Brazil and Argentina. Therefore, to meet domestic demands, local soybean production and processing need to be modified with a projected land increase of 121% in hectares up to the year 2018 (NAMC, 2011). The state of South Africa recognises the importance of soybean in the economy and has an Industrial Policy and Action Plan (IPAP) for 2012/2013 to 2014/15 (DTI, 2010). This document recognises soybean production as having the prospects for job creation and investment opportunities. The South African bureau for food and agricultural policy (BFAP) has projected an increase in land assigned for commercial soybean (BFAP, 2013). However, despite the bright outlook of production, not all provinces are suitable for soybean production.

1.2.1.3. Soybean Growth

Soybean plants are vulnerable to changes in the environment, for example temperature; nutrient availability; water content. Hence these crops require specific climatic and soil conditions. In South Africa, overall optimum conditions for all growth stages include: Temperature (25^{o}C); Rainfall (500-900mm); Soil (pH6-6.5, moist loose soils) for all growth stages except for germination which can occur at a lower temperature optimum of 15^{o}C (DAFF, 2010; Panner, 2006). One of the most susceptible stages of soybean plant growth to low temperatures is the seedling growth stage as stated earlier in chapter 1.1

The standard rate of soybean seedling development occurs at estimated timeframes stated in Table 1. Other factors like weeds, diseases and pests can affect yield hence mitigation practices must be included in the production to maximise seedling growth.

Table 1: Estimated growth characteristics of Soybean seedlings.

Growth Stage	Estimated Days	Characteristics
Germination	7 - 10	Imbibition (36-48 hours after sowing)
Seedling Emergence	7 - 10	Radicle emergence and hypocotyl pulls cotyledons out of soil
Vegetative emergence (VE)	5 - 21	Photosynthesis by green cotyledons
Vegetative cotyledon (VC)	5 - 21	Unifoliate and Cotyledons are fully expanded; Cotyledons above the soil surface
Vegetative First Trifoliate (V1)	5 - 21	Unifoliate fully developed and unrolled trifoliate present

Adapted from DAFF (2010); Casteel (2010) and Ohyama (2013).

1.2.2. Leaf structure & Function

Leaves are plant organs specifically adapted for the process of photosynthesis and play a vital long-term adaptation to the environment through various plant functions. Hans and Bassham (2017) summarises adequately the process of photosynthesis that mainly occurs in leaves. It can be described as the process by which photosynthetic organisms use the energy from sunlight in the presence of carbon dioxide (CO_2) and water (H_2O) to produce mainly carbohydrates and oxygen (O_2) and to a minor extent amino acids, proteins, lipids and pigments. During cellular respiration adenosine triphosphate (ATP) is produced when glucose ($C_6H_{12}O_6$ -building block of carbohydrate) is converted to pyruvate.

The upper epidermis has an upper cuticle (waxy barrier), cells lack chloroplasts. The lower epidermis contains specialised cells called guard cells which enclose a pore called a stoma. Stomata are present to allow for CO_2 and transpiration (Yamori, Hikosaka & Way, 2013).

Hairs may be present that may alter air flow across leaf surface (Gates, 1980). The palisade mesophyll is mainly responsible for photosynthesis as the palisade cells have many chloroplasts with respect to the spongy mesophyll. Chloroplasts contain chlorophyll and the location of all photosynthetic reactions. Spongy mesophyll contains large air spaces which are linked to lower epidermis for gaseous exchange (Tholena, Booma & Zhua, 2012). The vascular bundle contains xylem which are responsible for H_2O and minerals transportation to leaf cells as well as phloem that transports products of photosynthesis away from leaf cells (Khan, 2002; Riedell, Khoo & Inglett, 1985).

Leaf thickness is often minute; thin to facilitate gaseous exchange and light penetration to relevant cells. It is generally considered that leaves show observable differences in thickness and surface area because of phylogenetic relationships and adaptation to specific environments. These have important implications for leaf photosynthesis capacity (Royer, McElwain, Adams, Wilf, 2008; Terashima, Hanba, Tholen, Ninemets, 2011). According to Xu, Salih, Ghannoum, David (2012) this illustrates that leaf anatomy and physiology can affect leaf characteristics and functioning which are closely linked to their structure. Several researchers have studied the relation between leaf thickness (LT), structure and functional traits with respect to salinity and drought stress (Giuliani, Koteyeva, Voznesenskaya, Evans, Cousins, Edwards, 2013; Scoffoni, Vuong, Diep, Cochard, Sack., 2014; Afzal, Yar, Ullah, Ali, Ali, Ahmad, Fu, 2017).

A strong correlation between leaf thickness and RWC has been established however this is dependent on the leaf structure, plant characteristics, plant species and environmental variables (Taiz & Zelger, 2006; Scoffoni *et al*., 2014). Furthermore, environmental factors affect leaf thickness variations through their role in transpiration as stated by Giuliani *et al*. (2013). Initial leaf thickness is established during early leaf development, following a phase of rapid thickening according to Maksymmonych & Maksymmonych (1973).

RWC has been reported by Ghosh, Rai, Tyagi, Challam (2016) in rice seedlings to be a good indicator for dehydration as it is the best representation of plant water status. RWC it is not well documented as a physiological indicator during LT despite a direct correlation to cellular dehydration.

1.2.2.1. ROS Production in Leaf cells.

Mittler, Miller, Suzuki, Ciftci-Yilmaz (2010) and Komatsu, Kamal, Hossain (2014) state that ROS is part of the fundamental process of signal transduction by acting as an arbitrator

during abiotic stress, infection by pathogens and programmed cell development or death. Naturally ROS are produced during aerobic metabolism in intracellular organelles with chloroplasts being the main production site in plant cells. Plant responds by activating an antioxidant defence system which comprises of enzymatic and non-enzymatic components (Kim and Portis, 2004; Mittler, 2006; Borowski and Blamowski, 2009, Komatsu *et al*., 2014).

Heightened concentrations of poly-unsaturated fatty acids (PUFA) and the process of photosynthesis that occur in the chloroplasts and electron transport chain in complex I and Q zone of the mitochondria can increase the risk of oxidative injury in plants according to Komatsu *et al*. (2014). These organelles are primary ROS production sites due to immense levels of oxidation during metabolic activities (Awasthi, Bandari, Nayyor, 2015). ROS production also occurs in peroxisomes through β-oxidation and photorespiration of fatty acids. H_2O_2 can be produced by 2 processes, either through glycolate oxidation by glycolate oxidase or directly by xanthine oxidase from O_2 (Awasthi *et al*., 2015).

However, when ROS threshold are exceeded oxidative injury can be aggravated and many processes have been postulated to contribute. Komatsu *et al*. (2014) specifies that antioxidants localized in plant cell organelles such as superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPX) and ascorbate peroxidase (APX) facilitate reduction of free radicles to water (H_2O). Hasanuzzaman, Hossain, Teixeira da Silva, Fujita (2012) states that SOD acts as the first line of enzymatic defence against oxidative stress by simultaneous redox reactions whereby O_2^{-} is converted to hydrogen peroxide (H_2O_2). Although H_2O_2 is relatively stable, it will still be enzymatically controlled by catalase and peroxidase. In the event of lipid peroxidation (LPO) and mitochondrial dysfunction, hydroxyl radicals are produced from H_2O_2 which can lead to oxidative damage (Komatsu *et al*., 2014). However even though O_2^{-} and H_2O_2 can trigger LPO in membranes, it is often initiated when H atom is generated from OH• due its reactivity character in the PUFA's unsaturated fatty acyl chain (Montillet, Chamnongpol, Rustérucci, Dat, van de Cotte, Agnel, Battesti, Inzé, Breusegem, Triantaphylidès, 2005).

1.2.3. Chilling Temperatures

Tolerance of plants to low temperature stress varies among species which can result in chilling and freezing damage due to suffocation and heaving of internal structures and cells of plants. Chilling injury is a serious problem especially during germination and early seedling growth in chilling sensitive plant species (Solanke & Sharma, 2008; Kratsch & Wise, 2000). A number of studies confirm that the primary site for chilling injury in plants is cellular

membranes of organelles i.e. chloroplasts and mitochondria (Musser *et al*., 1984; Lukaktin *et al.*, 2012). A review done by Yadav (2010) superbly condenses studies that have been done on plant responses to cold stress. These include increase reactive oxygen species (ROS), growth suppression of plants, necrosis and discolouration of leaves, reduced photosynthetic capacity and increase in abscisic acid levels. Temperature stress can induce toxic accumulation of compounds in cells such as ROS that lead to oxidative stress (Suzuki and Mittler, 2006). Water deficit and cellular dehydration can also happen which will probably cause a gross disruption of metabolism and cell structure (Smirnoff, 1993; Steponkus, 1984).

