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Determining the frost tolerance potential of commercially important South African eucalypts.

By

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A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

School of Animal, Plant and Environmental Sciences,

University of the Witwatersrand,

Johannesburg, South Africa.





signed on the 3rd day of June 2016 in Johannesburg.

DECLARATION

The experimental work described in this dissertation was carried out at the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg, South Africa, under the supervision of Dr Kershree Padayachee and Professor David J Mycock (School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, Johannesburg).

I declare that this Dissertation is my own original and unaided work, and has not been submitted in any form for any degree or diploma at any other tertiary institution. Any research work conducted by other investigators has been duly acknowledged in the following text, as evidenced by citations and references.



YAKIRA BAHADUR

3rd day of June 2016 in Johannesburg.

DEDICATION

For my loving parents, my strong grandmother, my role-model sister and my grandparents who have passed. Your sacrifices and generosity have led me to realize my potential today.

“One of the first steps to accomplishing great things in your life is to cease dwelling on the negative things in your past. Carefully assess your present strengths, successes, and achievements. Dwell on those positive events in your life, and quit limiting your potential by constantly thinking about what you have done poorly.” – A lesson from the Mad Hatter.

For me. I am proud of my determination and commitment in accomplishing this.

“To become a master at any skill, it takes the total effort of your heart, mind and soul working together in tandem.” – Maurice Young.

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LIST OF SYMBOLS AND NOMENCLATURE

CF: chlorophyll fluorescence

CS: cold shock

DNA: deoxyribonucleic acid

DWB: dry weight basis

FC: Folin-Ciocalteau reagent

FWB: fresh weight basis

GAE: gallic acid equivalent

GFG: glucose, fructose and galactose

GN: *E.grandis* x *E.nitens* hybrid

GOPOD: glucose oxidase plus peroxidase and 4-aminoantipyrine

≥: greater or equal to

>: greater than

<: less than

×: hybrid cross (breeding)

MSc: Master of Science

NADPH: nicotinamide adenine dinucleotide phosphate

PA: phenolic acids

PCA: principal component analysis

Rboh: respiratory burst oxidase homologs

ROS: reactive oxygen species

REC: relative electrolyte conductance

rpm: revolutions rep minute

TSS: total soluble sugars

ABSTRACT

Currently *Eucalyptus* plantations in the warm and cool temperate parts of South Africa are being exposed to damaging temperature extremes and unseasonal frost events that, in particular, have detrimental effects on juvenile plantations. To accommodate these conditions, *E. grandis* and *E. nitens* have been selected for hybridization in efforts to identify and select clones suitable for successful plantation establishment in affected areas. Biochemical and physiological responses of plants to cold shock and simulated frost conditions offers a means for this type of selection. In this study, the responses of *E. grandis*, *E. nitens* and 8 characterized *E. grandis* x *E. nitens* (GN) hybrid clones to cold shock and simulated frost conditions were evaluated. The responses elicited were used as an indication of the eucalypts' low temperature and frost tolerance potential, based on levels of: reactive oxygen species (ROS), phenolic acids (PA), starch, total soluble sugars (TSS), chlorophyll fluorescence (CF) and relative electrolyte conductance (REC). Plants were subjected to standard growth conditions of 25°C day/14°C night temperature and a 12h photoperiod for 7 days and subsequently cold shocked at 5°C for 24h. Frost conditions were simulated by freezing excised leaf discs from 2°C to -6°C at a rate of -4°C/h with a one hour hold at -6°C. The results showed an up-regulation of ROS in *E. grandis*, GN 1, GN 4 and GN 6, 30-90 minutes into the cold shock; and levels were highest in *E. nitens*, GN 3 and GN 7 only 24h after the cold shock exposure. PA levels changed marginally under cold shock conditions, with levels of GN 4 increasing the most by 58%. Starch levels of GN 6 were the most affected by the cold shock, where a 33% increase in levels was recorded. TSS levels of *E. grandis* and GN 6 increased by 201% and 409% respectively, while TSS levels of GN 2 and GN 3 decreased by 41% and 76% respectively. CF levels of *E. nitens* and two GNs were most affected by the cold shock, however, all the eucalypts tested, except GN 2, GN 3 and GN 6, displayed a high recovery potential to the cold shock. REC levels fluctuated slightly between unfrozen and frozen samples under standard and cold shock conditions and it was found that *E. grandis*, GN 1 and GN 3 were the least frost tolerant; and GN 4, GN 7 and GN 8 were the most frost tolerant according to REC levels under cold shock and simulated frost conditions. The results indicate that of all the tested eucalypts, only three GNs were not tolerant to the cold shock and *E. grandis* and two GNs were not tolerant to the simulated frost. Therefore, it was concluded that all of the eucalypts investigated, apart from *E. grandis*, GN 1 and GN 3, may be suitable for plantation establishment in areas prone to frost in South Africa.

CHAPTER 1. INTRODUCTION AND CONTEXTUALIZATION OF THE RESEARCH

1.1. *Eucalyptus*

The genus *Eucalyptus* L'Heritier (1789) of the family Myrtaceae is native to Australia and surrounding islands, with over 700 species of shrubs and hardwood trees (Grattapaglia and Sederoff 1994). Numerous *Eucalyptus* species and hybrids are grown extensively as commercial plantations in temperate and subtropical regions of the world such as Australia, Uruguay, Argentina, Portugal, Brazil, Morocco, USA and South Africa, collectively covering over 20 million ha (Watt *et al*. 2003). *Eucalyptus* is one of the most widely propagated genuses in terms of its variety of uses and it is grown in each region according to the end product requirements and the climatic and geographic conditions able to sustain a plantation (Eldridge *et al*. 1994, Watt *et al*. 2003). Some of the end products from eucalypts include saw timber, fuel wood for locomotives, firewood, poles, mine props, honey, charcoal, tannin, ornamentals, pulp and paper (Turnbull 1999). The fast growth and wide range of adaptability of eucalypts aid in their plantation establishment in tropical and subtropical regions, enabling their constitution as one of the world's main sources of biomass with high economic and commercial importance (Eldridge *et al*. 1994, Grattapaglia and Sederoff 1994). One of the most widely propagated *Eucalyptus* species globally is *Eucalyptus grandis*; however, *Eucalyptus nitens* is sometimes selected for establishment instead of *E.grandis* in conditions unconducive to the successful establishment of *E.grandis*.

1.1.1. *Eucalyptus grandis* and *Eucalyptus nitens* native distribution

Eucalyptus grandis (W. Hill ex Maiden) has a natural distribution along the eastern coastal and sub-coastal regions of Australia, from Newcastle in New South Wales towards northern Queensland (Figure 1, Floyd *et al.* 2002, Hudson *et al.* 2015). *Eucalyptus grandis* grows best under sub-tropical conditions and is sensitive to low temperatures, frost and snow events (Brink 2011, Otim 2008). However, this species has been intensively studied and selected for hybridization due to its fast growth, with various successful plantations now extending to regions outside of the species' natural distribution (Floyd *et al.* 2002).

Eucalyptus nitens (Deane & Maiden) Maiden has a natural distribution in south eastern Australia that spreads from the Central Highlands of Victoria to the Dorrigo area of New South Wales (Figure 1, Hamilton *et al.* 2008, Hudson *et al.* 2015). However, *E. nitens* has also been established in regions outside of its usual distribution in Australia, indicating the species' suitability to bioclimatic conditions outside of its natural range (Hamilton *et al.* 2008). One of these regions includes areas of Tasmania where temperatures are very low (Hamilton *et al.* 2008, Tibbits and Hodge 2003). *Eucalyptus nitens* is most commonly selected for establishment in areas prone to wind, snow and frost

events to replace other *Eucalyptus* species that are too sensitive to survive under these conditions (Tibbits and Hodge 2003).

