Screening *Acacia mearnsii* (black wattle) seedlings for frost tolerance using an artificial frost technique, combined with proteomic analysis.

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Thesis Summary

Acacia mearnsii de Wild. (commonly known as black wattle), was first introduced to South Africa in the 1800's. Today, it is one of the leading commercially grown forestry crops in South Africa due to its usefulness and versatility. Black wattle is a source of high-quality tannin and raw material for wood pulp. It is also excellent for building and as a source of firewood. These uses have contributed to its popularity as a crop for both commercial farmers and small growers. Since its introduction to South Africa, abiotic stresses such as frost damage have affected the silviculture of black wattle, and this has resulted in major financial losses for the forestry industry. Over several decades, breeding research has been conducted with the aim of developing genetically improved seed for frost prone areas. However, this has yet to be achieved. Screening for frost tolerance in black wattle has mainly been conducted using field trials. While this provides the most realistic means of screening, it has several challenges. Field trials are time consuming, expensive and require much effort. Furthermore, frost events are unpredictable in timing and in magnitude, thus affecting frost damage screening trials, with levels of zero to extreme frost occurring in any one year at a selected site. This does not allow for a productive plant breeding program to consistently screen for frost tolerance. The current study focussed on the development of an artificial frost screening method for black wattle. A protocol comprising of eight days of moderately cold temperatures (adaptation) and one day of extremely cold temperature (frost tolerance) was established using a temperature-controlled chamber. The frost damage that resulted from the implementation of this protocol produced similar levels of damage as those experienced in previous field trials. This protocol was thereafter tested in two separate trials involving 100 families of wattle accessions that had previously been ranked in field trials that had been run in frost prone areas. A weak correlation was observed between the results of the artificial frost screening trials and the corresponding field results (r=0.24 to 0.28, r_s=0.20 to 0.28). Kruskal-Wallis tests showed a statistical similarity between the medians of one of the artificial frost screening trials and the field frost damage evaluations, and a significant difference between the medians of the two artificial frost screening trials that were conducted.

The understanding of the molecular aspects that contribute towards frost tolerance in black wattle is extremely limited. Several studies involving a molecular approach to understanding this trait in other woody species have shown promising results. Frost tolerance is a multigenic trait and therefore a proteomic approach was chosen as the best option to identify biomarkers

associated with it. Protein extraction from black wattle has not been previously conducted and therefore a protocol that dealt with the interference of phenolics and that was compatible with downstream proteomic techniques was developed. During the protein extraction protocol development, three different protein extraction methods were compared. These methods differed in terms of their precipitation agent combinations. These combinations included acetone and methanol, phenol and ammonium acetate and ammonium acetate and methanol. The combination of phenol and ammonium acetate produced the highest protein yield, as well as the most distinct protein spots after separation by two-dimensional gel electrophoresis. This method was used to extract proteins from 40 black wattle families with varying levels of frost tolerance, before and after cold-stress treatment. These extracted proteins were thereafter separated using two-dimensional gel electrophoresis so that changes in protein expression as a result of cold exposure could be analysed. Multivariate analyses of the proteomic data revealed that six proteins were upregulated in frost-tolerant black wattle families. The identified proteins were: two isoforms of oxygen-evolving enhancer protein 1, probable 1-acyl-sn-glycerol-3-phosphate acyltransferase 4, ribulose bisphosphate carboxylase/oxygenase activase, chaperonin 60 subunit alpha 1 and stromal 70 kDa heat shock-related protein. After identification by mass spectrometry, it was established that these proteins have previously been shown to contribute to the protection of cellular membranes, maintenance of photosynthetic processes and the prevention of protein misfolding and aggregation. These proteomic functions have been observed in previous studies to be associated with the process of cold acclimation in plants and thus seem to play a role in frost tolerance in black wattle.

The establishment of an artificial frost screening protocol and the proteomic profiling of black wattle for frost tolerance are important tools for preliminary screening of families prior to field trials. By using these protocols fewer black wattle families will require field testing. This will be beneficial both in terms of cost, effort and time associated with field trials. The development of artificial methods for the induction of stresses and the proteomic changes that result from these are valuable tools for understanding the mechanisms that plants use to cope with abiotic and biotic threats.

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I, Mayuri Jugmohan declare that:

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Dr. J. Moreno Chan (Co-Supervisor)

Declaration 2: Publications

The following work has been submitted to a peer-reviewed, ISI-accredited scientific journal and is currently under review:

1. Changes in protein expression in *Acacia mearnsii* de Wild (black wattle) as a result of exposure to frost-inducing temperatures. Submitted to Southern Forests: a Journal of Forest Science (Manuscript ID: 230303784).

I (Mayuri Jugmohan), have established the hypotheses, identified the research problems, chosen the experimental approaches, conducted the experimental work, collected the data, performed the statistical analyses and wrote the documents for the publication that I have submitted.

The co-authors have assisted with the experimental design, statistical analyses and review of the submitted documents.

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Thesis Introduction

Acacia mearnsii de Wild, commonly referred to as 'black wattle' is native to Australia and was introduced to South Africa in the 1800's (Mathura *et al*., 2006). This species has become increasingly important to the South African forestry industry because it is a source of high-quality tannin (Gujrathi and Babu, 2007). Furthermore, black wattle wood is used as building material for rural communities, mining and farming. The pulp from this species is also used by the paper industry (Beck *et al*., 2003). Black wattle silviculture has been plagued by problems with frost damage being a major concern (Chan *et al*., 2015). To date the current black wattle planting stock cannot survive temperatures below -4°C and therefore there is an urgent need to develop frost tolerant seed (Moreno Chan and Isik, 2021). This will greatly help the silviculture of black wattle in frost prone areas in South Africa such as south-eastern Mpumalanga and northern KwaZulu-Natal (Chan *et al*., 2015). The screening for frost tolerance in black wattle has traditionally involved the use of field trials. While this is the most realistic method of screening, it does have several disadvantages. Frost events are unpredictable both in terms of their severity and frequency (Augspurger, 2009). This greatly impacts field screening trials with harsh winters resulting in the death of most black wattle families regardless of their level of frost tolerance. On the other hand, mild winters result in little or no frost damage. In both cases very little information is gathered with regards to levels of frost tolerance. Therefore, there is a need for the development of a controlled and predictable method of simulating frost damage in black wattle and thus forms the basis of this current PhD study. In addition to this, the actual molecular mechanisms that control frost tolerance are also important for screening purposes. Over the years several genes that are associated with cold tolerance in plants have been identified (Rihan *et al*., 2017). While this research is of immense value it is important to note that cold tolerance is a multigenic trait (Janmohammadi *et al*., 2015). Therefore, the study of individual cold tolerance genes does not provide a holistic understanding of this trait. Proteomics (the study of proteins) bridges the gap between the genotypic and phenotypic levels (Renaut *et al*., 2006). Protein expression is dynamic and is affected by biotic and abiotic stresses (Roberts *et al*., 2002). The upregulation of proteins that are involved in functions such as the maintenance of proper protein structure and cell membrane integrity have been reported as responses to cold stress (Kjellsen *et al*., 2010; Rawat *et al*., 2021). Other upregulated proteins include those that combat oxidative stress and pathogens (Liu *et al*., 2003; Renaut *et al*., 2004).

The molecular aspect of frost tolerance in black wattle is poorly understood and is therefore also explored in this current PhD study using proteomic techniques.

This dissertation is comprised of five chapters the first of which focusses on a review of literature on cold tolerance in plants as a background to frost tolerance in black wattle. This is followed by four research chapters. The first research chapter involved the development of an artificial frost damage protocol for black wattle involving the use of a temperature-controlled chamber. This protocol was tested in many black wattle families of varying frost tolerance levels in the second research chapter. The third research chapter involved the development of a protein extraction protocol for black wattle that was compatible with proteomic techniques such as SDS-PAGE and two-dimensional gel electrophoresis. The final research chapter identified those proteins that were upregulated in frost tolerant black wattle families in response to cold temperature exposure. Each research chapter has been written in the form of a semi-independent scientific paper and as a result some information and references have been repeated. The University of KwaZulu-Natal uses this as a standard format.

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Chapter 1: Literature Review

Biochemical and physiological changes that take place in woody and non-woody plants during exposure to cold temperatures - a background to frost tolerance in *Acacia mearnsii* (black wattle)

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1.1 Abstract

The ability of plant species to adapt to low temperatures has a major impact on their distribution and survival. Responses to an abiotic stress involve changes in a plant's genome, transcriptome, proteome, metabolome and lipidome. The process of cold acclimation has been established as one of the main mechanisms used by plants to achieve freezing tolerance. Although much research has been conducted in this field the process is still not fully understood. In this review, the various aspects of how woody and non-woody plants respond to low temperatures is discussed, with emphasis on their physiological and biochemical changes as a background to understanding cold tolerance in *Acacia mearnsii* (black wattle). Extremely low temperatures often bring about frost damage in trees such as black wattle, which has a major impact on their survival and productivity. Frost tolerant cultivars of black wattle are needed to reduce the risk of crop losses by wattle growers. However, it is difficult to phenotype large numbers of tree progeny for frost tolerance. Marker assisted selection would enable breeders to screen large numbers of wattle progeny for frost tolerance, if appropriate biochemical markers could be identified.

1.2 Introduction

Approximately two thirds of the world's landmass experience temperatures below freezing point annually and around half of the landmass is subjected to temperatures below -20°C (Beck

et al., 2004). One of the damaging effects of such low temperatures is caused by frost. This phenomenon refers to the formation of ice crystals on surfaces, either by freezing of dew or the conversion of water vapour to ice. The term "frost" has also been used interchangeably with "freeze" by plant growers as well as by the general world population to describe a weather event that causes plants to experience freezing injury. Frost events have been categorized into two types; advection and radiation (Tait and Zheng, 2003; Hu *et al*., 2018). Advection frost develops as a result of a horizontal delivery of cold air (either during the day or night) into an area to replace warmer air that was present before a change in temperature. This type of frost is associated with a well-mixed, windy atmosphere; a temperature that is usually sub-zero, with cloudy conditions, low humidity and no temperature inversions (Laughlin and Kalma, 1990; Snyder and Melo-Abreu, 2005; Lu *et al*., 2019). At high altitudes advection frosts have an increased potential to cause damage (Dittmar *et al*., 2006). Radiation frosts result from longwave radiation cooling under calm, clear and dry atmospheric conditions. They are associated with clear skies, temperature inversion, low dew-point temperatures and atmospheric temperatures that are below 0°C at night but above 0°C during the day. Dry regions such as deserts and semi-deserts, and features of land such as slopes, valleys and depressions are strongly affected by radiation frosts (Snyder and Melo-Abreu, 2005; Wang *et al*., 2021). The response to cold temperatures, which often brings about frost damage, vary among plant species; with some species being killed the moment they freeze. Others, such as herbaceous annuals, use seasonal avoidance in which they survive as seeds, roots or rhizomes buried deep in the soil to avoid sub-zero temperatures (Burke *et al*., 1976; Hincha and Zuther, 2014; Lubbe and Henry, 2019). Another response of plants to cold involves the process of cold acclimation (Thomashow, 1999; Kosmala *et al*., 2009). It has been established that such plants develop an enhanced tolerance to sub-zero temperatures after exposure to low but above zero temperatures (Hughes and Dunn, 1996; Smallwood and Bowles, 2002; Chang *et al*., 2021). Cold acclimation increases the ability of a plant to attain maximum freezing tolerance (Huner *et al*., 1998; Li *et al*., 2020). When cold acclimated plants are exposed to warmer temperatures, they usually undergo a gradual loss of cold tolerance during a process referred to as deacclimation (Gusta and Weiser, 1972; Chen and Li, 1980; Mahfoozi *et al*., 2001a; Vyse *et al*., 2019). Understanding of the mechanisms used by plants to survive low temperatures is important because of the impact that freezing injury has on the yield and quality of several crop species, affecting both food security as well as the economy of many countries (Pyke *et al*., 1986; Thomashow, 2001; Mahajan and Tuteja, 2005; Sharma *et al*., 2005; Ehlert and Hinch,

2008; Dhankher and Foyer, 2018). Furthermore, this knowledge has potential application in terms of expanding the geographical locations and seasons in which crops can be grown (Smallwood and Bowles, 2002; Hannah *et al*., 2005; Ambroise *et al*., 2020). In order to understand the mechanisms used by plants to survive low temperatures, it is important to first understand the effect of cold stress on plants.

The impact of low-temperature on plants may be divided into two aspects:

1) the effects of low temperature alone
2) freeze-induced dehydration (Beck *et al*., 2004; Miki *et al*., 2019)

When low temperatures occur above 0°C, tropical and subtropical plants that are not able to acclimatize to such conditions experience chilling injury. This type of damage results mainly from a loss of function of biomembranes due to a decrease in membrane fluidity and a loss of or reduced activity of membrane-bound ion pumps. A consequence of this is the production of reactive oxygen species (ROS), which are highly stimulated in light-induced photo-oxidation (Aroca *et al*., 2001; Baek and Skinner, 2012). When temperatures drop below 0°C to a range of -1 and -3°C frost-sensitive plants experience frost damage (Burke *et al*., 1976; Beck *et al*., 2004; Leske and Biddulp, 2022). The risk and extent of cold related damage to plants depends on several factors such as the plant's developmental stage, the duration and severity of frost, the minimum temperatures experienced and the rate of temperature change (Beck *et al*., 2004; Neuner, 2014; Moreno Chan, 2019). During the early stages of frost damage, the temperature of both the cells and their suspending medium are extremely low. The process of ice nucleation (transition of water to ice) takes place in the suspending medium at a temperature that is dependent on the freezing point of the solution as well as the presence of ice-nucleation facilitating particles/agents (Suzuki *et al*., 2017). In the absence of ice nucleating agents, i.e., in pure water, ice formation takes place at -40°C and this is referred to as the homogenous ice nucleation point. This is regarded as the "absolute limit to the phenomenon of biological freeze resistance" (Franks, 2003). Pure water rarely exists under natural conditions, and it is rather in the form of an ionic or colloidal solution. Heterogeneous ice nucleation occurs in such solutions and is brought about on the surface of objects or particles in suspension (Wisniewski *et al*., 2001; Franks, 2003).

One of the consequences of ice formation is an exclusion of solutes, which results in a concentration of the partially frozen solution (Choat *et al*., 2009). Ice formation and the concentration of solutes continue to take place until the chemical potential of water in the unfrozen portion is in equilibrium with that of the frozen portion (Uemura and Hausman, 2013). The chemical potential of the intracellular solution must also be brought to a state of equilibrium with the partially frozen suspending medium. This may be achieved either by cell dehydration (movement of water out of the cell into the extracellular domains) or intracellular ice formation (Eurich *et al*., 2022). During cell dehydration, the semi-permeability of the plasma membrane allows the cell to dehydrate in response to the lower chemical potential of the extracellular solution (Mazur, 1969; Steponkus, 1984; Thomashow, 1999). This process results in increased intracellular solute concentrations, decreases in cell volume and area, and pH changes because of differing solubility of buffering compounds (Mazur, 1969; Steponkus, 1984). Intracellular ice formation is usually prevented by the lack of effective ice-nucleating facilitating particles and an intact plasma membrane that serves as a barrier to the extracellular ice (Chambers and Hale, 1932; Mazur, 1977; Steponkus, 1984). If intracellular ice formation does take place, then cell integrity is compromised and this invariably causes cell death (Asahina, 1956; Burke *et al*., 1976). The stability of the plasma membrane plays a key role in determining how chemical potential equilibrium is achieved between the intracellular and extracellular domains (Mazur, 1970; Steponkus, 1984). Ironically, the plasma membrane is the primary site affected during the freeze-thaw cycle/ freeze-induced injury (Steponkus, 1984; Steponkus and Lynch, 1989; Yoshida, 1984; Thomashow, 1999; Miki *et al*., 2019).

In order to understand the nature and impact of freeze-induced injury on the plasma membrane several research groups have used cryomicroscopy studies of isolated protoplasts from different crop species (Siminovitch, 1979; Uemura *et al*., 1995; Webb and Steponkus, 1993; Webb *et al*., 1994; Arora, 2018). The use of isolated protoplasts allows for the direct study of the plasma membrane because these cells lack cell walls as a result of isolation techniques involving such procedures as enzymatic digestion (Galbraith and Northcote, 1977). The spherical shape maintained by isolated protoplasts over a broad range of osmolytes allows for cryomicroscopic studies of their osmotic behaviour and for studying the extent of freeze-induced dehydration (Dowgert and Steponkus, 1984; Gordon-Kamm and Steponkus, 1984; Steponkus, 1984; Davey *et al*., 2005). The use of protoplasts as a model system has also allowed for the study of the destabilization of the plasma membrane during freezing injury from a molecular and cellular

perspective (Wolfe and Steponkus, 1983; Gordon-Kamm and Steponkus, 1984; Webb *et al*., 1994; Uemura *et al*., 1995).

Cellular membrane damage has been observed to be due mainly to severe dehydration that is associated with freezing and mechanical stress (Steponkus, 1984; Uemura *et al*., 2006; Manasa *et al*., 2021). Studies conducted by several research groups have demonstrated that freezing-induced injury results in three different cellular membrane lesions: expansion-induced-lysis, hexagonal-II phase transitions and fracture jump lesions (Webb *et al*., 1994; Gordon-Kamm and Steponkus, 1984; Uemura *et al*., 1995; Thomashow, 1999; Uemura *et al.*, 2006; Arora, 2018). The occurrence of these different lesions depends on the lowest temperatures at which the samples were frozen, the stage of exposure to freezing conditions and the degree of freeze-induced dehydration (Webb *et al*., 1994; Uemura *et al*., 1995; Xin and Browse, 2000; Uemura *et al*., 2006).

During expansion-induced-lysis protoplasts of non-acclimated leaves experience a reduction in cell volume due to freeze-induced dehydration. This reduction is associated with an irreversible loss of plasma membrane surface area as a result of endocytic vesiculation of the plasma membrane. This occurs in non-acclimated protoplasts that have been cooled to -5°C (minimal temperature for 50% survival, referred to as LT_{50}) (Dowgert and Steponkus, 1984). Endocytic vesiculation is not injurious by itself; however, during thawing conditions water re-enters the protoplast but the cell lyses before it regains its original volume as a result of hydrostatic pressure created by the incoming water (Webb *et al*., 1994; Uemura and Steponkus, 1999; Xin and Browse, 2000). In protoplasts of cold acclimated leaves, freeze-induced cellular volume reduction results in the formation of exocytic extrusions of the plasma membrane. These exocytic extrusions are continuous with the plasma membrane and are incorporated back into the membrane during thawing. As a result, expansion-induced-lysis does not occur (Dowgert and Steponkus, 1984; Webb *et al*., 1994; Uemura and Steponkus, 1999; Xin and Browse, 2000).

During colder conditions, (approximately -10°C) a greater degree of dehydration takes place, freeze-induced injury is more severe, and the injury occurs as a complete loss of osmotic responsiveness (Gordon-Kamm and Steponkus, 1984; Webb *et al*., 1994). At such low temperatures and with such extreme dehydration, the plasma membrane is brought into very

close contact with various endomembranes. The lipid molecules in the different membranes are exposed to a very thin aqueous layer and are subjected to strong-short range forces between the different membrane surfaces (Uemura and Steponkus, 1999). As the aqueous layer between the different membranes becomes thinner, the free energy of the lipid molecules within the membranes increases greatly. The free energy of lipid molecules, which have changed their orientation such that they become inverted lipid tubes, is greatly lowered and at some point, during the transition, it becomes more energetically stable for the lipid molecules to take on a cylindrical formation (Kirk *et al*., 1984). The packing symmetry of these cylindrical tubes takes on a three-dimensional hexagonal formation and thus this phase is referred to as the Hexagonal (H_{II}) phase. In this phase there is an interbilayer interaction involving two or more bilayers in which water is sequestered in the core of each cylinder, bringing about destabilization of the plasma membrane (Gordon-Kamm and Steponkus, 1984; Uemura *et al*., 1995). The H_{II} phase has been frequently observed in regions where the plasma membrane is brought into close contact with the outer membrane of chloroplasts, resulting in a lamella-to-H_{II} phase transition. However, the H_{II} phase transition has also been observed to involve tonoplasts, though less frequently (Uemura *et al*., 1995). Several studies have shown that cellular membrane injury due to the formation of the H_{II} phase is not observed in cold-acclimated plant tissue over a range of -3 to -35°C (Cudd and Steponkus, 1988; Webb and Steponkus, 1993; Uemura *et al*., 1995; Gordon-Kamm and Steponkus, 1984).

Freeze-induced injury in cold-acclimated protoplasts have been associated with "fracture-jump lesions" (Fujikawa and Steponkus, 1990). In a study conducted by Webb *et al*. (1994), the plasma membrane of cold-acclimated oat protoplasts had very few membrane alterations at -3°C. They observed that the plasma membrane had a bilayer configuration with randomly distributed intra-membrane particles. At -5°C they observed that 33% of the examined cold-acclimated protoplasts displayed fracture-jump lesions in their plasma membranes and subtending lamella. The term "fracture-jump lesion" is used to describe a localized deviation in the fracture plane in aparticulate domains (intramembrane, particle-free areas) of membranes. In this type of lesion, the fracture plane usually "jumps" from the plasma membrane to aparticulate lamella subtending the plasma membrane (Fujikawa and Steponkus, 1990; Uemura and Steponkus, 1999). At -10°C Webb *et al*. (1994), found that 88% of the examined cold-acclimated protoplasts displayed fracture-jump-lesions in their plasma membranes and subtending intracellular membranes, indicating that this was the principal type

of lesion at this temperature. The exact cause of fracture jump lesions is not fully understood but there are two theories to explain their origin. The first involves the formation of interlamellar attachments and the fusion of areas of the plasma membrane and subtending endomembranes. The second involves the interdigitation of lipids that have changed from a liquid crystalline state to a gel phase in areas of the plasma membrane (Webb and Steponkus, 1993; Uemura and Steponkus, 1994).

Although using isolated protoplasts as a model to understand the effects of freezing on the cellular membrane has been extremely informative, there have been comparatively few studies using intact plant tissues (Webb and Steponkus, 1993; Carter *et al*., 2001; Nagao *et al*., 2008; Aslamarz *et al*., 2010). It has been suggested that freezing responses in intact tissues may differ from isolated protoplasts. Differences in response to freezing in the presence or absence of cell walls have also been reported (Tao *et al*., 1983; Murai and Yoshida, 1998).

Webb and Steponkus (1993), observed that the freeze-induced ultrastructural changes observed in non–cold acclimated and cold acclimated protoplasts also occurred in the leaves from which these protoplasts are isolated from. In addition, the study also demonstrated that the formation of H_{II} phases in non-acclimated leaves required a significantly longer period than previously reported by Gordon-Kamm and Steponkus (1984). Webb and Steponkus (1993), provided an explanation for this based on the longer cell dehydration times for intact tissue as compared to isolated protoplasts. In a study conducted by Nagao *et al*. (2008), it was observed that ultrastructural changes associated with short-term freezing (samples frozen at a slow cooling rate to the desired sub-zero temperature over the course of a few hours) differed between intact tissue cells and isolated protoplasts. These studies suggested that it is not accurate to extrapolate findings from studies involving isolated protoplasts for the purpose of understanding freeze-induced injury in whole plant systems. Steponkus (1984), argued that isolated protoplasts facilitate the direct study of changes in the plasma membrane during the freeze-thaw cycle and that such information is required prior to considering the added effects of the cell wall.

Besides the major impact of freezing injury on the plasma membrane, low temperatures also affect the stability of RNA and DNA secondary structures, activity of enzymes (some of which play a role in transcription and translation) and many metabolic processes (Heber, 1968; Smallwood and Bowles, 2002). Photosynthesis is also affected by low temperatures because

the rate of photosynthetic electron transport is reduced because of a decrease in CO_2 fixation (Rizza *et al*., 2001; Xu *et al*., 2022). Freeze-induced production of ROS such as superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals take place at a higher rate as a result of excessive excitation of the respiratory and photosynthetic electron transport systems. These ROS are destructive because they cause lipid peroxidation, membrane deterioration, protein degradation and chlorophyll quenching (Scandalios, 1993; Scebba *et al*., 1999; Baek, 2012). Visible effects of freezing injury include frost splitting of tree trunks, blackheart in stems of trees and shrubs, winter burn on conifer foliage, crown kill of winter cereals and herbaceous perennials, die-back in citrus and midwinter killing of dormant flower buds (Weiser, 1970; Yu and Lee, 2020).

In order to cope with the negative side effects of low temperatures such as frost, plants have evolved adaptations that provide resistance or tolerance to frost (Agrawal *et al*., 2004; Ding *et al*., 2019). Breeding for frost tolerance is difficult to achieve in plants either by classical breeding using pedigree breeding or gene transfer because cold resistance is a multigenic trait, occurring as a combination of processes, and it involves many aspects of cell biology such as the fluidity of biomembranes, the production of cyroprotectants and the ability to cope with oxidative stress (Jaglo-Ottosen *et al*., 1998; Steponkus *et al*., 1998; Nanjo *et al*., 1999; Fowler and Thomashow, 2002; Beck *et al*., 2004; Oberschelp *et al*., 2020). The study of frost resistance is made even more difficult because it can vary between different organs and tissues within a plant as well as seasonally (Beck *et al*., 2004; Neuner, 2014; Yazdanpanah *et al*., 2021). Furthermore, frost tolerance in some plants may vary during their life cycle. For example, the ability of wheat plants to maintain their levels of frost tolerance decreases after the transition from the vegetative to the reproductive phase (Mahfoozi *et al*., 2001b). The state of frost resistance in many plants is developed via the process of cold acclimation (Hällgren and Öquist, 1990; Juurakko and Walker, 2021). Hannah *et al*. (2005), described the process of cold acclimation as "a multigenic and quantitative trait with complex physiological and biochemical changes." Such changes are due to adjustments of the plant's transcriptome, proteome, metabolome and lipidome (Hannah *et al*., 2005; Griffith *et al*., 2005; Guy *et al*., 2008; Sandve *et al*., 2008; Degenkolbe *et al*., 2012; Thalhammer *et al*., 2014). In order to activate the mechanisms of cold acclimation, a plant needs to perceive cold temperatures and the resultant stress of such conditions. Prior to cold acclimation, the disturbances of a plant's internal environment as a result of exposure to low temperatures usually serve as perception points for

the initiation of a signalling cascade that ultimately allows the plant to bring about internal changes so that it may cope with such adverse external environmental conditions (Huner *et al*., 1998; Smallwood and Bowles, 2002).

The induction of cold acclimation is a complex process and to date is not fully understood. Its molecular basis involves a complex interplay of many genes, proteins and signalling pathways. The complexity of the signalling pathways makes it difficult to attribute the activation and control of cold acclimation to individual factors because their roles overlap and are strongly interconnected. Two such factors that have been identified include the phytohormone abscisic acid (ABA) and the C-repeat binding factors (Rehman *et al*., 2021). Signalling pathways that are dependent on and independent of these factors appear to control most of the identified aspects of cold acclimation (Thomashow, 1999; Sharma *et al*., 2005; Jiang *et al*., 2013; Schubert *et al*., 2019).

Events which have been observed to induce cold acclimation have also been studied extensively. The main trigger event for the induction of cold acclimation are low non-freezing temperatures. In natural environments light and temperature reduction often occur together since the hours of daylight decrease continuously prior to and during winter (Lindlöf, 2010). These shorter days result in decreasing photoperiods often leading to the end of growth. This is referred to as growth cessation and has been well documented in woody deciduous trees (Weiser, 1970; Lee and Thomashow, 2021; Chang *et al*., 2021). Photoperiods are detected by light-absorbing photoreceptors known as phytochromes in plants. This activity has been observed to be an important factor in the transcription of cold responsive genes (CORs) (Kim *et al*., 2002; Lindlöf, 2010; Roeber *et al*., 2021). Another event that is involved in triggering the cold acclimation response is the loss of plasma membrane fluidity (Alonso *et al*., 1997; Cano-Ramirez *et al*., 2021). During exposure to low temperatures, the plasma membrane changes from a liquid crystalline state to a rigid gel phase (Chinnusamy *et al*., 2010). Based on a study conducted by Örvar *et al*. (2000), it was suggested that cold signalling in plants is a result of membrane rigidification followed by actin microfilament re-organisation, leading to the induction of calcium channels, an influx of calcium into the cytoplasm and cold acclimation gene expression (such as the expression of cold-responsive genes (CORs)). The influx of calcium into the cytosol is an early activity that takes place during cold acclimation (Wang *et al*., 2019). Calcium has been observed to be a secondary messenger in the transduction of

environmental stimuli in plants (Anil and Rao, 2001). Prevention of this has been observed to prevent cold-gene expression and ultimately the development of freezing tolerance in alfalfa (*Medicago sativa* L.) cells (Monroy *et al*., 1993; Monroy and Dhindsa, 1995). Other secondary messengers include calmodulin, cyclic AMP, cyclic GMP, cyclic ADP-ribose, inositol 1,4,5-triphosphate and reactive oxygen species (ROS), calcium sensors, transient receptor potential ion channels and phospholipases. These secondary messengers bring about a complex signalling cascade that is mediated by kinases or phosphatases (Sharma *et al*., 2005; Shi *et al*., 2018).

1.3 Changes that occur during cold acclimation.

Cold acclimation has been shown to result in several changes intra- and extracellularly and even within cell walls. One of the changes includes an osmotic adjustment of the cytoplasm through the accumulation of compatible solutes that decrease the water potential of cell compartments (Juurakko and Walker, 2021; Sigala *et al*., 2021). These compounds include monosaccharides; oligosaccharides such as sucrose, raffinose, stachyose, verbascose, disaccharide mellobiose; sugar alcohols such as sorbitol, mannitol and pinitol; polyamines such as spermine, spermidine and putrescine; and quaternary ammonium compounds and proline (Kosová *et al*., 2007).

Besides resulting in an adjustment of the osmotic potential of the cytoplasm, accumulation of soluble sugars can act as protective agents that stabilize biomembranes (Janmohammadi *et al*., 2018; Ouyang *et al*., 2021). Research conducted by Olien (1967), showed that carbohydrates found in the apoplast interfered with ice crystal growth and significantly reduced freezing injury. In a study conducted on red osier dogwood (*Cornus sericea L*.) by Ashworth *et al*. (1993), it was established that starch concentration was highest in autumn and lowest in midwinter, while soluble sugars were at their highest concentrations in midwinter, coinciding with the breakdown of starch. During early spring the concentration of soluble sugars decreased while that of starch increased. In this study it was also established that the predominant sugars were glucose, fructose, sucrose and raffinose, and that these were present in both the bark and wood tissues. A study conducted by Sasaki *et al*. (1996), demonstrated that an accumulation of sugar in cabbage leaves positively correlated with freezing tolerance. The accumulation of fructose polymers such as fructan has also been associated with cold acclimation of certain grass species (Vijn and Smeekens, 1999; Dionne *et al*., 2001). Although the accumulation of

carbohydrates is an important energy source required for the colder months, their membrane-protective characteristics indicate that they play a role in freezing tolerance (Balamurugan *et al*., 2018; Joudmand *et al*., 2019).