A few reviews on chilling effects in organelle ultrastructure have been published stating that although there are a number of variables affecting the severity and time course of chilling injury, symptoms are comparable among plant species (Kratsch & Wise, 2000; Mohammed, 2011; Lukaktin *et al*., 2012). These include swelling and disorganization of both chloroplast and mitochondria; reduced size and number of starch granules; dilation of thylakoids; unstacking of grana; formation of small vesicles of chloroplasts and condensation of chromatin in nucleus. Chloroplasts are stated to be more sensitive to chilling temperatures and show the earliest signs of injury. Mitochondria are apparently more resistant to chilling even during periods of chloroplast deterioration. However, a study by Ishikawa, (1996) mentioned enlarged cristae and vacuolation; swollen nuclei; enlargement of golgi apparatus specifically vesicles and bulging of endoplasmic reticulum in chilled plant cells. Effects on nucleus are not widely reported because visible changes rarely occur or not pronounced (Kratsch & Wise 2000; Mohammed, 2011).

Plants that grow in warm climates exhibit symptoms of injury when exposed to chilling temperatures. These include soybean (glycine max) and tomato (*Lycopersicon esculentum*) which have been shown to exhibit signs of injury from 48hours to 72hours, between 10°C and 15°C (Lynch, 1990). However, symptoms have been shown to vary due to duration and different plant adaption mechanisms (Shilpi & Narendra, 2005).

Low temperatures are very likely to lower soybean biomass and yield (Gaynor, Lawn, and James, 2011; Kurosaki & Yumoto, 2011). Yamaguchi Yamazaki, Ohnishi, Suzuki, Hagihara, Miyoshi & Senda, (2014) states that yields of soybean may be affected by low temperature either early or late in plant growth. However other studies have shown that the reproductive stage of plant growth is more sensitive to temperature stress than vegetative growth process (Prasad, Boote, Allen, Thomas, 2003; Singh, Redońa & Referzo, 2010; Hall, 2001).

A decrease in the photosynthesis capacity has been identified by Ohnishi, Miyoshi, and Shirai (2010); Board & Kahlon (2011) during chilling stress of soybean as partly due to chilling oxidative damage of chloroplast components. Tambussi, Baartoli, Gulamet, Beitrano, Araus (2004) stated that the majority of damage is attributed to lipid peroxidation that occurs when cellular or organelle membranes lose their integrity. Ishikawa (1996) further elaborates on the former that if the chilling condition progresses, disorganization of total plastids occurs due to accumulation and swelling of lipid drops.

Experiments have been conducted on effects of cold temperature on the soybean growth. Some of the literature includes experiments done by Borowski & Michalek (2014) in Poland who investigated the effect of chilling temperatures on germination and early growth of domestic and Canadian cultivars. The authors have shown how two-week-old soybean plants of different cultivars respond to a 6-day chilling period at day/night conditions of 25/^{o}0C and 10/0^{o}C. Catalase activity, proline and leaf electrolyte leakage increased when compared with the control. It was established that soybean seedlings are sensitive to chilling temperatures from 0^{o}C to 10^{o}C. This is supported by a review done by Lukaktin *et al*. (2012) that chilling temperatures of 1^{o}C to 10^{o}C lead to physiological disturbances in cells of chilled vegetative species of subtropical origin i.e. cucumber, beans and cotton.

Effect of chilling temperature on seedlings was further investigated in France by Posmyk, Bailly, Szafranska, Janas and Corbineau (1997) by exposing seedlings to 1^{o}C in a growth chamber for 8days. Inhibition of hypocotyl and root elongation growth occurred after transferring test subjects to control conditions (25^{o}C), catalase activity and superoxide dismutase activity was observed in hypocotyls with capacity for growth recovery stronger in hypocotyls than in roots. This demonstrates that chilling injury is usually evident after the transfer of tissue to non-chilling conditions according to Wright and Simon, 1973; Jennings and Saltveit, 1994. Musser *et al*. (1984) illustrated soybean sensitivity to night temperatures of 10^{o}C with respect to growth, metabolism, development and yield. Chloroplast grana unstacking with the envelop membrane developing protrusions; alteration of lipids and reduction in chlorophyll fluorescence was observed.

Studies conducted in South Africa include experiments on effects of dark chilling on carbon dioxide assimilation, kinetics of chlorophyll fluorescence and nitrogen fixation in the vegetative stage of soybean. Soybeans that grow in tropics are more sensitive to night temperatures below 15°C (Van Heerden, Kruger, Tsimili-Michael & Strauss, 2003; Van Heerden & Kruger, 2004; Strauss & Van Heerden, 2001). The agricultural research focused

more on weed and disease management. Cultivar's used are Roundup® Ready cultivars that are generally gene tolerant to herbicide glycophosphate for weed control management with variations in yield potential, height of pod, length of growing plants, utilisation and plant vigour (Panner, 2006). However, none of the cultivars are listed for chilling temperature tolerance of 5^{o}C and 15^{o}C. What is currently known by DAFF is that soybean yields are adversely affected by low temperatures below 13^{o}C for extended periods during reproductive period thus inhibiting flower and seed formation. 25^{o}C is considered optimum for all growth stages. Soil temperature must preferably be in the region of 15^{o}C to stimulate germination (DAFF, 2010).

The demand of soybean products in Southern Africa is increasing with South Africa being the largest market in Southern Africa (NAMC, 2013). This demand creates a favourable context for increasing soybean production (Gasparri, Kuemmerle, Meyfroidt, De Waroux and Kreft, 2015), to sustain the growing population and combat food security that has become an area of concern. Therefore, to meet market and production demand, the understanding of factors that affect one of the important agricultural crop in South Africa, soybean, is essential

1.3. Problem Statement

As mentioned in literature review, soybean is originally adapted for warm climates hence it will have sensitivity to chilling temperatures. It is a fast-growing species that makes it likely to be strongly responsive to variations in temperature. This is supported by a study done by Fatichin, Zheng, and Arima (2013) that observed a faster dry matter accumulation during soybean seeding growth as compared to other legumes making soybean a suitable model species for study of chilling stress on leaf structure. Extensive research has been done which contributes to our understanding of physiological effects on plant growth. However previous studies paid little attention to the histological, and ultrastructure responses. Most studies have focused on soybean plant growth and physiology and none focused on soybean leaf structural attributes that are also known to contribute to the regulation of leaf anatomy. Therefore, an investigation on leaf structural attributes i.e. LT, mesophyll layers, cell arrangement of leaf and organelle integrity are essential. Low temperature stress has been known to accelerate the generation of ROS which includes superoxide radical ($O^{\bullet-}_2$) inducing oxidative stress. SOD an antioxidant localized in plant cell organelles acts as the first line of enzymatic defence against oxidative stress by simultaneous redox reactions whereby $O_2^{\bullet-}$ is converted to H_2O_2. Hence antioxidant status will be determined from SOD assay.

1.3.1. Aim

To investigate potential effects of chilling temperatures (5ºC and 15ºC) on the leaves of soybean seedlings.

1.3.2. Objectives of research study

- To determine leaf fresh weight, dry weight and relative water content in soybean seedlings under 5ºC; 15ºC and 25ºC (control) for 72hours
- To determine the leaf histological and ultrastructure responses of soybean seedlings under 5ºC; 15ºC and 25ºC (control) for 72hours.
- To determine the level of oxidative stress in the leaves of soybean seedlings under 5ºC; 15ºC and 25ºC (control) for 72hours by measuring superoxide ($O^{\cdot-}_2$) using superoxide dismutase (SOD) assay.

Chapter 2

2.1 Materials and Methodology

2.1.1. Plant Material and Growth conditions

Soybean (*Glycine max (L.) Merr.*) seeds were purchased from a local farmers market and sterilised prior to germination with 50% ethanol for 1 minute and rinsed with distilled water. The seeds were grown in polystyrene cups filled with organic potting soil at a density of 2 seedlings per pot, from August to October for growth indexes, leaf functional traits (colour, vigour, thickness) and ROS analysis. Seedlings were grown and germinated in a controlled growth chamber at 23±2°C; 50-70% humidity; photoperiod of 14hours and a light Intensity of 200µmol.m^{-2}.s^{-}1. A watering regime was scheduled with 20ml of tap water every 2days. After 2$\frac{1}{2}$weeks, seedlings with similar length were subjected to 5°C and 15°C chilling stress for 3days under the same regime as indicated above. For comparison purposes, plants not exposed to chilling stress were grown in a growth chamber at 25°C (control). 30 seedlings were allocated each to growth analysis, microscopy, and SOD assay, with 10 seedlings used per temperature treatment [25°C (control); 5°C and 15°C (treatments)]. Methods used were dependent on apparatus and chemical availability.

2.1.2. Leaf Growth Indexes

General leaf changes i.e. vigour, shape, colour were observed on captured images between treatments.

Growth indexes that include fresh weight and dry weight were measured on seedlings for each treatment every 24hours for 3days. The fresh weight (FW) was weighed immediately after harvesting the leaves. Leaves were dried at 80°C for 24hours to determine the dry weight (DW).