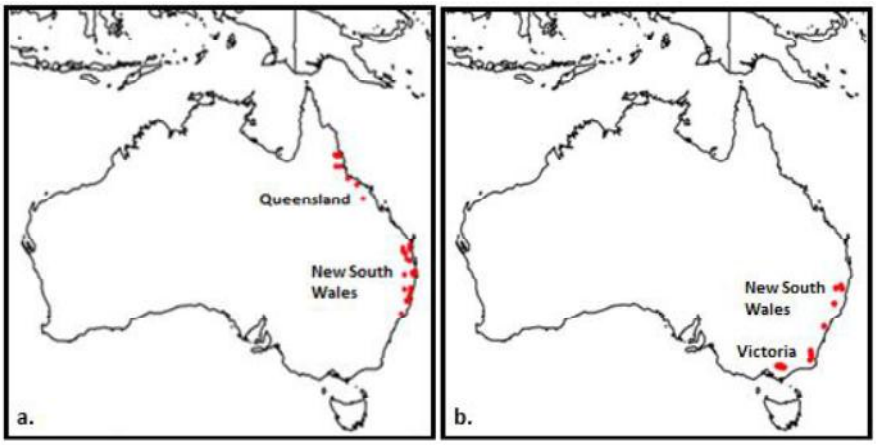

Figure 1. The natural distributions of a. *Eucalyptus grandis* and b. *Eucalyptus nitens* in Australia (Adapted from Hudson *et al.* 2015).

1.2. Commercial forestry in South Africa

The forestry industry in South Africa contributes significantly to the country's gross domestic product and employment. *Eucalyptus* accounts for 40% of the plantation forestry area in the country and expands over 516 000ha (Godsmark 2013, Hunter *et al.* 2004, Pogue 2008). These plantations are grown primarily for the production of paper, pulp and timber products (Godsmark 2013, Hunter *et al.* 2004, Pogue 2008). There are a number of eucalypts that are grown commercially in South Africa, however, *E. grandis*, *E. nitens* and their hybrids are among the most commercially important in the South African forestry sector. Ideal growing conditions for *E. grandis* and *E. nitens* include the summer rainfall regions of South Africa (Brink 2011). *Eucalyptus grandis* grows optimally under a mean annual temperature >17^{0}C and a mean annual precipitation >900mm (Brink 2011). *Eucalyptus nitens* prefers a mean annual temperature no greater than 15^{0}C and a mean annual precipitation >810mm (Brink 2011).

In South Africa, *Eucalyptus* plantations have been established in three major climatic regions – the cool temperate zone, the warm temperate zone and the subtropical zone (Figure 2, Sappi 2014). *Eucalyptus grandis* has traditionally been the most commonly cultivated hardwood species because of its rapid growth and favourable wood properties (Poynton 1979). However, over the past two decades, commercial plantation ranges have expanded to include the cool temperate zones (Figure 2, Sappi 2014) where extreme high and low temperatures and severe frost events occur (Swain and Gardner 2004); zones which are unsuitable for *E. grandis*. Consequently, these sites were classified as low productivity sites due to these conditions and the unsuitable site species matching for *E. grandis* as it

does not grow successfully on these sites. It should also be noted that the establishment of plantations in more favourable conditions at new sites is not an option as they are prohibited by legislation under the Afforestation Permit System, the National Water Act and the National Environmental Management Act (DWAF 1999, van der Zel 1995). In an effort to improve yield on these sites, other species with greater inherent tolerance for cold and frost (such as *E. nitens*) as well as hybrids of *E. grandis* and *E. nitens* (*E. grandis* x *E. nitens*) have been tested on these sites (Swain and Gardner 2004).

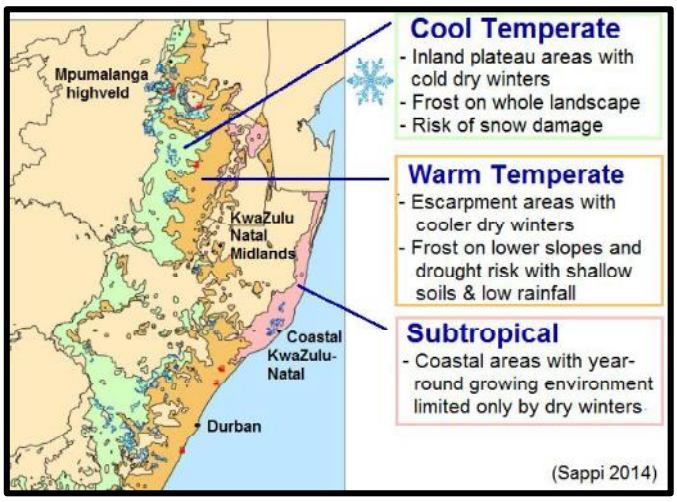

Figure 2. *Eucalyptus* plantations have been established in three climatic regions of South Africa (Adapted from Sappi 2014).

1.2.1. Hybrid intensive forestry in South Africa

Hybridization of various tree species has become a necessity in many parts of the world where traditional species cannot perform optimally under changing environmental conditions. Hybridization can bring about hybrid vigour which is the "superior performance of a hybrid organism compared with either of their parents" (Birchler 2015). In terms of commercial forestry, hybridization offers the advantage of combining selected desirable traits from each parent where there is an increased expression of the desired trait (O'Reilly-Wapstra *et al*. 2014). It is also a possibility that the hybrids may express novel traits that are not present in either parent (O'Reilly-Wapstra *et al*. 2014, Stelkens and Seehausen 2009).

Hybrid intensive forestry in South Africa began in the early nineteen nineties with *E. grandis* being crossed with *E. urophylla*, *E. camaldulensis* or *E. tereticornis* (Denison and Kietzka 1993). These hybrids generally outperform the pure species due to their higher disease, pest, drought, heat or cold resistance (Denison and Kietzka 1993). More recently, *E. nitens* has been selected for hybridization with *E. grandis* due to the contributing complementary traits of these two species,

selected for possible commercial viability under present and predicted climate conditions (Thompson 2013). The contributing traits of *E. grandis* include favourable wood properties, fast growth and tolerance to *Mycosphaerella* leaf blotch disease that *E. nitens* is susceptible to and strong rooting ability (Thompson 2013). The contributing trait of *E. nitens*, on the other hand, includes tolerance to lower temperatures in areas prone to snow and frost that *E. grandis* is sensitive to (Thompson 2013). However, to fully recognize the need for hybrid intensive forestry and the selection of these two species for hybridization, current and future climate conditions in South Africa need to be explored.

1.3. Climate change in South Africa

During the past and current century, heightened atmospheric concentrations of carbon dioxide and other greenhouse gasses have and will continue to result in intensified global warming, a phenomenon aggravated by increasing unsustainable anthropogenic activities (Hughes *et al*. 1996, IPCC 2007, Minorsky 2002). These activities have been predicted to lead to significant changes in seasonality and it is anticipated that such changes will be more prominent in temperate regions, such as South Africa, than in tropical regions (Hughes *et al*. 1996). The International Panel for Climate Change (IPCC) has speculated that the southern African region is becoming warmer, although there is also a strong possibility of an increase in the occurrence of extreme weather events such as sudden heat waves and droughts or unseasonal, severe frosts (Barlow *et al*. 2015, Easterling *et al*. 2000, Germishuizen 2013). An example of extreme temperatures can be seen in an analysis of temperature data from a plot of a trial plantation conducted by the Sappi Shaw Research Centre in KwaZulu-Natal. High and low temperature extremes were seen particularly during early July 2014 where, over a period of four hours, there are records of a temperature change of 24^{0}C (Figure 3, Sappi 2015). There have also been changes in the duration and timing of growing seasons due to climate change which increases the vulnerability of plants, in particular, to freezing damage caused by early or late season frosts (Norby *et al*. 2003, Woldendorp *et al*. 2008).