Low temperatures have also been observed to induce the synthesis of proteins that display cryoprotectant qualities. Such proteins have been observed in plants such as cabbage (*Brassica oleracea* L.) (Hincha *et al*., 1990; Sieg *et al*., 1996), spinach (*Spinacia oleracea* L.) (Hincha *et al*., 1990) and *Nothofagus dombeyi* (Mirb.) Oerst. (a Chilean rain forest woody evergreen) (Rosas *et al*., 1986). The accumulation of a specific group of proteins known as dehydrins (part of the late embryogenesis abundant proteins (LEA) group II) has also been associated with cold acclimation (Yu *et al*., 2018; Vítámvás *et al*., 2019). These proteins are usually induced by conditions that cause dehydration (Kosová *et al*., 2007). It has been proposed that these proteins serve as emulsifiers or chaperones in which they interact with cell endomembraneous systems or partially unfolded proteins, protecting them against adverse changes during dehydration (Close, 1997; Allagulova *et al*., 2003; Kosová *et al*., 2007).

The secretion of antifreeze proteins (AFPs) in apoplastic spaces have been reported to be induced during cold acclimation (Juurakko *et al*., 2021). These proteins bind irreversibly to ice and thereby inhibit the growth of extracellular ice (Griffith and Yaish, 2004; Wisniewski *et al*., 2020). Extraction of AFPs from cold acclimated leaves has been observed to result in greater injury after freezing and thawing than in cold acclimated leaves that did not undergo extraction and they have also been observed to lower the ice nucleation temperature of winter rye leaves (Marentes *et al*., 1993; Griffith *et al*., 2005). One of the main mechanisms used by AFPs to decrease freeze-induced injury is called thermal hysteresis, in which they decrease the temperature at which ice is formed but do not have any effect on the melting point (Thomashow, 1998; Kiran-Yildirim and Gaukel, 2020). Another important activity of AFPs is ice recrystallization inhibition that results in the prevention of ice growth. In the absence of ice recrystallization inhibition, larger molecules of ice grow at the expense of the smaller ones. These larger ice molecules inflict physical stress on the plasma membrane, often leading to rupture of the membrane and cell death (Gupta and Deswal, 2014). It is interesting to note that in a study conducted by Antikainen and Griffith (1997), AFPs did not accumulate in cold-treated maize or tobacco, both of which are freezing-sensitive plants that do not cold acclimate

(Thomashow, 1998). This may also indicate that these proteins play a significant role in reducing freeze-induced injury.

The accumulation of several enzymes that scavenge reactive oxygen species (ROS) and antioxidant enzymes also occurs during cold acclimation, which is interesting because oxidative stress caused by ROS is one of the consequences of both freezing and chilling injury (Aroca *et al*., 2001; Baek and Skinner, 2012; Dreyer and Dietz, 2018). Degand *et al*. (2009), reported an increase in superoxide dismutase (an antioxidant enzyme) during cold acclimation. Research conducted by Scebba *et al*. (1999), also reported an increase in the activity of the antioxidants: superoxide dismutase, catalase and guaiacol peroxidase in cold acclimated roots.

Besides extra- and intracellular changes, changes in cell wall composition have also been observed during cold acclimation. Thickening of the cell wall in rye leaves and the accumulation of cell wall components such as callose, cellulose, soluble polysaccharides and pectic polysaccharides have been reported as a result of cold acclimation (Huner *et al*., 1981; Fujikawa *et al*., 1999a; Parrotta *et al*., 2019). Increased rigidity of plant cell walls has been observed and this may be due to certain biochemical changes such as phenolic crosslinking between cell wall polymers and the deposition of lipids and extensin on the wall (Bartolo *et al*., 1987; Fujikawa *et al*., 1999b). It has been suggested that an increase in cell wall rigidity may prevent cell deformation during extracellular freezing whereas fragile cell walls may cause deformation that results in membranes being brought near each other, ultimately leading to cellular membrane damage (Fujikawa *et al*., 1999b; Takahashi *et al*., 2021).

Changes in the protein and lipid composition of the plasma membrane is another important activity that takes place during cold acclimation (Kawamura and Uemura, 2003; Ghassemi *et al*., 2021). Goodwin *et al*. (1996), observed that the cell wall-plasma membrane linker protein increases at the mRNA level in rapeseed (*Brassica napus* L.) during cold acclimation. In a study conducted by Breton *et al.* (2000), two isoforms of wheat annexins were identified as intrinsic membrane proteins whose levels increased rapidly during cold acclimation. The natural lipid composition of the plasma membrane includes phospholipids, sterols (made up of free sterols, sterylglycosides and acylated sterylglycosides) and cerebrosides (Lapshin *et al*., 2021). The main phospholipid classes are phosphatidylcholine, phosphatidylethanolamine and smaller proportions of phosphatidylinositol, phosphatidylglycerol, phosphatidylserine and

phosphatidic acid (Yoshida and Uemura, 1986; Lynch and Steponkus, 1987; Uemura and Steponkus, 1994; Uemura *et al*., 1995; Uemura and Steponkus, 1999). During cold acclimation an increase in the proportion of phospholipids occurs (Chang *et al*., 2021). This is due to an increase in the proportion of di-unsaturated forms of phosphatidylcholine, and phosphatidylethanolamine (Cheong *et al*., 2022). The proportion of cerebrosides has also been observed to decrease (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994; Uemura *et al*., 1995; Chen *et al*., 2020). This change in lipid composition results in an alteration of the cryobehaviour of the plasma membrane (Steponkus *et al*., 1990). By using membrane engineering techniques involving the artificial enrichment of the plasma membrane with mono- or di-unsaturated forms of phosphatidylcholine, Steponkus *et al*. (1988), observed that non-acclimated protoplasts did not display endocytic vesiculation as a result of freezing but rather the formation of exocytic vesicles, as observed in cold acclimated protoplasts, thereby preventing expansion-induced lysis. The enrichment of the plasma membrane with unsaturated forms of phosphatidylcholine has also been observed to be one of the factors that reduce the participation of the plasma membrane in the formation of the H_{II} phase (Sugawara and Steponkus, 1990; Uemura and Steponkus, 1999). The exact sequence of changes in the plasma membrane lipid composition on the formation of fracture-jump lesions is still to be determined. However, Uemura and Steponkus (1994), have suggested that there is a relationship between the ratio of plasma membrane phospholipids to cerebrosides and the temperature range over which this type of lesion occurs.

The identification of genes expressed during cold acclimation is an ever-evolving field of research. One of the most well researched groups of genes are the cold responsive genes (COR) expressed in the widely used model plant *Arabidopsis thaliana* L. The key COR genes identified include COR15a, COR78, COR6.6 and COR47 (Thomashow, 1998; Liu *et al*., 2019). The COR15a gene is of particular interest because it encodes a protein that is associated with chloroplasts and has been shown to play a role in reducing freeze-induced injury by increasing the cryostability of the plasma membrane (Artus *et al*., 1996; Thomashow, 1998). Although expression of COR15a has been observed to play a role in freezing tolerance at the cellular level, expression of other COR genes is required for freezing tolerance to be detectable in whole plants thus further emphasizing that cold acclimation is a polygenic trait (Jaglo-Ottosen *et al*., 1998; Wang and Szmidt, 2001; Ritonga and Chen, 2020).

1.4 Mechanisms of frost tolerance

One of the ways in which frost tolerant plants survive low temperatures is by avoiding freezing via supercooling, a phenomenon in which water in cells remains liquid even when the temperature is close to the homogeneous nucleation temperature of water (-40°C) (Fujikawa *et al*., 1996; Franks, 2003; Neuner *et al*., 2019). During homogenous nucleation, water molecules come together spontaneously to form a stable ice nucleus. However, if the reaction is not spontaneous and is catalysed by another substance, then it is referred to as heterogeneous nucleation. Heterogeneous ice nucleating agents/ particles found in nature include ice nucleation-active bacteria, biological molecules, and organic and inorganic debris (Schwidetzky *et al*., 2021).

Ice nucleating agents may be found on the plant surface or within the plant, and require contact with water to function (Pearce, 2001). At temperatures below -40°C disruption of supercooling occurs, leading to intracellular ice formation and frost injury. The presence of supercooling varies in plant tissues and species (Neuner *et al*., 2019; Kovaleski *et al*., 2019). According to Wisniewski and Davis (1989), for supercooling to occur, plant tissues must have a barrier that prevents the rapid loss of cellular water to extracellular ice. This barrier should also prohibit the growth of ice crystals inside cells because this can lead to the nucleation of intracellular water. Furthermore, such tissues must not have heterogeneous ice nucleating agents that would promote the formation of intracellular ice.

In colder regions below -40°C plants survive by moving cellular water into apoplastic spaces as a response to prior extracellular ice formation, which results in a chemical potential difference between the intra- and extracellular domains (Atıcı and Nalbantoğlu, 2003; Arora, 2018; Petruccelli *et al*., 2022). The formation of extracellular ice is not lethal if the cell wall integrity is maintained and does not separate from the plasma membrane. In addition to this, the flexibility of the cell-wall matrix must allow for sufficient contraction and expansion during dehydration and rehydration (Takahashi *et al*., 2021). If this does not take place, cytorrhysis (collapse of the cell wall) may occur (Burke *et al*., 1976; Carpita *et al*., 1979; Yamada *et al*., 2002). Unlike supercooling, extracellular freezing has no temperature limit. Some plant organs as well as cells have been observed to survive the temperature of liquid nitrogen, if they were fully acclimated and almost all their freezable cellular water had been withdrawn by sufficient

extracellular freezing (Parker, 1959; Sakai, 1965; Sugawara and Sakai, 1974; Burke *et al*., 1976; Kuroda *et al*., 2003).

In order to study frost tolerance in plants, several researchers have used xylem ray parenchyma cells (XRPCs) as research tools (Ristic and Ashworth, 1994; Wisniewski and Davis, 1989; Kuroda *et al*., 2003; Wu *et al*., 2019). These cells have been reported to survive low temperatures by deep supercooling or by extracellular freezing (Burke *et al*., 1976; Kuroda *et al*., 1997 and Kuroda *et al*., 1999). It has been suggested that the freezing response of XRPCs of hardwood species may be understood as a continuum and that the specific response chosen by the organism may be dependent on the temperatures of the growing conditions and may change seasonally (Fujikawa *et al*., 1996; Fujikawa *et al*., 1997).

1.5 Methods for studying freezing in plants.

Over the years several methods have been developed to study freezing in plants because this knowledge is fundamental to understanding frost tolerance (Table 1.1). These methods differ in terms of cost, time requirements, visual resolution and the degree of information obtained. Using these methods, ice is either directly visualized or indirectly detected by determining the amount of remaining liquid water or by measuring freezing exotherms (measurable heat that is released during the phase transition from liquid water to ice).

Table 1.1. Methods used for studying freezing in plants.

Method	Description	Reference
Nuclear magnetic resonance microscopy	Characterizes the physical changes of water at different temperatures in plants.	Burke *et al*., 1976; Borompichaichartkul *et al*., 2005; Yu and Lee, 2020
Magnetic resonance imaging	Detects the change in signal intensity when water changes from a liquid to a solid state. Enables the visualization of ice growth and distribution.	Stegner *et al*., 2020; Villouta *et al*., 2021
Differential Scanning Calorimetry	Measures heat flows that take place during endothermic and exothermic events, ice nucleation temperatures and amounts of freezable water.	Vertucci and Stushnoff, 1992; Bilavčík *et al*., 2019; Han *et al*., 2021; Impe *et al*., 2022
Differential thermal analysis (DTA)	Detects the temperature difference between a reference (usually dry or dead) and a sample (living) using thermocouples.	George *et al*., 1974; Mancuso, 2000; Kaya *et al*., 2021
Infrared video thermography (IRVT)	Used to determine the location and growth of ice in intact plants at the organ level. This helps to avoid artefacts that may be created by thermocouples during DTA.	Wisniewski *et al*., 1997; Hacker and Neuner, 2007; Kuprian *et al*., 2014; Bertel *et al*., 2021
Infrared differential thermal analysis (IDTA)	Modification of DTA and IRVT. Infrared thermography used to observe tissue specific freezing by comparing reference and sample thermal images. Can detect ice nucleation, growth pattern and speed.	Wisniewski *et al*., 1997; Hacker and Neuner, 2007; Livingston *et al*., 2018; Stegner *et al*., 2020
Cryo-scanning electron microscopy	Visualization of anatomical changes of plant tissue during freezing and thawing. Ice formation on and within plants can also be observed.	Stegner *et al*., 2020; Schott *et al*., 2020; Gorb and Gorb, 2022
X-Ray phase contrast imaging	Different densities within plant tissues cause x-rays to scatter resulting in phase contrast images. This allows for the phase transition of liquid water to ice to be visualized.	Kovaleski *et al*., 2019; Kaya and Kose, 2022

1.6 Methods of studying frost tolerance in plants

The various methods that have previously been used to evaluate cold tolerance in plants have certain advantages and disadvantages associated with their use, therefore it has been difficult to establish a single, ideal screening method for every plant species (Mabaso *et al*., 2019; Atucha Zamkova *et al*., 2021; Peng *et al*., 2021).

Field investigations involving the assessment of progeny tests after natural frost-induced damage have commonly been used as a measure of cold sensitivity (Andersson, 1994; Anekonda *et al*., 2000; De Waal *et al*., 2018; Prevéy *et al*., 2018). However, such damages take place irregularly in terms of location in field test sites, time periods and severity. This results in uneven testing and is statistically unsatisfactory (Frederiks *et al*., 2012; Murphy *et al*., 2020). Furthermore, it is difficult to distinguish between frost damage and other factors such as drought, disease and insects that also bring about injury (Anekonda *et al*., 2000). Due to these limitations several researchers have used artificial freeze testing as a means of assessing cold-induced injury (Repo, 1992; Aho, 1994; Atucha Zamkova *et al*., 2021). This ensures that freeze-inducing temperatures are properly controlled and applied in a uniform manner (Anekonda *et al*., 2000). Although artificial freezing and controlled growth environments offer a means of standardization for the study of cold tolerance, it has been recognized that such environments may fall short of mimicking the complex field conditions associated with cold acclimation (Gusta and Wisniewski, 2013). Thus, a true reflection of cold tolerance may not be possible to create using artificial methods as described in studies conducted by Dhanaraj *et al*. (2007) and Li *et al*. (2011). It is also important to note that factors such as the rate and duration of freezing as well as whether plants are dry or wet can affect the assessment of freeze-induced injury (Gusta and Wisniewski, 2013).

Electrolyte leakage measurements after artificial freezing is a common way of assessing frost sensitivity in plants and is based on the effect that freezing temperatures have on cellular membranes (Dexter *et al*., 1930; Dexter *et al*., 1932; Sutinen *et al*., 1992; Bucher and Rosbakh, 2021). During such temperatures, cellular membranes are disrupted thus hindering a cell's ability to retain solutes such as ions (Maier *et al*., 1994; Demidchik *et al*., 2014). Other stresses that may bring about electrolyte leakage include pathogen attacks (Brisset and Paulin, 1991; Ghanbary *et al*., 2021), high salinity (Nassery *et al*., 1975; Chen *et al*., 1999; Hoang *et al*.,

2020), heavy metals (Xu *et al*., 2008; Hall, 2002; Shirani Bidabadi, 2020), oxidative stress (Demidchik *et al*., 2010; Ding *et al*., 2022), acidic environments (Baziramakenga *et al*., 1995; Miri Nargesi *et al*., 2022), waterlogging (Yan *et al*., 1996; Toral-Juárez *et al*., 2021), drought (Bajji *et al*., 2002; Bijanzadeh *et al*., 2019) and heat (Martineau *et al*., 1979; Ali *et al*., 2020). Electrolyte leakage may be determined by measuring the electrical conductivity of the bathing solution in which the plant tissue is placed (Prášil and Zámečník, 1998). This may be performed almost immediately after the application of the relevant stress factor, with the conductivity value of the efflux being a direct measure of the injury produced (Reitsma, 1994; Demidchik *et al*., 2014). Since this method is numerically based, it allows for results to be analysed statistically (Murray *et al*., 1989). Furthermore, it is a relatively quick, inexpensive method that is not destructive to whole plants and may be applied to plant material from many different cultural systems (Whitlow *et al*., 1992; Bajji *et al*., 2002). Although the measurement of electrolyte leakage is a valuable indicator of the extent of injury experienced by plant material, there are some caveats associated with the use of this method. In a study conducted by Reitsma (1994), difficulties were encountered when measuring the electrolyte leakage for *Nothofagus solandri* (black beech), var *cliffortioides* (mountain beech), *Dracophyllum subulatum* (monoao) and *Phyllocladus alpinus* (mountain celery pine), all of which have thick cuticles. It is believed that this characteristic prevented electrolytes contained within internally damaged cells from moving into the bathing solution. However, the thick cuticle does not prevent leaves from freezing. Thus, in such a case the measurement of electrical conductivity is not a true reflection of freezing resistance. In earlier studies the use of electrolyte leakage in plant studies required the use of uniform plant samples for purposes of standardization. This was time consuming and cumbersome (Dexter *et al*., 1932; Wilner *et al*., 1960; Prášil and Zámečník, 1998). Furthermore, different concentrations of electrolytes in plant tissue samples may also be affected by specific treatments (Prášil and Zámečník, 1998). In order to deal with such issues and bring about standardization, various ways of expressing electrolyte leakage have been developed over the years (Stuart, 1940; Flint *et al*., 1967; Driessche, 1976; Read and Hill, 1989; Whitlow *et al*., 1992). One of the most common ways was developed by Flint *et al.* (1967), who introduced an "Index of Injury" in which the conductivity of a control or treated sample is expressed relative to the conductivity of the same sample when it is completely killed for example by freezing. This method of expression has been used in studies conducted by Burr *et al*. (1990) and Bajji *et al*. (2002) while modifications of the original method have been used in studies such as those conducted by Whitlow *et al*. (1992).

Whole-plant freezing tests have also been used by several researchers to establish cold hardiness of plants (Burr *et al*., 1990; Otgonsuren and Lee, 2013; Atucha Zamkova *et al*., 2021). Although this test has been deemed to be reliable, it is time consuming (often requiring seven to fourteen days for a result) and requires destructive sampling (Burr *et al*., 1990). The measurement of chlorophyll fluorescence has been used as a more rapid screening method for cold tolerance (Perks *et al*., 2004; Zhou *et al*., 2018). However, it is unclear whether freezing damage develops evenly over the whole leaf. Thus, measurements taken at small, single points in leaves may increase the chance of erroneous results (Ehlert and Hincha, 2008). An increase in the concentration of soluble sugars has also been used as an indication of cold tolerance (Tinus *et al*., 2000; Omidifard and Gharaghani, 2022). However, caution is advised when using this as a means of screening for cold tolerance as a positive correlation does not always result as indicated in a study by Hincha *et al*. (1996).

1.7 Frost tolerance in trees

Most overwintering (plants that survive/ pass through the winter) perennial trees prepare in advance for winter. In response to shortening day length activity (reduced photoperiod), activities of the apical bud meristem ceases, and growth cessation occurs, followed by a period of dormancy (Rinne *et al*., 2001; Welling and Palva, 2006; Nilsson, 2022). As discussed earlier, phytochromes are responsible for the detection of photoperiods and in a study conducted by Welling *et al*. (2002), in hybrid aspen trees, phytochrome A was observed to be essential in this activity. Freezing tolerance of trees increases in parallel to growth cessation and exposure to low temperatures. Although the exact mechanisms used by frost tolerant woody perennials is not fully understood, it is believed that the cellular changes associated with the dormancy period play a crucial role (Welling and Palva, 2006). In studies conducted by Welling *et al*. (1997) and Rinne *et al*. (1998) in birch (*Betula pubescens* Ehrh.), it was observed that on the onset of cold acclimation the level of the plant hormone abscisic acid increased, tissue desiccation and an accumulation of dehydrins also occurred. Sauter *et al*. (1996) observed an increase in intracellular sucrose concentration and development of an elaborate system of vesicular and cisternal endoplasmic reticulum (ER) in ray parenchyma cells of poplar wood during low temperatures. It was suggested in this study that the elaborate system of vesicular and cisternal ER may play a role in dehydration of the cells based on the accumulation of sucrose within this system as well as its spatial relation to the plasma membrane. This

controlled form of dehydration during the cold acclimation process is characteristic to overwintering tissue and has been suggested to protect the cells from forthcoming injuries induced by freeze-induced dehydration and intracellular ice formation (Welling and Palva, 2006).

Another cellular change that is common in frost tolerant woody plants is the formation of metastable cell solutions called glassed cell solutions which increase the stability of the cells and result in them being relatively unaffected by the stresses associated with low temperatures and ice formation (Buchner and Neuner, 2011). These solutions are highly viscous and form as a result of a high solute concentration (for example sugars) at sufficiently low temperatures (Wisniewski *et al*., 2003; Welling and Palva, 2006). Although the other characteristics of glassed cell solutions include their high degree of supercooling and high hydrostatic tension, they are not subject to ice nucleation, solute crystallization or water vapour cavitation, provided that the solution remains below the melting temperature of the glassed cell solution (Wisniewski *et al*., 2003).

The many cellular changes that occur during the increase of frost tolerance in woody plants may be explained by studying changes in the transcriptome (Yu *et al*., 2020), proteome (Baniulis *et al*., 2020), metabolome (Oberschelp *et al*., 2020) and lipidome (Watanabe *et al*., 2018). These three fields of study and their integration into systems biology have been suggested to provide a holistic analysis of cell biology and the development of ways to alter cellular characteristics in a controlled manner (Renaut *et al*., 2006). Proteomics, the study of changes in the proteome has been described by Renaut *et al*. (2006), as an "essential bridge between the transcriptome and the metabolome".

In a study conducted by Renaut *et al*. (2004), changes in the protein levels of poplar were studied during exposure to low temperatures. The most noticeable changes were associated with defences against reactive oxygen species, accumulation of dehydrins, chaperon-like proteins and transcription factors involved in cold signalling (Table 1.2). The increase in chaperone-like proteins, especially heat shock proteins (HSPs) is interesting because these proteins are usually upregulated during both high and low temperature extremes (ul Haq *et al*., 2019). HSPs play a role in the prevention of irreversible protein inactivation and aggregation associated with stress conditions, and promote productive folding of polypeptides (Sung *et al*.,

2001; Wang *et al*., 2004; Welling and Palva, 2006). The accumulation of small heat shock proteins (sHSPs) has been also observed in woody species such as sweet chestnut (*Castanea sativa* Mill.) and mulberry (*Morus bombycis* Koidz) during cold acclimation (Ukaji *et al.*, 1999; Lopez-Matas *et al*., 2004)

In another study conducted by Renaut *et al*. (2008) in bark tissues of peach **(***Prunus persica* L. Batsch) an upregulation in the following proteins was reported in response to low temperatures (Table 1.2): dehydrins, enzymes involved in carbohydrate metabolism such as enolase, UDP-glucose pyrophosphorylase and malate dehydrogenase; proteins that promote proper protein assembly such as HSP70s; stress protective proteins such as quinone oxidoreductase and β-cyanoalanine synthase; and metabolic enzymes such as S-adenosyl-$_L$-Methionine (SAM) synthetase and thiazole biosynthetic enzyme (Goward and Nicholls, 1994; Forsthoefel *et al*., 1995; Ross *et al*., 2000; Ciereszko *et al*., 2001; Sung *et al*., 2001; Settembre *et al*., 2003; Siegel *et al*., 2004; Machingura and Ebbs, 2010; Chu *et al*., 2013).

Studies conducted by Kjellsen *et al*. (2010) in *Picea obovate* Ledeb. (Siberian spruce) showed that the following proteins were upregulated during cold acclimation (Table 1.2): dehydrins, HSP 70s, the AAA^+ family of proteins, lipocalin-like proteins, cyclophilins, glycine-rich RNA-binding proteins, scavengers of ROS, proteins related to cellular metabolism such as nucleoside-diphosphate-sugar epimerases, proteasome subunit alpha type-2-B, PsbP and dihydroflavonol 4-reductase. Some of the AAA^+ family of proteins that were upregulated were identified as the FtsH class, which are membrane bound ATP dependent metalloproteases. These proteins have chaperone roles and are involved in assembly, operation and disassembly of protein complexes (Snider *et al*., 2008). The upregulation of lipocalin-like proteins is interesting because studies conducted by Charron *et al.* (2005) and (2008) in *A. thaliana* found that lipocalins and lipocalin-like proteins were associated with the plasma membrane, were also upregulated during cold acclimation and played a role in the response to oxidative stress. Cyclophilins have been involved in stress responses to several adverse conditions such as low CO_2 conditions, heavy metal pollution, salt, heat, cold, hormonal, osmotic stresses and fungal infection (Bozhko *et al*., 2003; Salika and Riffat, 2021). The presence of pathogenesis-related proteins (PR proteins) has also been observed to increase during cold winter months indicating that these proteins may protect plants against certain adverse conditions encountered during these months (Welling and Palva, 2006). In a study conducted by Liu *et al*. (2003) in *Pinus*

monticola Douglas ex D. Don (Western white pine) it was observed that the PR protein PMPR10 was induced to the highest-level during winter cold-hardening conditions. Ukaji *et al*. (2004) observed the accumulation of the WAP18, a winter accumulating protein belonging to the PR10 protein family, in the cortical parenchyma cells of mulberry during winter months. A summary of up-regulated protein during cold acclimation in woody plants is provided in Table 1.2.

Table 1.2: List of up-regulated proteins during cold acclimation in woody plants

Tissue/s	Species	Protein	Function	Reference
Leaves, bark needles, floral buds	Poplar, Peach, Siberian spruce, Blueberry	Dehydrins/ dehydrin-like proteins	Responsive to water stress, stabilization of proteins in membranes and cellular matrix	Arora and Wisniewski, 1994; Muthalif and Rowland, 1994; Renaut *et al*., 2004; Renaut *et al*., 2008; Kjellsen *et al*., 2010; Kjellsen *et al*., 2013
Leaves, bark, needles	Poplar, Peach, Siberian spruce, Sweet chestnut, Mulberry	HSP70, sHSPs	Prevention of irreversible protein inactivation and aggregation, facilitates proper folding of polypeptides	Ukaji *et al*., 1999; Lopez-Matas *et al*., 2004; Renaut *et al*., 2004; Renaut *et al*., 2008; Kjellsen *et al*., 2010
Leaves, Bark, needles	Poplar, Peach, Siberian spruce, Scots pine, Red spruce	ROS defence proteins	Combat oxidative stress	Hausladen and Alscher, 1994; Tao *et al*., 1998; Renaut *et al*., 2004; Renaut *et al*., 2008; Kjellsen *et al*., 2010
Bark, needles	Peach	Carbohydrate metabolic enzymes	Metabolism of carbohydrates	Renaut *et al*., 2008; Kjellsen *et al*., 2010
Bark, needles	Peach	Cellular metabolic enzymes	Control of cellular metabolic reactions	Renaut *et al*., 2008; Kjellsen *et al*., 2010
Needles, stems	Western white pine	Pathogenesis-related proteins	Defence against pathogens	Liu *et al*., 2003; Ukaji *et al*., 2004

1.8 Concluding Remarks

Although frost tolerance mechanisms in trees have been widely researched, they are still not completely understood. As research in this field continues it is hoped that the integration of frost tolerance into breeding programs will be improved. This review was written as a background for a research project on the development of biochemical markers for frost/cold tolerance in black wattle (*Acacia mearnsii* de Wild.) in South Africa. The following section therefor, provides an overview of frost tolerance research and its importance in black wattle.

Injury in wattle plantations in South Africa due to cold are either as a result of radiation frosts or extremely cold winds (Sherry, 1971; Moreno Chan, 2019). In the case of radiation frosts damage is limited to valleys or depressions in which cold air accumulates (Wang *et al*., 2021). However, frost damage may also occur at higher points where the normal down-hill flow of cold air has been prevented due to some obstruction. Frost damage due to cold winds usually takes place on ridges or plateaux that are exposed to winds from close mountain ranges that are snow-capped during winter. This rarely takes place at altitudes below 4000 feet (1219 m). Black wattle in South Africa is usually killed by frost when minimum temperatures fall below -4°C. However, in Australia this species can tolerate temperatures far below this at altitudes above 609.6 meters (Sherry, 1971).

During exposure to temperatures below freezing point the younger and more succulent tissues of the leading shoot and the distal ends of lateral branches of black wattle are usually the first to be injured. Injury to the stem cambium may result in swellings near the base of the stem. Temperatures below the tolerance limit of black wattle trees results in trees being totally killed or killed back to ground level. When black wattle trees are killed to ground level, new growth from the root collar may inhibit the original frosted shoot, which then forms a slender core of dead wood within the basal area of the stem when the tree is felled (Sherry, 1971).

One of the earliest studies conducted in frost tolerance in black wattle in South Africa was based on seed collections arranged by S.P Sherry from Australia in 1957. These included seed collected from 25 locations in New South Wales, Victoria, South Australia and Tasmania (Moreno Chan and Isik, 2021). Almost all these collections were from single trees. Much effort was taken in the selection of potentially frost-tolerant trees by sampling populations from high

altitudes and latitudes. Trial results using these seed collections indicated that most Australian plant material generally had thinner bark, lower tannin concentrations and were less vigorous than South African plant material. However, three Australian families were observed to be quite vigorous, and they were later included in a breeding program conducted by the Institute for Commercial Forestry Research (ICFR) previously known as the Wattle Research Institute (WRI) (Searle *et al*., 1992). Results of these earlier trials also influenced an ICFR-funded collection in 1985 that placed emphasis on northern New South Wales provenances that were used for a series of frost tolerance trials planted in 1986, as described in Hagedorn (1987). These trials included nine Australian provenances with four South African controls planted on three sites: Bloemendaal Research Farm in KwaZulu-Natal, and sites at Iswepe and Commondale in Mpumalanga. Heavy frosts caused severe damage to the trial at Iswepe during the first winter, resulting in assessment of frost tolerance being conducted after winter. An index to rank the different genotypes was developed based on the percentage of trees left alive after the first winter. Results from the Iswepe trial indicated that the most frost tolerant provenances were collected from the highest altitudes, Lake George and Mittagong in New South Wales, while the least frost tolerant provenances were taken from low altitude sites (Hagedorn, 1987; Dunlop *et al*., 2003).

While the study of frost tolerance in black wattle in South Africa was based on assessing patterns of genetic variation as a result of field trials, earlier frost tolerance analysis in black wattle in Australia was focused on laboratory-based screening of genotypes using a non-destructive, electrical conductivity screening technique (Searle *et al*., 1991). In a study conducted by Searle *et al*. (1991) electrical conductivity was used to examine within provenance variation in frost tolerance of black wattle using two provenances from the following sites: Mount Gladstone located 4km west of Cooma (925-1070 m above sea level); and 8-23 km west of Bodalla (15-40 m above sea level). These represented altitude extremes of the species distributed at the same latitude. The higher altitude provenance (Mount Gladstone) was observed to be more frost tolerant than the lower (Bodalla). In another study conducted by Searle *et al*. (1992) frost tolerance variation amongst twenty-five provenances of black wattle were examined based on frost damage assessed by measuring electrical conductivity. The selected provenances were chosen because they represented the climatic and geographic range of the natural distribution of the species in Australia. It was observed that the least tolerant provenances were from the most northerly coastal locations in New South Wales,

while the most tolerant provenances were from inland, high-altitude sites in New South Wales. The high altitude, inland sites of New South Wales are the coldest locations within the natural distribution of black wattle in Australia (Searle *et al*., 1992). The fact that the most tolerant provenances were observed in these sites tie in with the concept of cold acclimation as discussed earlier. In another study conducted by Pollock *et al*. (1986) involving eighteen Australian *Acacia* species it was observed that seedlots originating from inland sites of higher altitudes or latitudes were consistently more frost tolerant.