Relative water content (RWC) was calculated to determine levels of dehydration. RWC was expressed as a percentage according to the following formulae adapted from Balestrasse *et al*. (2010).

$$RWC(\%) = \frac{(FW-DW)100}{DW} *0.1$$

2.1.3. Microscopy Analysis

The microscopic study was used to assess the response of seedling leaves on chilling treatments (5^{o}C and 15^{o}C) as compared with the control (25^{o}C) at cellular level after 24hours. Light microscopy (LM) was sampled for comparative study on leaf morphology parameters. Transmission electron microscopy (TEM) was done for a ultrastructural analysis of organelles. Hand cut sections of less than 3mm in width were made at selected locations as indicated in figure 1. The hand-cut sections were prepared for microscopy using a standard gluteraldehyde-osmium method and embedded in Spurr's epoxy resin (Spurr, 1969). Refer to annexure 1 for detailed method. Semi-thin (0.5µm – 0.8µm) and ultra-thin (60nm-70nm) sections of the seedling leaves were obtained using a Reichert- Jung Ultracut C Ultramicrotome. Semi-thin sections were heat-fixed onto 76 x 26mm glass slides and stained with 0.01% (w/v) Toluidine Blue (stains nucleic acids blue and polysachharides purple, increasing clarity of histology slide images). These where mounted in *p*-xylene*bis*(*N*-pyridinium bromide) (DPX) and covered with glass cover slips, and thereafter viewed and photographed with an Olympus BX 63 OM/FM compound microscope. Data captured by LM was further quantified using measuring tool under CellSens Dimensions software by measuring leaf parameters using image analysis software (leaf thickness, palisade thickness, spongy mesophyll thickness, upper and lower epidermis layers) at multiple points. All measurements were recorded in micrometres (µm) on transverse sections of leaves using the light microscope calibrated reticule at 40X & 60X magnification as illustrated in figure 1. Results were recorded as means and standard deviation of 7 replications. If F-statistic of one-way ANOVA was lower than 0.05, follow up assessment were verified by a Tukey HSD, post-hoc test. Ultra-thin sections of leaf seedlings were collected on 300µm mesh copper grids and stained with lead citrate (Reynolds, 1963)]. These sections were viewed and photographed with a FEI Spirit 120kV transmission electron microscope with focus emphasis on the mesophyll cells as illustrated in Figure 1b. Results were quantified using micrographs using differential interference contrast imaging to increase visibility of fixed cells. The structural integrity of organelles i.e. chloroplast, cell membrane, vacuole was observed.

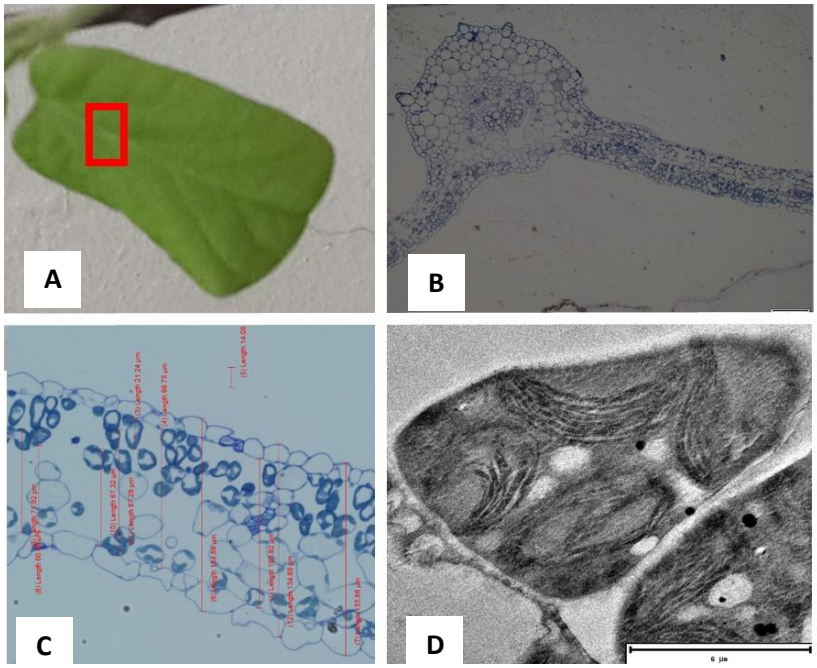


Figure 1: Histological, ultrastructural and leaf thickness assessments. (A)Leaf sample showing an area where hand-cut sections were obtained (red square). (B) Light microscope micrograph showing a focal area for leaf thickness determination and cellular response observation (red square). (C) Micrograph showing how leaf thickness was measured (red markings/indication/lines. (D)TEM micrograph used for ultrastructural observations.

2.1.4. Determination of Superoxide Production

ROS production analysed using SOD assay according to the method by Misra and Fridovich (1972). Superoxide ($O^{\cdot -}_2$) production was determined using a colorimetric assay that measured the oxidation of epinephrine to adrenochrome spectrophotometrically at 485 nm. Leaves from both control and treated seedlings were incubated in 4ml of 1mM epinephrine solution (pH 7) and shaken at 45 revolutions/minute on a rotary shaker in the dark for 15minutes. Superoxide production was calculated by using the molar extinction coefficient for adrenochrome (4020 $lmol^{-1}cm^{-1}$).

2.1.5. Statistical analysis

Results were statistically evaluated; Inter-treatment data differences were subjected to one-factor analysis of variance (ANOVA) with post-hoc tests for statistical significant groups, for leaf functional parameters tested after 24 hours. Two-factor ANOVA for physical and chemical traits observed every 24 hours for 3days was scrutinised. $P<0.05$ was used to access statistically significant data. Full statistical analysis results are indicated on annexure 1. Representatives of histological and ultrastructural data are presented in micrographs, and each experiment was replicated three times

Chapter 3

3. Results & Discussion

Experiments were carried out to assess the possible effects of chilling temperatures (5°C and 15°C) and compared with the control (25°C) on soybean leaves at seedling stage. Leaf functional parameters which included general appearance of leaves (leaf vigour, shape, colour) were analysed for chilling treatment effects. Physical parameters, DW and FW were analysed to determine RWC which indicated the level of dehydration. Microscopic analysis was done to determine leaf cellular changes using the LM and TEM. Data captured by LM was further quantified using measuring tool under CellSens Dimensions software by measuring the leaf thickness, assessing the responses of the palisade mesophyll and spongy mesophyll layers, adaxial and abaxial layers on the leaf cross sections. ROS analysis was also carried out to establish physiological changes in leaf by assessing superoxide concentrations.

3.1. General Leaf Changes

Morphological changes were observed between treatments (15°C and 5°C) and compared with the control, 25°C. Visual changes were captured as illustrated in Figure 2 below. Variations in leaf shape were evident on leave seedlings under both 15°C and 5°C assessments. Control leaves had a more elliptical or ovoid shape while low temperature treated leaves, 15°C and 5°C tipped between an oblong and obovate shape. Nicotra, Cosgrove, Cowling, Schlichting, and Jones (2008) noted that the variations in the leaf shape are likely to be a product of ecological context. The leaf shape can vary between oval; ovate, lanceolate and linear. Therefore, leaflet shape is considered as a qualitative trait may be influenced by environmental conditions (Sujata, Basavaraj & Salimath, 2011). Colour differences were detected under the 15°C when compared to the control, plant samples seemed to exhibit a light green colour as compared with the control. This response may possibly be associated with the reduction of the pigment in the treated leaves. This response was demonstrated by Crang and Noble (1974) who stated that distinct differences are present with light green leaves processing pleomorphic chloroplasts which exhibit reduced thickness of grana and lack of starch grains with respect to dark green leaves. Discoloration of leaves and internal tissue was also observed by Tsuda, Niimura and Katoh (2003); Sharom, Willemot and Thompson (1994) and Yoshida, Kanno and Kaтeya (1996). This was attributed to reduction in chlorophyll accumulation in actively growing leaves (Balestrasse et al., 2010;

Sanghera Wani, Huisain & Singh, 2011). However, there was no significant colour change under 5°C therefore it is possible that the amount of tissue discoloration may not necessarily be a result of injury but another reason i.e. some seedlings may be genetically predisposed to produce seedlings with lighter leaves than others (Govindarao, 2010).