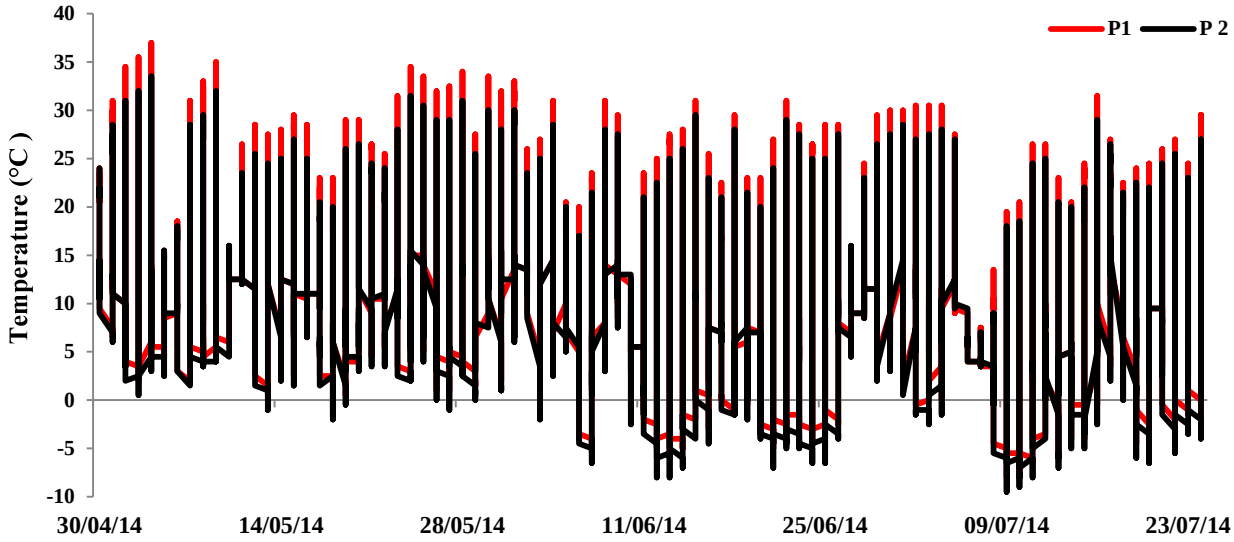

Figure 3. Temperature (°C) data at two week intervals from a 2014 field trial in KwaZulu-Natal, South Africa, conducted by the Sappi Shaw Research Centre. Various GN clones were used in the trial, including the GNs investigated in this study. P1represents the temperature from the bottom left corner of the alpha lattice design trial plot. P2 represents the temperature from the top right corner of the alpha lattice design trial plot (Adapted from Sappi 2015).

1.3.1. Frost

"Frost" refers to the formation of ice crystals as water vapour undergoes a phase change to ice or as the freezing of dew occurs (Snyder and Melo-Abreu 2005). However, frost is more widely used to describe the meteorological event that causes freezing injury to a variety of plant species (Snyder and Melo-Abreu 2005). Kalma *et al*. (1992) and Snyder *et al.* (1987) have categorized two types of frosts, *viz*. advective and radiative frosts. Advective (advection) frosts occur when there is movement of cold air into a windy, well-mixed atmosphere with a sub-zero temperature (Snyder and Melo-Abreu 2005). Radiative (radiation) frosts occur as a result of cooling due to energy loss through radiant exchange between the earth's surface and the sky (Snyder and Melo-Abreu 2005). In South Africa, radiation frosts seem to occur more frequently and have a more severely negative effect on plants compared with advection frosts.

1.3.1.1. Radiation frost

Radiation frosts occur typically during a clear night sky when there is minimal wind and the air temperature falls below 0^0C but is above 0^0C during the day (Snyder and Melo-Abreu 2005). When night-time skies are clear, more heat is radiated away from the surface of the earth rather than received (Figure 4) (Snyder and Melo-Abreu 2005). This results in a decrease in temperature which occurs faster near the radiating surface of the earth, causing a temperature inversion since temperature

increases with height above ground (Snyder and Melo-Abreu 2005). An example of a radiation frost and its effects in a plantation forest is represented in Figure 4: 1) night-time air is cooled by radiation heat loss; 2) the cold, dense air moves below the trees and 3) displaces the warm air, causing it to move upwards; 4) the displacement of the warm air by the cold air results in a warm inversion layer above the cold surface of the earth where ice crystals may form on the leaf surfaces of the trees (Taylor 2012). These radiation frost events tend to occur during early autumn or early spring, at points when plants are usually not fully cold acclimated (cold acclimation referring to the process by which plants increase their ability to withstand subsequent freezing temperatures in response to a period of low but non-freezing temperatures) (Norby *et al*. 2003, Woldendorp *et al*. 2008, Thomashow 1999, Xin and Browse 2000).

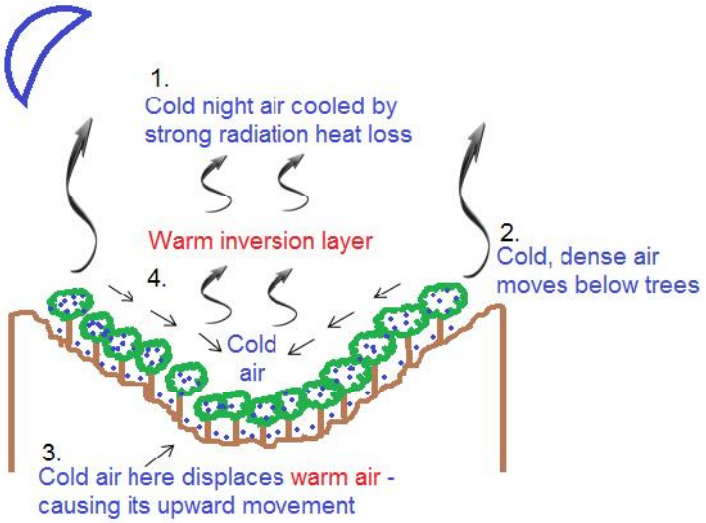

Figure 4. The process of a radiation frost event (Adapted from Taylor 2012).

1.3.2. Frost effects on forestry in South Africa

Commercial *Eucalyptus* plantations in Mpumalanga and KwaZulu-Natal have been affected to a great extent by erratic temperatures and radiation frosts brought upon by climate change (Blignaut *et al*. 2009, Germishuizen 2013, Koyro *et al*. 2012). Germishuizen (2013) reported that although the average temperatures in Mpumalanga did not change significantly between 1950 and 2007, there are records of temperatures being more erratic with serious consequences. For example, extensive damage was caused to commercial *Eucalyptus* plantations in 2011 and 2013 as juvenile eucalypts were exposed to extreme temperatures and a sudden onset of frost conditions (Figure 5, Sappi 2011, Kanzler and Maritz 2015).

a)  b) 

Figure 5. Frost damaged eucalypts in a) a *Eucalyptus nitens* plantation on 7 August 2011 in Mpumalanga, South Africa; and b) a trial plantation on 6 May 2013 in Howick, KwaZulu-Natal, South Africa (Sappi 2011, Kanzler and Maritz 2015).

1.4. Biological effects of low temperature and frost conditions on plants

Each plant has an optimum temperature range in which it performs best. However, when exposed to temperature extremes outside of this range, growth and development may be hampered (Tuteja 2009). In terms of a low temperature stress, there are two types of extremes: temperatures above freezing (i.e. non-freezing temperatures of 10°C – 15°C), that is termed 'chilling' for tropical plants; and below freezing (sub-zero temperatures), that is termed 'freezing' for tropical and temperate plants (Tuteja 2009). Tolerance to these conditions is usually gained through the process of cold acclimation, but, if the period of cold acclimation is insufficient or non-existent, damage to the plant may occur (Tuteja 2009).

Plant productivity in terms of metabolism and photosynthesis is often limited by extreme low temperatures, including unseasonal frost events when plants have not fully cold acclimated (Tuteja 2009, von Fircks and Verwijst 1993). These impacts include membrane destabilization in the case of chilling and freeze-induced cellular dehydration in the case of freezing (Tuteja 2009, von Fircks and Verwijst 1993). In both instances, essential growth and developmental processes in the plant are affected since most of these processes occur in membranous structures (Binder and Fielder 1996, Palta and Li 1980, Pearce 2001, Rizza *et al.* 2001, Wolfe and Bryant 1992). As a result, it is important to take note of the precise effects that chilling and freezing have on plant membranes.

1.4.1. Chilling and freezing effects on membranes

One of the earliest direct and detectible effects of an extreme alteration in temperature on plant cells is a change in membrane fluidity (Levitt 1980, Orvar *et al*. 2000). An increase in temperature results in increased membrane fluidity while a decrease in temperature results in reduced membrane

fluidity (Figure 6, Los and Murata 2004). The level of fluidity is also dependent on the degree of fatty acid unsaturation where the synthesis and incorporation of unsaturated fatty acids into the membrane serves to compensate for the decrease in membrane fluidity under low temperatures (Los and Murata 2004).