Conventional methods for the screening of frost tolerance in black wattle have focussed on the physical assessments of frost damage and not much emphasis has been placed on the biochemical aspects of frost tolerance. Current methods used for the screening of frost tolerance in South Africa often rely on expensive and time-consuming field trials. Although there has been a great amount of time and effort invested in breeding research for frost tolerance in black wattle by the WRI/ ICFR, there is still a great demand for the development of seed for frost-prone areas in South Africa (Chan *et al*., 2015; Bairu *et al*., 2020). An efficient, low-cost method for screening for frost tolerance that can be incorporated into breeding programs is required. The use of proteomics and artificial screening as breeding tools offers this possibility and thus forms the basis of the ongoing research project.

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Chapter 2: Development of an artificial frost screening protocol for *Acacia mearnsii* (black wattle)

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2.1 Abstract

Frost damage is a severe problem that affects the successful silviculture of *Acacia mearnsii* de Wild (black wattle) in South Africa. This abiotic stress has resulted in major financial losses for the wattle industry and has thus been an ongoing area of research. Previously, the screening of frost tolerance in black wattle germplasm has been based on field trials. However, the unpredictability of frost events, both in terms of occurrence and severity, has often resulted in insufficient information with regards to levels of frost tolerance in black wattle families. The current study aimed to develop an artificial frost screening protocol under controlled environmental conditions. This protocol was capable of inducing frost damage in black wattle, and the severity of frost damage could be calibrated to generate frost-tolerance results in black wattle seedlings similar to those obtained in previous field trials.

2.2 Introduction

Black wattle *(Acacia mearnsii* de Wild) is an important commercial forestry species in South Africa. The bark extract of black wattle is a high-quality source of tannin and adhesives, and its timber is used to produce woodchips for export markets (Griffin *et al*., 2011). The current planted area for black wattle is 110 000 ha in the provinces of KwaZulu-Natal and south-eastern Mpumalanga at latitudes 25-33°S, longitudes 27-30°E, and altitudes between 400-1500 m (Moreno Chan *et al*., 2015; Smith, 2002). The climate where black wattle is grown ranges from warm subtropical to cold temperate with mean annual temperatures of 16 to 20°C. The annual rainfall in these areas is between 800-1200 mm (summer distribution) (Smith, 2002). There is a limited availability of land and water for commercial forestry in South Africa and it is unlikely that more planting land will be made available for black wattle because of government restrictions (regulation 15B,DWAF, 1996; Govender, 2014).

This has led to a change in the silviculture of black wattle from an extensive approach to an intensive one (Govender, 2014). Frost damage is an important abiotic risk that affects approximately 29% of black wattle plantations in South Africa. Current black wattle planting stock cannot tolerate temperatures below -4°C. Despite breeding research over several decades at the Wattle Research Institute (WRI) (now the Institute for Commercial Forestry Research (ICFR)), genetically improved seed of *A. mearnsii* for frost prone areas is not yet available to wattle growers (Moreno Chan, 2015a). If such seed was available it would increase plantation productivity. Furthermore, with the erratic weather patterns associated with climate change, frost tolerant black wattle seed may also be planted in growing areas that have developed into frost prone zones.

In order to test black wattle families for their suitability for cultivation in frost prone areas, tree breeders usually plant trials in these locations and assess the levels of frost damage that may occur after a frost event (Skulason *et al*., 2018; Moreno Chan, 2019). This is the most realistic representation of frost tolerance because plants can undergo cold acclimation and deacclimation in response to natural conditions (Gusta and Wisniewski, 2013). However, this method has limitations because frost events are unpredictable, both in terms of their occurrence and severity. Plants that possess moderate levels of frost tolerance may be wiped out completely if an abnormally severe frost event occurs, while in mild winters, frost-sensitive plants may go undamaged. In both cases the quality of information gathered with regards to differences in frost tolerance levels is poor (de Waal *et al*., 2018). In addition, seasons change from year to year such that no two autumns or winters are the same at a given site. These seasonal changes result in levels of cold acclimation that can vary, depending on the length and severity of the cold seasons preceding frost events. Therefore the "level" of cold acclimation achieved, together with genetic potential for frost tolerance, play a major role in the ability of plants to survive a frost event. The most recent black wattle frost field trials took place in northern KwaZulu-Natal and south-eastern Mpumalanga (Smith, 2005; Moreno Chan, 2019; Moreno Chan and Isik, 2021).

In an effort to obtain predictable frost conditions several approaches have been developed for controlled frost studies (Arora and Rowland, 2011). These range from standard commercial cold rooms and freezing cabinets to specialized frost units that can simulate specific types of frost such as radiation frost (Warrington and Rook, 1980; Lu *et al*., 2019).

Whole plant and tissue freeze testing, electrolyte leakage and chlorophyll fluorescence analysis are among the most commonly used methods to quantify frost tolerance in artificial cold screening in plants. Whole plant frost testing involves the placement of the entire above-ground portions of plants in a programmable freezer in which the temperature is decreased gradually until a targeted subfreezing temperature is reached (Tanaka *et al*., 1997). This temperature is uniformly maintained for a specified time period, aiming to create an environment that is similar to natural conditions. After the plants are removed from the freezer, they are placed in a moderate and natural environment for a specified period before they are visually assessed for browning of damaged plant tissue. This is referred to as the "browning test" (Burr *et al*., 2001).

Exposing plant tissue such as needles/leaves and shoots to freezing temperatures is commonly used in combination with electrolyte leakage methods to determine the level of cold damage on leaf tissue material (Murray *et al*., 1989; Mabaso *et al*., 2019). Injury to the cell membrane results in permeability and leakage of intracellular electrolytes. This leakage can be measured and used as an indicator for the extent of cell death in plant tissues. This method was originally developed by Dexter *et al*., (1932) and later included variations such as the ninhydrin reaction test (Siminovitch *et al*., 1964) and a UV-absorbing test (Redmann *et al*.,1986).

Chlorophyll fluorescence measurements have also been used as a non-invasive indication of frost tolerance in plants that have leaves with sufficient surface area, such as strawberries (Khanizadeh and Deell, 2001). Environmental stresses such as frost cause the photosystem II (PS II) in leaves to become damaged, and since chlorophyll fluorescence responds to changes in PS II phytochemistry, this provides a tool to measure plant responses to such stresses (Francko *et al*., 2011).

To date, all breeding research for frost tolerance in black wattle at the ICFR has been carried out in field trials, which have the limitations discussed above. An artificial frost screening protocol for black wattle seedlings would be more accurate and cost effective than exposing the material to natural frosts in field trials. Thus, the objective of this study was to develop an artificial frost induction protocol for screening black wattle seedlings of different genotypes for frost tolerance.

2.3 Materials and methods

Six temperature schedules were tested in a programmable frost chamber located at the Ukulinga Research Facility, University of KwaZulu-Natal, Pietermaritzburg (Figure 2.1). This chamber

was manufactured in Indonesia by Mitsui O.S.K Lines, fitted with a refrigeration unit by Daikin and further modified with a Danfoss ERC 21 X, smart multipurpose refrigeration controller. Besides studies with forest species, the chamber has also been used for the vernalization of cabbage plants and sugar beet.

The sequence of temperature schedules that were tested is shown in Table 2.1. Temperature schedules were 7 to 12 days long, divided into 2 to 4 stages, with each stage varying in length and temperature conditions (Table 2.2). The different temperature schedules consisted of an acclimation stage during which time the seedlings became adjusted to the chamber environment. This was followed by a day/s of significant differences between day and night temperatures, and thereafter in some of the schedules, a period where the seedlings were exposed to moderately cool temperatures before they were removed and placed in a nursery environment with warm conditions. These different stages were chosen in an attempt to mimic the natural process of cold acclimation in a shortened period of time as compared to natural conditions. Several studies have used a similar approach, although they differed in terms of the specific temperatures and timing of the stages (Fowler *et al*., 1973; Harrison *et al*., 1978; Persson *et al*., 2010).

Figure 2.1. The UKZN Ukulinga frost chamber utilized in the study

Temperature Schedules 1-2 were applied to plants from two black wattle seedlots with previously determined differences in frost tolerance. The frost tolerance levels of the seedlots were not revealed prior to the frost tests, to create a blind screening of the protocol. Eight plants per seedlot were used for each schedule. Both schedules were repeated twice. Plant material consisted of seedlings grown in 90 cm^3 tubes placed in seedling trays, leaving empty spaces between the plants.

Temperature Schedules 3-6 were applied to plants from eight black wattle seedlots of varying frost tolerance levels. The experimental design was randomised complete blocks with 25 replicates, with each seedlot represented by one plant in each replicate. Plant material were also seedlings in 90 cm^3 tubes. Plants were placed in a checker-board arrangement to ensure uniform cold air distribution across the material. There were six replicates per tray, thus each experiment utilised four full trays and one partially full tray. Schedule 4 was repeated twice, while the other schedules were carried out once. As with schedules 1-2, the frost tolerance levels of the seedlots were not revealed before the frost screening (blind screening).

Inside the chamber, light conditions were maintained at 550 µmol photons m^{-2} s^{-1} with a photoperiod of 13 hours. Relative humidity was kept at 80%.

Table 2.3: Sequence of temperature schedules applied to black wattle plants in a temperature-controlled chamber

Schedule		Repeat	No. Seedlots	No. plants	Schedule length (days)
1		1	2	16	8
		2	2	16	8
2		1	2	16	12
		2	2	16	10
3		1	8	200	10
4		1	8	200	9
		2	8	200	9
5		1	8	200	9
6		1	8	200	7

For the first repeat of Schedules 1-3, air temperature inside the frost chamber was measured with miniature sensors (Maxim iButtons ®, Fairbridge Technologies, Johannesburg, South Africa). The iButtons are an accurate and relatively low-cost system for temperature measurements in cold environments/freezing conditions (Hubbart *et al*., 2005; Lundberg *et al*., 2016). Five iButtons were used in each of the experiments in Schedules 1-3. The iButtons were programmed to record temperatures at 15 min intervals.

The temperatures programmed in the chamber were compared to the temperatures measured by the iButtons for Schedules 1-3. Unexpectedly, the iButton temperatures differed considerably from the temperatures programmed in the chamber for Schedule 1 and to a lesser extent for Schedule 2. In contrast, the iButton temperatures were fairly similar to those programmed in the chamber for Schedule 3. The iButton mean temperatures are reported for Schedules 1-3 in Table 2.2.

Due to logistic limitations, temperatures inside the chamber were not measured for the experiments in Schedules 4-6. It is thus not known if there were any differences between the temperatures programmed and the actual temperatures inside the chamber for these schedules.

Table 4.2: Description of temperature schedules tested on black wattle plants in a temperature-controlled chamber. Schedules 1-3 are the mean temperatures measured inside the chamber, while for Schedules 4-6 the reported temperatures are the temperatures programmed for the chamber.

Schedule 1		**Schedule 2**	
Stage	Temperature (°C) Day / Night	Stage	Temperature (°C) Day / Night
Day 1	12 / 1	Day 1-4	11 / 0
Day 2-7	16 / 0	Day 5	11 / -4
Day 8	12 / -2	Day 6-8	22 / -8
		Day 9-12	22 / -12
Schedule 3		**Schedule 4**	
Stage	Temperature (°C) Day / Night	Stage	Temperature (°C) Day / Night
Day 1-4	21 / 2	Day 1-3	18 / 4
Day 5-10	26 / -8	Day 4-6	12 / 2
		Day 7	22 / -3
		Day 8-9	18 / 4
Schedule 5		**Schedule 6**	
Stage	Temperature (°C) Day / Night	Stage	Temperature (°C) Day / Night
Day 1-3	18 / 4	Day 1-2	12 / 2
Day 4-6	12 / 2	Day 3-5	22 / 0
Day 7	22 / 0	Day 6-7	18 / 4
Day 8-9	18 / 4		

The lowest temperature (-12°C) of Schedule 2 simulated actual minimum temperatures observed in a field breeding trial during the winters of 2014 and 2015 (Moreno Chan, 2015b). Furthermore, the temperature gradients used in the coldest stages of Schedules 2-6 approximated the large diurnal temperature fluctuations observed in forestry sites of South Africa, and in ongoing field trials (Moreno Chan, 2015b). Although actual winter day

temperatures can exceed the upper temperature limits (22 or 26°C) used in this study, these were the highest temperatures supported by the frost chamber.

2.3.1 Plant material and frost damage scoring

The black wattle seedlots used in the study were sourced from the seed archives of the Institute for Commercial Forestry Research (ICFR), Pietermaritzburg, and are described in Table 2.3. *Acacia dealbata* was included as a control seedlot because of its high frost tolerance (Pollock *et al*., 1986). Seedlots were selected based on geographic origin and/or cold hardiness performance in previous trials. Seedlots 3 and 8 were tested in Schedules 1-2 and all the seedlots were tested in Schedules 3-6. Seedlots were sown and reared in 90 cm^3 Unigro® tubes under a black net-shaded tunnel (shade factor of 60%) at the ICFR, University of KwaZulu-Natal, Pietermaritzburg (29.6265° S, 30.4050° E, 670 masl). Plants were sown approximately five months prior to the frost tests. Height and collar diameter of the plants were measured before the frost tests as broad indicators of plant hardiness.

After the artificial frost test, the plants were transported back to the ICFR nursery and placed at ambient temperature conditions for one week before the frost damage assessments were carried out (Pollock *et al*., 1986; Sakai and Larcher, 1987). A visual scoring system based on foliage changes/symptoms and extent of damage was used to assess frost damage (Moreno Chan, 2019). Scores were: 0, no damage; 1, light foliage discolouration, almost normal; 2, foliage discoloured, pinnules closed; 3, topmost/distal leaves and/or apical shoot only damaged; 4, marked discolouration, pinnules closed and desiccated, 50% foliage damaged; 5, same foliage changes as (4) but 100% foliage damaged; 6, pinnules closed, very hard and desiccated, leaves curled, sometimes plant scorched, 50% foliage damaged; 7, same foliage changes as (6) but 100% foliage damaged.

Table 2.5: Black wattle (*Acacia mearnsii*) seedlots from Australian sites (New South Wales [NSW] and Victoria) and South Africa tested in the study (approximate altitudes in meters are indicated in brackets). *Acacia dealbata* was included as a control seedlot. Frost tolerance levels indicated are based on performance in ICFR field trials. During the frost tests only the seedlot numbers were known (blind screening). Seedlots 3 and 8 were used in temperature Schedules 1-2 and all the seedlots were used in Schedules 3-6.

Seedlot No	Seedlot ID	Description	Frost tolerance level
1	LG	A85-16, Lake George, NSW (700 m)	Tolerant
2	AD	L2_27-2, *Acacia dealbata,* control seedlot	Tolerant
3	Cooma	Sher_9-1, Cooma, NSW (884 m)	Tolerant
4	LalLal	Sher_15-1, Lal, Victoria (381 m)	Susceptible
5	Omeo	A85-81, Omeo Highway, Victoria (200 – 550 m)	Susceptible
6	Sheepmoor	Sheepmoor, Mpumalanga, South Africa (1458 m)	Intermediate
7	Liff	Karkloof, Kwazulu-Natal, South Africa (1290 m)	Susceptible
8	PSO5	Seed orchard, control seedlot	Susceptible

2.3.2 Statistical analysis

Summary statistics and graphs for the six temperature schedules were prepared in SAS 9.4 (SAS Institute Inc., Cary, NC, USA, 2002-2012). The data of the best schedule was examined by analysis of variance using Proc GLM in SAS to test the statistical contribution of all the sources of variation in the experiment. Artificial frost score means for the seedlots tested and statistical differences between seedlot means were estimated with the GLM model.

2.4 Results

2.4.1 Temperature schedules

Means and standard deviations of frost score, plant height and collar diameter for the six temperature schedules tested are shown in Table 2.4. Plant height and collar diameter were uniform across schedules, indicating that differences in frost damage between schedules were not based by differences in plant size.

Schedule 1, whose minimum temperatures were 0°C and -2°C, caused no frost damage (all plants had scores of 0), irrespective of the frost tolerance level of the seedlots (Figure 2.2 A) and was deemed to be too gentle a treatment regime. In contrast, Schedule 2 was too severe (minimum temperatures of -8°C and -12°C) and all the plants were killed by frost, irrespective of the seedlot (Figure 2.2 D). Results of Schedule 3 (minimum temperature of -8°C) were similar to Schedule 2, namely, all the plants had scores of 7 and were killed by frost. Schedule 4 (minimum temperature of -3°C) produced the best results, with the broadest response in frost damage (Figure 2.2 B and C). The mean values (4.2 and 4.7) of both repeats of Schedule 4 were similar, indicating good experimental repeatability (Table 2.4). In addition, the mean values were close to the midpoint of the scale used to assess frost damage (0-7) (Figure 2.3). Under Schedule 5 (minimum temperature of 0°C), the majority of plants showed no response (score = 0), whereas some plants were killed by frost (score = 7) and a few others showed frost damage only on the topmost leaves (score = 3). Consequently, the mean frost score of Schedule 5 was very low (0.79) (Table 2.4). It was surprising that schedule 5 caused slightly more frost damage to the plants than Schedule 1, considering that the latter had lower temperatures (Figure 2.3). Finally, under Schedule 6, whose minimum temperature was 0°C as in Schedule 5 but with a longer exposure (3 days vs. 1 day for Schedule 5), all the plants had frost damage scores of 0. The reduced response to Schedule 6 compared to that of Schedule 5 was unexpected.

Table 2.6: Summary statistics of six temperature schedules tested on black wattle plants. In Schedules 1-3 and 6 all plants had the same score (0, no damage or 7, extreme frost damage) and thus their standard deviations (std dev) were 0. Schedules 1-2 included two seedlots with eight plants each, Schedules 3-6 included eight seedlots with 25 plants each. Data shows that the height and collar diameter of the plants used were uniform across schedules.

Schedule	Repeat	No plants	Frost score		Height (cm)		Collar diameter (mm)	
			mean	std dev	mean	std dev	mean	std dev
1	1	16	0	0	47.6	9.0	4.1	0.39
	2	16	0	0	46.5	6.3	4.2	0.34
2	1	16	7	0	47.3	8.9	4.1	0.40
	2	16	7	0	48.6	6.3	4.3	0.37
3	1	200	7	0	49.6	9.5	4.0	0.46
4	1	200	4.22	3.01	54.4	11.2	4.0	0.76
	2	200	4.71	3.18	48.4	8.7	3.9	0.45
5	1	200	0.79	2.14	51.7	9.1	4.0	0.42
6	1	200	0	0	52.0	8.8	4.0	0.44

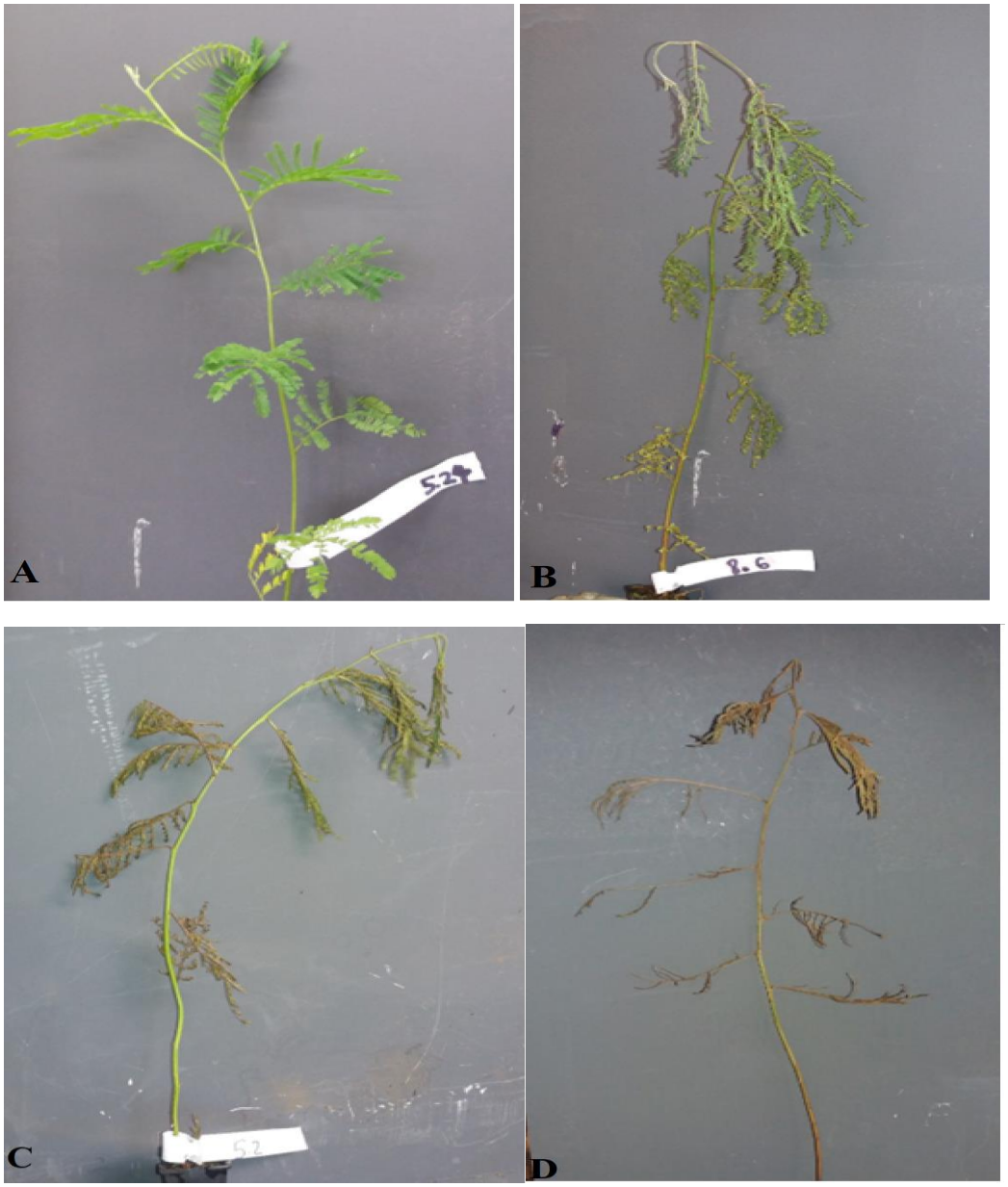
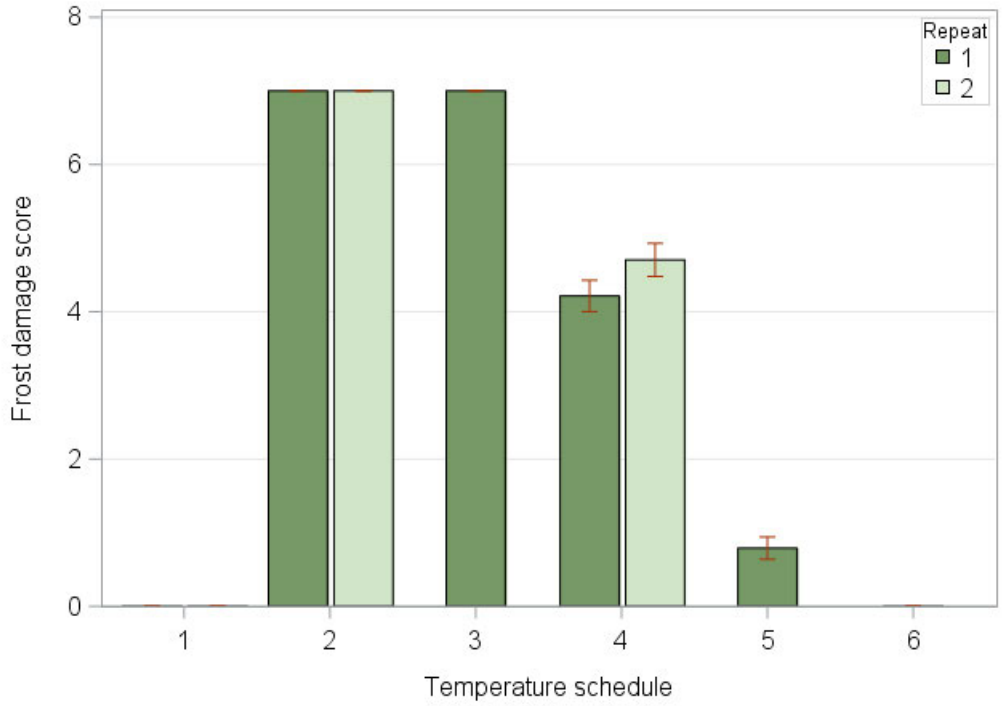


Figure 2.2. Photos showing frost damage on black wattle plants after artificial frost exposure: top left, no damage (A); top right (B) and bottom left (C), intermediate damage; bottom right, extreme damage (D).



Score of 1 = no damage; Score of 7 = dead seedlings

Figure 2.3. Frost damage score means and standard error bars of six temperature schedules tested on black wattle seedlots. Schedules 1,2 and 4 were repeated twice, while the other schedules were carried out once. Schedule 4 showed the best results, with mean values close to the midpoint of the frost damage scale. Schedules 1 and 6 were too mild and caused no damage to the plants (all plants had a score of 0), whereas Schedules 2 and 3 were too severe and killed all the plants (all plants had a score of 7). The standard errors for Schedules 1-3 and 6 were zero.

2.4.2 Effectiveness of temperature Schedule 4 to evaluate frost tolerance

The Schedule 4 treatment revealed considerable differences in frost score means between the two experiments for some seedlots (Figure 2.4). In addition, there were differences in plant size between repeats across seedlots (illustrated for plant height in Figure 2.5). Differences in plant size between seedlots were less pronounced than differences between repeats within seedlots. In particular, the plants of Seedlots 2 and 8 were smaller than the rest of seedlots (Figure 2.5).

The possible effects of experiment repeats and plant size, together with the other sources of variation in the experiment were assessed by analysis of variance using a generalised linear model (GLM) (Table 2.5). The effects of plant height and collar diameter were included separately in the GLM model. Both variables produced similar results, thus plant height was chosen for presentation of results. The effects of experiment repeat, plant height and collar diameter were not statistically significant, despite the variation in these variables between and within seedlots. The effect of seedlot was by far the most significant parameter ($P<0.01$), implying that Schedule 4 was an effective protocol to evaluate frost damage in black wattle seedlots of varying frost tolerance. The interaction Repeat × Seedlot was significant ($P<0.05$), indicating that there were some changes in frost damage scores between experiment repeats.

Table 2.7: Analysis of variance of frost damage score for temperature Schedule 4. The error term for testing the significance of Repeat and Seedlot effects was the interaction Repeat × Seedlot. The effects of plant height and collar diameter were tested separately in the GLM model. Plant height was chosen for presentation of results.

Source of variation	Degrees of freedom	Mean square	Variance ratio	F prob.
Plant height	1	0.002	0.00	0.9851
Repeat	1	20.327	1.49	0.2621
Seedlot	7	158.342	11.59	0.0022
Repeat × Seedlot	7	13.667	2.25	0.0301
Replication	24	8.852	1.45	0.0790
Residual	359	6.084		
Total	399			

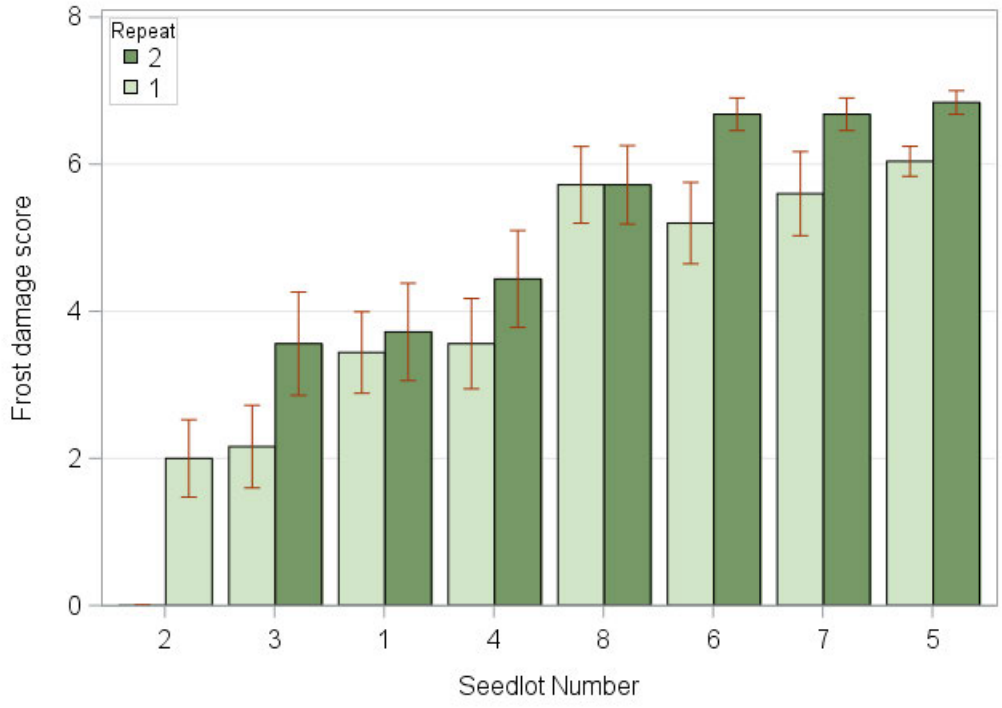


Figure 2.4. Frost score means and standard error bars of eight seedlots for temperature schedule 4. Separate bars are shown for each experiment repeat.

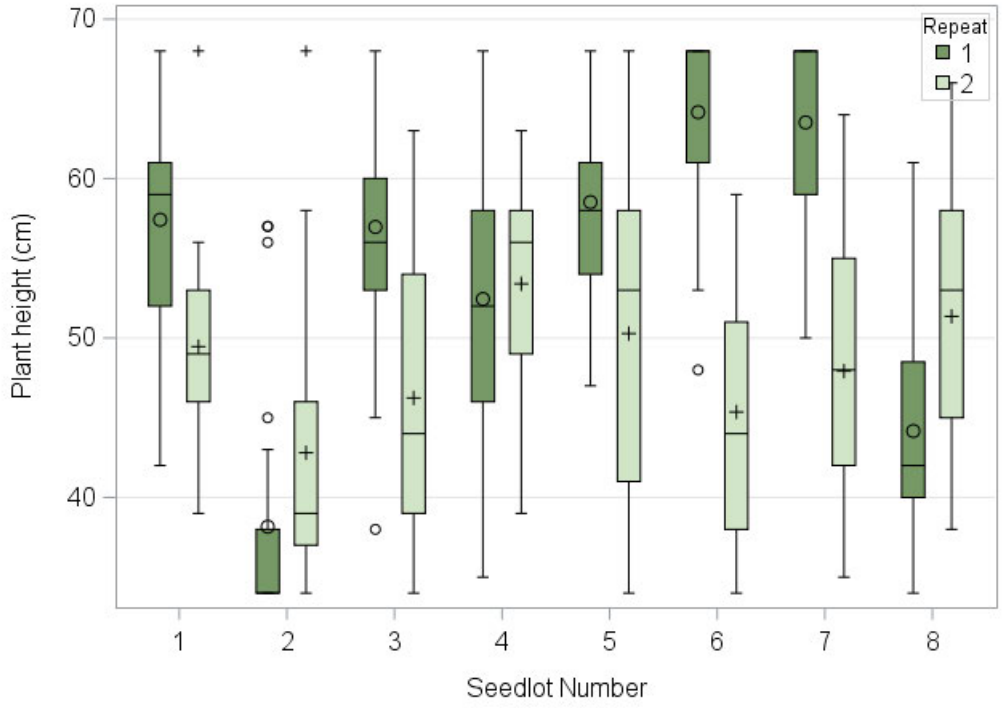


Figure 2.5. Variation in plant height between seedlots and between experiment repeats for temperature Schedule 4.