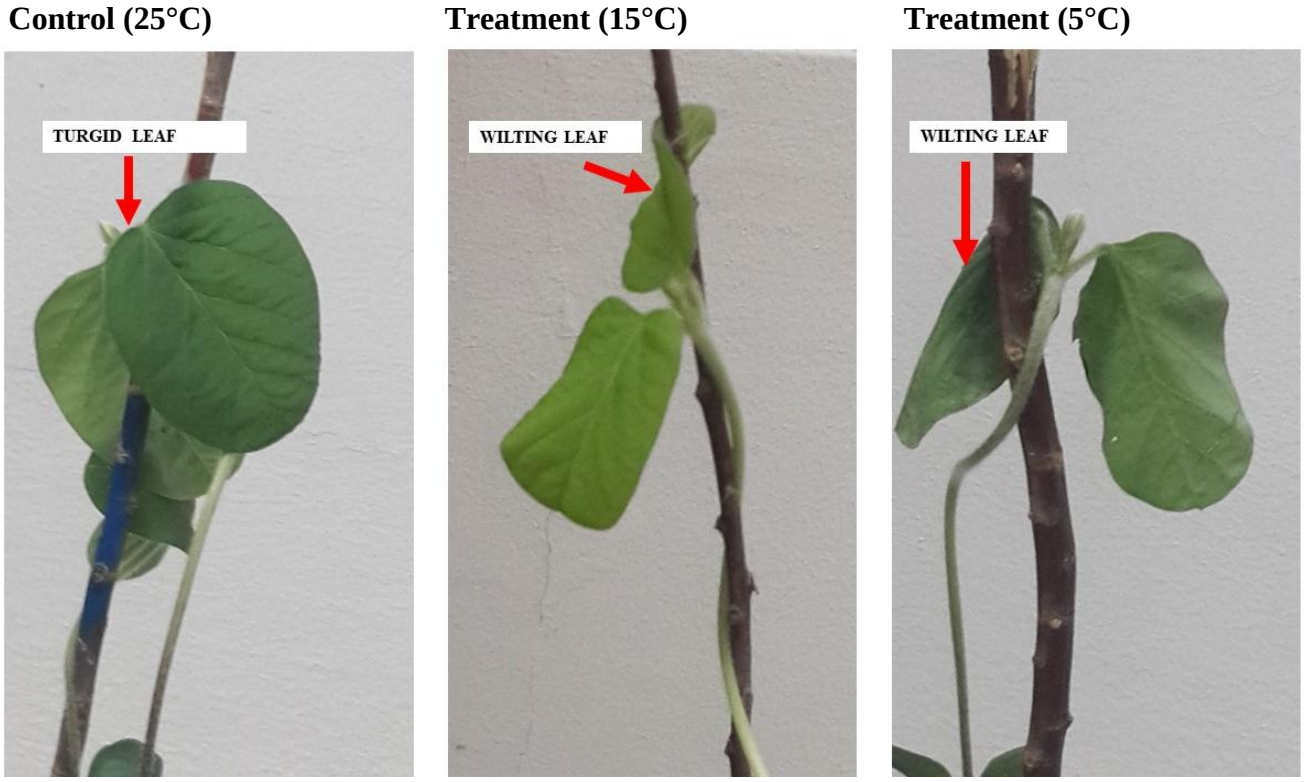


Figure 2: Visual changes observed on the leaf appearance of soybean seedlings after 24hour incubation at low temperatures 15°C and 5°C. Treatments were compared with the control (25°C).

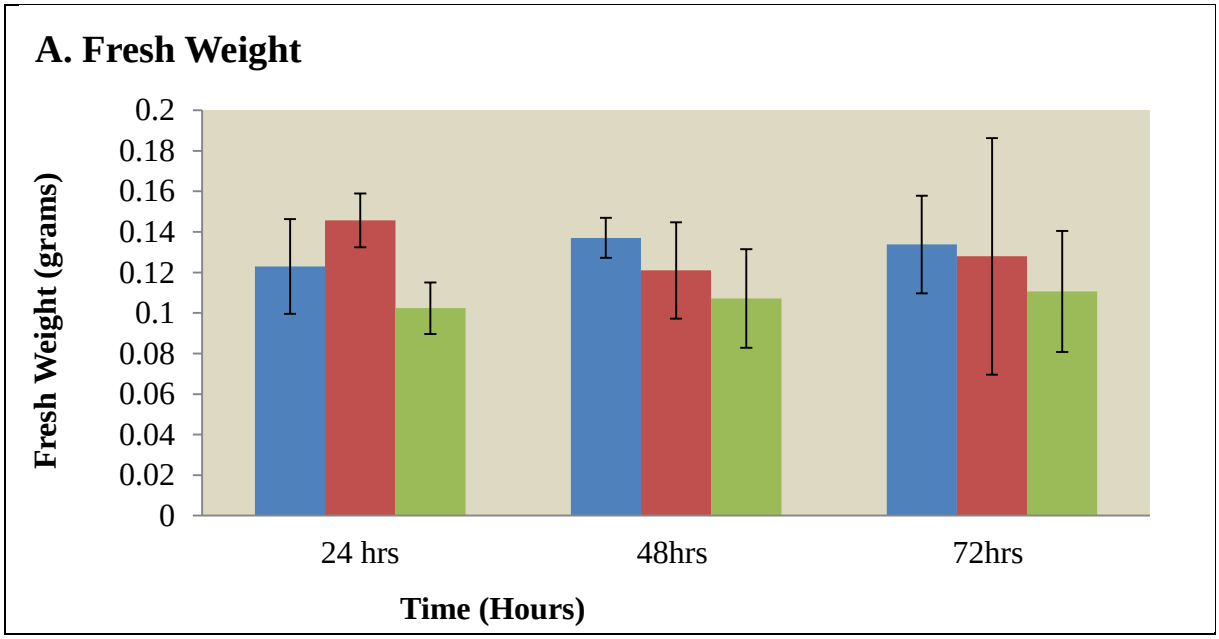
Leaf vigour varied amongst treatments**,** firm leaves were evident in the control with leaves holding a flat position which is a result of turgid cells according to Kadioglu & Terzi (2007). Leaves looked firm under 15°C but interestingly leaf margin of leaves slightly curled. Demiralay, Saglam and Kadioglu (2011) stated that leaf curling is used as a defence mechanism in response to water loss to effectively reduce leaf area for transpiration. Therefore, curling of leaf margin reflected a strategy of protection from chilling temperatures; water loss was simply a symptom of low temperature stress which is an agreement with studies done by Lianopoula, Bosabalidis, Patakas, Lazari, and Panteris (2014). This is further illustrated in Figure 3C, where RWC declined with chilling treatments.

Wilting was observed on leaves under 5°C. Some structural integrity seemed to be compromised and leaves looked flaccid after 24hours. This can be based on a few reasons which include rapid decline in the ability by roots to absorb water and transport it to shoots

(Bolger, Upchurch & Mcmichael, 1992). Pardossi, Vernieri and Tognoni (1992) and Bloom, Zwieniecki, Passioura, Randall, Holbrook and Clair (2004) have attributed wilting to the reduced ability to close stomata in response to subsequent water deficit. Visible wilting was also reported by Wang, Reddy and Quebedeaux (1997) who looked at the soybean leaves response to 8°C after 24hours. Wilting of leaves due to water loss has been reported by Skog (1998); Bloom *et al*. (2004) and Bagnall, Wolfe and King (1983). After 48hours and 72hours all treatments had a similar appearance to their respective 24 hours test observations (data not shown).

3.2. Leaf Growth indexes

Growth indexes that include fresh weight, dry weight was measured on seedlings for each treatment every 24hours for 3days. Relative water content (RWC) was calculated to access possible level of water loss (dehydration). Results are illustrated in figure 3 below.



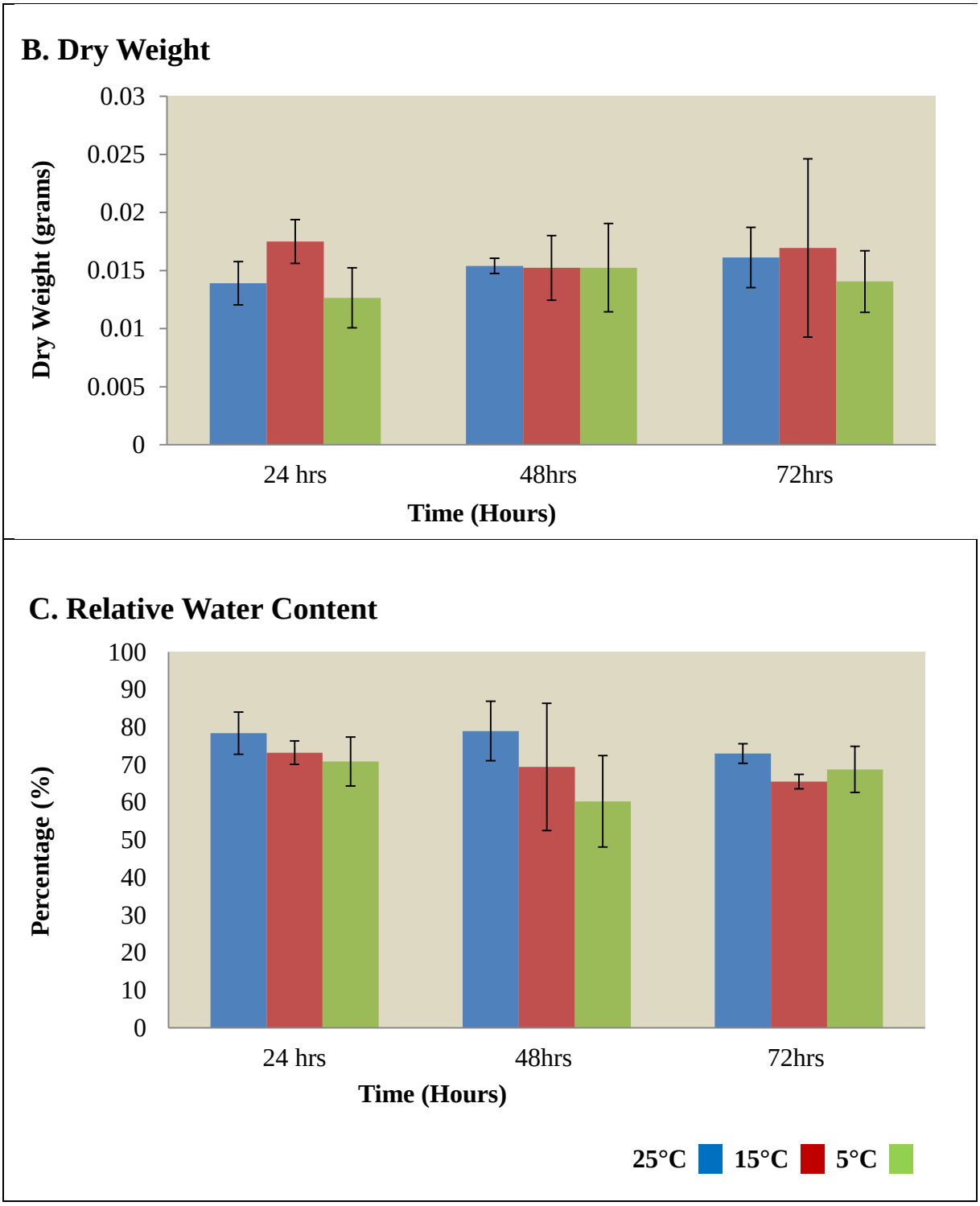


Figure 3: Differences in (A) Fresh Weight(FW); (B) Dry Weight (DW) and (C) Relative Water Content (RWC) levels for chilling temperature treatments (15°C and 5°C) as compared with the control (25°). Error bars represent the standard deviations of respective means.