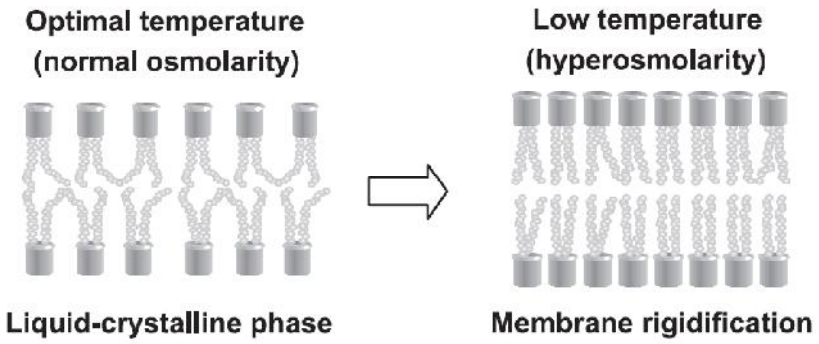

Figure 6. A schematic representation of the changes that occur in membrane structure and the behaviour of the lipid bilayers under low temperature stress (Taken from Los and Murata 2004).

Maintaining the precise level of fluidity is important to facilitate the movement of essential biomolecules in and out of the cell because the cell membrane itself is the interface between the internal workings of the plant and the external environment (Wolfe and Bryant 1992). The structure of the cell membrane is maintained and kept fluid by water in its liquid form (Wolfe and Bryant 1992). However, when plants are exposed to temperatures below their ability to prevent freezing, this liquid state is converted to an unfavourable ice form (Pearce 2001, Christersson 1971). When ice nucleation occurs – a process whereby water molecules come together to form a stable ice nucleus – certain parts of the plant freeze and are unable to avoid the continued formation and growth of ice (Pearce 2001).

A number of potentially lethal stresses occur as a result of freezing, including: osmotic, chemical, thermal and mechanical damage (Steponkus and Webb 1992). These effects are caused by intracellular ice crystal formation and freeze-induced cell dehydration (Steponkus and Webb 1992, Muldrew and McGann 1990, Pearce 2001). Extracellular ice crystal growth draws water out from the cells until the water potential of the ice and the cells are equal – resulting in freeze-induced dehydrated cells (Figure 7, Pearce 2001). However, the water potential of ice decreases as temperature decreases, therefore, cellular dehydration intensifies as temperature decreases (Figure 7, Farrant *et al.* 1977, Pearce 2001). Additionally, ice crystals could also puncture cell membranes, particularly plasma membranes, and key biomolecules held within these cells may consequently leak out (Figure 7, Pearce 2001, Steponkus and Webb 1992).

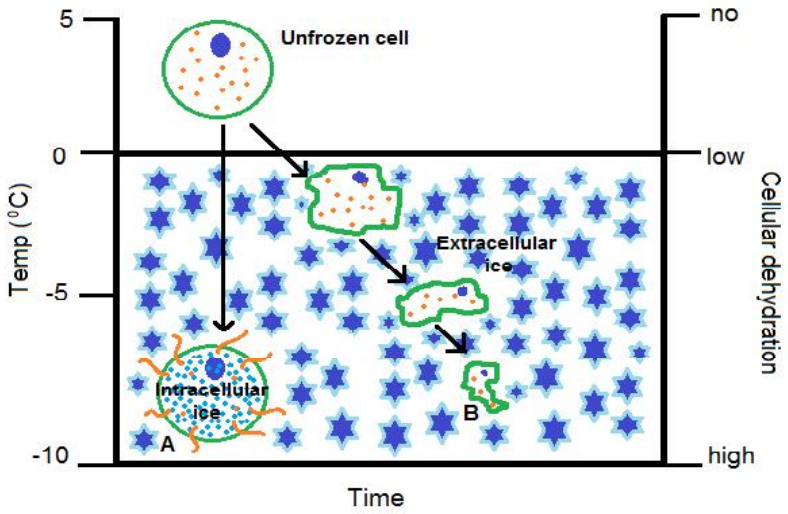

Figure 7. Extracellular and intracellular ice crystal formation as temperature decreases and plant cell dehydration increases over time. Key biomolecules are represented in orange (Adapted from Farrant *et al.* 1977).

The ultrastructure of membranes are altered in response to dehydration and are demonstrated by: 1) injury at a cellular level which entails the flux of bulk water (cytoplasmic and extracellular water that determines the osmotic homeostasis of a plant, the transport of important biomolecules, membrane fluidity and the rate of reactions) and large osmotic release (Hoekstra *et al.* 2001) and 2) the removal of water closely associated with membranes and other large biomolecules (Pearce 2001, Steponkus and Webb 1992). The fate of the cell is determined by the degree of freeze-induced dehydration where inadequate dehydration causes injury due to ice crystal formation and excessive dehydration causes injury due to membrane destabilization (Steponkus and Webb 1992).

In addition, the photosynthetic apparatus of the two photosystems in plants are predominantly membranous and are consequently also susceptible to the damages detailed above (Muldrew and McGann 1990, Pearce 2001, Rizza *et al.* 2001, Wolfe and Bryant 1992, Steponkus and Webb 1992). Damage or destabilization of chloroplast thylakoid membranes of photosystems I and II caused by low temperature or frost may, therefore, be measured by a change in chlorophyll fluorescence, a technique that is detailed further below (Binder and Fielder 1996).

1.5. Determining frost tolerance potential

Considering the type of damage that can be caused by extreme frost events and the consequent potential economic implications, low temperature and frost tolerance is clearly an important criterion to plant breeders in the forestry sector, particularly in South Africa. The industry now faces the issue of identifying frost tolerance characteristics of the selected GNs to help identify frost tolerant genotypes for possible plantation establishment. Part of the identification process includes screening

the selected GNs for low temperature and subsequent frost tolerance by determining their cold shock and simulated frost responses. Intrinsic to this determination however, is the understanding of how plants respond to abiotic stresses in general, and knowledge of the techniques that can be used in this determination is equally necessary.

1.5.1. Stress syndrome response of plants

The response of a plant to an abiotic stress is prompted through a series of biochemical alterations within the plant when certain biomolecules are up-regulated, or stress-specific signalling cascades and pathways are induced (Kaur and Gupta 2005). These processes are dependent on the type, intensity and duration of the stress (i.e., acute vs chronic), and the plant's tolerance or sensitivity to the stress (Lichtenthaler 1996). Lichtenthaler (1996) described the stress responses of plants in a graphical representation of the phase sequences and responses that are prompted when plants are exposed to a stress (Figure 8).

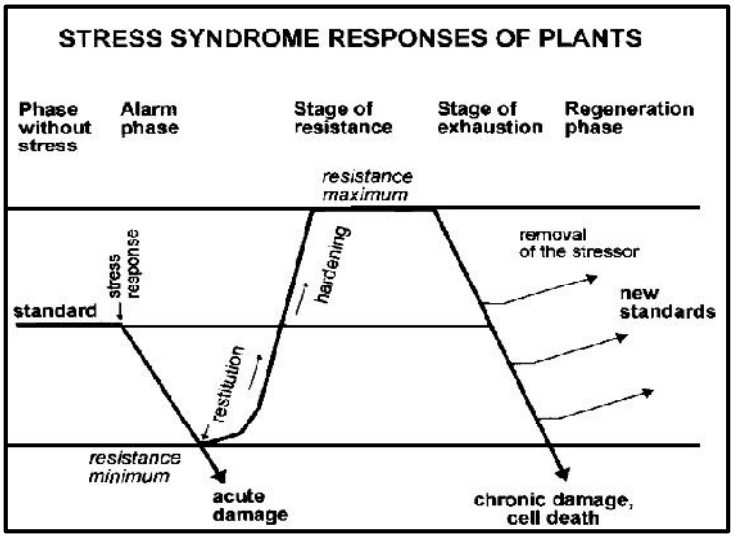

Figure 8. The general concept of the phase sequences and responses induced in plants by stress exposure (Adapted from Lichtenthaler 1996).