Artificial frost score means and seedlot rankings obtained with temperature Schedule 4 are shown in Table 2.6. With exception of Seedlots 4 and 6, results produced by the artificial frost protocol were in agreement with results from recent field trials at the ICFR. As expected, seedlots of *Acacia dealbata* Link (AD), Cooma and Lake George (LG) showed the highest frost tolerance, while PSO5, Sheepmoor, Liff and Omeo were all highly susceptible to frost damage. Seedlot 4 (LalLal) showed relatively good frost tolerance, which was unexpected because this seedlot had shown poor frost hardiness in ICFR field trials (Moreno Chan and Isik, 2021). On the other hand, the poor performance of Sheepmoor was surprising because this seedlot was more frost hardy than seed orchard seedlots in a recent study (Moreno Chan, 2019). Despite these two discrepancies, it can be concluded that Schedule 4 was effective for screening frost damage in black wattle seedlots of varying frost tolerance levels.

Table 2.8: Artificial frost score means and seedlot ranking obtained with temperature schedule 4. Seedlots ranked from most to least frost hardy. Means with the same letter are not statistically different (P < 0.05).

Seedlot No	Seedlot ID	Frost tolerance level in ICFR field trials	Frost damage score by artificial frost	Rank
2	*A.dealbata*	Tolerant	0.99 d	1
3	Cooma	Tolerant	2.86 cd	2
1	LG	Tolerant	3.58 c	3
4	LalLal	Susceptible	4.00 bc	4
8	PSO5	Susceptible	5.72 ab	5
6	Sheepmoor	Intermediate	5.94 a	6
7	Liff	Susceptible	6.14 a	7
5	Omeo	Susceptible	6.44 a	8

2.5 Discussion

As expected, temperature Schedules 1 and 6, with minimum temperatures between 0°C and -2°C, were too mild and caused no damage at all to the plants. On the other hand, Schedules 2 and 3, with minimum temperatures between -8°C and -12°C applied over several consecutive days, were too severe and killed all the plants. These results are comparable with the known frost tolerance levels of black wattle young seedlings in field conditions in South Africa, i.e. -3.3°C to -4°C (Beard, 1949; Beard, 1957; Sherry, 1971). In recent field trials with material of varying levels of frost tolerance, minimum temperatures of between -6.5°C and -8.7°C caused extensive frost damage to black wattle seedlings (Moreno Chan, 2019; Moreno Chan and Isik, 2021).

The expectation was that relatively small plants grown in small plugs, and thus with small root systems, would have been more sensitive to mild subfreezing temperatures compared to young seedlings grown in the field. In other words, if the temperature threshold to damage field seedlings of average frost tolerance is -4°C, then the threshold to damage plants in small plugs should have been just below 0°C. While the plants grown in 90 cm^3 plugs were more cold-hardy than expected at -2°C, none of the seedlots could tolerate the -8°C of Schedule 3. It is then clear that the type of plant material used in the study cannot tolerate temperatures beyond -8°C (Schedules 2 and 3), regardless of the frost tolerance of the seedlots employed.

Out of the six temperature schedules assayed, only Schedule 4 produced frost scores other than 0 or 7 (no damage/extreme damage) in a significant number of plants. This suggested that although the minimum temperature of -3°C used in Schedule 4 lies within the tolerance threshold of planted seedlings, this temperature is close to the frost damage threshold for the spectrum of black wattle germplasm used in the study.

In retrospective, the current study placed too much emphasis on trying to simulate field frost conditions inside the chamber and this reflected on the structure and the length of the schedules assayed. In these experiments, the artificial frost chamber was used for 8-12 days, making it a relatively slow and expensive process. In comparison, Pollock *et al*. (1986) employed a four-stage frost screening schedule run over 24 h in a controlled environment room for plants in 1.2 L pots of several temperate *Acacia* species, including black wattle. However, other frost protocols are similar in length to the protocol of the current study. In a study involving the development of a protocol for the frost-tolerance evaluation of rapeseed (*Brassica napus* L.), a period of 7 days at 4°C was established as the acclimation period and this was followed by frost treatment at -4°C for 16 hours (Fiebelkorn and Rahman, 2016). Lorenzetti *et al*. (1971) established that a cold acclimation period of 14 days at 2°C followed by 2-4 days freezing at -8°C was a suitable protocol for frost screening of *Lolium perenne* L. (perennial ryegrass).

We consider the frost protocol developed here only as a preliminary protocol. There are several factors that need to be refined through further systematic study:

- Optimum stage lengths and threshold temperatures in each stage;
- Temperature thresholds for the type of plant material screened, e.g., plants in nursery plugs, potted plants, etc;
- Establish whether air temperature in the temperature-controlled chamber is significantly different to the temperature of plug/root system of the seedlings being tested;
- The use of “live” temperature sensors instead of iButtons so that temperatures can be monitored more efficiently;
- Replace the electromechanical temperature controller with a more sophisticated proportional integral derivative (PID) controller;
- Investigate whether day/night temperature gradients are relevant for frost screening in a controlled room. We know that temperature gradients are important factors affecting frost hardiness under South African winter field conditions, but we do not know whether they matter for frost testing in a controlled environment.
- Photoperiod and light intensity;

- Number of plants per seedlot required for accurate, cost-effective screening;
- Repeatability of the protocol.

2.6 Conclusions

The establishment of an artificial frost screening method for black wattle was more complex than was initially expected. Although understanding frost damage in field trials is important, the duplication of field conditions for artificial frost screening does not necessarily provide an effective frost screening protocol. In an attempt to produce a rapid screening method, it is possible that some factors may have been overlooked. While there are several aspects of the current protocol (Schedule 4) to refine, it was encouraging that clear differences in frost tolerance among the various seedlots were observed with this preliminary protocol. An optimized frost screening protocol will be of considerable value to the black wattle industry in accelerating the breeding of frost tolerant wattle germplasm.

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Chapter 3: Application of an artificial frost tolerance protocol to screen accessions of *Acacia mearnsii* de Wild (black wattle)

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3.1 Abstract

A previously established protocol for the artificial frost screening of *Acacia mearnsii* de Wild. (black wattle) (by author) was tested in two separate trials involving over 100 families of wattle accessions. The 100 families had previously been ranked using field trials in typically frost prone areas in South Africa (insert a reference). Two different rating scales were used to phenotypically assess frost damage in the black wattle seedlings. A weak correlation was observed between the results of the artificial frost screening trials and the corresponding field results (r=0.24 to 0.28, r_s=0.20 to 0.28). Kruskal-Wallis tests showed statistical similarity between the medians of one of the artificial frost screening trials and the field frost damage evaluations and a significant difference between the medians of the two artificial frost screening trials that were conducted.

3.2 Introduction

To date, all breeding research in frost tolerance of black wattle (*Acacia mearnsii* de Wild.) at the Institute for Commercial Forestry Research (ICFR) and its predecessor, the Wattle Research Institute (WRI), has been carried out in field trials where young seedlings are exposed to natural frosts. One of the main limitations of this method is the unpredictability of occurrence and intensity of frost events. An artificial frost screening protocol for black wattle seedlings would be more accurate and cost-effective, and it would also allow for accelerated testing cycles. Frost damage evaluation in field trials takes several months from the establishment of the trials in autumn followed by the entire winter season, while artificial frost screening takes only a few days.

Variation in frost hardiness of black wattle at the population level has been investigated in several field and artificial frost studies (Searle *et al*., 1992, 1998). The most cold-hardy natural populations of black wattle are from high altitude sites in New South Wales and some sites in

Tasmania, whereas the least cold-hardy populations are from low elevation sites along the southern coast of New South Wales and the eastern coast of Victoria in Australia (Sherry, 1958; Hagedorn, 1990, 1993; Searle *et al*., 1992, 1998). Past research at the WRI/ICFR has shown that only a very few Australian provenances can tolerate the extreme temperatures of -8°C to -10°C that regularly occur in areas affected by intense frosts in south-eastern Mpumalanga, South Africa (Moreno Chan, 2015). In contrast, published studies on the additive genetic control of frost damage in black wattle are very limited. The one study on the subject reported individual tree heritability for relative electrical conductivity (a measure of cold injury) as 18% and within group family heritability as 37% (Searle *et al*., 1998).

A recent study by Moreno Chan and Isik (2021) investigated genetic variation in frost hardiness, rust resistance, growth and other traits of economic importance in 110 open-pollinated families of black wattle sourced from mid/high altitude sites in New South Wales and Victoria, Tasmanian sites and locally grown F1 families whose parents were from cold-hardy Australian provenances or local landraces from cold sites. Families were tested across six field trials established during 2014-2015 in northern KwaZulu-Natal and south-eastern Mpumalanga. The minimum temperatures across the field trials ranged from -6.1°C to -8.7°C and the number of frost events below -4°C varied between four and nine from the start of winter to the date of frost damage assessment (Moreno Chan and Isik, 2021). Considerable genetic variation for frost damage was observed between families within seed sources for frost damage, heritability = 77%. The objective of this study was to validate the artificial frost protocol developed in Chapter 2 through a proof of concept experiment with black wattle families tested recently in ICFR field trials.

3.3 Materials and methods

3.3.1 Plant material and experimental design

The artificial frost protocol developed in Chapter 2 was applied to the black wattle families that were tested for frost tolerance, rust resistance, growth and other traits in ICFR field trials established in 2014-2015 (Moreno Chan and Isik, 2021). Frost damage in these trials was evaluated during the winters of 2014-2016. The reference data to validate the artificial frost trials were the predictions of parental (family) breeding values produced by fitting a linear mixed model to the frost damage data from the field trials.

For the current study, seeds from over 100 black wattle families were sown into a composted pine bark medium in Unigro® 90 cm^3 tubes in June 2016 and grown under a black net-shaded tunnel (shade factor of 60%) at the ICFR nursery, University of KwaZulu-Natal, Pietermaritzburg. The germplasm consisted of open pollinated progeny of maternal parent trees (half-sib families). Due to variations in germination levels, growth rates between families and the logistics involved with the usage of the frost chamber, two separate trials were run (Table 3.1). The first one was carried out with 40 families and the second one with 96 families. Height and collar diameter of the plants were measured before the frost tests as broad indicators of plant hardiness. The experimental design was randomised complete blocks with 15 and 20 replicates for Trial 1 and 2, respectively. Each family was represented by one plant in each replicate. Plants were placed in a checker-board arrangement to ensure uniform, cold air distribution across the plants.

Table 3.9: Description of trials to validate an artificial frost protocol developed to screen black wattle families for frost tolerance.

Trial	No. Families	No. Replicates	Total No. plants	Schedule length (days)
1	40	15	600	9
2	96	20	1920	9

3.3.2 Artificial frost protocol and frost damage scoring

The two screening trials were carried out in the Ukulinga frost chamber, University of KwaZulu-Natal, Pietermaritzburg (Figure 3.1) (a modified 6m shipping container for frozen meat (Daikin Carrier Thermo King)). The temperature schedule shown in Table 3.2 was applied at cooling and warming rates of 4ºC/hour and 7ºC/ hour, respectively. Light conditions inside the chamber were set at 550 µmol photons m^{-2} s^{-1} for a photoperiod of 13 hours. A timer switched the lights on and off at specified times. The lights were switched on at 5 am and switched off at 6 pm. Relative humidity was kept at 80%.

Air temperature inside the chamber was measured at 15 min intervals with miniature sensors, Maxim iButtons® (Fairbridge Technologies, Johannesburg, South Africa). One sensor was used in Trial 1 and three sensors in Trial 2. With exception of Day 7, the temperatures programmed in the chamber were fairly similar to the temperatures measured by the sensors in both trials (Table 3.2, Figure 3.2). On Day 7, the actual minimum temperatures in the chamber

were lower than those programmed by 2-3°C and the actual maximum temperatures were higher than those programmed by 2-4°C (Table 3.2).

After completion of the temperature schedule, plants were transported back to the ICFR nursery and placed at ambient temperature conditions for 2-4 days before the frost damage assessments were carried out.

Figure 3.2. Inside view of one of the screening trials at the UKZN Ukulinga frost chamber.

Table 3.10: Temperature schedule applied to two screening trials with black wattle families in the UKZN Ukulinga frost chamber. Approximate temperature means measured inside the chamber at temperature stabilization periods are shown for each of the trials.

Stage	Temperature (°C) Day / Night		
	Programmed in the chamber	Actual temperatures	
		Trial 1	Trial 2
Day 1-3	18 / 4	18 / 3	17.5 / 3
Day 4-6	12 / 2	10 / 1.5	9 / 2
Day 7	22 / -3	26 / -5	24 / -6
Day 8-9	18 / 4	18 / 3.5	17.5 / 3

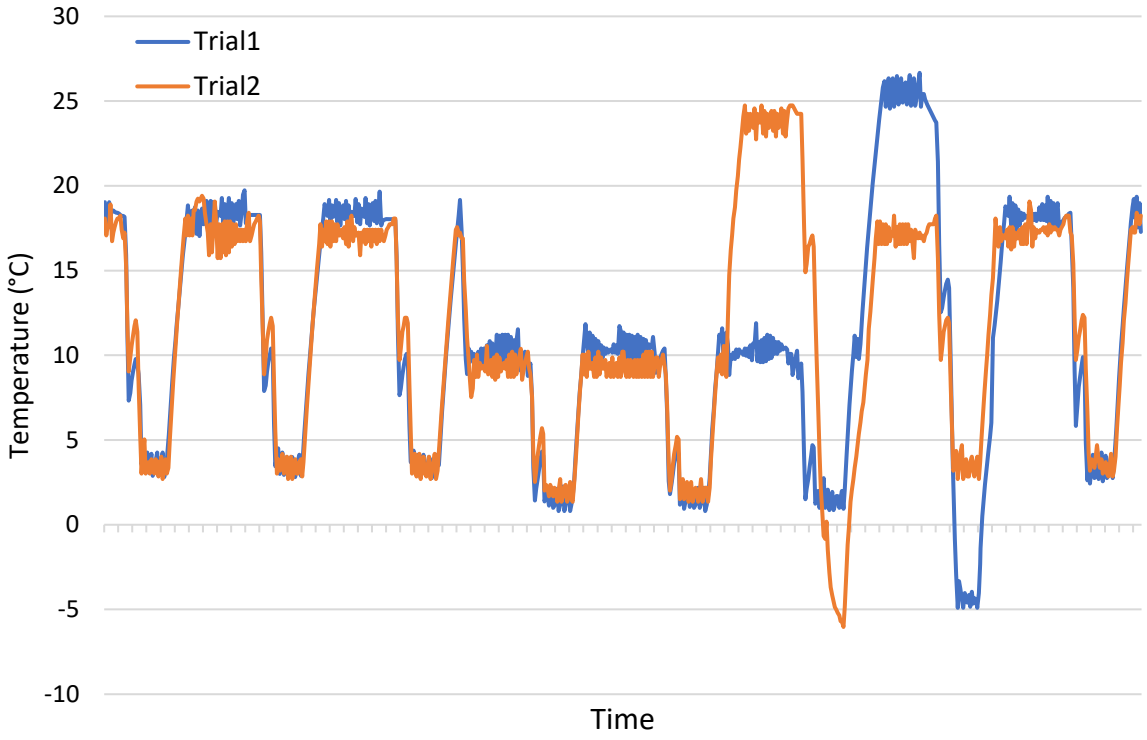

Figure 3.3. Temperatures measured inside the cold chamber for each of the screening trials. One sensor was used for Trial 1 and three sensors for Trial 2. Data shown for Trial 2 is the mean of the three sensors.

Two scores, referred to as Scale A (Moreno Chan, 2019) and Scale B (adapted from Pollock *et al*., 1986), were employed to assess frost damage. In the current study, Scale A was expanded to include 75% of foliage damaged by frost and thus the frost damage scale ranged from 0 to 9 (compared to 0 to 7 in the scale used in Chapter 2). The frost damage scores were: 0, no damage; 1, light foliage discolouration; 2, foliage discoloured and pinnules closed; 3, topmost/distal leaves and/or apical shoot only damaged; 4-6, marked discolouration and pinnules closed and desiccated in 50%, 75% or 100% of the foliage, respectively; 7-9, pinnules closed, very hard and desiccated and leaves curled in 50%, 75% or 100% of the foliage, respectively.

The frost damage scores of Scale B were as follows: 0, < 1% foliage damage; 1, 1-20% foliage and stem damaged, terminal bud undamaged; 2, 21-50% foliage and stem damaged, terminal bud damaged; 3, 51-80% foliage and stem damaged, terminal bud damaged; 4, > 80% foliage and stem damaged or entire plant killed.

3.3.3 Data analysis

SAS version 9.4 (SAS Institute Inc., Cary, North Carolina, USA) was used to derive the summary statistics of the two artificial frost trials, histograms with frost score distribution of individual-plant data and family means with associated standard errors for each trial. Pearson and Spearman correlation coefficients (r, r_s), together with scatter plots of the family means were used to make comparisons between artificial frost and field data, between frost damage scores and between artificial frost trials. In addition, graphs were prepared to compare family performance in frost tolerance between artificial frost and field data and between artificial frost trials. Minitab version 17.3.1 (Minitab Inc., State College, Pennsylvania, USA) was used to conduct Kruskal-Wallis analyses to test for the equality of medians between artificial frost trials and the field frost data as well between each artificial frost trial. In order to perform the Kruskal-Wallis analyses, Scale A was normalized from 0-9 to 0-7 (same as field frost trials). Scale A and B had strong correlations (r=0.90 to 0.91, r_s=0.87 to 0.93), (Table 3.4) and thus only Scale A was used in these analyses.

3.4 Results

3.4.1. Overall trends

Summary statistics of frost Scales A and B, plant height and collar diameter for the two screening trials are shown in Table 3.3. Score means were larger for Scale A due to the new

rating scale. Frost damage score variations relative to the mean were greater in Trial 1 than in Trial 2 for both frost scores. Frost damage was greater in Trial 2 due to the accidental lower minimum temperature in the trial (-6°C) compared to Trial 1 (-5°C). Interestingly, despite the larger size of the plants in Trial 2 (due to their longer growing period at the nursery, approximately two months), they showed greater frost damage, suggesting that minimum temperature played a more important role than plant size in terms of frost damage.

As shown in Table 3.3, plants in Trial 1 were considerably smaller than those in Trial 2. The difference was simply because the plants used in Trial 1 were grown for five months, whereas those in Trial 2 were grown for 7 months. The differences in plant size between trials did not seem to affect the results. In a similar temperature schedule carried out in Chapter 2 (Table 2.4), plants had a mean height of 48.4-54.4 cm and a mean collar diameter of 3.9-4.0 mm and the mean frost damage scores obtained were comparable to the mean scores (Frost Scale A) obtained here, suggesting that researchers have some flexibility in plant size when using this artificial frost screening protocol.

Table 3.11: Trial means with standard deviations (in brackets) for the two screening trials.

Trait	Scoring	Trial 1	Trial 2
No. Families		40	96
No. Plants		600	1920
Frost Scale A	0-9	3.0 (3.24)	4.7 (3.3)
Frost Scale B	0-4	1.8 (1.71)	2.2 (1.57)
Height	cm	23.3 (5.1)	38.2 (10.6)
Collar diameter	mm	3.3 (0.43)	4.1 (0.98)

3.4.2. Frost damage score distributions

Individual-plant score distribution of frost scores in Scale A in both trials indicated that the most frequent scores were 0, 3 and 7-9 (Figure 3.3). Scores 1-2 and 4-6 were rarely observed (Figure 3.3). Thus, most plants had either no frost injury at all (score 0), injury only at the topmost leaves and/or apical shoot (score 3) or extreme frost damage symptoms (defined as pinnules closed, very hard and desiccated, leaves curled) in 50%, 75% or 100% of the foliage (scores 7, 8 and 9). In addition, a score of 9 was considerably more frequent than scores 7 and 8.

The fact that scores 1-2 and 4-6 did not occur indicated that some symptoms were not observed and thus Scale A must be reviewed for future artificial frost work. Scores not observed were defined as follows: 1, light foliage discolouration; 2, foliage discoloured and pinnules closed; scores 4-6, marked discolouration and pinnules closed and desiccated in 50%, 75% or 100% of the foliage, respectively.

Regarding frost Scale B, individual-plant score distribution was different between trials (Figure 3.4). In Trial 1 most plants showed either no damage or extreme frost damage (scores 0 and 4, respectively) with scores 1-3 being less frequent. In contrast, in Trial 2, score 4 was the most frequent, which was related to the higher mean level of frost damage in the trial. Score 4 was followed by scores 0, 1 and 2 which showed similar frequencies. Score 3 showed the lowest occurrence in Trial 2 (Figure 3.4).

Unlike Scale A, there were no gaps in the plant score distributions due to the simpler damage of Scale B, namely, increasing the % of plants damaged by frost (0, <1%; 1, 1-20%; 2, 21-50%; 3, 51-80%; 4, >80%).

While the lack of occurrence of plants for several scores can be seen as a disadvantage of Scale A over Scale B, score distribution is not the only factor to consider for selecting the best scale. Both scales were compared in terms of their ability to rank families for frost tolerance subsequently.

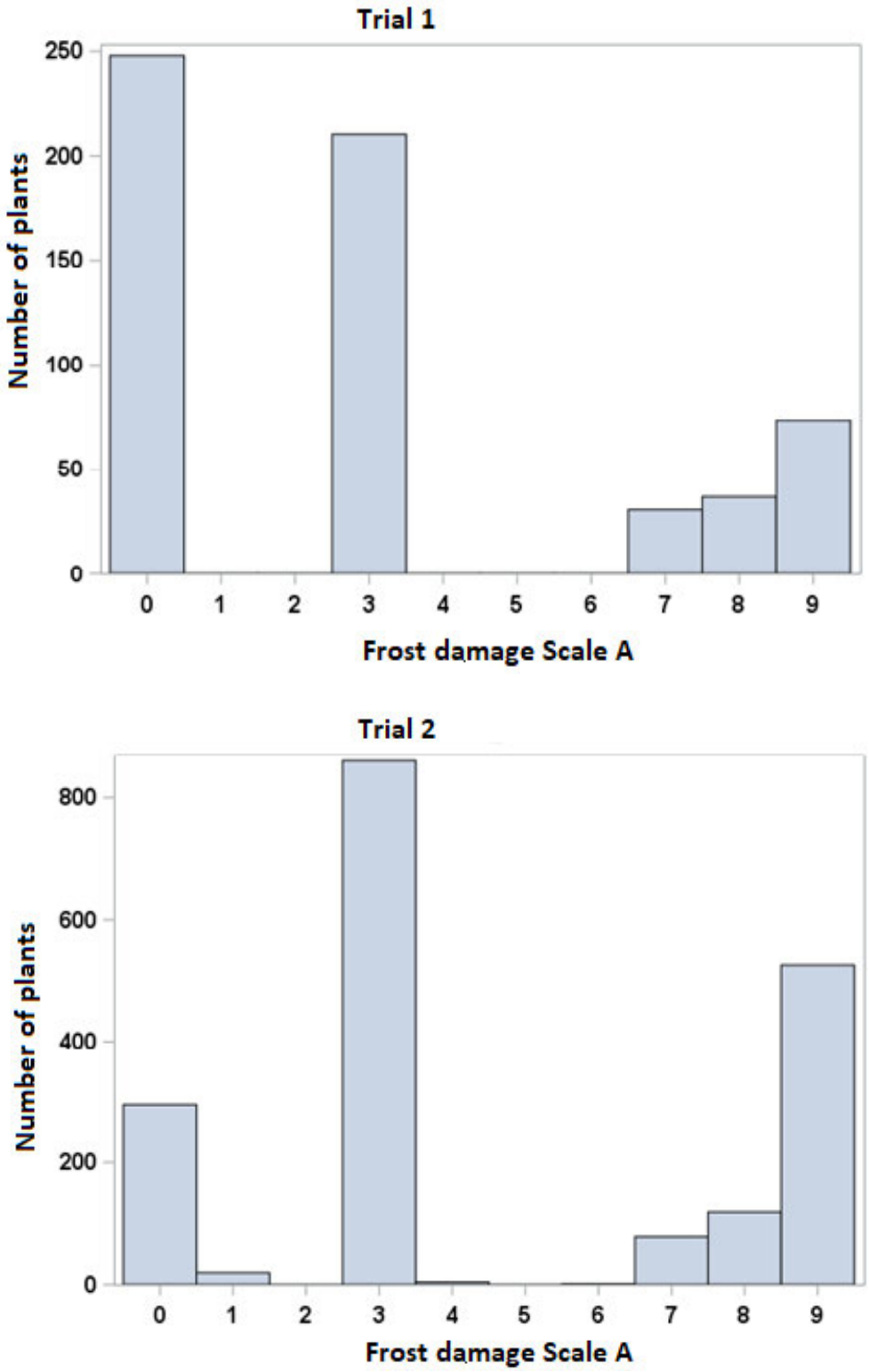

Figure 3.4. Score frequency of Frost damage Scale A. Data is shown separately for each artificial frost trial.

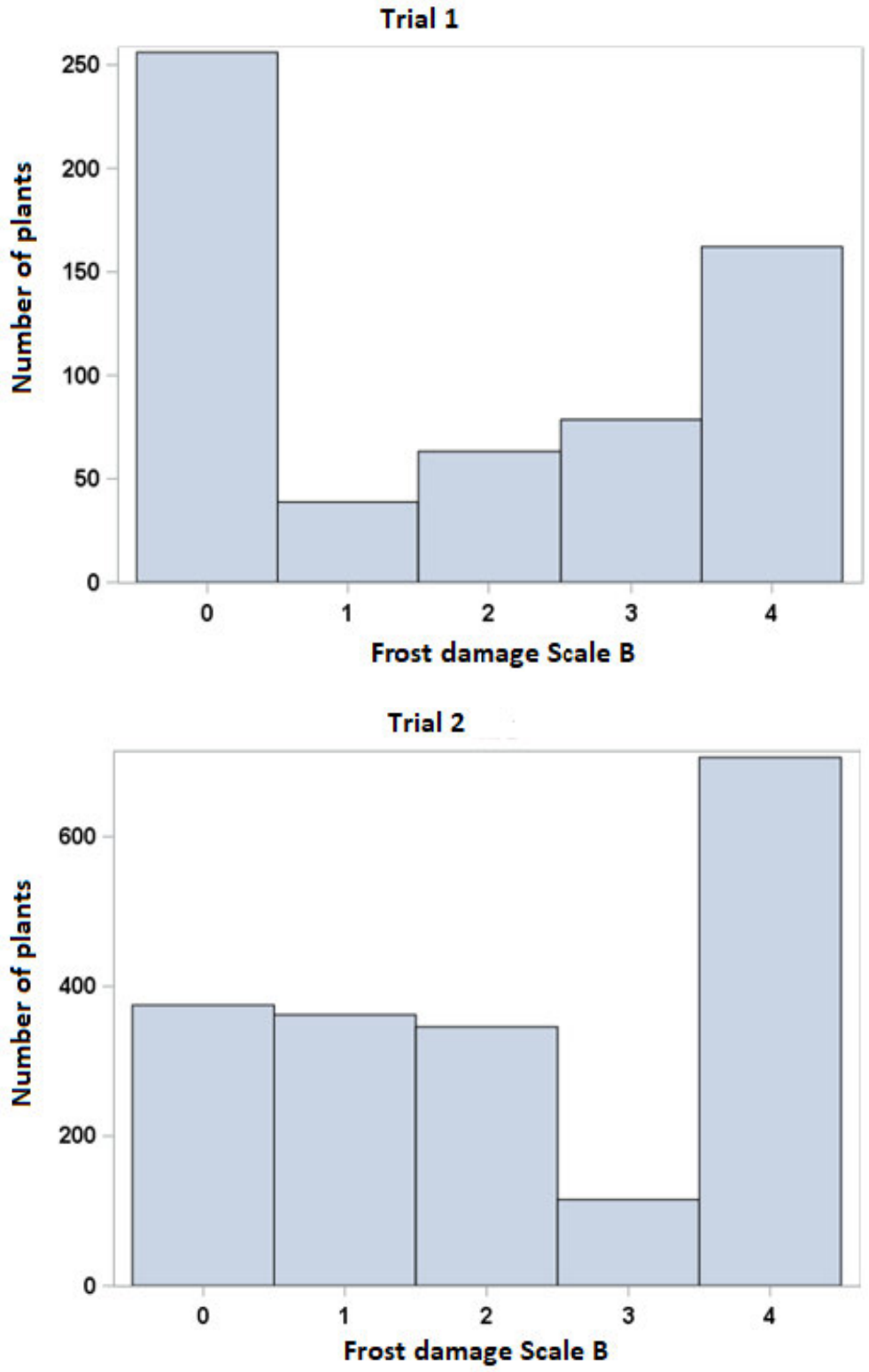

Figure 3.5. Score frequency of Frost damage Scale B. Data is shown separately for each artificial frost trial.

3.4.3. Comparison between artificial frost and field data and between frost damage scores

Correlation analysis between artificial frost and field data and between frost damage scales is shown in Table 3.4 and Figures 3.5 and 3.6. The scores from the two artificial frost screening trials were weakly correlated with the scores from the field data (r=0.24 to 0.28, r_s=0.20 to 0.28). In both trials, the frost damage scores showed similar weak correlations with field data. The weak agreement between artificial frost and field data did not seem to be influenced by the number of families used for the correlation analysis (35 families in Trial 1 compared to 82 families in Trial 2). On the other hand, there were strong correlations between frost damage scales across trials (r=0.90 to 0.91, r_s=0.87 to 0.93), regardless of the number of families tested.

Table 3.12: Pearson and Spearman correlation coefficients (r, r_s) with the probability of significance values in brackets. Comparisons were carried out between artificial frost and field data, between frost damage scales and between artificial frost trials. FSA = Frost damage Scale A, FSB = Frost damage Scale B. The suffixes T1 and T2 indicate trial number.