In both low temperatures treatments, FW of leaves was significantly affected as illustrated in figure 3A. FW of leaves declined under 5°C temperature at an average decrease of 17%±2 at 24hour intervals during the 72 hour period. FW of leaves under 15°C treatment had similar

responses as compared with the 5°C treatment with an average decrease of 16%±8. Statistically significant differences were observed between the control and 5°C treatment as well as 15°C and 5°C treatments; p-value (0.0049<0.05) and p-value (0.040402) as illustrated in Table 3(Annexure 2).

Figure 3c shows that RWC decreased at both chilling temperature treatments by 9%±2 (15°C) and 13%±2 (5°C) as compared with the control. Both treatments were statistically significant at p<0.05, p-values obtained across the 3day period were 0.03 and 0.0006 for 15°C and 5°C respectively. P-value (0.132792 > 0.05=α) was obtained for DW therefore differences between temperature treatments was statistically insignificant. As samples were exposed to lower temperatures, FW and RWC declined during extended periods of exposure. Effects on growth indexes can be observed from as early as 24 hours to 72hours.

Bloom *et al*. (2004) stated that chilling temperatures affects all components of water regime and causes strong wilting which was noted at 5°C in figure 1. Insufficient water supply to shoots could be correlated to a decline in roots ability to absorb water and transport to leaves as well and reduced ability to close stomata in response to subsequent water deficit (Bolger *et al*., 1992; Bloom at al., 2004). Smirnoff (1993) also supported this notion by stating that when RWC falls between 30% - 70%, photosynthesis was affected because of inhibition of dark reactions. But nonetheless chloroplasts were able to undergo osmotic adjustment to enable them to maintain their volume and avoided inhibition of photosynthesis by increasing stomatal conductance (Yadav, 2010; Silvente, Sobolev & Lara, 2012; Filová & Krivosudská, 2017).

Our study confirms that chilling stress can cause water deficit and will likely result in leaf dehydration as this was evidenced in decrease of RWC between 15°C and 5°C by 9%±2 and 13%±2 respectively and this can be correlated to the physical manifestation of leaf curling at 15°C and wilting of leaves at 5°C.

3.3. Microscopy

3.3.1. Light microscopy

Soybean leaf structure is that of a dicotyledonous plant and this is evident by the presence of palisade cells located right below the thin waxy cuticle and the adaxial layer (upper epidermis comprised of one layer of cells). The tightly packed palisade cells are vertically elongated and densely packed with chloroplasts to facilitate absorption of light energy for photosynthesis. The spongy mesophyll cells beneath are different with circular cells that have

large intracellular spaces connected to stomata for gaseous exchange. Stomata are located on the lower abaxial layer (lower epidermis comprised of one layer of cells). This structural distribution was observed in the control (25°C) in figure 4 below.

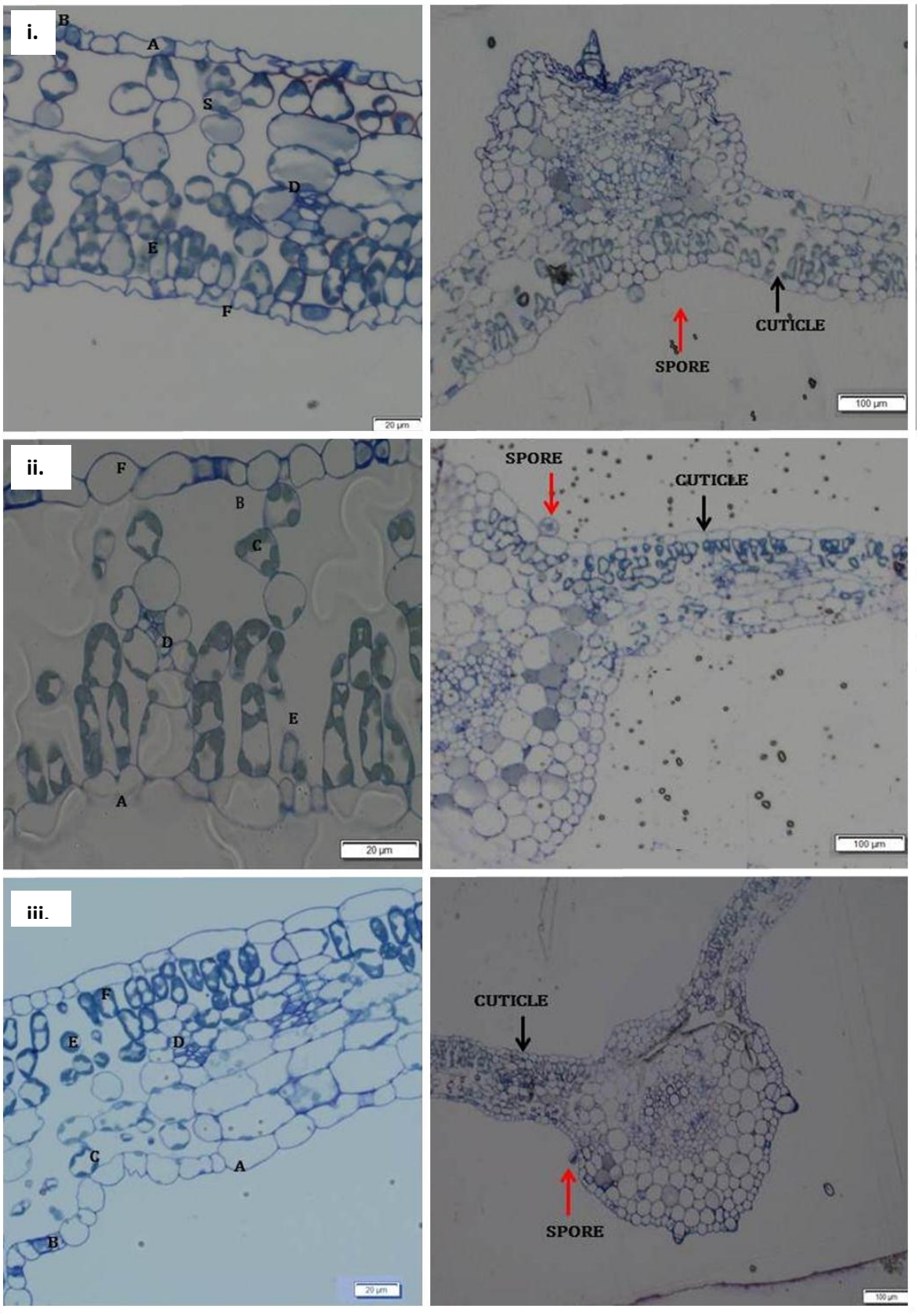


Figure 4: Micrographs of soybean seedling visualized at scale bars 20µm for the expanded inserts and 100µm for the leaf section on LM. Magnification of X40 and X60 was employed to capture anatomical changes in leaf under (i) Control (25°C); (ii)Low temperature treatment 15°C and (iii) Low temperature treatment 5°C conditions. Structures of interest are represented as follows: A-Abaxial layer (lower epidermis); B-Guard cells; C- Spongy Mesophyll Layer; D- Secondary Veins; E- Palisade layer; F-Adaxial layer (upper layer). The control (25°C) treated leaf structure had elongated, tightly packed palisade cells located right below the thin waxy cuticle layer of adaxial cells. The circular spongy mesophyll cells beneath had large intracellular spaces connected to stomata for gaseous exchange. Stomata are located on the abaxial layer. 15°C treatment had loosely packed elongated palisade cells while 5°C palisade cells were mostly oval shaped and loosely packed. Both chilling treatments showed an increase in chloroplast density of the palisade cells and spongy intracellular space. Periphery of adaxial layer became smoother and levelled at both treatments. Presence of fungal spores were noted in all treatments but enlargement of spore was noted at 15°C and 5°C.