In this representation, 'Standard' refers to the normal physiological standard of a plant before the application of a stress (Figure 8, Lichtenthaler 1996). During the "Alarm phase", plants detect the applied stress through signalling (e.g. reactive oxygen species signalling) and react with a down-regulation of physiological functions, including a decline in metabolic and photosynthetic activities (Figure 8, Lichtenthaler 1996). The degree to which this occurs is dependent on the speed of, and extent to which the plant responds, based on the efficiency of the sensing, signalling and response mechanisms activated (Lichtenthaler 1996, Cramer *et al*. 2011, Li *et al*. 2011). These mechanisms determine if the plant is acutely damaged or has the ability to undergo "Restitution" under the applied stress conditions (Figure 8, Lichtenthaler 1996). Restitution refers to the restoration of previous

physiological functions of the plant before the stress was applied (Lichtenthaler 1996). Acute damage is likely to occur in plants with no or low stress response or tolerance mechanisms and these plants would consequently have a low 'Resistance minimum' (Figure 8, Lichtenthaler 1996). The resistance minimum of a plant is the ideal physiological stage the plant reaches under the application of the stress (Figure 8, Lichtenthaler 1996). If the stress persists beyond the coping mechanisms of the plant's resistance minimum, or if the plant does not respond to the extended application of the stress, further acute and even fatal damage may occur (Figure 8, Lichtenthaler 1996).

Some plants have the ability to activate stress coping mechanisms by alterations of metabolic fluxes and can activate certain repair processes if faced with an extended application of a stress (Figure 8, Lichtenthaler 1996). These repair processes and adaptations lead to the 'restitution' of the standard physiological functions and also 'harden' plants by establishing new physiological standards, corresponding to a 'Resistance maximum' (Figure 8, Lichtenthaler 1996). If there is a long-term application or overdose of the stress, a 'Stage of exhaustion' is reached when the physiology and the vitality of the plant progressively declines and sometimes causes cell death (Figure 8, Lichtenthaler 1996). However, if the stress is removed before senescence processes occur, the plant will transition into the 'Regeneration phase' and new physiological standards may be reached (Figure 8, Lichtenthaler 1996). It is important to note that a plant's ability to undergo these phase sequences depends on its inherent tolerance to the stress and the efficient activation of response mechanisms in reaction to the applied stress (Lichtenthaler 1996). These processes also aim to maintain membrane integrity as most metabolic and photosynthetic processes take place in membranous structures that are often compromised under the application of a stress (Lichtenthaler 1996).

1.6. Techniques to assess abiotic stress responses

There are a variety of techniques that can be employed to assess the responses of a plant to an abiotic stress that can be carried out at the molecular, genetic, physiological or biochemical level. With respect to assessing the cold shock responses of a plant, investigating the sensing, signalling and response mechanisms at the biochemical level may be one of the most useful approaches in determining the frost tolerance potential of the plant.

1.6.1. Sensing, signalling and response mechanisms

In the case of an acute stress such as a cold shock, the response time and levels of stress biochemicals regulated would differ based on the efficiency of the sensing, signalling and response mechanisms of the plant, which should also correspond with the plant's possible level of tolerance to low temperatures or frost (Cramer *et al*. 2011, Li *et al*. 2011, Lichtenthaler 1996). Some of the sensing, signalling and response biomolecules associated with cold stress in plants include reactive

oxygen species (ROS) (Miller *et al*. 2008), phenolic acids (PA) (Pennycooke *et al*. 2005), starch and total soluble sugars (TSS) (Yuanyuan *et al*. 2009). The efficiency with which these biomolecules are regulated may also determine how well cell membrane integrity is maintained (measured by relative electrolyte conductance (REC)) (Murray *et al*. 1989, Tsarouhas *et al*. 2000). This, in turn, also provides an indication and how the plants' photosynthetic processes (measured by chlorophyll fluorescence (CF)) perform during and post application of the stress (Binder and Fielder 1996, Rizza *et al*. 2001).

1.6.2. Reactive oxygen species (extracellular superoxide)

Oxygen has a high oxidizing ability that is required for its use in respiration and other chemical reactions during which 'reactive oxygen species' (ROS) are formed (Beckett *et al.* 2005). These radicles are highly reactive atoms or molecules that possess an unpaired electron that is available for donation (Beckett *et al*. 2005). The following radicles are classified as ROS: hydrogen peroxide, singlet oxygen, superoxide, nitric oxide radical and hydroxyl radical (Beckett *et al.* 2005). Biologically, ROS are constantly produced in chloroplasts, mitochondria and peroxisomes in plants during photosynthesis and respiration, however, certain abiotic stresses increase their production (Apel and Hirt 2004, Beckett *et al.* 2005). To identify the role of specific ROS, it is critical to first understand the various pathways through which ROS are generated.

1.6.2.1. Reactive oxygen species production pathways

When considering plants under temperature stress specifically, the production of ROS occurs through various pathways. A model detailing these possible pathways triggered by temperature stress has been proposed by Suzuki and Mittler (2006) (Figure 9). Pathway 1 involves the enhancement of ROS production within cells where cellular homeostasis and metabolic processes are disrupted by temperature stress (b). Pathway 2 involves temperature stress sensors (c) which sense the stress, leading to the enhanced production of ROS generation by respiratory burst oxidase homologs (Rboh) (d). Pathway 3 involves ROS sensors detecting ROS produced by stress-generated ROS (b) or Rboh-generated ROS (d), and ROS defense mechanisms (f) are then activated, or, ROS production is further enhanced (d) to strengthen the ROS signal. In Pathway 4, both ROS sensors (e) and temperature sensors (c) could activate the temperature defense pathway (g) that results in the activation of certain protective mechanisms and/or the ROS defense pathway (f) that results in ROS-scavenging activities. These pathways are activated during temperature stress, although, due to their converging nature, they suppress each other when the stress is removed or when the plant cells have achieved a new state of homeostasis, where it is able to tolerate the temperature stress (Suzuki and Mittler 2006). In the latter instance, the rate of ROS production is reduced (Suzuki and Mittler 2006).

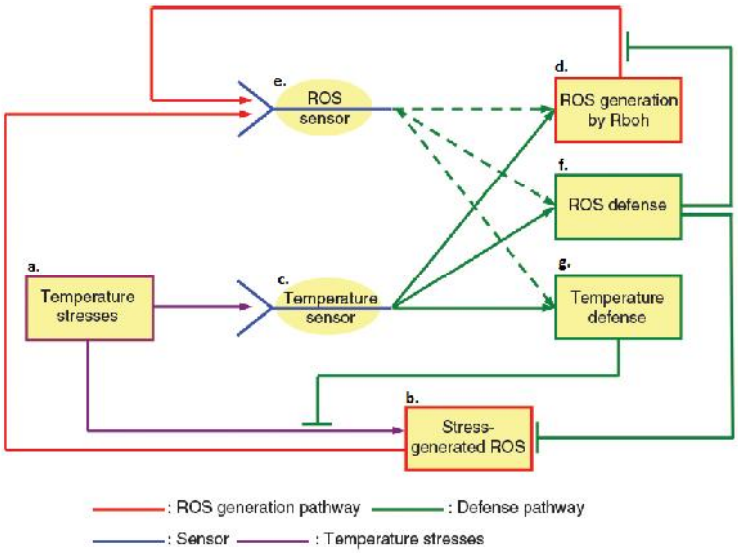

Figure 9. A proposed model for the involvement of reactive oxygen species (ROS) in the sensing of and protection from temperature stress (Rboh = respiratory burst oxidase homologs) (Adapted from Suzuki and Mittler 2006).

Furthermore, it is important to identify the site/s of ROS production as this determines the type of ROS produced, with each ROS playing a different role under certain stress conditions (Beckett *et al.* 2005). Sites of ROS production vary depending on the type, intensity and duration of the applied stress.

1.6.2.2. Intracellular and extracellular reactive oxygen species production

The major sources of ROS production in abiotically-stressed plants are at the sites of electron transport in chloroplasts and mitochondria (Apel and Hirt 2004, Mittler *et al.* 2004). In chloroplasts, a combination of limited CO_2 fixation and over-reduction of the electron transport chain are the primary cause of intracellular ROS production and in mitochondria, over-reduction of the electron transport chain is the primary cause of ROS production (Suzuki and Mittler 2006). Extracellular ROS is formed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, peroxidases, polyaminooxidases, diaminooxidases and laccases in the cell walls and plasma membranes (Beckett *et al.* 2005). High extracellular concentrations of ROS have the ability to increase intracellular ROS to toxic levels with the potential to damage DNA, lipids and proteins; however, this is controlled through the up-regulation of ROS scavenging mechanisms (Apel and Hirt 2004, e Silva *et al.* 2008). Despite their potential to disrupt molecular and physiological process, it has been discovered that ROS may also play a role in the activation of stress signalling mechanisms that are facilitated by the intricate balance of ROS production and scavenging (Apel and Hirt 2004, Miller *et al.* 2008, Suzuki and Mittler 2006).