Trial	No. Families	Comparison	r	r_s
1	35	FSA vs. field	0.28 (0.110)	0.20 (0.243)
		FSB vs. field	0.28 (0.098)	0.21 (0.223)
		FSA vs. FSB	0.91(<0.001)	0.93 (<0.001)
2	82	FSA vs. field	0.25 (0.026)	0.28 (0.011)
		FSB vs. field	0.24 (0.028)	0.25 (0.026)
		FSA vs. FSB	0.90 (<0.001)	0.87 (<0.001)
1 vs. 2	30	FSA_T1 vs. FSA_T2	0.00	-0.06 (0.752)
		FSB_T1 vs. FSB_T2	-0.11 (0.578)	-0.16 (0.397)

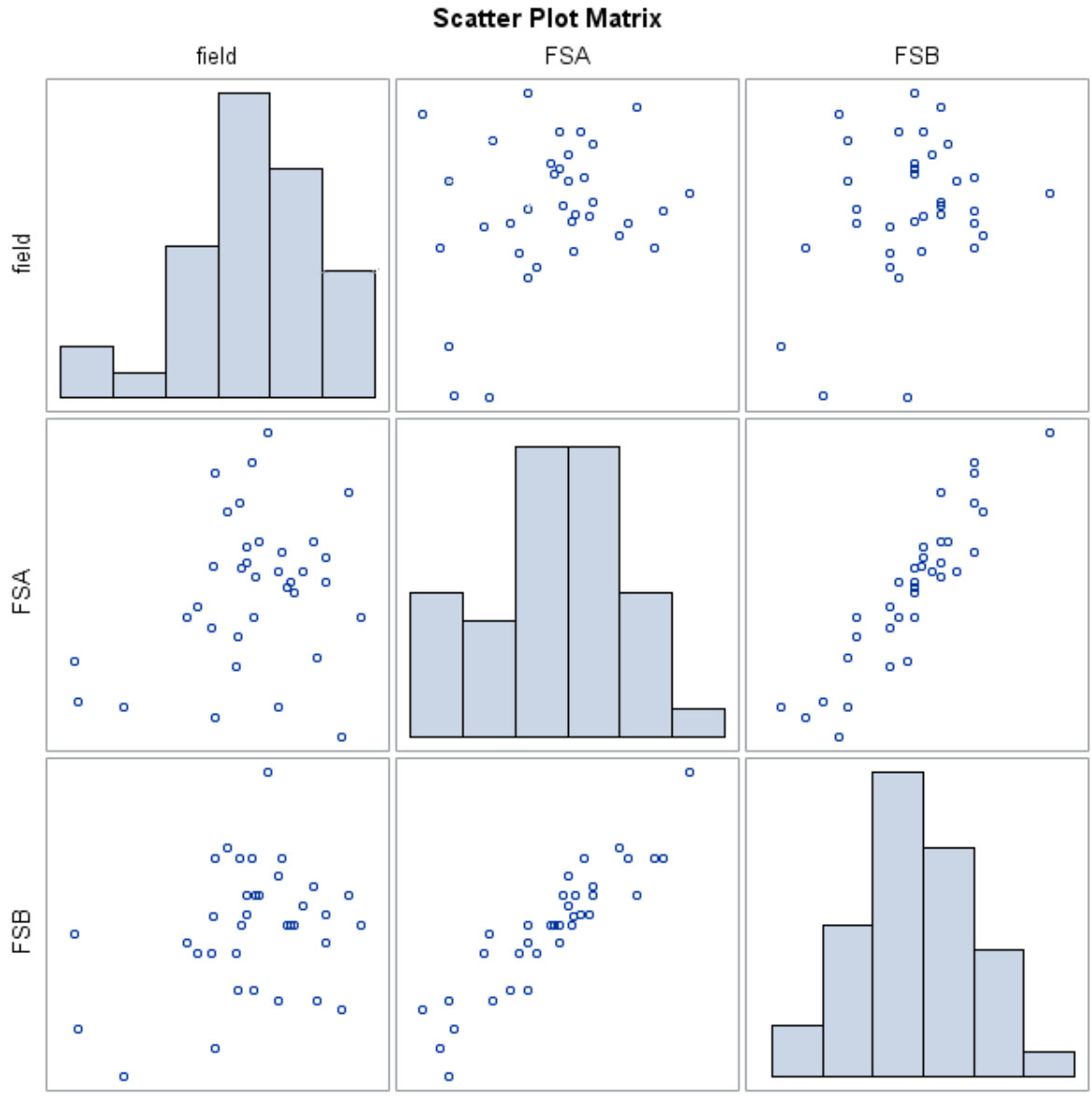

Figure 3.6. Comparison between artificial frost and field data and between frost damage scales for Trial 1. Points represent family means from 35 families tested both in the field and artificial frost. FSA = Frost damage Scale A, FSB = Frost damage Scale B, field = field data.

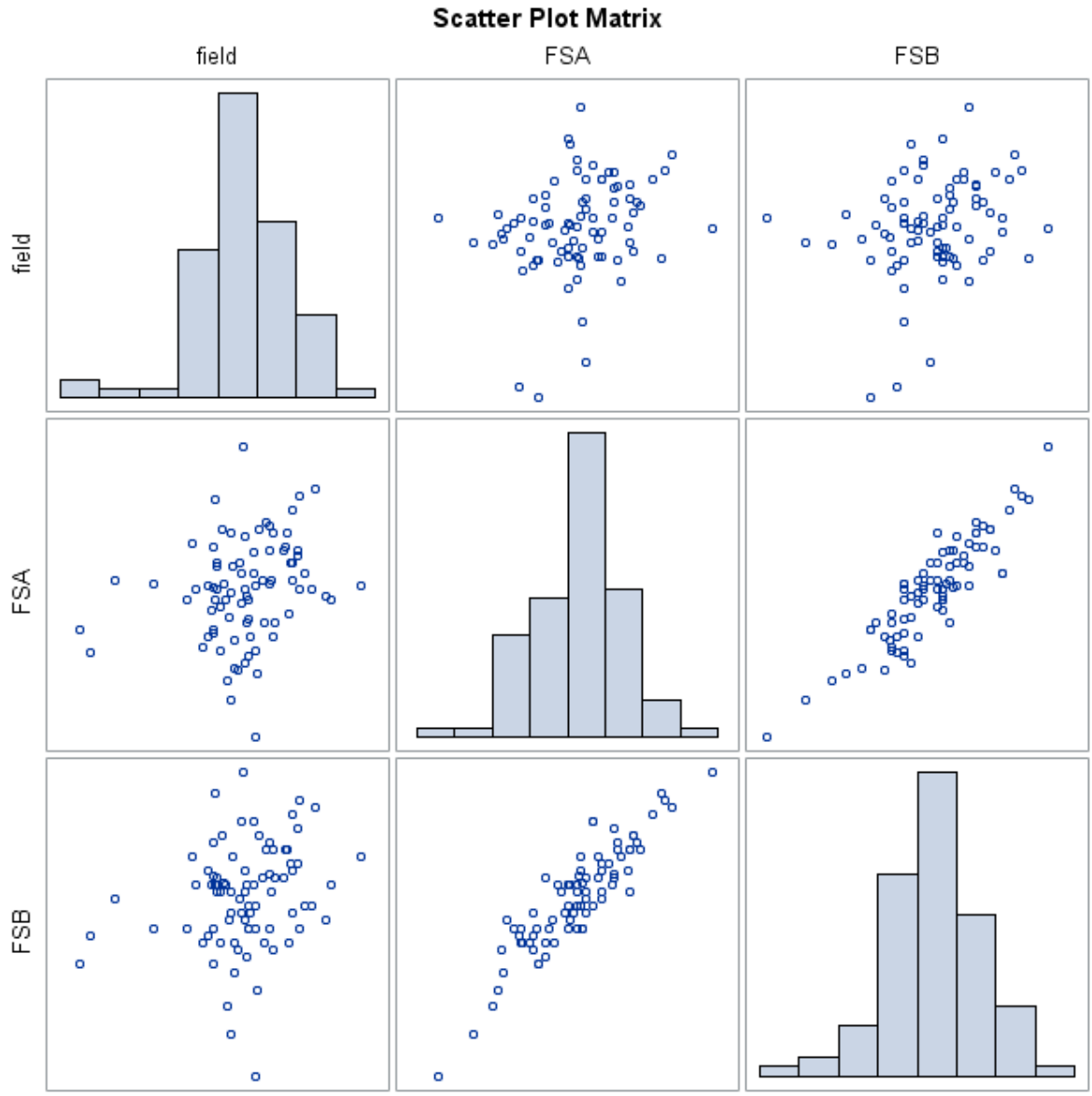

Figure 3.7. Comparison between artificial frost and field data and between frost damage scales for Trial 2. Points represent family means from 82 families tested both in the field and by artificial frost. FSA = Frost damage Scale A, FSB = Frost damage Scale B, field = field data.

In addition to the weak correlations between artificial frost and field data, both artificial frost trials did not identify frost tolerant and frost susceptible families that were identified in the field evaluations. For example, in the artificial frost Trial 1, six families showed very high frost tolerance. However, in the field data, out of the six families, three were labelled as truly frost hardy, two had intermediate frost tolerance and the other one was highly susceptible to frost (Figure 3.7). In Trial 1, all the families identified as frost susceptible in the field evaluations, showed average or high levels of frost tolerance when measured by artificial frost (Figure 3.7). Similar results were observed in the artificial frost Trial 2. Specifically, three families showed very high frost tolerance using artificial frost screening, whereas they had only average or less than average frost tolerance in the field evaluations (Figure 3.8). In Trial 2, the four frost hardiest families in the field evaluations, showed lower than average frost tolerance when measured by artificial frost (Figure 3.8). In particular, the Sher_9-2 family, which had been rated as having high frost hardiness in the field trial showed only average frost tolerance using artificial frost screening. Finally, several families identified as frost susceptible in the field evaluations, showed average tolerance when using artificial frost screening (Figure 3.8).

The black wattle families used in the current study performed differently when tested as young plants in a frost chamber compared to their performances as young seedlings exposed to natural frost in field trials. Given the good results obtained with a temperature schedule in Chapter 2, the current study produced unexpected results.

The large standard error bars in Figures 3.7 and 3.8 denoted significant variation in frost damage scores within families but they could also be created by assessment errors. Standard errors were larger in Trial 1 than in Trial 2,and were equally large for both frost damage Scales A and B. Frost score variation within families could have reduced precision in the assessments and would have contributed to the uneven results obtained.

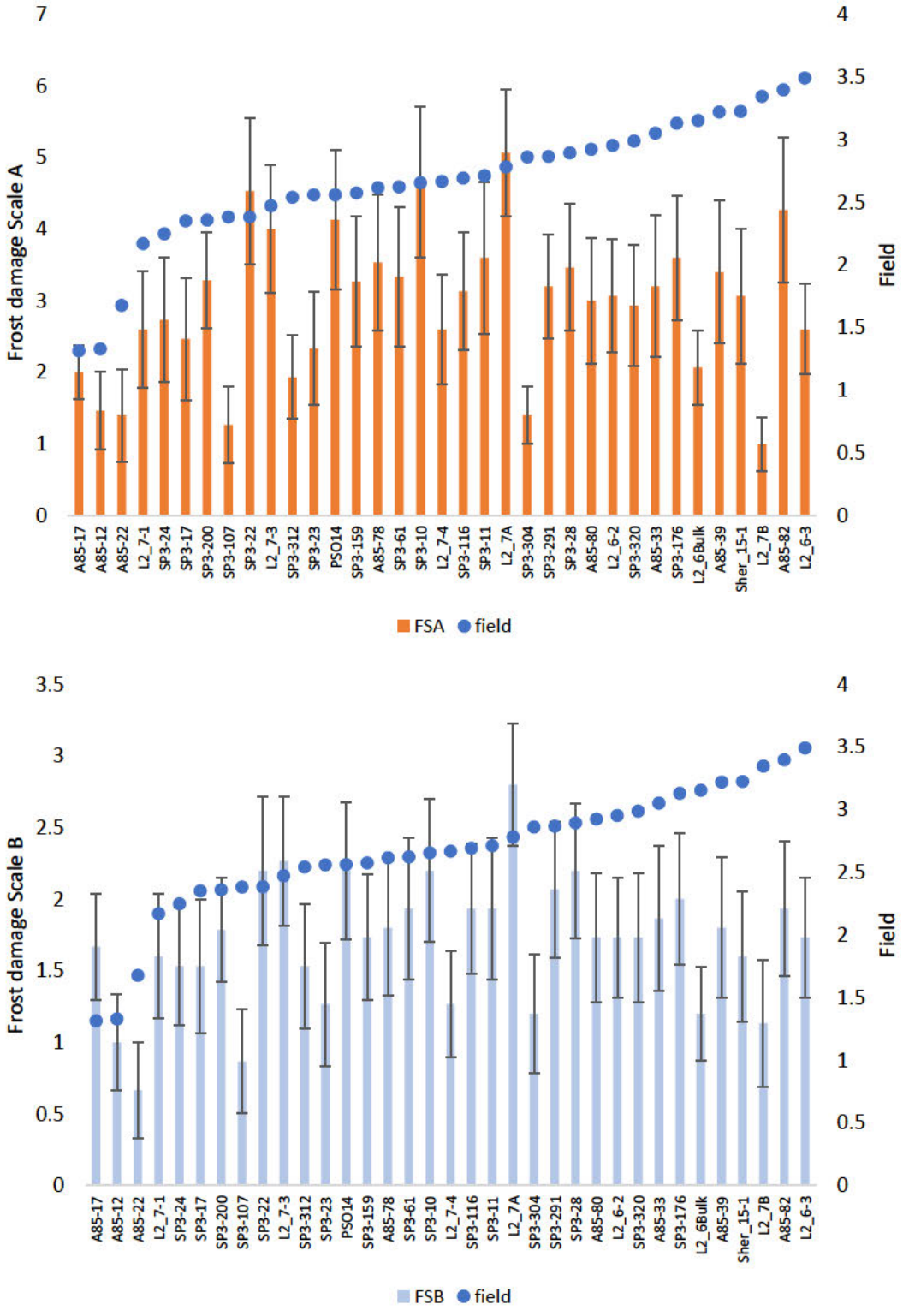

Figure 3.8. Trial 1 artificial frost family means with standard error bars for each of the two frost damage scales (FSA and FSB) compared to field data. Data includes 35 families tested both in the field and by artificial frost.

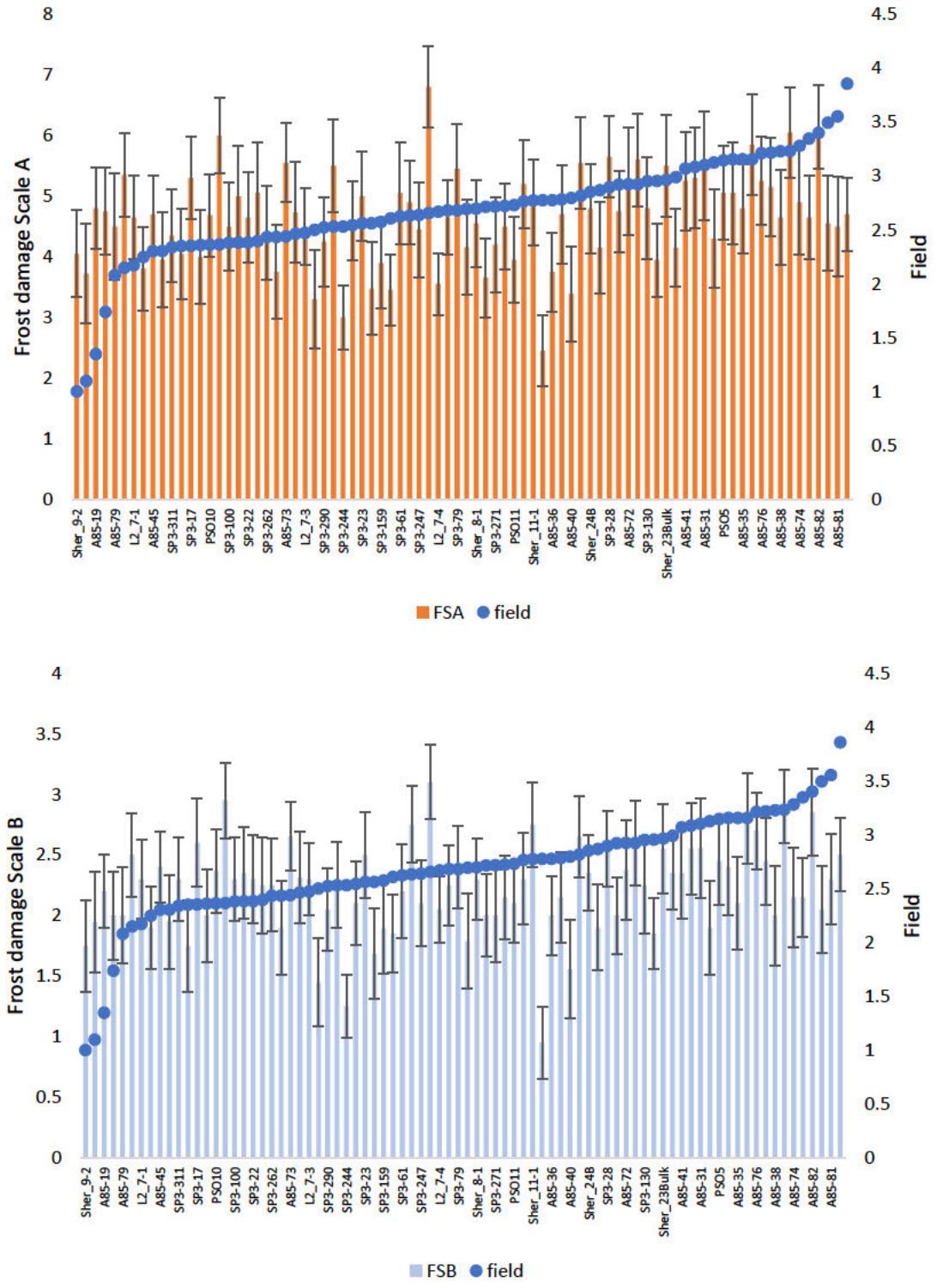

Figure 3.9. Trial 2 artificial frost family means with standard error bars for each of the two frost damage scales (FSA and FSB) compared to field data. Data includes 82 families tested both in the field and by artificial frost. Not all family IDs are shown.

3.4.4. Comparison between artificial frost trials

Unexpectedly, there were poor correlations between the artificial frost trials (r=0.00 to -0.11, r_s=-0.06 to -0.16). Frost damage Scale B showed slightly better correlations than frost Scale A, yet the agreement between trials was poor overall, indicating poor experiment repeatability (Table 3.4 and Figure 3.9).

The poor correlations between artificial frost trials were likely driven by the accidental difference in minimum temperature between trials (-5°C in Trial 1 vs. -6°C in Trial 2). Such a difference caused higher levels of frost damage in Trial 2 and caused significant differences in family performance across trials (Figure 3.10). Namely, several families that showed high frost tolerance in Trial 1, displayed either low or average frost tolerance in Trial 2 (Figure 3.10). Results suggested that small changes in minimum temperature can produce very different results when screening material.

Since both Frost damage Scales A and B were ordinal scales, the non-parametric, Kruskal-Wallis test was used to test the differences between: field and trial 1 scores; field and trial 2 scores; and trial 1 and trial 2 scores (Table 3.5). Frost damage Scale A was chosen for these analyses because it was the only scale used in the field frost damage assessments, furthermore, Scale A and B had a strong correlation (r=0.90 to 0.91, r_s=0.87 to 0.93) (Table 3.4). The medians for the field and trial 1 frost damage scores did not differ statistically (p=0.07), based on a 95% confidence interval. However, the medians of the field and trial 2 frost damage scores as well as those of trial 1 and 2 did differ significantly ($<2.2 \times 10^{-}16$ and 1.372×10^{-5} respectively).

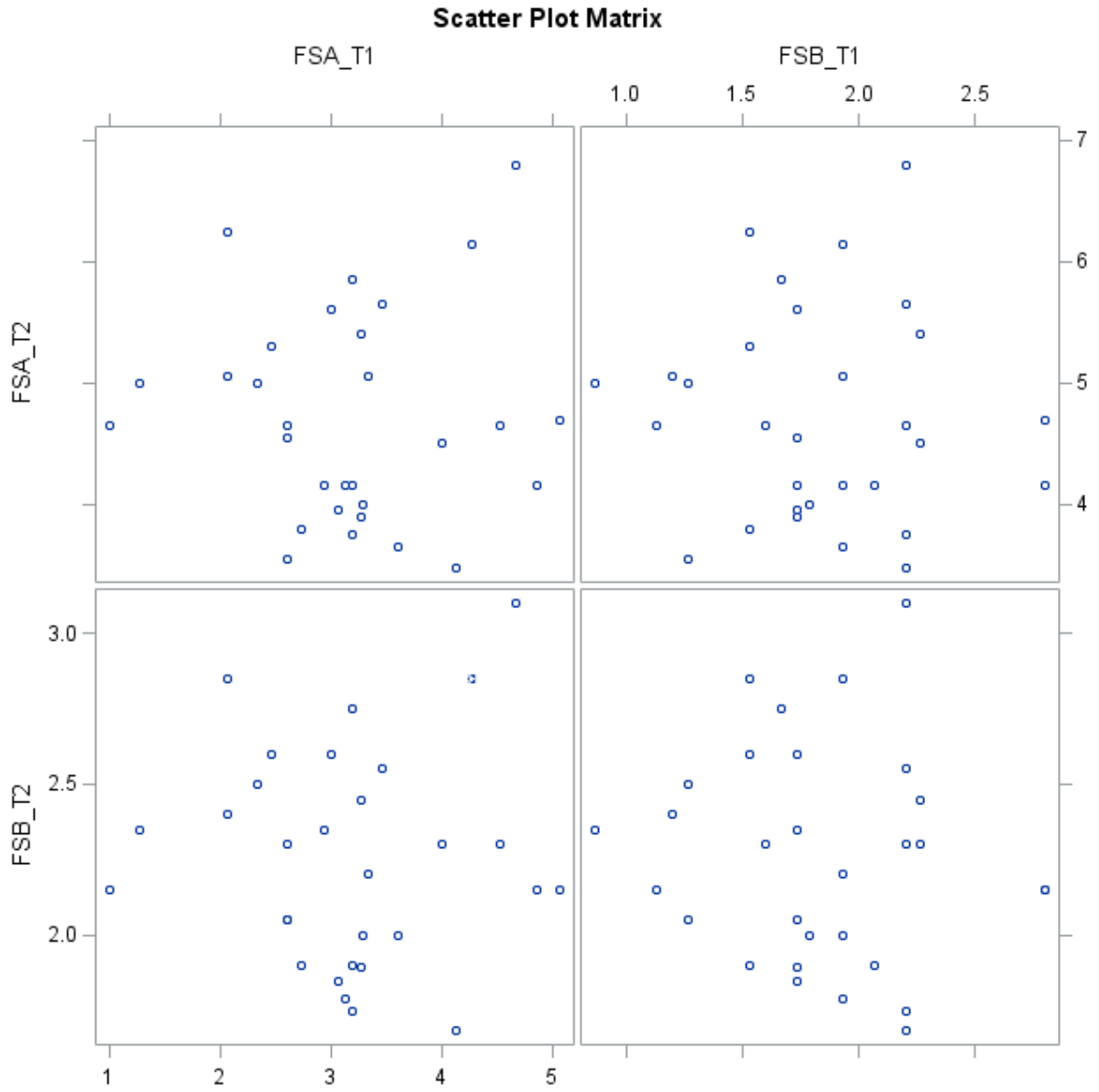

Figure 3.10. Comparison between artificial frost trials. Points represent family means from 30 families tested in both trials. FSA = Frost damage Scale A, FSB = Frost damage Scale B. The suffixes _T1 and _T2 indicate trial number.

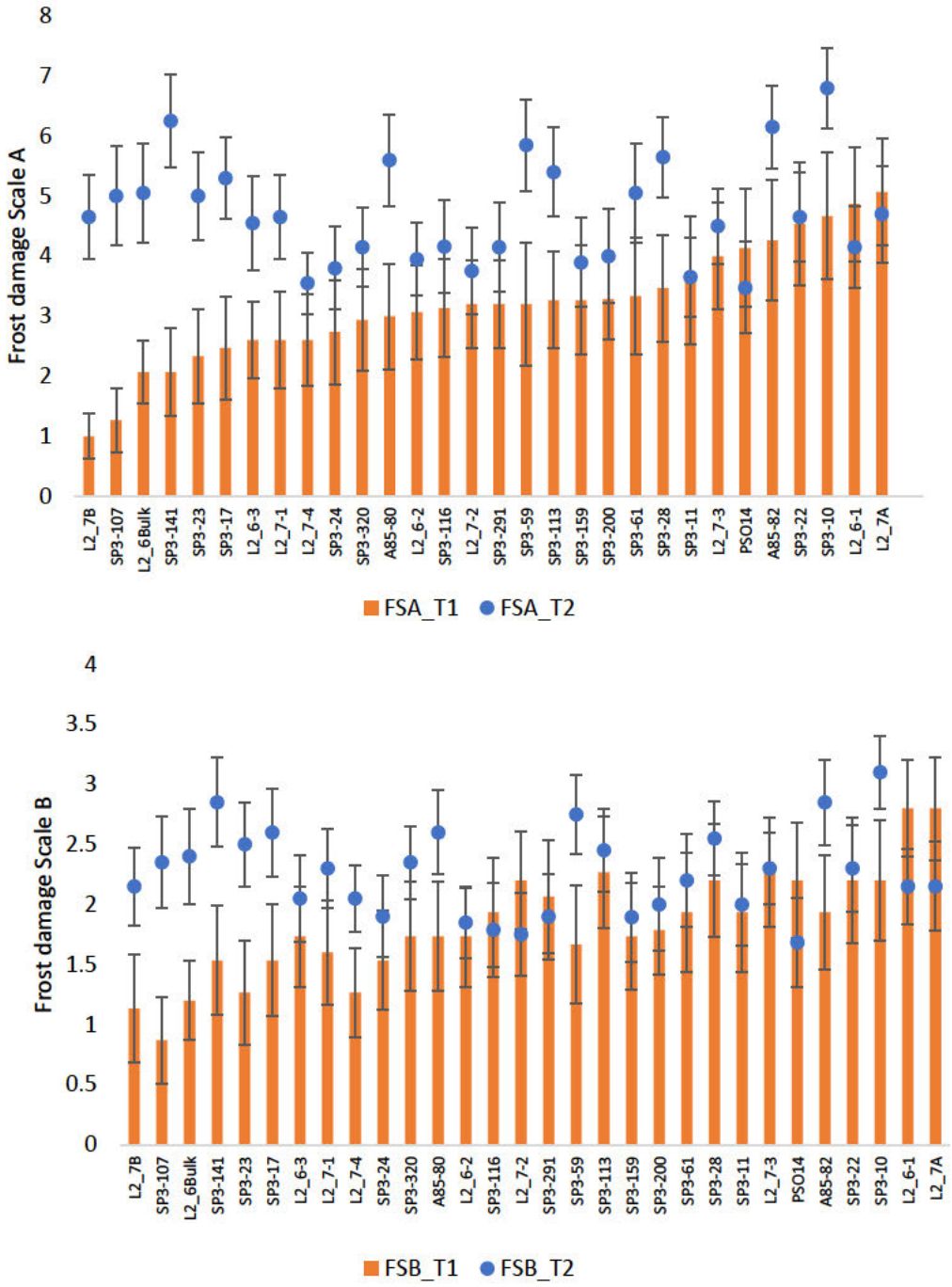

Figure 3.11. Family means and standard error bars of 30 families tested in two artificial frost trials. FSA= frost damage Scale A, FSB= frost damage Scale B. The suffixes _T1 and _T2 indicate trial number. Data shows higher frost damage in Trial 2 and differences in family frost tolerance across trials.

Table 3.5: Kruskal-Wallis analysis of field and artificial frost trials.

	Field vs Trial 1	Field vs Trial 2	Trial 1 vs Trial 2
Common families	35	82	30
H-statistic	3.29	77.98	18.90
DF	1	1	1
p-value	0.07	$<2.2 \times 10^{-16}$	1.372×10^{-5}

*$\alpha \leq 0.05$
*Frost damage Scale A scores used in analysis

3.5. Discussion

There was a general lack of agreement between artificial frost and field data based on the correlation analysis. The artificial frost trials did not distinguish between frost tolerant, and frost susceptible families as identified in the field evaluations. Seedlings in the field trials had longer exposures to "shortened day length" and low, non-frost temperatures, factors that trigger cold acclimation and enhance frost tolerance (Welling and Palva, 2006). This may have contributed to different levels of cold acclimation achieved by the seedlings in the field as compared to those in the artificial frost trials, hence the difference in the data between the two. This issue could be dealt with by increasing the acclimatization period of seedlings used in the artificial screening protocol.

Another factor that should be taken into consideration is desiccation stress, which would have been greater under the field conditions as compared to the artificial frost conditions. Incomplete desiccation and supercooling have shown to be the mechanisms that xylem ray parenchyma cells in boreal hardwoods use to survive sub frost temperatures (Kuroda *et al*., 2003). Therefore, the greater desiccation stress that is experienced by seedlings in the field trials may result in a greater level of frost tolerance as compared to those in the artificial frost chamber.

Another worrying result was the poor correlation between the two artificial frost trials as this denoted poor test repeatability. This may have been due to a difference in the number of seedlings tested in each trial due to their suitability at the time of testing. The larger number of seedlings that were used in Trial 2 may have affected the distribution of cold air in the frost chamber thus affecting the frost damage results. Interestingly, promising results were obtained with Schedule 4 in Chapter 2 when fewer seedlings (compared to Trial 1 and 2) were used.

The accidental difference in minimum temperature between the trials (-5°C in Trial 1 vs. -6°C in Trial 2) may have also been the reason for the poor correlation since the other temperatures

of the schedule were similar. This suggested that relatively small changes in minimum temperature can affect family performance and screening repeatability.

Interestingly, the results from the non-parametric, Kruskal-Wallis test showed that the medians from Trial 1 and the field evaluations were slightly similar, while that of Trial 2 vs the field evaluations and Trial 1 vs Trial 2 differed statistically. The possible reasons for the weak correlation between Trial 1 and Trial 2 (as discussed earlier) may be why there is a greater similarity between the medians of Trial 1 and the field evaluations as compared to Trial 2 vs the field evaluations. It might be possible that the lower number of seedlings used in Trial 1 (as compared to the greater number in Trial 2) might have created a similar spatial effect to the field conditions. Trial 2 showed possible signs of overcrowding and non-uniform distribution of cold air. This should be further investigated in future work.

It is necessary that the artificial frost protocol for black wattle should be improved in future trials. The first step would be to maintain the same temperature conditions between tests. Another related technical issue that needs attention is the differences observed between temperatures programmed and actual temperatures in the frost chamber. The minimum temperature programmed in the chamber for the two trials of the current study was -3°C while the actual minimum temperatures were -5°C and -6°C.

A simple solution to fix the artificial frost protocol is to work back empirically the temperatures used in Schedule 4 of Chapter 2 using 30 seedlots/families with varying levels of frost tolerance out of the families tested here. It is reasonable to expect that Schedule 4 will work provided the correct temperatures are used. What is needed in the end, is that the artificial frost protocol can distinguish between frost tolerant and frost susceptible material and that it can rank families/clones for frost tolerance. In other words the artificial frost simulating protocol should produce the same trend in discrimination with regards to frost tolerance susceptibility as the field of the same genotypes. The objective is not to produce frost damage scores similar to those obtained in field trials.

Regarding the frost damage scales used in the current study, results showed that both scales produced similar results despite the differences in the nature and scale of frost damage between scores. Individual-plant score distribution showed that scores 1-2 and 4-6 of Scale A did not occur because the symptoms associated with those scores were not observed, and thus Scale A must be reviewed for future artificial frost work. Intermediate frost damage scores/symptoms

were also less frequent in field trials with young black wattle seedlings using a similar frost score (Moreno Chan, 2019). Regarding frost Scale B, there were no gaps in the plant score distributions due to the simpler frost damage scale and symptoms of this score.

Finally, for future artificial frost studies, it is proposed to screen material in batches of a maximum of 500-600 plants at a time in order to minimise assessment errors caused by visual fatigue.

3.6. Conclusion

It would appear that the temperature schedules used in the current study were not appropriate and produced unexpected results. The most concerning result was that the artificial frost trials could not distinguish between frost tolerant and frost susceptible families identified in field evaluations. A second unexpected result was the poor agreement of the frost damage scores of the accessions tested in both artificial frost trials. The artificial frost protocol, therefore, needs to be improved to ensure consistency of outcomes before it can be used as a practical tool to screen families for frost tolerance. Once this issue is resolved, the use of artificial frost screening will accelerate the primary screening of families for frost tolerance, and this will reduce the number of families that need to be tested in field trials.

3.7. Literature references

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Chapter 4: Comparison of three protein extraction methods from *Acacia mearnsii* de Wild (black wattle) for the analysis of frost tolerance using two-dimensional electrophoresis

Jugmohan M, Moreno Chan J, Bairu MW and Laing MD

4.1 Abstract

Acacia mearnsii (de Wild) (black wattle) is an important forestry crop in South Africa as both its timber and bark are used locally and are also exported to foreign markets. The silviculture of this species has been plagued by problems such as pests, diseases and frost damage. In this study, three methods of protein extraction from black wattle were compared for the purposes of studying protein expression in response to frost conditions. The three methods involved the following precipitation agent combinations: acetone and methanol, phenol and ammonium acetate and ammonium acetate and methanol. The phenol and ammonium acetate precipitation method produced the highest protein yield as well as the most distinct protein spots after separation by two-dimensional gel electrophoresis. This method was chosen for subsequent black wattle proteomic studies involving two-dimensional gel electrophoresis.