When compared to the control, leaves had elongated palisade cells in the where well defined under the 15°C treatment but cells were not tightly packed. However, palisade cells were loosely packed under the 5°C treatment and majority of cells had more oval shapes and were shortened. Table 2 illustrates a statistically significant decrease in palisade layer thickness under the 15°C treatment when compared to the control with 27±3µm. These results indicate that changes in leaf thickness may have been attributed to increase modifications in cell size. However, both 5°C and 15°C samples showed an increase in chloroplast density of the palisade cells as compared with the control sample. Increase in chloroplasts density has been observed during cloudy days to compensate for less light availability according to Forghany, Khodabandeh, Habidi and Bankeshaz (2010) as cited in Jenabiyan, Pirdashti, Yaghoubian (2014). Stomata were observed on either abaxial or adaxial and irregularity of cell size and shape was observed which increased in both treatments (15°C and 5°C). A well-developed spongy mesophyll layer was observed in the control, cells were loosely packed, however percentage of intracellular space increased at 15°C and 5°C treatment samples making the spongy mesophyll appear looser with respect to control.

In figure 4i, adaxial layer had one layer of cells with periphery having protrusions; cells were not levelled and roughly under 15°C treatment. However, in figure 4.ii under the 5°C treatment; cells appeared levelled and smoother which could be an indication of plant responding to chilling temperatures by thickening the cuticle. Our results in table 2 show that adaxial layer thickness increased and was significant under 5°C (12±2µm) when compared to

the control (8±2µm). Although it is not certain how environmental factors illicit wax deposition on the cuticle surface; Hietala, Mozes, Genet, Rosenqvist and Laakso (1997) established that willow leaf surfaces responded to low temperatures by increasing quantities of cuticular wax as a tolerance mechanism to frost.

Images of leaf sections were quantified with analysis software to determine a series of leaf parameters at multiple locations which include Leaf thickness, Spongy mesophyll and Palisade mesophyll layer, Adaxial and Abaxial layers in table 2.

Table 2: Response of leaf parameters to chilling temperatures after 24hours

	Temperature		
Leaf parameter	**Control (25°C)**	**15°C**	**5°C**
Leaf Thickness (µm)	115±10	121±7	109±15
Spongy Mesophyll Layer(µm)	60±6	60±8	56±6
Palisade Mesophyll Layer(µm)	40±4	27±3	34±7
Abaxial Layer(µm)	8±2	11±2	10±3
Adaxial Layer(µm)	9±1	10±2	12±2

When compared to the control statistically significant findings were observed for the PM layer was statistically significant under 15°C treatment (27±3µm), p-value ($0.001 > 0.05=\alpha$) and the adaxial layers under 5°C treatment with 12±2µm, p-value ($0.01 > 0.05=\alpha$). This suggests that the upper epidermis was possibly the most affected part and it has been suggested by Stefanowska, Kuras, Kubacka-Zebalska and Kacperska (1999) that this is a result of accumulation of pectin's in leaf cell walls and lipid polymers.

These results indicated that changes in leaf thickness were attributed to modifications in cell structure of adaxial and palisade layers. Although leaf thickness increased between the control and 15°C treatment, the difference was not significant. However, Poorter and Perez-Soba (2002) have illustrated that leaf thickness increases during low temperature stress as a result of carbohydrate accumulation. Interestingly, studies done by Wilson and Couper (1969) at 9°C, 15°C and 20°C indicated that temperature effects on mesophyll cell size or leaf thickness had no effect on photosynthesis. It was still uncertain how most of the environmentally induced morphological differences were established during leaf growth but what is known so far is that spongy mesophyll layer contributes more to leaf thickness and that the palisade mesophyll layer was established earlier than in spongy mesophyll layer

(Coneva, Frank, de Luis Balaguer, Li, Sozzani, & Chitwood, 2017). Leaves of soybean seedlings are still developing which may also account for some of the visual changes observed, i.e. vascular tissue were not transcurrent but surrounded by sclerenchyma sheath which are not strongly developed.

Thickness of all leaf parameters and increase in intercellular spaces confirms the sensitivity of soybean to cold temperatures. However, measuring leaf thickness was not straight forward but an estimate, in the case of this research; it was estimated from microscopical images. Errors could have occurred due to intercellular variation; heterogenicity within leaf tissue.

Presence of fungi was suspected under all treatments as spores may have attached to epidermis of leaf as indicated on figure 4. However, enlargement of spore was noted in the chilling treatments (15°C and 5°C) which may indicate spore germination. Chilling temperatures likely caused destruction to cellular structure and lead to the leakage of plant metabolites which created idea conditions for germination. Cabrera, Saltveit and Owens (1992) and Wilner (1959) stated that one of the indicators of low temperature injury is attack by saprophytic fungi.

3.3.2. Transmission Electron Microscopy

Ultra-thin sections of leaf seedlings were viewed under TEM with focus emphasis on the mesophyll and palisade cells. The structural integrity of organelles i.e. chloroplast, cell membrane, vacuole, nuclei and mitochondria was observed, and results illustrated in Figure 3.4. Kratsch & Wise (2000) stated that damaging effects are often revealed in destruction of the cellular. i.e. chloroplast swelling and structural changes due to destruction of membrane, disintegration of starch grains, reduction of ribosomes numbers, formation of peripheral reticulum (small vesicles of envelop); accumulation of lipid bodies; enlargement of cristae and vacuolation of mitochondria; swollen nuclei.

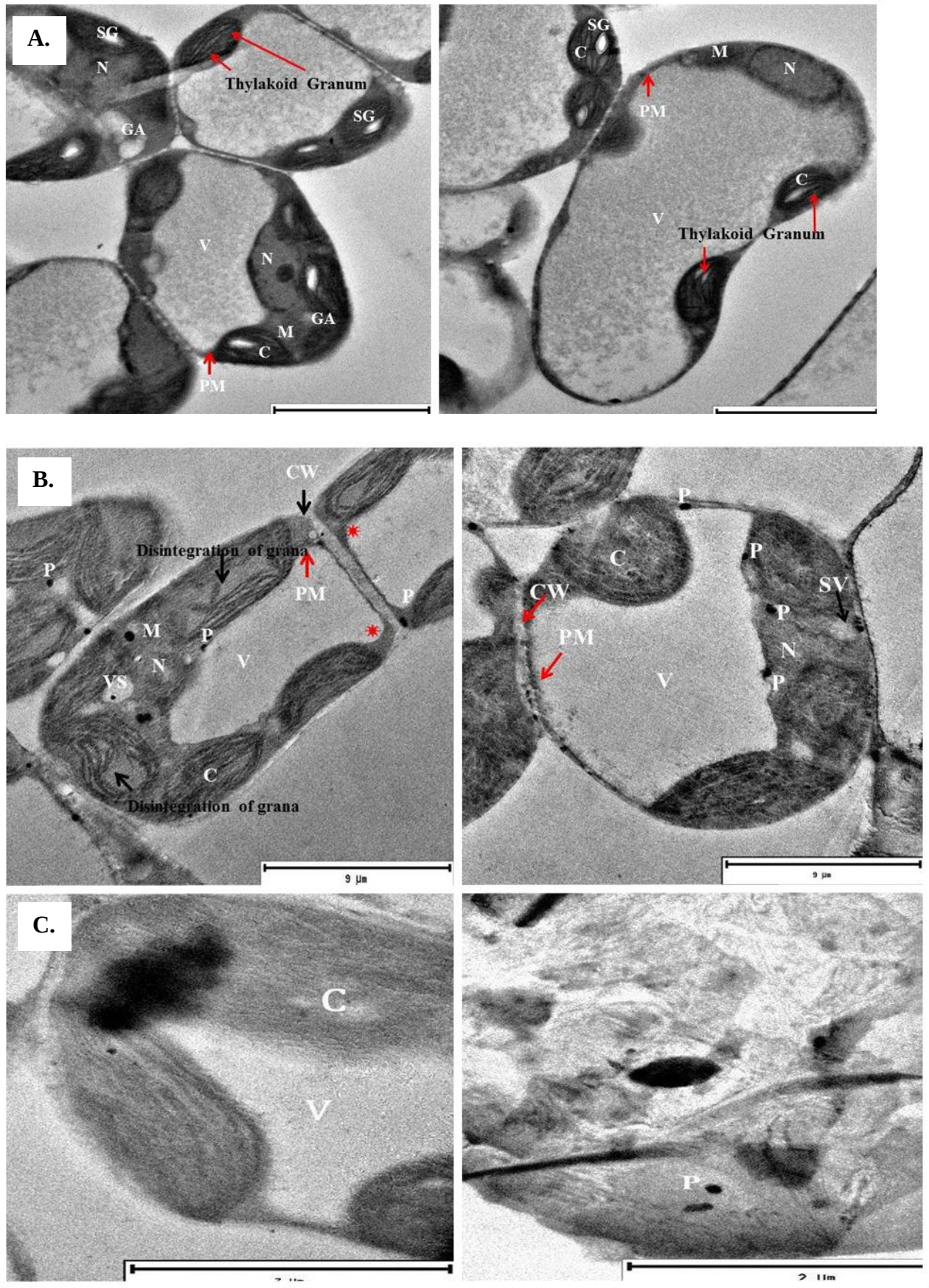


Figure 5: Micrographs of soybean seedling visualized at scale bars between 2µm and 10 µm on the TEM. The images of ultrastructural changes in leaf under (A) Control (25°C); (B)Low

temperature treatment 15°C and (C) Low temperature treatment 5°C conditions were captured. Structures of interest are represented as follows: C-Chloroplast; V-Vacuole; PM-Plasma Membrane; M- Mitochondria; N- Nucleus; SG-Starch granule; GA-Golgi Apparatus; SM-Small Vesicles; CW-Cell Wall.