1.6.2.3. The role of reactive oxygen species in abiotic stress signalling

The first level of ROS production occurs upon the perception of the stress when there is a peak in ROS, known as the 'oxidative burst' (Desikan *et al*. 2005, Suzuki and Mittler 2006). This peak serves as a secondary signalling mechanism to co-ordinate the plant's responses to the stress by initiating various signal transduction pathways (Desikan *et al*. 2005, Kaur and Gupta 2005, Miller *et al*. 2008). The activation of the stress-specific signal transduction pathways lead to the activation of certain genes that generate beneficial physiological and biological alterations in the plant (Desikan *et al*. 2005). These processes are diagrammatically represented in Figure 10 where the role of ROS in abiotic stress signalling is shown (Desikan *et al.* 2005).

The second peak in ROS occurs when extracellular ROS flood cellular compartments where antioxidants (molecules that scavenge ROS) are contained and there is a subsequent "overspill" of ROS into various regions of the cell (Figure 10, Desikan *et al*. 2005). This second peak serves to intensify the ROS signal if eliminated effectively, or, results in lipid peroxidation or the degradation of proteins and DNA (Apel and Hirt 2004, Pennycooke *et al*. 2005). Therefore, it is important that plants prone to abiotic stresses such as a cold shock or frost events have the ability to produce antioxidants to minimize or eliminate this type of ROS damage. The ability of a plant to regulate these activations effectively under the stressed conditions may be used as an indication of their level of possible tolerance to the applied stress. This ability is primarily determined by the regulation of antioxidants (e.g., PA) in the plant.

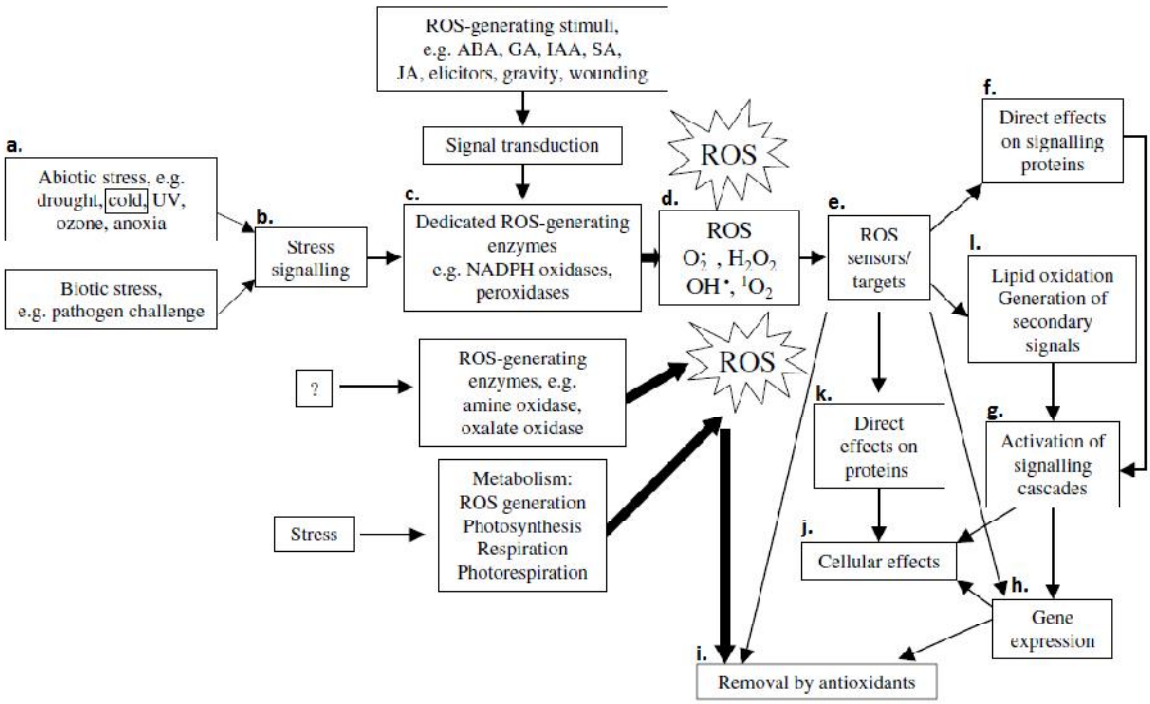

Figure 10. The role of reactive oxygen species (ROS) in signalling. The abiotic stress signalling pathways are referred to by letters a-l (Adapted from Desikan *et al*. 2005).

1.6.3. The role of phenolic acids in stress response

Plants contain a number of antioxidants with phenolic acids (PA) (phenylpropanoids) being one of the most effective because of their ability to absorb and neutralize ROS (Grace 2005, Rivero *et al*. 2001, Pennycooke *et al*. 2005). Phenolic acids are secondary metabolites that can be divided into four categories: tannins, hydroxycinnamic acids, flavonoids and anthocyanins (Ainsworth and Gillespie 2007, Blokhina *et al*. 2003). The structural chemistry of PA is ideal for the scavenging of free radicals, with their high reactivity as electron or hydrogen donors (Blokhina *et al.* 2003). This allows for these secondary metabolites to maintain the intricate balance of ROS in stressed plants (Pennycooke *et al.* 2005). Consequently, plants that are able to tolerate certain abiotic stress conditions have the ability to produce PA to scavenge ROS timeously and effectively, preventing subsequent oxidative damage (Pennycooke *et al*. 2005).

1.6.4. The role of starch in stress response

Starch is one of the major carbohydrates produced in spongy mesophyll, photosynthetic palisade cells, stomatal guard cells, epidermal cells, and bundle sheath cells of plants (Streb and Zeeman 2012). Leaf starch is usually produced during the day in large quantities and stored for utilization during the night as a source of carbohydrates in the absence of photosynthesis (Streb and Zeeman 2012). Starch accumulation under abiotic stress conditions is often classified as transitory where increase in starch levels occurs; such as during exposure to low temperature stress (Streb and Zeeman 2012). This transient accumulation serves to facilitate the 'normal' functioning of photosynthetic and metabolic processes under 'abnormal' conditions. Additionally, leaf starch also serves as a temporary store for assimilates that are metabolized into sugars under low temperatures by the process of carbohydrate metabolism (Ashworth *et al*. 1993, Bornke and Sonnewald 2011, Yuanyuan *et al*. 2009).

The physiological role of starch under normal and cold stress conditions in the chloroplast and mitochondria are represented in Figure 11 (Peng *et al.* 2015). Under normal conditions, CO_2 is produced from the tricarboxylic acid cycle during photorespiration in the mitochondrion (Figure 11, Peng *et al.* 2015). This CO_2 aids in the synthesis of starch in the chloroplast as a product of the Calvin-cycle (Figure 11, Peng *et al.* 2015). Starch is then catalysed into triose phosphate which is transported out of the chloroplast and used in the synthesis of sucrose (Figure 11, Peng *et al.* 2015). However, this carefully equilibrated process is disrupted under cold stress (Figure 11, Peng *et al.* 2015). When plants are exposed to low temperatures, photorespiration is inhibited, affecting the transport of triose phosphate and impeding the synthesis of sucrose (Figure 11, Peng *et al.* 2015). As a result, starch is accumulated in the chloroplast (Figure 11, Peng *et al.* 2015). In addition, it should

also be noted that a reduction in energy capture during photosynthesis caused by cold stress could decreases the rate at which starch is synthesized in the chloroplast (Figure 11, Peng *et al.* 2015).