4.2 Introduction

Acacia mearnsii de Wild, also known as black wattle is a quick growing, leguminous tree that is indigenous to Australia. It was introduced to South Africa in 1864 and has become one of the most important commercially grown forestry crops in the country (Beck *et al*., 2003). Black wattle bark is a well-known source of good quality tannin while its wood serves as the raw material for pulp production, wood chips and timber (Griffin *et al*., 2011). Frost damage is one of the major problems affecting the silviculture of black wattle in South Africa and has been the cause of great financial loss for the country's forestry sector (Moreno Chan, 2019). The development of improved seed for frost prone areas is an ongoing task for black wattle breeders. Thus, a deeper understanding of frost tolerance with the aim of developing a quick screening method for this trait would be most beneficial for black wattle growers.

The different aspects of frost tolerance have been researched over the years in order to gain a holistic understanding of this quantitative trait. These include studies that have been conducted at the physiological, cellular and molecular levels. The study of quantitative traits at the molecular level has been dominated by approaches such as quantitative trait loci mapping (Mackay *et al*., 2009). Although this approach has produced valuable information, it does not provide a complete understanding of the gene products, namely the proteins associated with quantitative traits. The study of proteins (proteomics) aims to bridge the gap between genotypic and phenotypic levels. This is especially important for quantitative traits which involve the accumulative action of many genes (Thelen and Peck, 2007). Improvements in plant protein extraction methods and resolution techniques such as two-dimensional gel electrophoresis has enabled the analysis of protein expression profiles (Jorrin-Novo *et al*., 2018). This is useful as protein expression changes in relation to stresses such as cold conditions. Proteins such as anti-freeze proteins, heat shock proteins and late embryogenesis abundant proteins have been observed to accumulate in relation to cold stress while conformational changes to membrane proteins also occur (Mishra *et al*., 2018; Guo *et al*., 2018; Ding *et al*., 2019). A comparison of protein expression profiles of differentially tolerant genotypes may serve as a method of identifying those important proteins involved in frost tolerance and may in turn be used as markers for identifying the level of frost tolerance in plant varieties of unknown genotypes (Kosová *et al*., 2014).

The extraction of protein from plant material is more difficult as compared to other organisms because plant tissues do not have a comparatively high protein content. Furthermore, they are also rich in proteases, lipids and phenolic compounds. In order to access plant protein, breaking of the cellular wall is also necessary (Patole and Bindschedler, 2019). The aim of this current study is to develop a protein extraction method for black wattle leaf proteome analysis for frost tolerance screening. For this purpose, three different protein extraction methods differing mostly in their precipitating agent combinations were compared. These combinations included acetone and methanol, phenol and ammonium acetate and ammonium acetate and methanol.

4.3 Materials and methods

4.3.1 Plant material and cold temperature stress treatment for the simulation of frost conditions

Black wattle seedlings were grown under a shaded netting (shade factor of 60%) structure at the Institute of Commercial Forestry Research (ICFR) based at the University of KwaZulu-

Natal (Pietermaritzburg). The seedlings were grown from the SP3 seedlots which were South African-grown F1 families from open-pollinated seed that was collected at a progeny trial at Liff, Karkloof, KwaZulu-Natal (South Africa) (Moreno Chan and Isik, 2021). Irrigation was supplied by an Aquarius-timer based system and implemented for 5 min, once a day. After six months of growth, half of the black wattle seedlings were placed in a low-temperature, controlled environment chamber and exposed to the following temperature schedule: 18^0C (days 1-3), 4^0C (nights 1-3); 12^0C (days 4-6), 2^0C (nights 4-6); 22^0C (day 7), -3^0C (night 7). The air temperature of the cold chamber was measured with Maxim iButtons DS1923L ® (Fairbridge Technologies, Johannesburg, South Africa). Light conditions were maintained at 550 µmol photons m^{-2} s^{-1} for a photoperiod of 13 hours a day and relative humidity was maintained at 80%. After night 7 the seedlings were removed and subjected to protein extraction.

4.3.2 Protein Extraction

Black wattle leaves were collected from seedlings that had undergone cold stress treatment (as described) and from those that were not exposed. Leaf tissue was ground with polyvinylpolypyrrolidone (PVPP) (0.05g/g leaf tissue) and liquid nitrogen using a pestle and mortar. The use of PVPP was important as it removes phenolic compounds which hinder protein extraction from plants and protein resolution methods such as two-dimensional gel electrophoresis (Charmont *et al*., 2005). The finely ground samples were stored at -80^0C prior to being subjected to one of the following protein extraction protocols:

4.3.2.1 Acetone and methanol precipitation

This protein extraction protocol was based on da Silva *et al*. (2010), with minor adjustments. Ground leaf material (with PVPP) (0.15 g) was placed into a plastic 2 ml tube and to this 1.25 ml of a solution containing 0.05 M Tris-HC, pH 8.8, 0.01 M dithiothreitol (DTT), 0.01M (PMSF), and 0.1% (w/v) SDS was added before vortexing for 20 min. The mixture was thereafter centrifuged at 16000 x g for 4 min at 4ºC to facilitate the removal of insoluble material. A precipitation solution (1.25 ml) containing acetone/methanol (3:1, v/v) was added and this mixture was stored at -20ºC overnight resulting in the formation of a pellet. The supernatant was thereafter removed following centrifugation at 16000 x g for 5 min. The protein pellet was left to air dry for 3 min and thereafter dissolved in 250 µl ReadyPrep 2D

starter kit rehydration buffer/sample buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte and 0.001% bromophenol blue) (Bio-Rad, Hercules, United States of America).

4.3.2.2 Phenol and ammonium acetate precipitation

This method of protein extraction was conducted according to Wang *et al*. (2006), with minor adjustments. Ground leaf material (with PVPP) (0.15 g) was transferred into a plastic 2 ml tube to which 1.25 ml of 10% trichloroacetic acid (TCA)/Acetone solution was added. The sample was vortexed and centrifuged at 16000 x g for 4 min at 4ºC. The supernatant was discarded and 1.25 ml of 0.1 M ammonium acetate in 80% methanol was added to the pellet. The sample was vortexed and centrifuged at 16000 x g for 4 min at 4ºC. The supernatant was removed and 1.25 ml of 80% acetone was added to the pellet. The sample was vortexed and centrifuged at 16000 x g for 4 min at 4ºC. The supernatant was removed, and the pellet was air dried for 20 min. Six hundred µl of 0.8 mL phenol (tris-buffered, pH 8.0) and 400 µl of dense sodium dodecyl sulfate (SDS) buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8, 5% 2-mercaptoethanol and 0.01 M phenylmethanesulfonylfluoride (PMSF)) was added to the pellet and vortexed. The mixture was left to incubate at room temperature for 15 min and thereafter centrifuged at 16000 x g for 4 min. The upper phenol layer was thereafter transferred to a new plastic 2 ml tube and 1.25 ml of 0.1 M ammonium acetate in 80% methanol was added. This mixture was then stored at -20ºC for two hours followed by centrifugation at 16000 x g for 5 min. The supernatant was discarded, and the resultant white pellet was washed once with 100% methanol followed by 80% acetone. Each wash step involved vortexing followed by centrifugation at 16000 x g for 4 min at 4ºC. The pellet was left to air dry for 3 min and thereafter dissolved in 250 µl Bio-Rad ReadyPrep 2D starter kit rehydration buffer/sample buffer.

4.3.2.3 Ammonium acetate and methanol precipitation

The third protein extraction was also based on da Silva *et al*. (2010) with a few modifications. A plastic tube containing ground leaf material (with PVPP) (0.15 g) and 1.25 ml of a solution containing 0.125 g Tris-HCl (pH 6.8), 1% (w/v) SDS, 10% (v/v) glycerol, 0.5 % (v/v) 2-mercaptoethanol and 0.01 M phenylmethanesulfonylfluoride (PMSF)) was mixed using a vortex for 20 min. Insoluble material contained in the mixture was removed after centrifugation at 16000 x g for 4 min at 4ºC. Thereafter 0.1 M ammonium acetate in cold methanol (1.25 ml) was added and the mixture was incubated over night at -20^{0}C. The mixture was then

centrifuged at 16000 x g for 4 min at 4ºC prior to the supernatant being removed. The pellet was then washed with a solution containing 0.2% (w/v) DTT dissolved in cold acetone and incubated at -20^{0}C for 1 hour. The supernatant was thereafter removed following centrifugation at 16000 x g for 4 min at 4ºC and the resultant pellet was dissolved in 250 µl Bio-Rad ReadyPrep 2D starter kit rehydration buffer/sample buffer.

4.3.3 Protein quantification

Protein concentration was determined using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad) with bovine serum albumin as the standard. Protein extraction was performed on black wattle leaf material three times per extraction method. Spectrometer readings were conducted in triplicate per extraction method. One-way ANOVA was used to determine whether there were any statistically significant differences between the means of the different extraction methods.

4.3.4 Electrophoresis

4.3.4.1 One dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method described by Laemmli (1970), with minor adjustments. A 3.75% stacking and 12.5% resolving gel system was used to separate black wattle proteins (20 µg) (from seedlings that were not exposed to the cold treatment) after denaturation at 95^{0}C for 2 min. This was conducted using a SE260 Mighty Small II Deluxe Mini Vertical SDS-PAGE gel system (Hoefer, Holliston, United States of America) at a constant current of 20 mA per gel. The gel was thereafter stained with Coomassie brilliant blue R-250 overnight. A protein ladder (P7703 -New England Biolabs) ranging in molecular weights from 10-250 kDa was used as a molecular weight marker.

4.3.4.2 Two-dimensional gel electrophoresis

Ninety micrograms of extracted protein were solubilized in 130 µl of Bio-Rad ReadyPrep 2D starter kit rehydration buffer/sample buffer and this was used to passively rehydrate 7 cm immobilized pH gradient (IPG) strips (Bio-Rad) with a linear pH gradient (4-7) for 13 hours. Isoelectric focusing (IEF) was performed at 20ºC using a Hoefer IEF100 isoelectric focusing unit as follows: 1) 0.1 W for 1 hour, 2) 0.5 W until 8000 Vh was reached, 3) 1000 V for 1 hour. After IEF, proteins were reduced with 2% DTT dissolved in an equilibration buffer (6 M urea,

2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl (pH 8.8); 20% glycerol) for 15 min and thereafter alkylated with 3.7% iodoacetamide dissolved in the equilibration buffer for 15 min. The IPG strips were thereafter rinsed in SDS-PAGE tank buffer (250 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) for 30 sec and transferred to a vertical SDS-PAGE gel system (Hoefer- SE260 Mighty Small II Deluxe Mini Vertical). After transfer, the IPG strips were sealed with Biorad ReadyPrep overlay agarose (0.5% low melting point agarose in 1 x Tris/glycine/SDS and 0.003% bromophenol blue). The agarose layer was allowed to solidify for 15 min prior to protein separation using a 12.5% polyacrylamide resolving gel as previously described. A precision plus protein unstained strep tagged standard plug (Bio-Rad) was used as a molecular weight marker (10-250kDa). Coomassie brilliant blue R-250 was used to stain the gel overnight.

4.3.5 Gel imaging and analysis

Stained gels were captured using the gel imaging system G:BOX EF2 (Syngene Ltd, Cambridge, United Kingdom) and analysed with PD Quest software (Bio-Rad). Triplicate gels were run for each of the protein extraction methods and for seedlings that were un/exposed to low temperature stress conditions. The gel image displaying the highest number of spots and the most distinct protein pattern was chosen for each condition.

4.4 Results

Three methods of protein extraction from black wattle leaf samples were compared in order to establish the most optimum method prior to protein resolution techniques such as two-dimensional gel electrophoresis. The three methods of extraction differed significantly at the precipitation step which involved one of the following combinations: acetone and methanol, phenol and ammonium acetate or ammonium acetate and methanol. The combination of phenol and ammonium acetate precipitation produced 1.4 and 1.2 times more protein than the methods involving the combinations of acetone and methanol and ammonium acetate and methanol respectively (Figure 4.1).

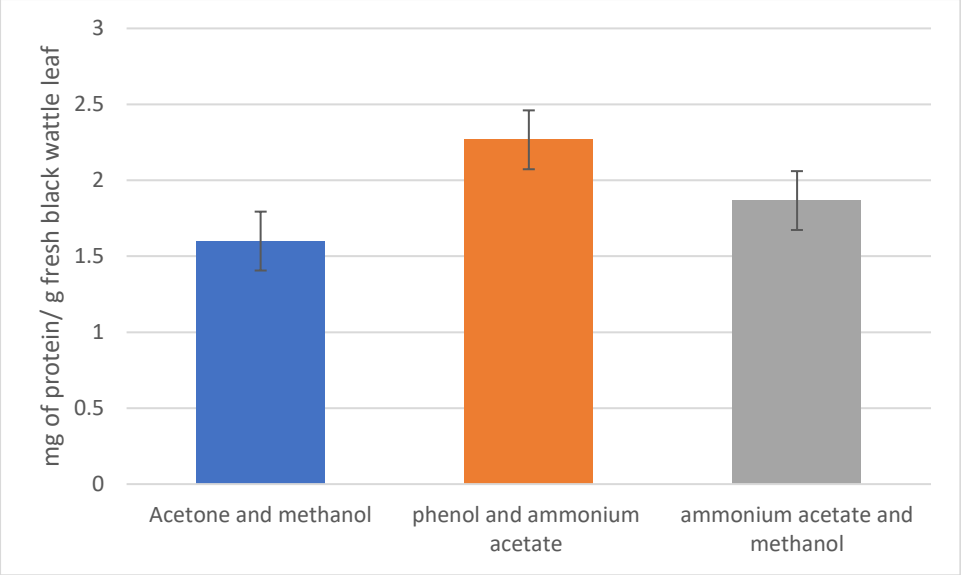


Figure 4.1. Protein yields obtained from three different methods of protein extraction from black wattle leaves. Three different methods of protein extraction from black wattle leaves were conducted and the resultant protein yields were quantified using the Bradford protein assay. The three different protein extraction methods involved the following protein precipitation agent combinations: acetone and methanol; phenol and ammonium acetate; ammonium acetate and methanol. Average protein yield values were obtained after each method was conducted three times.

The resolution of proteins by one dimensional SDS-PAGE (Figure 4.2) did not show a significant difference ($p > 0.05$) among the different protein extraction methods (Lanes A, B and C). The protein bands were separated in a similar way with very little difference in the densities of the corresponding bands.

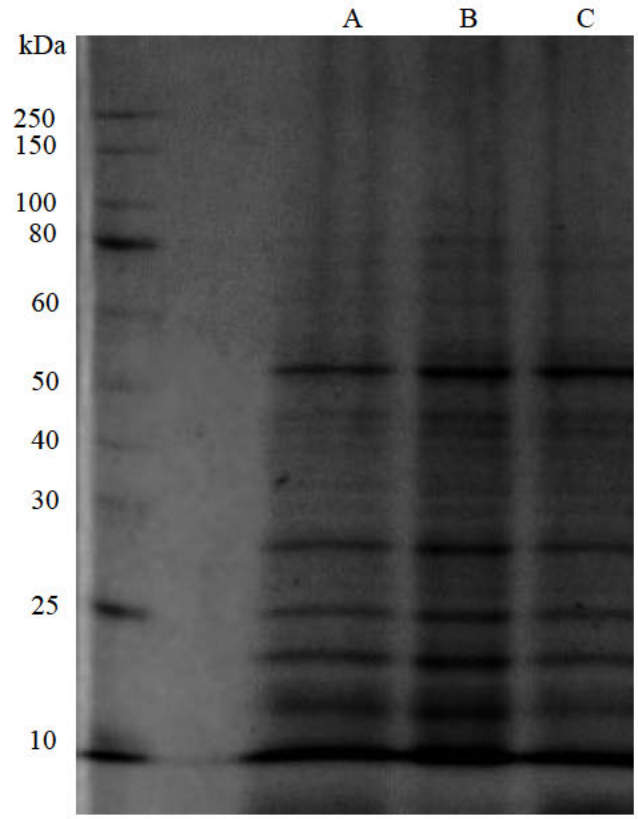
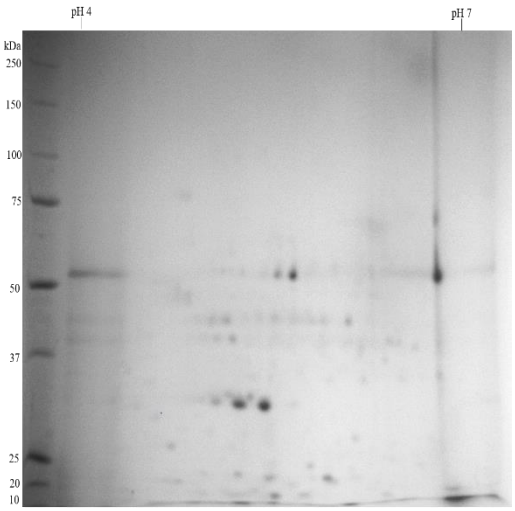


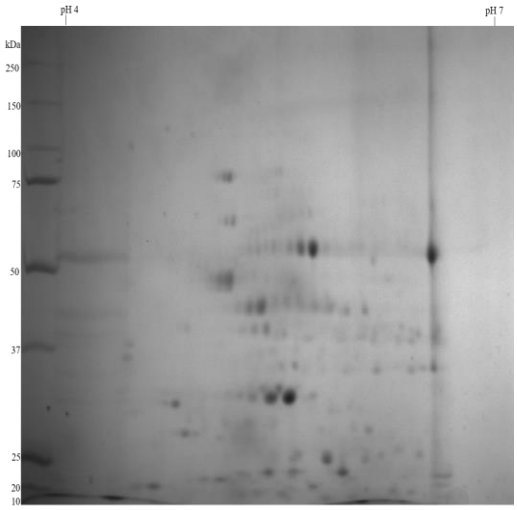
Figure 4.2. SDS-PAGE separation of black wattle leaf proteins extracted by three different methods. Black wattle leaf protein (20 µg) was extracted using three different protocols and resolved using a 12.5% polyacrylamide gel. Lane A- acetone and methanol protein precipitation, Lane B- phenol and ammonium acetate protein precipitation and Lane C- ammonium acetate and methanol protein precipitation. Protein molecular mass standards with sizes (in kDa) are indicated on the left. Coomassie brilliant blue R-250 staining was used for visualization of the protein bands.

Two-dimensional gel electrophoresis was conducted on the proteins that were extracted from black wattle seedlings using three different extraction methods (Figure 4.3). Proteins that were extracted from black wattle seedlings that were not exposed to cold treatment were observed in Gel A1, B1 and C1. Gel A2, B2 and C2 show those proteins that were extracted and resolved from black wattle seedlings that were exposed to the cold treatment regime. Resolved proteins that were extracted using: acetone and methanol were observed in Gel A1 and A2; phenol and ammonium acetate in Gel B1 and B2; ammonium acetate and methanol in Gel C1 and C2.

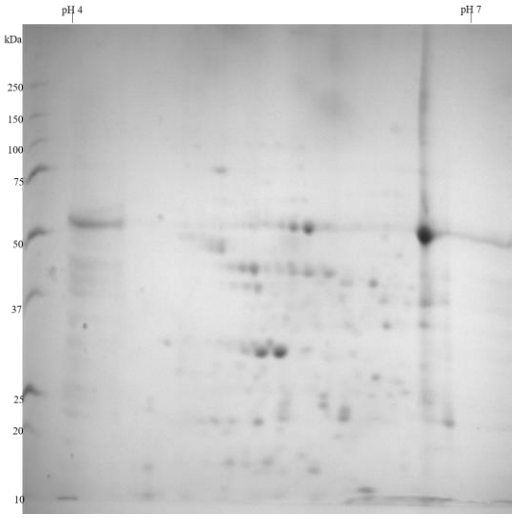
Gel A1



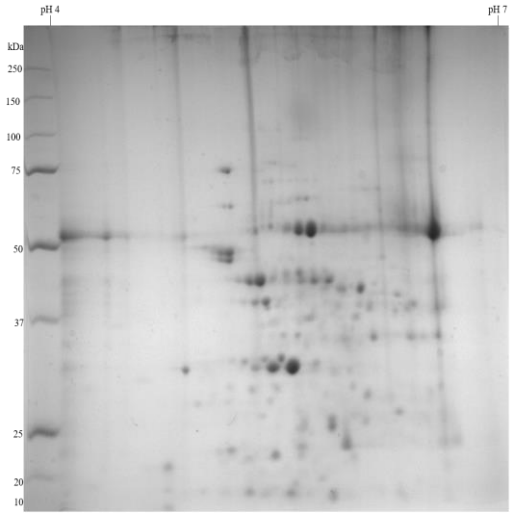
Gel A2



Gel B1



Gel B2



Gel C1 **Gel C2**

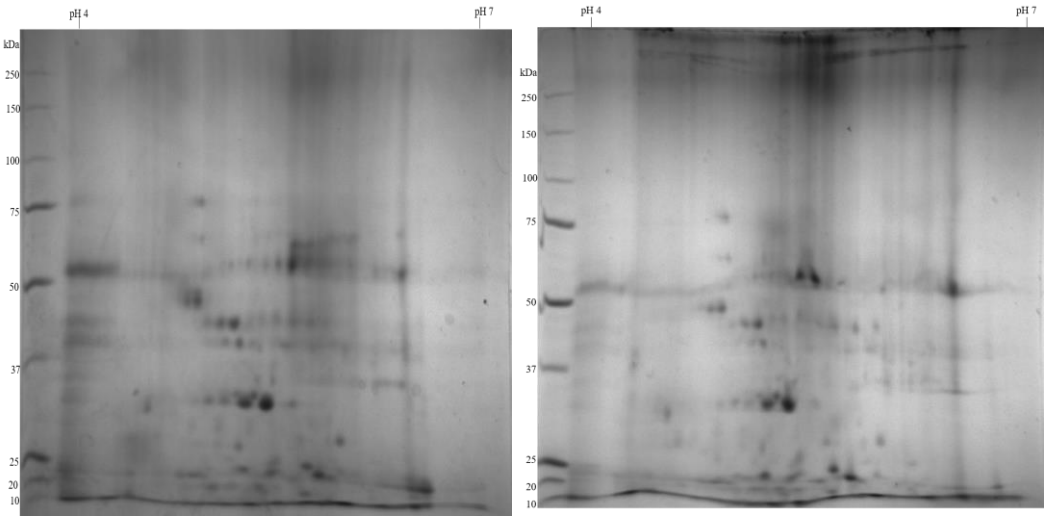


Figure 4.3. Comparison of two-dimensional electrophoresis patterns of black wattle leaf proteins extracted by three different protocols prior to and after cold stress treatment.
Proteins were extracted from black wattle seedlings that were not exposed to cold treatment (A1, B1 and C1) and seedlings that were exposed to cold treatment (A2, B2 and C2) using three different protocols and resolved by two-dimensional gel electrophoresis. Each protocol involved different protein precipitation agent combinations: acetone and methanol (A1 and A2), phenol and ammonium acetate (B1 and B2) ammonium acetate and methanol (C1 and C2). Gels were stained with Coomassie brilliant blue R-250. Protein molecular mass standards with sizes (in kDa) are indicated on the left and pH gradients at the top of the gels.

Image analysis of the two-dimensional gels showed that the phenol and ammonium acetate precipitation method resulted in more protein spots (Figure 4.3, Gel B1 and B2 and Table 4.1) and overall, a more distinct protein spot pattern as compared to the other extraction methods.

Table 4.1: A comparison of two-dimensional gel spot numbers after protein extraction from black wattle using three different extraction methods

Precipitating agent combinations	[a]Number of spots detected	
	Cold treated seedlings	Untreated seedlings
Acetone and methanol	44	28
Phenol and ammonium acetate	71	59
Ammonium acetate and methanol	40	36

[a] The number of spots detected in two-dimensional electrophoresis gels that were used to resolve proteins that were extracted from black wattle seedlings that were un/exposed to cold treatment.

4.5 Discussion

This study focussed on optimizing a protein extraction method for black wattle seedlings that would be compatible with gel electrophoresis techniques so that changes in protein expression could be analysed. Three protein extraction methods differing in their precipitation agent combinations were analysed. All three extraction methods resulted in the extraction of major leaf proteins such as ribulose-1,5-bisphosphate carboxylase (Rubisco-large subunit) at approximately 55 kDa as observed in SDS-PAGE (Wang *et al*., 2003). However, the precipitating agent combination of phenol and ammonium acetate was observed to produce the greatest number of protein spots as well as the most distinct protein spot pattern as observed in the two-dimensional gel electrophoresis images. This may be due to the ability of phenol to dissolve both cytosolic and membrane proteins (Wang *et al*., 2003). Furthermore, protein extraction methods involving phenol and TCA/ acetone have been observed to minimize protein degradation while at the same time removing contaminants such as pigments, salts, phenolics and DNA (Patole and Bindschedler, 2019). Contaminants such as proteases, nucleic acids, lipids and salts are known to hinder the separation and staining of proteins during two-dimensional gel electrophoresis (Kumar *et al*., 2017). These may have been less present in the phenol and ammonium acetate precipitation method as compared to the other methods. This may be due to phenol being a strong dissociating agent which decreases molecular interactions between proteins and other materials (Carpentier *et al*., 2005). Although the phenol and ammonium acetate precipitation method is quite time consuming, it may be completed in one day unlike the other two methods described in this study which require overnight precipitation. It is also important to note that due to the toxicity of phenol safety precautions should be taken

during its use and proper disposal protocols should be adhered to after protein extraction is completed.

4.6 Conclusion

Plant protein extraction is challenging due to the complexity and variation of plant tissues. There is no singular protein extraction method that can ensure the extraction of every single protein from a plant species. In this study, the protein extraction method for black wattle involving phenol and ammonium acetate precipitation resulted in better protein spot patterns after resolution by two-dimensional gel electrophoresis as compared to the other extraction methods that were investigated. New and improved methods of protein extraction from plants are developed on a continuous basis and should be tested on black wattle in future studies to improve knowledge of its proteomic profile.

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Chapter 5: Changes in protein expression in *Acacia mearnsii* de Wild (black wattle) as a result of exposure to frost-inducing temperatures

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5.1 Abstract

Acacia mearnsii de Wild (black wattle) is one of the most important forestry crops in South Africa. The bark of this species contains high-quality tannins, while its wood is used for wood chips, charcoal and mining timber. One of the main problems faced by black wattle farmers is frost damage, thus there is an urgent need to develop frost-tolerant black wattle varieties. A quick method for the screening of frost tolerance variation would be beneficial for black wattle breeders and would result in the need for fewer families to undergo field trials. Frost tolerance in plants is directly related to their ability to undergo cold acclimation in response to changing weather conditions. During the cold acclimation process, many physiological changes occur, including modifications in protein expression. In this study, two-dimensional gel electrophoresis was used to investigate changes in protein expression among 40 black wattle families with varying levels of frost tolerance before and after a cold-stress treatment. The proteomic data generated from this was analysed using multivariate analysis. Six proteins were observed to be upregulated in frost-tolerant black wattle families. The proteins were identified and have been previously shown to contribute to the protection of cellular membranes, maintenance of photosynthetic processes and the prevention of protein misfolding and aggregation.

5.2 Introduction

Acacia mearnsii de Wild, commonly known as black wattle, is indigenous to Australia and was first introduced to South Africa in 1864 (Sherry,1971). Black wattle is grown for its bark because of its high tannin content, and its wood that is used for a variety of purposes such as mining timber and pulp production (Midgley and Turnbull, 2003; Moreno Chan *et al*., 2015).

One of the main problems faced by black wattle breeders is frost damage, which causes regular financial losses for wattle growers in the frost-prone regions of South Africa. Research conducted in this area has focussed primarily on the improvement of wattle varieties through conventional tree breeding using field screening to select for frost tolerance. The molecular aspect of this problem has not been well researched. This study aimed to identify protein molecular markers that can be used to identify frost-tolerant black wattle varieties.

Plants adapt to cold/frost-inducing temperatures through a process known as cold acclimation. This process encompasses all the changes that take place at low positive temperatures that allow plants to survive below 0^0C (Thomashow, 1999). One of the key aspects of cold acclimation is the prevention of damage induced by cellular dehydration that results from ice formation. In addition, other cellular activities such as osmotic stabilization, changes to lipid membrane composition, induction of antioxidant defence mechanisms, changes to photosynthetic processes, and the accumulation of cryoprotective molecules such as sugars and proline also take place. It has also been well established that many cold-related genes are also induced. In studies involving *Arabidopsis thaliana* (L.) Heynh plants, more than one thousand genes have been reported to be cold-induced for up- or down-regulation (Thomashow, 2010). These include key regulators such as the transcriptional cascade ICE1-CBF (Chinnusamy *et al*., 2007).

Defence against abiotic stresses such as extreme heat, cold, drought and salinity are complex processes that involve a combination of appropriate gene expression, post-transcriptional regulation, expression of corresponding proteins and the regulation of various metabolites. It is necessary to have a holistic understanding of all these processes in order to breed for crop varieties that have high abiotic stress resistance, thus many omics-based techniques have been developed for this purpose. These include genomics (Shen *et al*., 2019), transcriptomics (Iyer *et al*., 2013), proteomics (Liu *et al*., 2015) metabolomics (Khan *et al*., 2019), lipidomics (Zhang *et al*., 2019), ionomics (Huang and Salt, 2016), miRNAomics (Song *et al*., 2017), interactomics (Shah *et al*., 2018), secretomics (Krause *et al*., 2013), phosphoproteomics (Zhang *et al*., 2014) and phenomics (Schnaubelt *et al*., 2013). The two most used omic-technologies for cold resistance investigations are proteomics and transcriptomics because both techniques assess the level of expressed proteins. Proteomics does this directly and normally involves techniques such as two-dimensional gel electrophoresis and different types of mass spectrometry (Chun *et al*., 2020). Transcriptomics analyses mRNA levels using techniques such as RNA- sequencing,

microarray platforms and digital gene expression profiling (Mehta *et al*., 2019). Several studies have reported a poor correlation between protein and mRNA expression levels (Hack, 2004; Soda *et al*., 2015; Xu *et al*., 2021). The reasons for this include alternative mRNA splicing, post-translational modifications, proteolytic processing activities and rapid mRNA decay in response to different stimuli (Hegde *et al*., 2003). Some modern RNA-sequencing techniques can identify sequence variations in transcripts that result from alternative splicing and other post transcriptional changes (Li *et al*., 2021; Liu *et al*., 2022). These techniques are generally not time-consuming and work well for large scale analyses because of the chemical nature of nucleotides (Mehmood *et al*., 2021). Even with these advancements, proteomics data currently correlates better with biological phenotypes as compared to mRNA data. This is because proteomics analyses protein production and degradation as well as identification and quantification of post-translational modifications such as glycosylation and phosphorylation that may be necessary for their functions, transport and activation (Rose *et al*., 2004; Zubarev, 2013). Furthermore, transcriptomics does not provide information on protein subcellular localization (Renaut *et al*., 2006). Large amounts of transcriptome data are much more easily obtained as compared to proteomic data, however, protein molecules are more relatable at the functional cellular level. Proteomics bridges the gaps between the genomic, transcriptomic and phenotypic levels (Renaut *et al*., 2006).