In this study however soybean leave cells had the following responses; chloroplasts in control (25°C) were well developed (visible thylakoid and grana), and lens shaped and were located near the cell wall and contain at least a starch grain. Nucleus and mitochondria were noted and located adjacent to chloroplast, large central vacuole was present. Plant cells were clearly separated by cell membranes and as illustrated in figure 5A.

However, in the chilling treatment 15°C, majority of chloroplasts were rounder than oblong. Structures were visible as well; however more prostaglandins were observed (small black dots flagged by a P in figure 5B. No starch grains were visible for 15°C and 5°C. Decrease in starch grain content in chloroplasts was stated by Jouve, Engelmann, Noirot, Charrier (1993) and Musser *et al*., (1984) attributed this observation to impaired metabolic processes (respiration, photosynthesis and activity on carbohydrate metabolism).

At 15°C, cells had an overall flaccid appearance. Incipient plasmolysis (indicated by✷) was observed in figure 5B and there was slight drawing back of cell membrane from cell wall. Shrinking of the central large vacuole was observed with respect to the control. Changes in shape could be attributed to water loss as RWC declined at both chilling treatments as illustrated in figure 3C. As stated earlier, RWC is directly correlated to cellular dehydration (Ghosh *et al*., 2016). Nucleus and mitochondria were noted but changes were not detected. Mitochondria and nuclei are apparently more resistant to chilling even during periods of chloroplast deterioration. Effects on nucleus are not widely reported because visible changes rarely occur or not pronounced but swelling has been observed (Kratsch & Wise 2000; Mohammed, 2011). An increase in vesicle number was noted and Ishikawa (1996) stated in his article that enlargement of golgi apparatus specifically vesicles and bulging of endoplasmic reticulum has been observed in plant cells exposed to chilling temperatures.

The 5°C treatment illustrated in figure 5C had cells whose structure was compromised due to severe cellular low temperature stress damage. Loss of cellular integrity and disintegration of cellular organelles was observed. Injury on membrane systems during low temperature stress is primarily associated with occurrence of cellular dehydration when period of stress surpasses capacity of plant to respond via osmotic adjustment (Steponkus, 1984; Farooq,

Aziz, Wahid, Lee & Siddique, 2009; Wang *et al*., 1997; Janmohammaddi, Zolla & Rinalducci, 2015). However, a few cells were visualized which showed chloroplasts with disintegrated grana. Similar results were observed by Musser *et al*. (1984) who compared effects of chilling between 25/10°C. Grana and thylakoid structure in chloroplast was compromised at 10°C and no cellular membranes were visible. Looking at Figure 5 it seemed like cells generally developed a mechanism to cope with temporarily non-permissive environment conditions as some cells could still be distinguished despite the lack of cell clarity in 5°C treatment. Kratsch &Wise (2000) explained that this was because it was linked to genetic programmed metabolic processes that salvaged and packaged of valuable resources for maintaining metabolic activities during short periods of low temperature exposure. Therefore, effects although detectable were not lethal (Musser *et al*., 1984).

The fixation protocol used in microscopy was not able to stabilize some of the membranes in 5°C chilled sections as cellular structure was not preserved under the 5°C treatment samples. This is supported by Musser *et al*. (1984) who stated that possible reasons of this could be organelle degradation in *vivo* during treatments before fixation and organelles being incapable of being fixed and stained.

3.4. Determination of Superoxide

Experiment was designed to look at levels superoxide ($O^{\cdot-}_2$) in soybean seedling leaves at 15°C and 5°C with respect to control (25°C).

Approximately 0.75-fold increase was observed after 24 hours under the 15°C treatment and a 0.4-fold increase was noted after 72hours under 5°C treatment when compared to the control. This can be illustrated in Figure 3.5. However, this enhancement was not statistically significant between temperature groups (p value=0.859081<0.05=α).

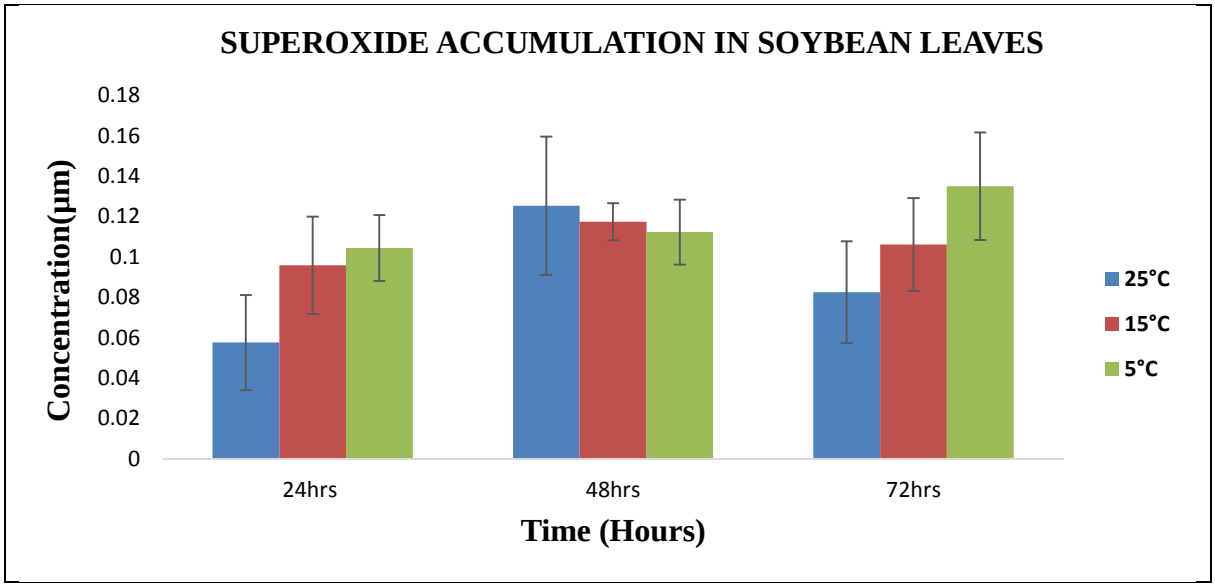


Figure 6: Differences in Superoxide Content levels for chilling temperature treatments (15°C and 5°C) with respect to control (25°). Error bars represent the standard deviations of respective means.

However, a point of interest in the interactions in ROS accumulation between temperature groups and time frame was statistically significant, p-value(time)=0.001041<0.05=α. This confirms that there was basal increase in ROS. Smirnoff (1993) and Halliwell & Gutteridge, (1989) elaborate that superoxide might not be highly reactive, and damage is more likely to arise from subsequent formation of H_2O_2. Luna, Badiani, Felice, Aitemi and Sermanni (1985) states that sunflower did not show an increase in SOD activity and remained constant in maize. Zhang Jiang, Peng, and Kerpelainen (2011) found that chilling temperatures resulted in slight increase in superoxide production in *populus cathayana* but it was not significant with respect to hydrogen peroxide increase. This indicates that oxidative stress could have occurred as result of hydrogen peroxide accumulation as SOD reacts with superoxide ($O^{\cdot-}_2$) to form hydrogen peroxide

$$O^{\cdot-}_2 \rightarrow H_2O_2$$

However, we will need to validate this notion by studying the relationship between superoxide and hydrogen peroxide under chilling stress in soybean seedlings and thither the metabolic activity during chilling stresses to establish the onset of injury.

An abnormal increase in ROS production due to biotic or abiotic stresses may cause lethal damage (Whitaker, Beckett, Minibayeva, & Kranner, 2010; Mittler *et al.*, 2010). Mitochondria and chloroplasts are important sources of ROS (which also acts as stress signals). These results correlate with the responses on the TEM study. The observed changes

in chloroplasts profiles, formation of vesicles, disappearance of starch grains, incipient plasmolysis could indicate that the low temperature treatments resulted in stresses and/or disruptions of the ATP generation processes which lead to more production of ROS and subsequently cellular damage, which was more pronounced on 5°C treatment. Therefore, environmental stresses can affect aspects of plant growth and development, consequently specific signalling pathways of plant metabolism (Johnova, Skalak, Saiz-Fernandez & Brzobohaty, 2016).

3.5. Future perspective

It should be noted though that physiological functions are not equally sensitive to chilling temperature and even though physiological functions are triggered the might not lead to visible manifestation of injury or changes in growth indexes since physiological processes are reversible until they become stable (Lyons *et al*., 1979). Therefore, it was important to extend this study to soybean seedling leaves that are still in the early vegetative stage of growth and that have not been extensively studied, however there are still other future perspectives that this study has incited.