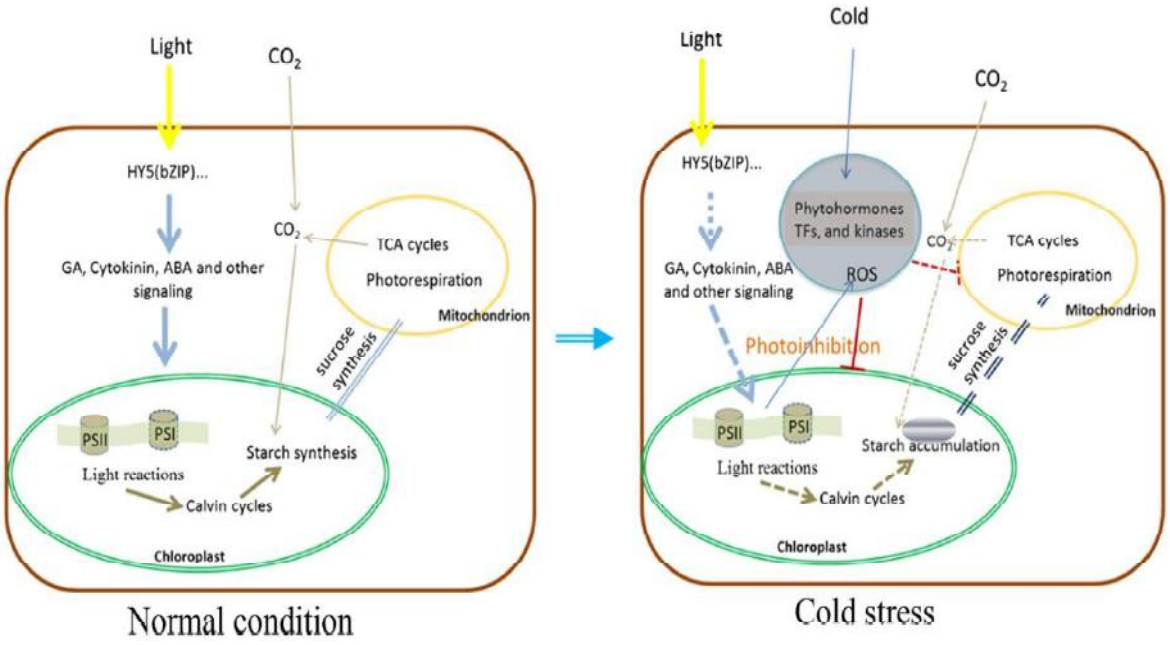

Figure 11. The physiological role of starch under normal and cold stress conditions in chloroplasts and mitochondria. Dashed lines represent slower processes and inverted T-shaped lines represent repressed physical processes (Taken from Peng *et al.* 2015).

1.6.5. The role of total soluble sugars in stress response

Total soluble sugars (TSS), such as glucose, fructose and galactose, play a number of roles in plants exposed to a low temperature stress. Total soluble sugar accumulation acts as a protective mechanism to reduce the susceptibility of cellular membranes to undergoing unfavourable low-temperature or cold-shock-induced non-bilayer phase formation, by contributing to the freeze tolerance of the membranes (Xin and Browse 2000, Yuanyuan *et al*. 2009). The bilayer formation of plant membranes is important as they facilitate the movement of essential biomolecules (Hoekstra *et al*. 2001). Being cryoprotectants, TSS also serve to lower the freezing point in plants when freeze-induced cellular dehydration might occur, thus lowering the temperature for the occurrence of ice nucleation (Xin and Browse 2000). Total soluble sugars also act as osmoprotectants by maintaining a favourable water potential gradient between the cell and the cytoplasm to ensure cell turgor which prevents the possible collapse of cells (Hoekstra *et al*. 2001). Total soluble sugars also inhibit the crystallization of important cellular compounds in the cytoplasm and prevent proteins from undergoing adverse conformational changes (Xin and Browse 2000). Recent studies have also focussed on the possible hormone-like role of TSS as signalling molecules where TSS may activate certain low temperature response genes (Xiao *et al.* 2009, Yuanyuan *et al.* 2009).

1.6.6. Chlorophyll fluorescence (*Fv/Fm*) as a measure of photosystem efficiency

Low temperatures affect photosynthetic processes by lowering stomatal conductance, decreasing the rate of thylakoid electron transport, reducing enzyme activity and carbon metabolism and decreasing the photochemical efficiency of photosystem II and membrane lipids (Campos *et al.* 2003, Suzuki *et al.* 2008). Huner *et al.* (1993) also note that the potential for an energy imbalance between metabolism, electron transport and photosynthesis is intensified by low temperatures. Low or freezing temperatures result in chloroplast thylakoid membrane damage or destabilization and this changes the way excitation energy of photosystem II is captured and consumed (Binder and Fielder 1996, Rizza *et al.* 2001, Roháček *et al.* 2008). Associated with this change in energy capture is a measurable change in leaf chlorophyll fluorescence (Binder and Fielder 1996, Rizza *et al.* 2001, Roháček *et al.* 2008).

When blue light with a short wavelength is absorbed by photosystem II, it excites chlorophyll to a higher energy state compared with the absorbance of red light with a longer wavelength (Figure 12) (Blankenship 2010, Taiz and Zeiger 2006). In this higher excited state, chlorophyll is exceedingly unstable and gives up some of its energy to its surroundings as heat and enters the lowest excited state (Figure 12) (Blankenship 2010, Taiz and Zeiger 2006). When chlorophyll enters this lowest excited state, it has four different pathways for disposing of its available energy and one of these pathways is known as 'fluorescence' (Figure 12) (Blankenship 2010, Taiz and Zeiger 2006). Chlorophylls fluoresce in the red region of the spectrum with a lower energy and longer wavelength compared with chlorophylls in the blue region of the spectrum that are in a higher energy state with a shorter wavelength (Figure 12, Taiz and Zeiger 2006).

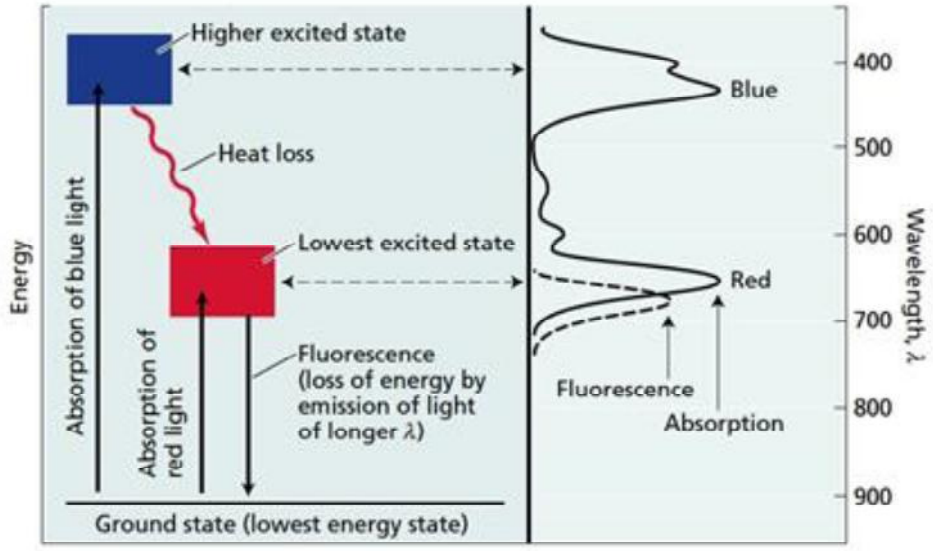

Figure 12. The absorption of light and electron excitation states of chlorophyll that relate to chlorophyll fluorescence (Taken from Taiz and Zeiger 2006).

In addition, photosynthetic electron transport and energy capture through lowered CO_2 fixation is reduced when plants are exposed to low temperatures which also correspond with a change in leaf chlorophyll fluorescence (Rizza *et al.* 2001). Consequently, there is a decrease in photochemical productivity and the efficiency of photosystem II is compromised (Binder and Fielder 1996, Rizza *et al.* 2001). The use of chlorophyll fluorescence to determine the effects of a variety of abiotic stresses on the photosynthetic efficiency of plants have been well documented (Baker 2008, Pietrini *et al*. 2005, Rizza *et al*. 2001). In particular, the maximum quantum yield of PSII photochemistry that can be measured by the ratio of variable fluorescence (*Fv*) to maximal fluorescence (*Fm*) in the dark-adapted state has been a widely used technique for determining cold-related stress-induced injury of photosynthesis in plants (Baker 2008, Pietrini *et al*. 2005, Rizza *et al*. 2001). According to Maxwell and Johnson (2000), *Fv/Fm* provides information about the processes underlying the altered quantum efficiency of PSII and dark-adapted *Fv/Fm* measurements are a particularly sensitive indicator of photosynthetic performance in plants.