Protein expression is dynamic and changes when plants are exposed to stressful conditions. Several studies have reported different groups of proteins that are associated with cold-stress responses. These include proteins involved in signalling, translation, host-defence mechanisms, and carbohydrate and amino acid metabolism. Examples of such proteins include anti-freeze proteins, pathogenesis-related proteins, heat shock proteins, dehydrins and cold-regulated (COR) proteins (Gusta and Wisniewski, 2013; Miura and Furumoto, 2013; and Awasthi *et al*., 2015). In this study, protein expression profiles of black wattle families with varying levels of frost tolerance were analysed to determine if those with higher levels of frost tolerance expressed specific proteins differently to those families with lower levels of frost tolerance after exposure to cold treatment. In families that had higher levels of frost tolerance, six specific proteins were observed to be upregulated after cold treatment. These proteins were identified, and their functions have been previously linked to the protection of cellular membranes, maintenance of photosynthetic processes and the prevention of protein misfolding and aggregation.

5.3 Materials and methods

5.3.1 Plant material and temperature stress treatment

Black wattle seedlings belonging to 40 different families (Appendix 5.1) were sown into a composted pine bark medium in Unigro® 90 cm^3 tubes and grown under a black net-shaded tunnel (shade factor of 60%) at the Institute of Commercial Forestry Research (ICFR), University of KwaZulu-Natal (Pietermaritzburg, South Africa). The seedlings consisted of open pollinated progeny of maternal parent trees (half-sib families). Irrigation was controlled by an Aquarius-timer based system and applied for 5 min, once a day. After six months of growth, 13 randomly selected seedlings from each of the 40 different families were placed in a low-temperature, controlled environment chamber and exposed to a previously established frost-inducing temperature schedule (Chapter 3, Table 3.2). The air temperature of the cold chamber was measured with Maxim iButtons DS1923L ® (Fairbridge Technologies, Johannesburg, South Africa). Light conditions were maintained at 550 µmol photons m^{-2} s^{-1} for a photoperiod of 13 hours a day and relative humidity was maintained at 80%. At the end of the frost-inducing temperature schedule, the seedlings were removed and subjected to protein extraction.

5.3.2 Protein extraction

Black wattle leaves were collected from seedlings that had undergone the above cold-stress treatment and from those that had not been exposed (control). The leaves of all the seedlings from each family were collated so that there would be enough leaf material per family, per treatment for protein extraction. Protein extraction was performed three times per family for both cold treated and untreated samples. Leaf tissue was ground with polyvinylpolypyrrolidone (PVPP) (0.05g/g leaf tissue) and liquid nitrogen using a pestle and mortar. The finely ground samples were stored at -80^{0}C before protein extraction.

Protein extraction was conducted according to the method of Wang *et al*. (2006), with minor adjustments. Finely crushed leaf material (0.15 g) was transferred into a plastic 2 ml tube to which 1.25 ml of a 10% trichloroacetic acid (TCA)/acetone solution was added. The sample was vortexed and centrifuged at 16000 x g for 4 min at 4ºC. The supernatant was discarded and 1.25 ml of 0.1 M ammonium acetate in 80% methanol was added to the pellet. The sample was vortexed and centrifuged at 16000 x g for 4 min at 4ºC. The supernatant was removed and 1.25 ml of 80% acetone was added to the pellet. The sample was vortexed and centrifuged at 16000

x g for 4 min at 4ºC. The supernatant was removed, and the pellet was air-dried for 20 min. Six hundred µl of 0.8 ml phenol (tris-buffered, pH 8.0) and 400 µl of dense SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-HCl, pH 8, 5% 2-mercaptoethanol and 0.01 M PMSF) was added to the pellet and vortexed. The mixture was left to incubate at room temperature for 15 min and thereafter centrifuged at 16000 x g for 4 min. The upper phenol layer was thereafter transferred to a new plastic 2 ml tube and 1.25 ml of 0.1 M ammonium acetate in 80% methanol was added. This mixture was then stored at -20ºC for two hours followed by centrifugation at 16000 x g for 5 min. The supernatant was discarded, and the resultant white pellet was washed once with 100% methanol followed by 80% acetone. Each wash step involved vortexing followed by centrifugation at 16000 x g for 4 min at 4ºC. The pellet was left to air dry for 3 min and thereafter dissolved in 250 µl of ReadyPrep 2D starter kit rehydration buffer/sample buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte and 0.001% bromophenol blue) (Bio-Rad, Hercules, United States of America).

5.3.3 Two-dimensional electrophoresis

Before 2D electrophoresis was performed, the total protein concentration was determined using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad) with bovine serum albumin as the standard. Ninety micrograms of extracted protein were solubilized in 130 µl of ReadyPrep 2D (Bio-Rad) starter kit rehydration buffer/sample buffer, and this was used to passively rehydrate 7 cm immobilized pH gradient (IPG) strips (Bio-Rad) with a linear pH gradient (4-7) for 13 hours. Isoelectric focusing (IEF) was performed at 20ºC using an IEF100 isoelectric focusing unit (Hoefer, Holliston, United States of America) as follows: 1) 0.1 W for 1 hour; 2) 0.5 W until 8000 Vh was reached; 3) 1000 V for 1 hour. After IEF, proteins were reduced with 2% dithiothreitol (DTT) dissolved in an equilibration buffer (6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl (pH 8.8), 20% glycerol) for 15 min and thereafter alkylated with 3.7% iodoacetamide (IAA) dissolved in the equilibration buffer for 15 min. The IPG strips were thereafter rinsed in SDS-PAGE tank buffer (250 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) for 30 sec and transferred to a vertical SDS-PAGE gel system, SE260 Mighty Small II Deluxe Mini Vertical, (Hoefer). After transfer, the IPG strips were sealed with ReadyPrep overlay agarose (0.5% low melting point agarose in 1 x Tris/glycine/SDS and 0.003% bromophenol blue) (Bio-Rad). The agarose layer was allowed to solidify for 15 min prior to protein separation using a 12.5% polyacrylamide resolving gel. A Precision Plus

Protein™ unstained, strep-tagged standard plug (Bio-Rad) was used as a molecular weight marker. Coomassie brilliant blue R-250 was used to stain the gel overnight.

5.3.4 Gel imaging and analysis

Stained gels were captured using the gel imaging system G:BOX EF2 (Syngene Ltd, Cambridge, United Kingdom) and analysed with PD Quest software, version 8.0.1 (Bio-Rad). Triplicate gels were run for each black wattle family, for seedlings that were cold-stressed and those that were not. The gel image displaying the highest number of spots and the most distinct protein pattern was used as a reference template (cold treated material). Seventy-one distinct protein spots were detected in this gel and were numbered 1-71. Spots in the other gels were then subjected to automated spot detection and matching followed by manual verification (using their pI and molecular weight) compared to the reference template. The optical densities of the protein spots were normalized in each gel relative to the total density of all the spots in the reference template, according to the method of Marengo *et al*. (2008). This resulted in the generation of relative spot volumes (RSVs), which were subjected to multivariate statistical analysis.

5.3.5 Multivariate statistical analysis

Principal component analysis (PCA) is a Euclidean distance-based eigenvector multivariate method that reduces the number of dimensions in a multivariate data set to identify and visualise strong patterns of variation in a multivariate set of measures (Marengo *et al*., 2007; Ringnér, 2008). The eigenvector scores of tree samples and spots were plotted along axes to represent the main, orthogonal, dimensions or gradients of variability in the data, with the relative size of the eigenvalues of axes indicating their relative importance for summarising pattern. PCA was initially used to identify outliers from the main pattern of protein spot variation among families; thereafter a single family (No. SP3-304) was removed because it had a few unique spots with very high RSV values.

Following preliminary data screening of gradients by PCA, redundancy analysis (RDA), was employed to focus on the multivariate pattern in the RSV matrix (39 families x 71 spots) that was most closely related to the Frost Sensitivity Index (FSI) ranking derived from the artificial frost screening study (Chapter 3). This FSI was previously referred to as Frost damage Scale B and was changed in this chapter for clarity purposes. RDA is a direct (canonical) ordination

method that is based on and extends PCA by identifying direction(s) of change most closely related to one or several explanatory variables, by using multiple regression during the derivation of the components or axes to constrain derived eigenvectors to be a direct function of the primary variable(s) of interest: FSI, in this case (van den Wollenberg, 1977; ter Braak and Šmilauer, 2015).

In this study, FSI values for families were fitted to their pattern of spot variation using RDA in Canoco 5 (ter Braak and Šmilauer, 2012). The association between the spot pattern and FSI was tested using a Monte Carlo Permutation test (n = 9999) (Šmilauer and Lepš, 2014). Separate RDAs were performed on the RSV matrices for the plants that were not exposed to the cold treatment, those that were, and the differences in RSVs (cold-stressed minus unexposed (control)) for each family. Gradients and disjunctions in the distribution of families, the direction of maximum variation of each protein spot, and the direction of correlation of FSI with the first two RDA axes were illustrated in triplots. Protein spots best described in the RDAs were identified by selecting those with 50% or more of their variation explained by the first and/or second RDA axes (Šmilauer and Lepš, 2014). RSV values were log-transformed prior to multivariate analysis to reduce the influence of very large values and RSVs for each spot were normalised (to mean = 0, sd = 1) across families to standardise their variance (Nedenskov Jensen *et al*., 2008). An alternative frost tolerance ranking system (Scale A, Chapter 3) was also tested in the RDAs but was found to have a weaker correlation than FSI with the familial pattern of protein spot variation (e.g., for RDA axis 1 of cold-stressed minus unexposed: r = 0.569 for FSI, r = 0.487 for Scale A) and was therefore not used any further.

5.3.6 Mass spectrometry and protein identification

5.3.6.1 In gel digestion and peptide extraction

Protein identification was performed at the Council for Scientific and Industrial Research-Biosciences Unit (Pretoria, South Africa). Each protein spot was excised using a sterile surgical blade. These gel protein spots were diced into small pieces (1x1 mm) to achieve maximum surface area during enzyme digestion. The gel pieces were destained and dehydrated with 50mM NH_4HCO_3 in methanol (50% v/v). Thereafter they were reduced with freshly prepared 10 mM DTT in 50 mM NH_4HCO_3 (prepared in dH_2O). This solution was replaced with freshly prepared 55 mM IAA in 25 mM NH_4HCO_3 (prepared in dH_2O). This was followed by the dehydration of the gel pieces with 25 mM NH_4HCO_3 in acetonitrile (50% v/v). Vacuum

dehydration was used to complete the drying process. The proteins in the gel pieces were then digested for 16-18 hours (at 37^0C) with a solution containing trypsin (10 ng/µl) and 25 mM NH_4HCO_3. The resultant peptides were extracted from the gel with a peptide extraction solution containing acetonitrile (50% v/v) and formic acid (50% v/v), vacuum dried and stored at -20^0C.

5.3.6.2 Ultra Performance Liquid Chromatography (UPLC) with mass spectrometry and protein identification

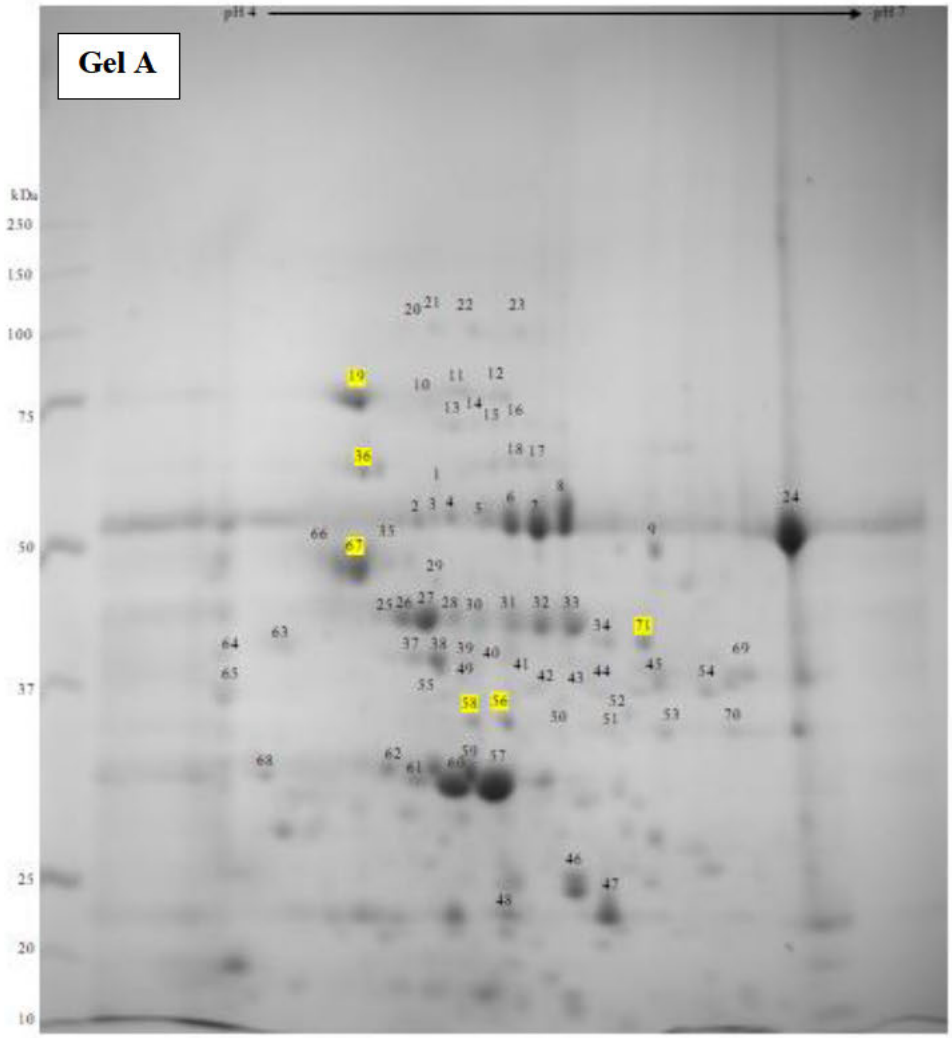
The extracted peptides were re-suspended in formic acid/ acetonitrile (0.2/2%) and this mixture was injected into a Waters Acquity UPLC M-class system (Waters, Milford, Massachusetts, United States of America). The peptides were desalted for 2 min using a Waters Acquity UPLC M-Class Symmetry C18 Trap Column (100Å pore, 5 µm particle size, 180 µm x 20 mm) and then introduced to a NanoEase M/Z Peptide BEH C18 (130Å, 1.7 µm, 300 µm X 150 mm) analytical column for chromatographic separation. The mobile phase consisted of deionized distilled water and 0.1% formic acid (A) and 100% acetonitrile and 0.1% formic acid (B). Peptide elution was achieved with a linear gradient of 5-35% (B) over 10 min at a flow rate of 8 µl/min.

The eluted peptides were introduced into a Waters Synapt G2-Si mass spectrometer through a LockSpray electrospray source at 100^0C and a capillary voltage of 2.5 kV. The mass spectrometry analysis was performed in positive ion mode with the time-of-flight (TOF) analyser set to detect ions in the 100-200 m/z range. The liquid chromatography-mass spectrometry (LC-MS) data was acquired in data independent (MS^E) mode and processed using Progenesis QI for proteomics software PD 2.0 (Nonlinear Dynamics, Newcastle, United Kingdom). Proteins were identified using MASCOT (Matrix Science, London, England) and searched against the UniProtKB/Swiss-Prot database (www.uniprot.org), where sequences of the eudicots clade were referenced and supplemented with sequences of common contaminating proteins.

5.4 Results

5.4.1 Separation of black wattle proteins after exposure to cold stress using two-dimensional gel electrophoresis

Changes in the protein profile of 40 black wattle families that had been exposed to a frost-inducing schedule (treated) and that had not been exposed (control) were observed using two-dimensional gel electrophoresis. Figure 5.1, Gel A and B show representative two-dimensional gel images for a black wattle family, A85-22, which had a high level of frost tolerance. Seventy-one distinct protein spots were detected ranging from 23 to 125 kDa.



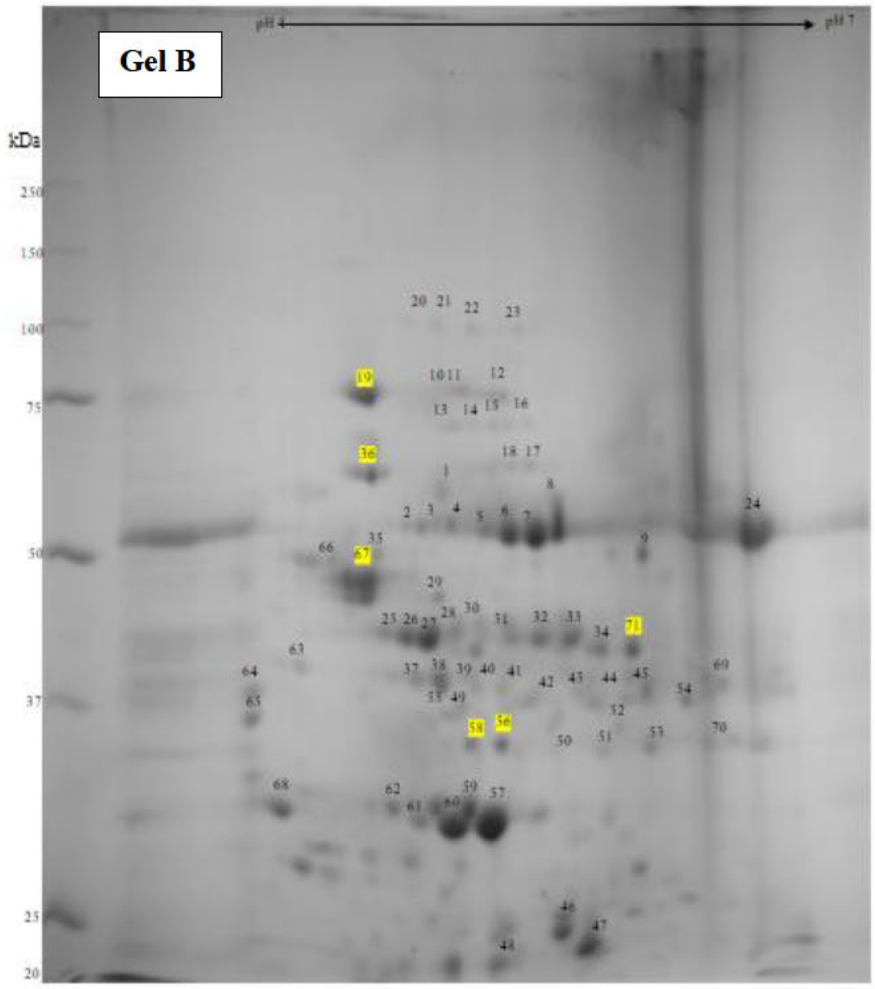


Figure 5.1. Two-dimensional gel electrophoresis for a black wattle family (A85-22) with high frost tolerance. Control sample (Gel A) and the sample that was exposed to cold/ frost-inducing temperature regime (Gel B). The proteins (90 µg) were separated using 7 cm, pH 4-7 linear IPG strips during IEF and thereafter with 12.5% polyacrylamide gels during SDS-PAGE. The polyacrylamide gels were stained with Coomassie brilliant blue R-250 and spot detection was performed using PD Quest software. The protein spots with the greatest upregulation are highlighted in yellow.

The RDA of protein spots before cold treatment (Figure 5.2 a) indicated that there was a pre-existing difference in the protein spot pattern in relation to the frost tolerance ranking order of the black wattle families (r = 0.8709). Some black wattle families that had moderate levels of frost tolerance A85-78, SP3-159 and 320 had a strong association with spots 23, 39, 20 and 32. Black wattle families that were less frost tolerant (upper and lower right quadrants) were

observed to be strongly associated with more protein spots as compared to the more frost tolerant families (upper and lower left quadrants) before cold treatment (Figure 5.2 a). After cold treatment (Figure 5.2 b), it was observed that even more protein spots were associated with frost-sensitive black wattle families as compared to the observation made in the pre-cold exposure RDA. Exposure to cold treatment also resulted in a larger difference in the proteomic expression pattern in the less frost-tolerant black wattle families as compared to the more tolerant families (Figure 5.2 c). The RDA conducted on the difference in spot volumes (before and after cold exposure) (Figure 5.2 c) showed that there were six protein spots that had the greatest upregulation and the strongest association with the most frost-tolerant families. These were protein spots 19, 36, 58, 67, 71 and 56 (also highlighted in Figure 5.1, Gel A and B).

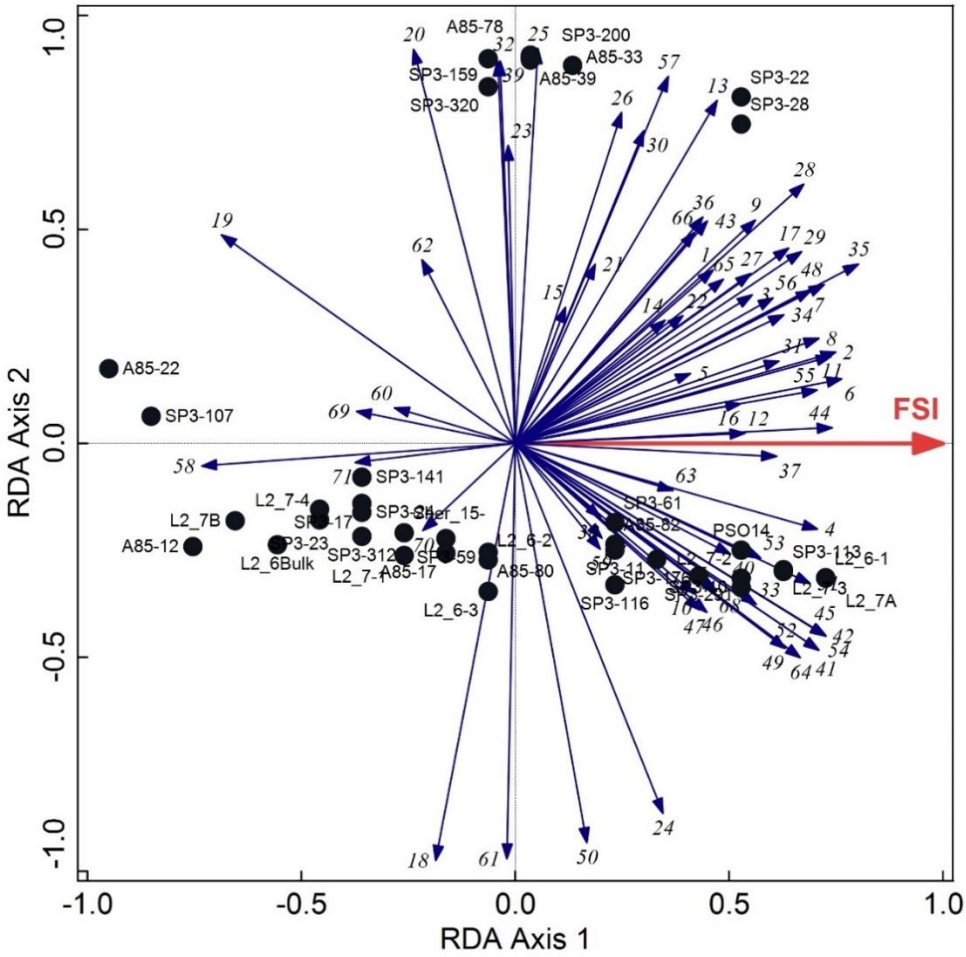


Figure 5.2a Redundancy analysis (RDA) biplot of the protein expression related to FSI (Frost sensitivity index) rank of black wattle seedlings that were not exposed to the cold-stress treatment (control). Proteins spots are numbered and illustrated as thin vectors, with protein expression increasing with the length of the vector. The dots represent the various black wattle families. Frost sensitive families are observed in the top and bottom right quadrants. The eigenvalue for RDA axis 1, is 0.2713; canonical correlation = 0.8709. Monte Carlo Permutation test (n = 9999) for all canonical axes: F-ratio = 13.8. P = 0.0001.

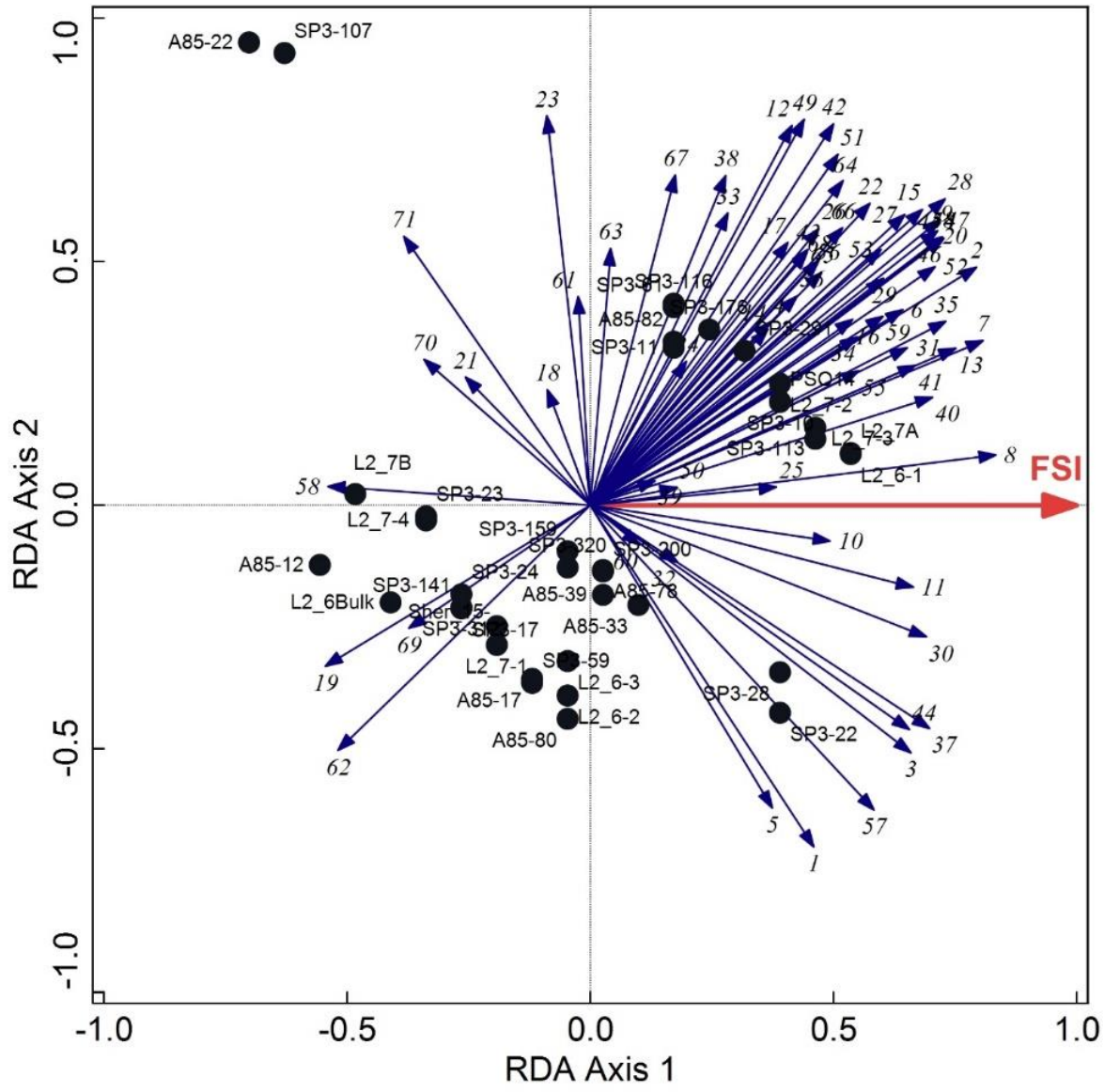


Figure 5.2b Redundancy analysis (RDA) biplot of the protein expression related to FSI of black wattle seedlings that were exposed to frost, in relation to the frost sensitivity index values of the families. Proteins spots are numbered and illustrated as thin vectors, with protein expression increasing with the length of the vector. The dots represent the various black wattle families. The eigenvalue for RDA axis 1 is 0.2936; canonical correlation =0.8585. Monte Carlo Permutation test (n = 9999) for all canonical axes: F-ratio = 15.4. P = 0.0001.