To reduce errors in leaves, a water regime was employed to eliminate external water stress. However, Barrs & Weatherly (1962) re-examined the RWC technique for estimating water deficit in leaves and established sources of error in change in DW; full turgidity changes and water accumulation in intercellular spaces. Therefore, turgidity (TW) was included in there RWC calculation and these formulae can be used for future studies to reduce experimental errors.

$$\boldsymbol{RWC}(\%) = \frac{\boldsymbol{(FW-DW)100}}{\boldsymbol{TW-DW}}$$

Ability of plants in the vegetative state to survive the action of chilling temperatures without harm to the future growth can confer cold resistance. Lukaktin *et al*. (2012) states that responses of plants to low temperatures are associated with a change in the rate of the transcription of low molecular weight proteins. Very brief exposures to chilling temperatures are sufficient for appearance of stress proteins which can be accessed after seedlings mature. The seeds produced can be accessed for cold resistance.

There were no significant differences between the effectiveness of chilling temperatures on superoxide accumulation in soybean seedling leaves. Suzuki and Mittler (2006) elaborated there is a need to understand how much ROS is produced because of stress for the purpose of

signalling. Symptoms of oxidative damage are not easily/reliably measured and they include lipid peroxidation, protein denaturing and nucleic acids which lead to breakdown of lipids and impairment of membrane functions. To analyse if superoxide is the ideal ROS species for this study we will have to analyse in-depth by teasing all aspects of ROS network and establish which component prevails as a significant indicator for chilling stress.

Chloroplasts apparently can prevent severe water deficit by osmotic adjustment, this reduces photosynthesis inhibition and increases stromal components as chloroplast volume is maintained. This is very critical as RWC can be used as an approximate of cell volume instead of water potential which has been widely used to measure water status of plant (Smirnoff, 1993). Thus, the degree of chilling damage of plants can be reduced by means of preventing the disturbance of water regime as stated by Boese *et al*. (1987). Therefore, further studies will need to be conducted to establish if a relation between ROS and RWC.

It was clear that further experiments need to be conducted; however with time being a limited factor in this research, this will be looked at in future studies.

3.6. Conclusion

Environmental stresses can affect aspects of plant growth and development, consequently specific morphology, anatomically and physiological parameters. In this study, they responses were a tool for evaluation of sensitivity of soybean seedlings to chilling stress.

Our study illustrated that a decrease in relative water content, curling of leaves and wilting of leaves as well as growth of microorganisms especially fungi are effects of chilling temperatures on soybean seedling leaves. The chilling injury was primarily associated with decrease in RWC which indicates cellular dehydration, which is also an indicator of drought stress. Therefore, chilling temperature stress studies may facilitate understanding of other abiotic stresses. It has been noted that one form of stress may confer some resistance to other stresses. Resistance may be induced following exposure of sub-lethal levels of stress. This illustrates interrelationship between different stress factors i.e. the resistance to low temperature and resistance to cellular dehydration (Shepherd & Griffith, 2006).

Anatomical or morphological parameters are conventional methods to identify resistant cultivars to stress making the use of physiological parameters such as RWC and ROS very important. It is important to determine how soybean phenology is influenced by short term chilling temperatures as these changes are directly associated with anatomical,

morphological, physiological changes. This gives us information on developmental changes that can be used for predicting soybean phenology under field conditions where short term exposure to chilling temperatures is likely to occur during seedling development.

Chapter 4

4. References

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Chapter 5

5. Appendix

5.1. Annexure 1

EM and LM Fixation Protocol

Fixation

Tissue is fixed overnight (approximately 8hours) at room temperature (20-25^{o}C) in 2% glutaraldehyde (fixative) in 0.05M Na phosphate buffer, pH 7.2.

- 2% glutaraldehyde -Dilute 25% gluteraldehyde to 4% in sterile ultra pure water. Store this stock solution at 4^{o}C.Dilute 4% gluteraldehyde to 2% using 0.1M Na Phosphate buffer pH 7.2
- 0.1M Na Phosphate buffer pH 7.2- Add 35 ml of 0.2 M NaOH (0.88 g. 100 ml^{-1}) to 50 ml 0.2 M NaH_2PO_4 (2.4 g. 100 ml^{-1}) and dilute to 100 ml.

Rinsing

Rinse 4 times in 0.05M buffer for 1 hour at room temp (15 min between each change of buffer).

- 0.05M buffer- Add 35 ml of 0.1 M NaOH (0.44 g. 100 ml^{-1}) to 50 ml 0.1 M NaH_2PO_4 (1.2 g. 100 ml^{-1}) and dilute to 100 ml

Post Fixation

Postfix in 2% OsO_4 in 0.05M buffer for 2 hours at room temp.

- Dilute 4% stock solution to 2% using 0.1M Na Phosphate buffer pH 7.2.
- 0.05M buffer- Add 35 ml of 0.1 M NaOH (0.44 g. 100 ml^{-1}) to 50 ml 0.1 M NaH_2PO_4 (1.2 g. 100 ml^{-1}) and dilute to 100 ml

Rinsing

Rinse 4 times in 0.05M buffer for 1 hour at room temp (15 min between each change of buffer).

Primary dehydration

Dehydrate in an ethanol series of 10, 25 and 50% ethanol, allowing 15 min in each EtOH concentration.

- Ethanol series (75%, 50%, 25% and 10%) v/v ethanol are made up from absolute ethanol using sterile distilled water.

Staining

Stain tissue in 1% uranyl acetate for 1 hour in the dark.

- 1% Uranyl Acetate- 0.1g uranyl acetate is dissolved in 10mls 75% EtOH. The solution is stored in a brown bottle and wrapped in tin foil as the uranyl acetate is light sensitive.

Secondary dehydration

The tissue is rinsed in 75% EtOH for 15 min to remove uranyl acetate, and then passed through 4 sequential dehydrations; 2 in 100% EtOH and 2 in propylene oxide (15 min each).

Embedding

The tissue is submerged in 1:1 mixture of epoxy resin and propylene oxide for 4 -5 hours. The mixture is replaced with pure resin and allowed to infiltrate overnight. The tissue is then polymerized in the resin in trays at 70^{o}C for 8 -12 hours.

5.2. Annexure 2

STATISTICAL ANALYSIS

Table 3: Two-Way ANOVA FW

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
25/15	0.000269	1	0.000269	0.328224	0.573795	4.413873
25/5	0.007225	1	0.007225	17.99636	0.00049*	4.413873
15/5	0.004707	1	0.004707	4.87835	0.040402*	4.413873

Table 4: ANOVA DW

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	2.34E-06	2	1.17E-06	0.096496	0.908325	3.354131
Columns	5.28E-05	2	2.64E-05	2.177758	0.132792	3.354131
Interaction	2.23E-05	4	5.58E-06	0.460331	0.764115	2.727765
Within	0.000327	27	1.21E-05			

Table 5: Two-Way ANOVA RWC

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
25/15	362.2707	1	362.2707	5.427779	0.031658*	4.413873
25/5	926.7937	1	926.7937	16.90163	0.000656*	4.413873
15/5	130.1853	1	130.1853	1.48023	0.239452	4.413873

*significant differences ($p<0.05$)

Table 6.1: One-way ANOVA on leaf parameters

	Temperature			**Statistical analysis (one-way anova)**						
Leaf parameter	**25°C**	**15°C**	**5°C**	***Source of Variation***	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Leaf Thickness (µm)	115±10	121±7	109±15	*Between Groups*	*489.6142*	*2*	*244.8071*	*2.370476*	*0.121948*	*3.554557*
Spongy Mesophyll Layer(µm)	60±6	60±8	56±6	*Between Groups*	*84.55547*	*2*	*42.27773*	*0.807431*	*0.461515*	*3.554557*
Palisade Mesophyll Layer(µm)	40±4	27±3	34±7	*Between Groups*	*539.6843*	*2*	*269.8421*	*9.185014*	*0.001781***	*3.554557*
Abaxial Layer(µm)	8±2	11±2	10±3	*Between Groups*	*35.15797*	*2*	*17.57899*	*2.521523*	*0.108291*	*3.554557*
Adaxial Layer(µm)	9±1	10±2	12±2	*Between Groups*	*32.80572*	*2*	*16.40286*	*4.997284*	*0.018784**	*3.554557*

Results were recorded as means and standard deviation of 7 replications.
Source of variation in the statistics tab refers to variation between the temperature groups (i.e. 25°C & 15°C; 25°C & 5°C; 15°C& 5°C).

Table 6.2: Tukey HSD results for PMT and Adaxial

	PMT layer			Adaxial layer		
Treatments Pair (°C)	**Q statistic**	**p-value**	**Interference**	**Q statistic**	**p-value**	**Interference**
25/15	6.0486	0.0012518	** p<0.01	1.9923	0.3581544	insignificant
25/5	2.6840	0.1679943	insignificant	4.4624	0.0143615	* p<0.05
15/5	3.3646	0.0700342	insignificant	2.4701	0.2157333	insignificant

*statistically significant

Table 7: ANOVA Analysis for superoxide accumulation.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Time	0.156912	2	0.078456	8.361228	0.001041	3.259446
Temperature	0.002863	2	0.001431	0.152535	0.859081	3.259446
Interaction (TxT)	0.156427	4	0.039107	4.1677	0.007097	2.633532