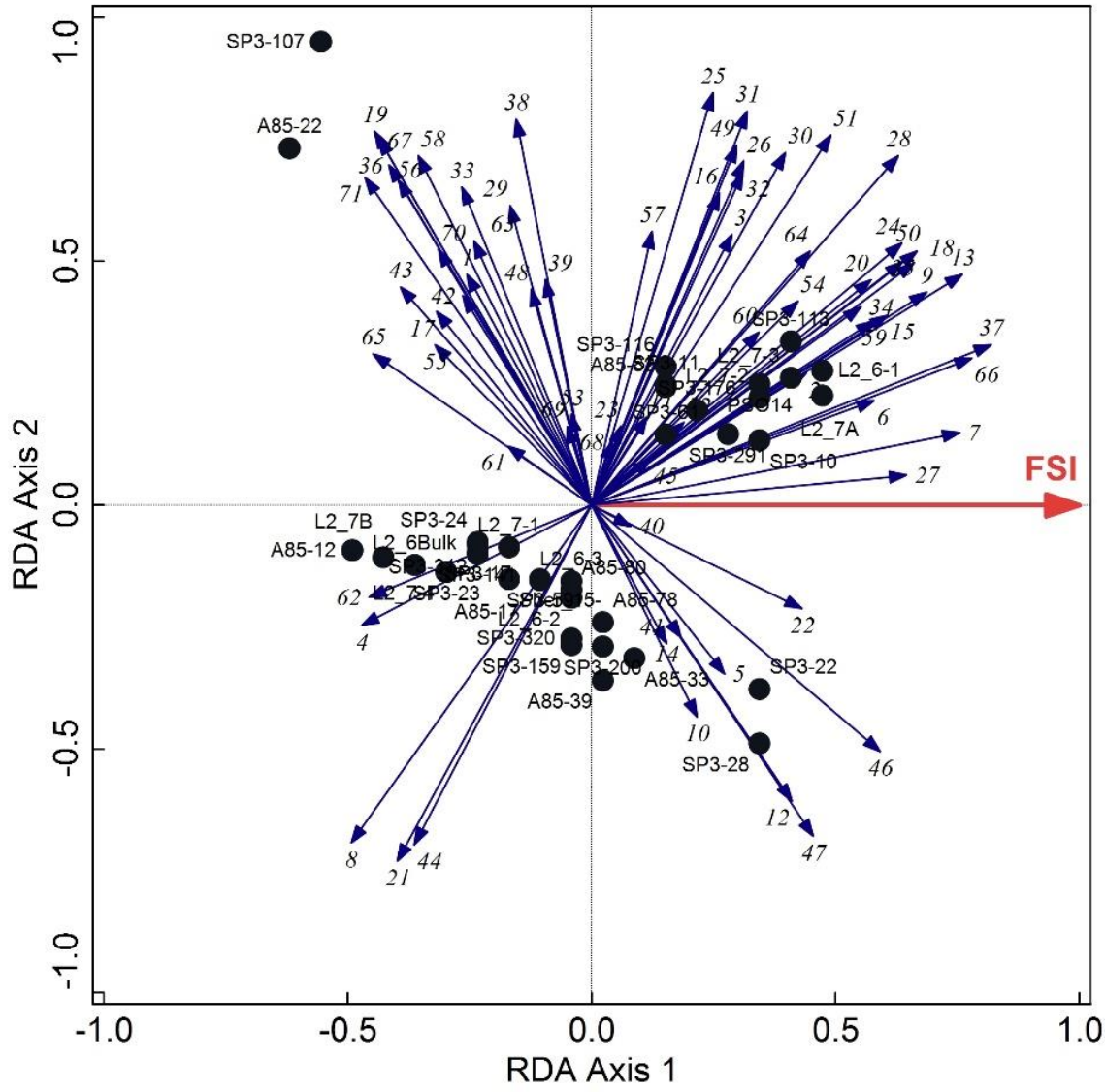
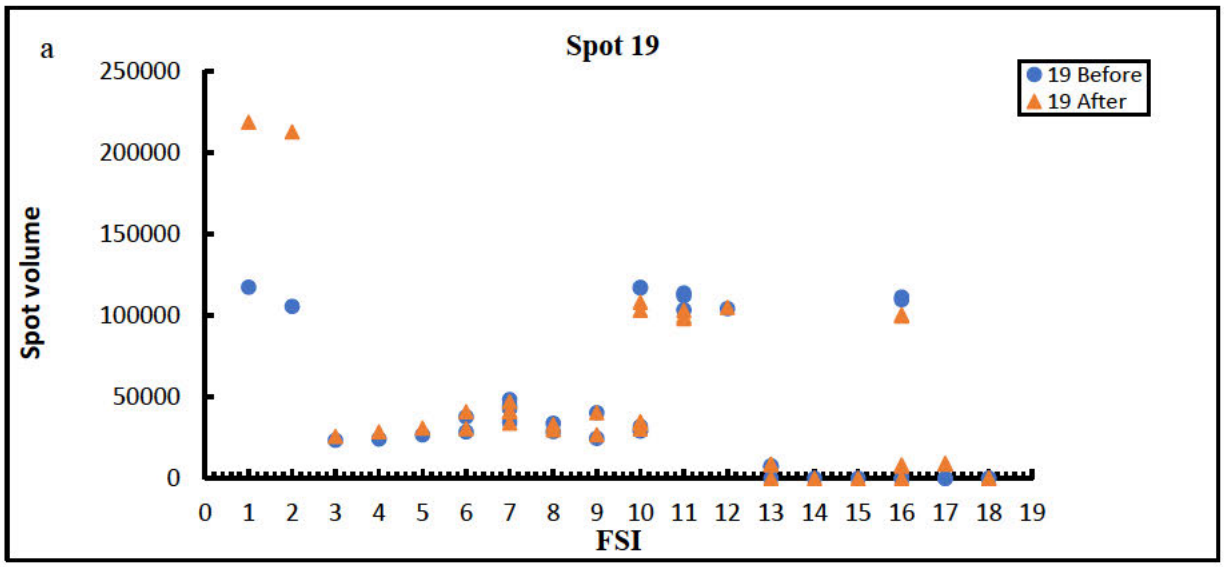
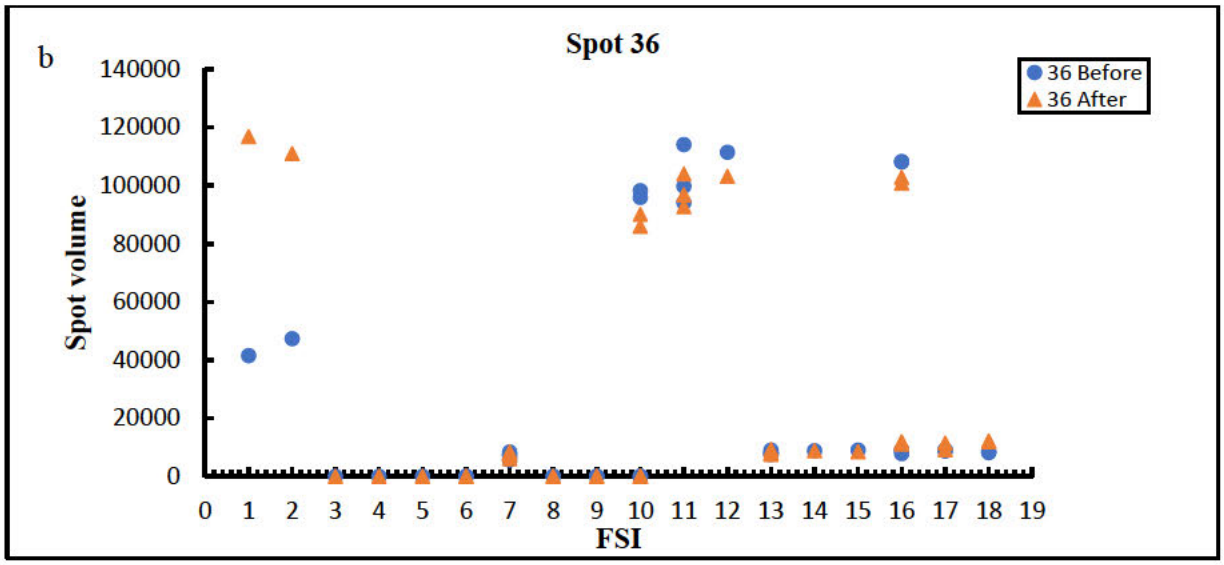
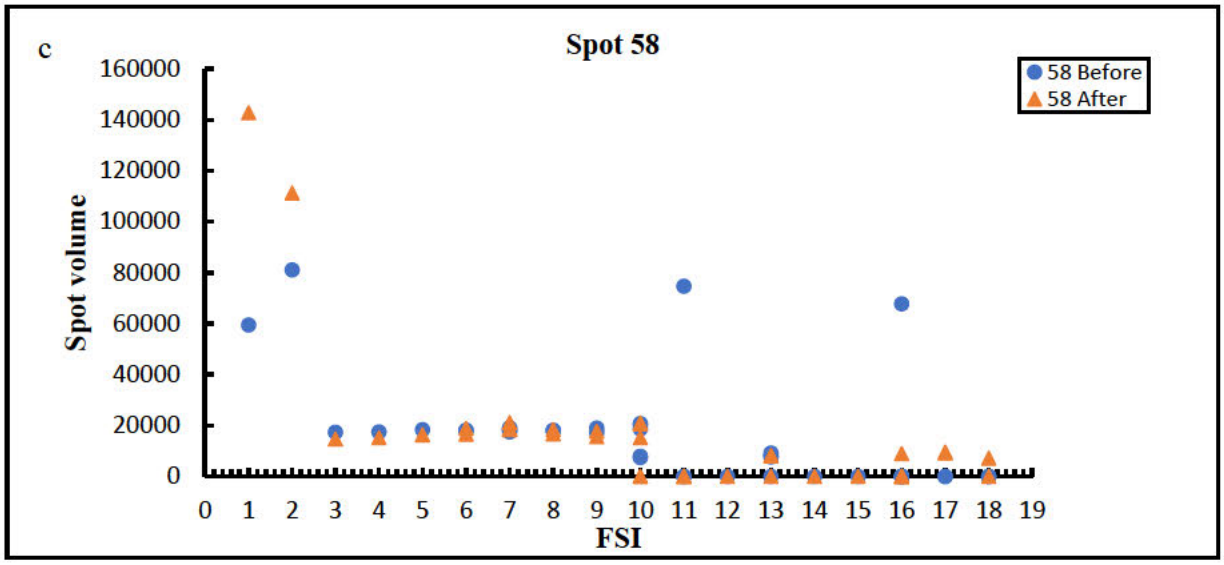


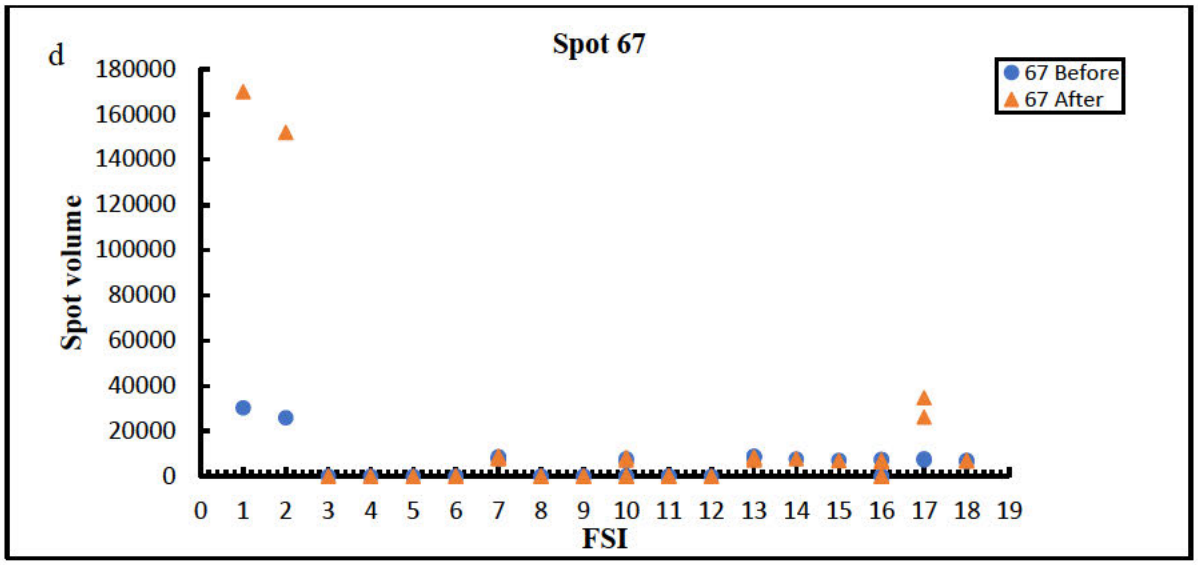
Figure 5.2c The RDA of the differential between the cold-stressed and unexposed families related to FSI. Proteins spots are numbered and illustrated as thin vectors, with protein expression increasing with the length of the vector. The dots represent the various black wattle families. The eigenvalue for RDA axis 1 is 0.1829; canonical correlation is 0.8687. Monte Carlo Permutation test (n = 9999) for all canonical axes: F-ratio = 8.3 P = 0.0001.

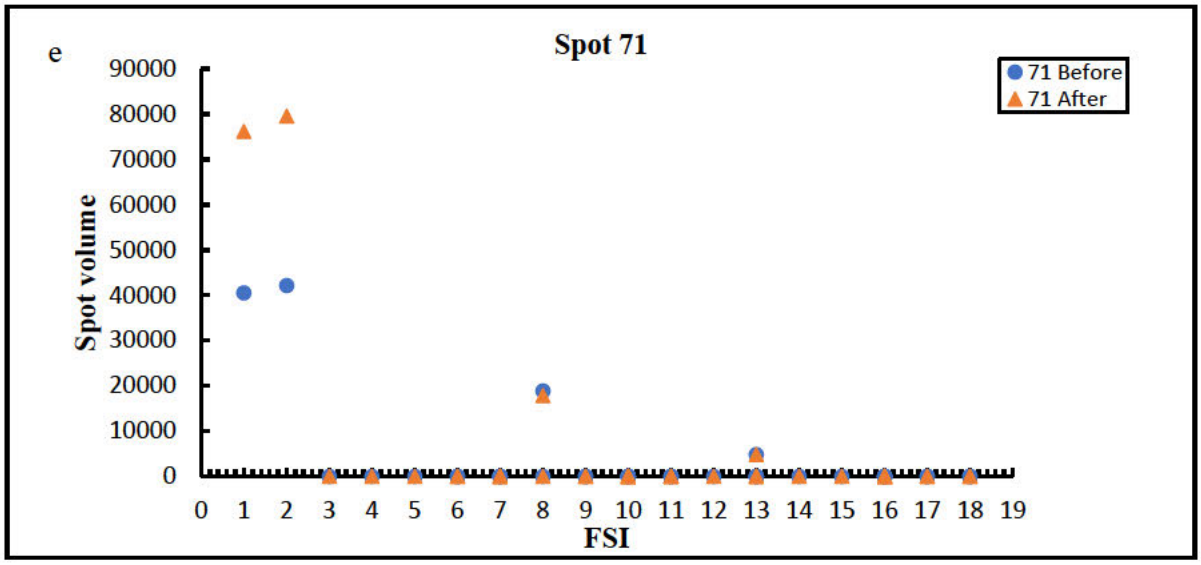
The variation of the protein spots (19, 36, 58, 67, 71 and 56) that had the greatest upregulation and the strongest association with the most frost-tolerant families after cold treatment are shown in Figure 5.3 a, b, c, d, e and f. Spot 58 (Figure 5.3 c) was also significantly upregulated in frost-tolerant families and showed noticeable down regulation in some frost sensitive families.


a
Spot 19
19 Before
19 After
Spot volume
250000
200000
150000
100000
50000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
FSI


b
Spot 36
36 Before
36 After
Spot volume
140000
120000
100000
80000
60000
40000
20000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
FSI


c
Spot 58
58 Before
58 After
Spot volume
160000
140000
120000
100000
80000
60000
40000
20000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
FSI


d
Spot 67
67 Before
67 After
Spot volume
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
FSI


e
Spot 71
71 Before
71 After
Spot volume
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
FSI

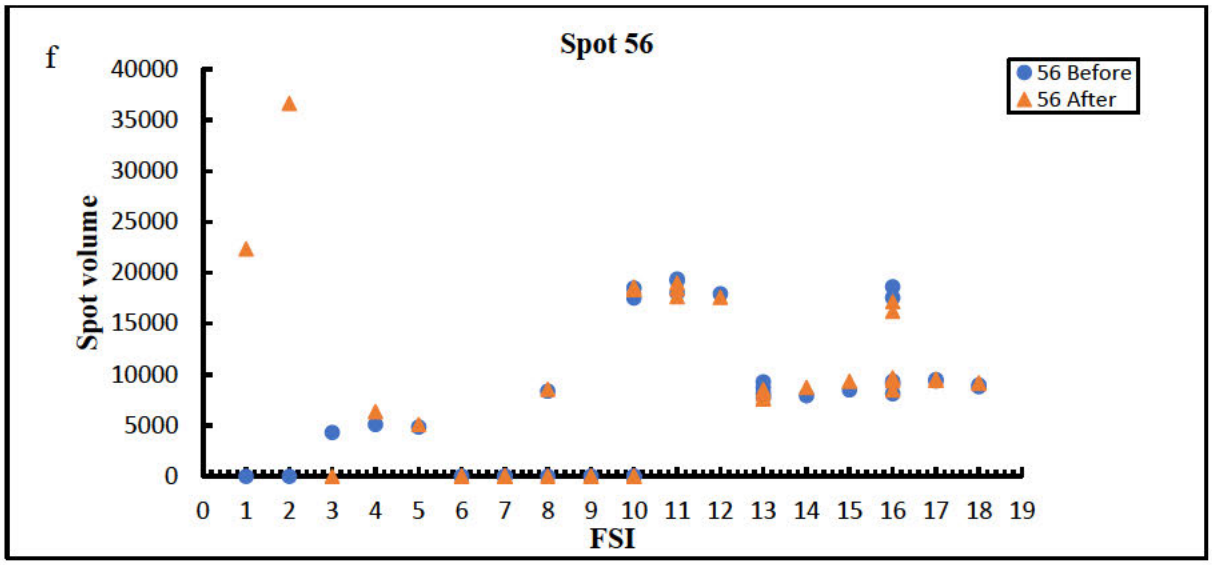


Figure 5.3 Protein spots that showed the greatest upregulation and association with frost tolerant black wattle families as a result of cold exposure. RDA conducted on the difference of protein spots (after cold exposure minus unexposed samples) identified protein spots 19 (a), 36 (b), 58 (c), 67 (d), 71 (e) and 56 (f) as being upregulated after cold treatment.

The amount of change resulting from cold exposure was the greatest among the most frost-sensitive black wattle families, followed by a smaller change in the most tolerant families, while the moderately tolerant families showed little change. This is illustrated in the Euclidean distance of protein spot patterns of black wattle families before and after cold treatment exposure (Figure 5.4).

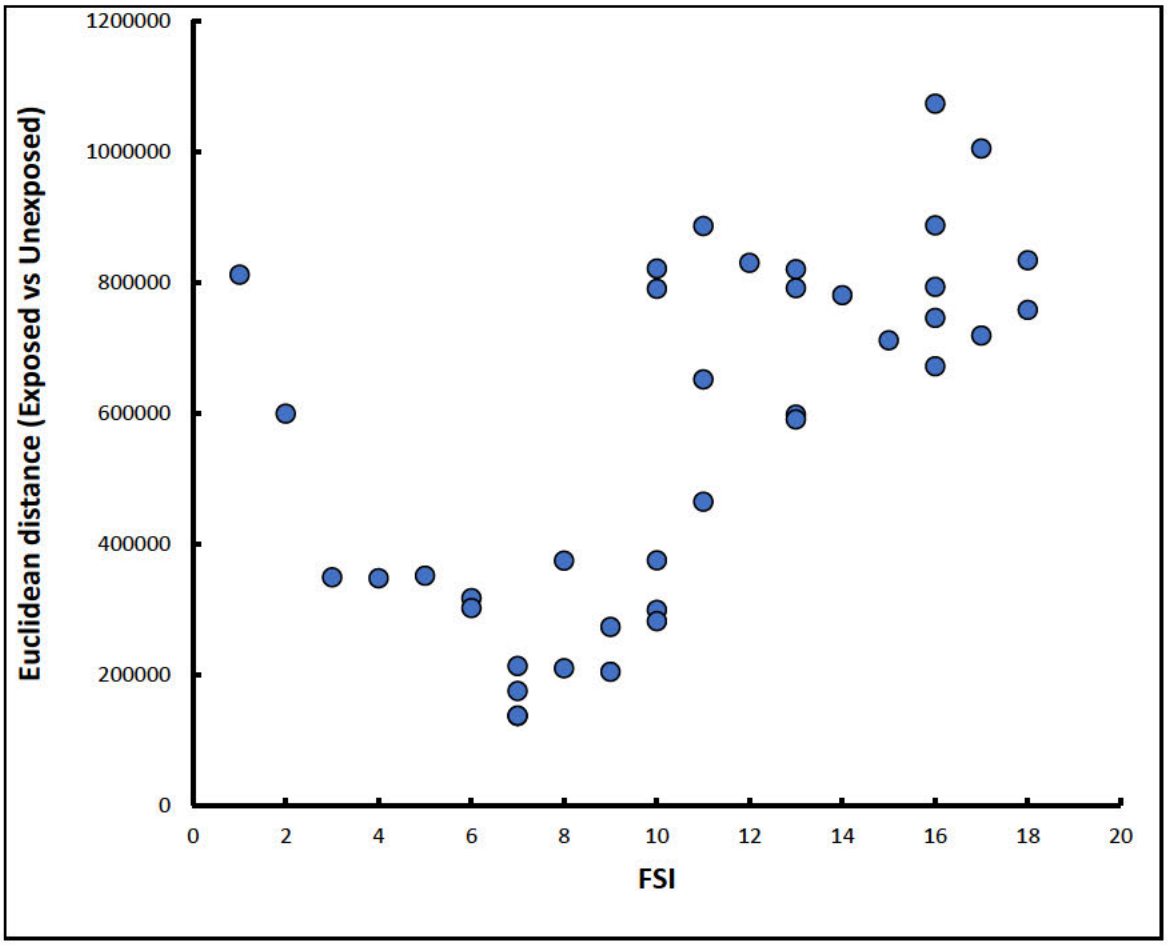


Figure 5.4. The magnitude of change in the protein spot pattern brought about by cold- stress treatment. The protein spot patterns of the black wattle families that were exposed to the cold-stress treatment were compared with that of the samples that were unexposed (control). The Euclidean distance is directly proportional to the amount of change observed in the protein spot patterns of each of the families. The black wattle families are arranged according to a previously established frost sensitivity index (FSI); 1- least frost-sensitive to 18- most frost-sensitive.

The protein spots (19, 36, 58, 67, 71 and 56) that had the greatest upregulation and the strongest association with the most frost-tolerant families after cold treatment were chosen for protein identification (Table 5.1). The identification of proteins was performed using MASCOT and searched against the UniProtKB/Swiss-Prot database. Due to only a small number of *Acacia mearnsii* protein sequences being available, the eudicots clade was used as a reference. The functions of the identified proteins included photosynthesis, proper protein translation, and folding and stabilization of cellular membranes (Testerink and Munnik, 2005). The identified spots ranged from: a molecular weight of 35.12 to 75.63 kDa, pI of 4.26 to 7.08 and protein sequence coverage of 21.69 to 45.77%.

Table 5.1: Identification of upregulated proteins in frost tolerant black wattle after cold treatment

Spot[a] number	Accession number (UniProt)	Protein identification (UniProt)	Function	MW(kDa)/ pI[b]	Protein sequence coverage (%)	Reference organism
56	P14226	Oxygen-evolving enhancer protein 1, chloroplastic	Stabilizes the tetra-manganese cluster and ionic environment for the water-splitting reaction of photosystem II	35.12/6.78	25,23	*Pisum sativum (Garden pea)*
58	P14226	Oxygen-evolving enhancer protein 1, chloroplastic	As described above	35.12/6.73	25,84	*Pisum sativum (Garden pea)*
71	Q8L4Y2	Probable 1-acyl-sn-glycerol-3-phosphate acyltransferase 4	May catalyze the conversion of lysophosphatidic acid into phosphatidic acid.	43.63/7.08	21,69	*Arabidopsis thaliana (Mouse-ear cress)*
67	Q40281	Ribulose bisphosphate carboxylase/ oxygenase activase	Activates RuBisCO from an inactive to an active form.	48.25/4.26	45,77	*Malus domestica (Apple) (Pyrus malus)*
36	P21238	Chaperonin 60 subunit alpha 1, chloroplastic	Binds RuBisCo small and large subunits. Protein translation, folding and degradation	62.24/4.31	34,81	*Arabidopsis thaliana (Mouse-ear cress)*
19	Q02028	Stromal 70 kDa heat shock-related protein_ chloroplastic	Protein folding and transport across membranes, cellular responses to abiotic stresses	75.63/4.26	32,86	*Pisum sativum*

[a] Excised spot number- identified by LC-MS as described in Material and Methods
[b] Experimental molecular weight (kDa) and pI

5.5 Discussion

Cold acclimation encompasses all the changes that occur in a plant that enable it to survive cold, stressful conditions. Changes in protein expression are some of the key activities that occur during cold acclimation (Brown *et al*., 2020). Two-dimensional gel electrophoresis was used to visualize the changes in protein expression in black wattle families chosen for their varying levels of frost tolerance after exposure to cold/ frost inducing temperature treatments. The gel images of the 40 black wattle families for both treated and control samples were analysed and 71 distinct protein spots were detected. In similar studies, two-dimensional gel electrophoresis has been used to identify changes in protein patterns in woody species as a result of cold exposure (Kjellsen *et al*., 2013; Wu *et al*., 2014; Baniulis *et al*., 2020). These changes in proteomics data have been analysed either by univariate or multivariate analytic methods (Grove *et al*., 2008). In the current study, a large, complex data matrix was developed from the relative spot volumes of individual protein spots for each black wattle family (ranked according to their frost sensitivity), and this necessitated the use of multivariate analysis methods, using PCA and RDA. PCA has been used in several two-dimensional gel electrophoresis studies in a similar way to this current study (Rogers *et al*., 2003; Mazzara *et al*., 2011; Vitova *et al*., 2017). However, to the authors' knowledge, RDA has not been used previously as a statistical tool for creating a predictive model for proteomic response to frost tolerance levels in forest crops. RDA analyses revealed six protein spots that were most strongly upregulated and had very close associations with the frost tolerant black wattle families, namely spots 19, 36, 56, 58, 67 and 71.

Spot 19 which had the highest molecular weight and lowest pI, 75.63 and 4.26, respectively, was identified as heat shock protein 70 (HSP 70). The upregulation of this protein has been reported in previous studies in response to low and high temperature extremes (Guy, 1999; Wang *et al*., 2004). It is involved in the three-dimensional folding of proteins, prevention of irreversible protein inactivation and aggregation, and protein translocation and degradation (Park and Seo, 2015). Chaperonin 60 subunit alpha 1 (Spot 36) has a similar function to HSP70. This protein binds to the large ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) subunit to prevent aggregation and misfolding. It is also required for proper plastid division (Suzuki *et al*., 2009; Zhao and Liu, 2018). This activity is important because during cold acclimation photosynthetic light reactions and the central carbohydrate metabolism of plants

are modulated to prevent imbalances that would lead to the production of reactive oxygen species, cell damage and/ cell death (Fürtauer *et al*., 2019). Another protein that is involved in photosynthesis, namely Oxygen evolving enhancer protein 1 was also upregulated in frost tolerant black wattle families (Spots 58 and 56). This occurred as two spots (isoforms). However, their structural differences were not further characterized. Oxygen evolving enhancer protein 1 is a cold and dehydration-stress related protein that belongs to the oxygen-evolving complex in Photosystem II. It stabilizes the manganese cluster and is required for the Photosystem II core assembly (Hu *et al*., 2013; Zadražnik *et al*., 2013; Liu *et al*., 2021). Ribulose bisphosphate carboxylase/ oxygenase activase (Spot 67) is another photosynthetic-related protein that was upregulated. This enzyme activates RuBisCO that is not carbamylated, by facilitating the dissociation of inhibitory sugar phosphates from the active site of RuBisCO (Portis, 2003).

The plant-cell membrane is extremely vulnerable during cold exposure and is the primary site of cold injury. Low temperatures decrease the fluidity of plant cellular membranes, which are needed to maintain cellular function and integrity (Steponkus *et al*., 1998). Adaptation of the plant cell membrane is one of the most important mechanisms for survival during extremely cold conditions (Takahashi *et al*., 2013). This is achieved by changes in lipid and protein compositions. One such change is the increase in unsaturated fatty acids and phospholipids such as phosphatidic acid. In the current study, there was an upregulation of 1-acyl-sn-glycerol-3-phosphate acyltransferase 4 (Spot 71), which is an enzyme that catalyses the conversion of lysophosphatidic acid into phosphatidic acid (Murata and Tasaka, 1997).

The proteins that were upregulated in frost-tolerant black wattle families in this study have three main functions: the maintenance of photosynthetic processes, the maintenance of cellular membrane integrity and the prevention of protein misfolding and aggregation. Previous proteomic cold acclimation studies in other plants have reported the upregulation of similar proteins (Renaut *et al*., 2004; Welling and Palva, 2006; Rocco *et al*., 2013). In future studies, it would be beneficial to identify those proteins that have been observed to have a pre-existing and strong association with frost-tolerant black wattle families. These proteins, together with those that were identified in this study, could be used to enhance predictive molecular models for frost tolerance in black wattle.

5.6 Conclusion

Changes in protein expression in black wattle offers an efficient and complementary tool for screening for frost tolerance in black wattle families with unknown frost tolerance levels. Molecular techniques such as proteomics integrated with other omic-technologies can be used in future studies so that a higher-throughput of informative and holistic data can be obtained. While this knowledge will contribute greatly to the understanding of cold tolerance in woody plants, the development of near infrared spectroscopy models based on proteomic results may offer an even quicker, cheaper and more efficient method of screening for plant breeders. This can greatly speed up recurrent selection and would positively impact preliminary screening so that fewer families would require field testing for frost tolerance.

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Thesis overview

Since its introduction to South Africa in the 19th century, the commercial and environmental importance of black wattle has increased exponentially. Over the years, the silviculture of this species has improved greatly. However, it has been plagued with several biotic and abiotic stress problems, one of which is frost damage. Breeding research for frost tolerance in black wattle has taken place over several decades at the Wattle Research Institute (now known as the Institute for Commercial Forestry Research). However, the production of genetically improved seed of this species for frost-prone areas is still lacking. Therefore, the breeding of frost tolerant black wattle varieties is still a priority for South African wattle growers. Screening for frost tolerance in black wattle has primarily been in the form of field trials. However, it is extremely challenging to breed for frost tolerance in a tree crop under field conditions. There is growing evidence that the ability of woody species to tolerate frost conditions is driven by multigenic, molecular mechanisms. These molecular mechanisms need to be researched and included into screening protocols to enhance their efficiency. Field screening for frost tolerance is also limited by environmental conditions and this has necessitated the development of an artificial protocol for inducing frost damage in black wattle.

In this current study, the first goal was to establish a reliable and reproduceable protocol for inducing frost damage in black wattle seedlings at approximately the stage that they would be planted in field trials. The use of an environmentally controlled chamber that could achieve extremely low temperatures was used for this purpose. Several temperature schedules were tested and ultimately a schedule was developed that provided conditions that artificially induced frost damage. The results obtained from the artificial frost screening were poorly correlated with those achieved in some of the previous field trials. This could have been due to the uneven distribution of cold air in field trials because of the topography of the land on which trials were established. The size of the inserts in the trays in which the black wattle seedlings were grown and later subjected to cold treatment also affected the distribution of coldness around the roots. Seedlings planted in field trials have greater soil surface area around their roots and this would result in a different level of frost damage in these seedlings compared to those that were subjected to the artificial frost treatment. There were also discrepancies between the programmed temperature of the chamber and the actual temperature that the seedlings were exposed to. This needs to be investigated in the future refinement of the protocol. Use of a digital controller of temperature for the cold chamber is probably essential.

These factors will need to be investigated in future work in optimizing the use of an artificial frost chamber. It would also be interesting to use cuttings of single genotypes because it would eliminate the experimental variable of siblings' genetic variability when comparing frost tolerance of different families.

The second goal was to understand the molecular aspects of frost tolerance in black wattle. This was challenging because research in this field is extremely limited. Some studies have focussed on the genetic aspect of cold tolerance in woody species, however, there have been few that have focused on the proteomics involved in this field of research. It is necessary to improve our understanding of the proteins involved in cold tolerance because it is a multigenic trait, and proteomics simplifies the numbers of molecules that need to be tracked, relative to genomics. One of the main challenges in this study was the development of a protein extraction method that would be suitable for black wattle leaves, due to their high phenolic content. An appropriate protocol that was compatible with the respective downstream technologies was established for this. This protocol involved the use of polyvinylpolypyrrolidone to trap phenolics so that they would not hinder the protein extraction protocol. Similar agents that could be used for this purpose should be looked at in future work to determine if protein extraction in black wattle could be improved further.

As part of the proteomics research, image analysis of the two-dimensional electrophoresis gels produced in this study generated many data points that required the use of multivariate analysis. Redundancy analysis (RDA) was used for the first time for this type of data analysis in forest crops. This approach should also work in studies of frost tolerance in other woody species. In addition, proteins that may be upregulated during resistance against diseases such as wattle rust could also be identified after protocols are developed involving artificial inoculation. The study of protein profiles in various black wattle families could also be used to improve our knowledge on other complex traits such as tannin production and lignin. As the number of protein data sets are expanded, future studies will benefit from the use of more advanced machine learning tools including deep learning techniques.

The identification of proteins in the study was performed after artificial cold treatments. However, the RDA results showed that there were pre-existing levels of some key proteins in frost tolerant families before cold treatment. It would be interesting to identify these proteins and to use the data generated from this to enhance molecular screening for frost tolerance in black wattle. In the long term, near infrared (NIR) spectroscopy models for frost tolerance

would be faster, cheaper and easier to work with than proteomics, which would allow plant breeders to screen far more progeny, far more quickly, so that fewer families would need to be planted during field trials.

Climate change has resulted in severely erratic weather patterns including extreme heat and cold events. This has had a major impact on agriculture and plant breeding. Technologies such as proteomics and the controlled, artificial simulation of weather conditions are techniques that can be used to understand the impact of climate change, and to rapidly adapt existing crops to more extreme conditions. As these and other technologies become more advanced and improve, so too will our ability to handle the challenges associated with rapidly changing weather patterns and their impact on crop production.

Appendix 5.1

Relative spot density means for 40 black wattle families and their relative FSI ranking. See attached Microsoft Excel file.