Most species have an optimal *Fv/Fm* of photosynthetic performance of 0.85 and values lower than these are indicative of a stressed plant (Björkman and Demmig 1987, DeEll *et al.* 1999, Maxwell and Johnson 2000). Chlorophyll fluorescence may also indicate the period and efficacy of recovery of the plant after exposure to a cold shock and has been used as a measure of recovery in winter barley and a number of cereals (Dai *et al*. 2007, Rizza *et al*. 2001). Healthy chlorophyll fluorescence levels, after the application of the stress, would indicate that the plant may have the ability to adapt to the applied stress conditions or is possibly tolerant to the applied stress (Dai *et al*. 2007, Rizza *et al*. 2001).

1.6.7. Relative electrolyte conductance as a measure of electrolyte leakage

Membranes could well be the most important part of a plant since they surround organelles such as chloroplasts and mitochondria where vital processes, including photosynthesis and respiration are carried out. However, these structures are most susceptible to damage or destabilization caused by temperature extremes such as cold shocks or frost events (Wolfe and Bryant 1992). These types of injuries results in the leakage of cytoplasmic contents that could be detrimental to the plant, depending on the degree of damage, which may be quantified using the electrolyte leakage method (Murray *et al*. 1989, Tsarouhas *et al*. 2000).

The electrolyte leakage method is based on the understanding that the chemical composition of injured cells cannot be maintained when stressed and, consequently, electrolytes are released through damaged or destabilized membranes (Murray *et al*. 1989). The extent of damage or destabilization caused by a cold shock or frost may be determined by quantifying the conductivity of water extracts of excised leaf tissue (Murray *et al*. 1989). For example, water extracts of excised leaf tissue with

high conductivity readings may indicate that the leaf membranes of the selected tissue are damaged or 'leaky'.

1.7. Other investigations of *Eucalyptus* screening methods

There have been two major studies conducted on screening *Eucalyptus* species and hybrids thereof to determine their frost tolerance potential in the field: one conducted by Hodge *et al.* (2013) for Camcore, South Africa and another at the University of the Witwatersrand, Johannesburg, South Africa (Bahadur 2013, pers. comm.). Hodge *et al.* (2013) investigated the effects of freezing on whole plants of *Eucalyptus benthamii*, *Eucalyptus badjensis*, *Eucalyptus dunnii*, *E. grandis* and *Eucalyptus urophylla.* The plants were firstly hardened off in a dark cold room set between 4°C and 6°C for three nights and were then maintained at ambient room temperature during the day (Hodge *et al.* 2013). The plants were then placed in a freezer set from 0°C to -3°C over two hours with a 6 hour hold at -3°C (Hodge *et al*. 2013). Thereafter, the plants were raised back to 0^0C over two hours and lastly placed in a cold room set between 4°C and 6°C for one night (Hodge *et al.* 2013). The following day, the plants were returned to ambient room temperature, watered and a visual assessment regarding the degree of crown damage was made 5 days later (Hodge *et al.* 2013). The results indicated the following ranking in terms of descending frost tolerance: *E. badjensis*, *E. benthamii*, *E. dunnii*, *E. grandis* and *E. urophylla* (Hodge *et al.* 2013).

The university study involved an induced cold acclimation period, followed by a simulated frost event by freezing leaf samples. The experiment demonstrated that *E. grandis*, *E. nitens* and *E. grandis* x *E. nitens* (GN) hybrids have the ability to successfully cold acclimate and displayed characteristics that indicated possible tolerance to low temperatures and frost conditions after exposure to a prolonged cold acclimation period (Bahadur 2013, pers. comm.) In that study, cold acclimation potential was demonstrated through the use of various screening methods, including electrolyte conductance and chlorophyll fluorescence (Bahadur 2013, pers. comm.). Electrolyte conductance and chlorophyll fluorescence values revealed that the initial response of *E. grandis*, *E. nitens* and the GNs may have differed, although, their ultimate response at the end of the induced cold acclimation period was very similar (Bahadur 2013, pers. comm.).

It was concluded that exposure to a chronic stress, such as the induced cold acclimation period, may have served as a successful priming process for *E. grandis*, *E. nitens* and the GNs to aid in their survival in the field under low temperature or frost conditions (Bahadur 2013, pers. comm.). However, the drawbacks of using induced cold acclimation during the process of screening for frost tolerance were also established. These drawbacks included the lengthy 6-8 weeks acclimation period, the requirements of appropriate and reliable growth room facilities, and the prolonged exposure to an extended mild stress (a chronic stress). The latter issue (i.e., chronic stress) resulted in acclimated

seedlings that were damaged to a lesser extent when exposed to a simulated frost event, and thus, demonstrated the response of plants to a chronic stress rather than an acute stress such as a sudden, severe frost as experienced in the field. Therefore, it was suggested in that study that future investigations should focus on screening unacclimated eucalypts for their response to a sudden, severe frost (in the form of a cold shock) as this would be most appropriate under current and future field conditions in light of climate changes in South Africa. It was also recommended that analysis of the biochemical and physiological responses of the eucalypts to cold shock should be considered to understand the biological mechanisms underlying their responses. These suggestions, therefore, formed the basis of the present study.

1.8. Aim

The aim of this study was to investigate the frost tolerance potential of *E. grandis*, *E. nitens* and *E. grandis* x *E. nitens* hybrids (GNs) based on their biochemical and physiological responses to cold shock.

1.9. Objectives

There were three key objectives:

1) To determine the response mechanisms of *E. grandis*, *E. nitens* and the GNs based on the levels of the following variables, before and after the cold shock treatment:

a. Reactive oxygen species (ROS)
b. Phenolic acids (PA)
c. Starch
d. Total soluble sugars (TSS)
e. Chlorophyll fluorescence (CF)
f. Relative electrolyte conductance (REC).

2) To determine the recovery potential of *E. grandis*, *E. nitens* and the GNs after the cold shock treatment based on CF.

3) To determine the effects of the cold shock and simulated frost treatments on *E. grandis*, *E. nitens* and the GNs based on REC.

CHAPTER 2. METHODS AND MATERIALS

2.1. Maintenance of plant material

Eight different young *E. grandis* x *E. nitens* hybrid clones (Figure 13) established from cuttings and juvenile *E. grandis* (W. Hill ex Maiden, Figure 14) and *E. nitens* (H. Deane and Maiden, Figure 15) seedlings were supplied by Sappi Forests South Africa Limited and used for the experiment. The GNs and pure species were 6-8 months old and the pure species were established from bulk seed collections from various orchards in KwaZulu-Natal. The plants were maintained in the greenhouse at the University of the Witwatersrand, Johannesburg for the duration of the experiment.



Figure 13. Young *Eucalyptus grandis* x *Eucalyptus nitens* hybrid clones used for the study.



Figure 14. Juvenile *Eucalyptus grandis* seedlings used for the study.



Figure 15. Juvenile *Eucalyptus nitens* seedlings used for the study.

All eucalypts were treated with fungicides and fertilizers as stipulated in Table 1 throughout the experimental period and were watered daily at 6:00 and 18:00 for 15 minutes by a sprinkler system in the greenhouse.

Table 1. Nutrient and fungicide treatment of experimental material on a weekly (rotating) basis.

Week	Day of the week	Chemical
One	Wednesday	Nitrosol (2ml/L)
	Friday	Previcure (1ml/L)
Two	Tuesday	Calmag (0.3mg/L)
	Friday	Bravo (2ml/L)

2.2. Experimental design

Sixteen plants of *E. grandis*, *E. nitens* and 8 GN clones were used for the experiment and two leaves per plant were used for each biochemical assay. Plants were grown under standard conditions (Table 2) for 7 days and baseline levels of reactive oxygen species (ROS), chlorophyll fluorescence (CF) and relative electrolyte conductance (REC) were then measured. Leaves for PA, starch and TSS were also selected and freeze dried for subsequent biochemical analysis. Selected leaf samples were also exposed to a simulated frost in a programmable freezer (Table 2) and REC measures of unfrozen and frozen samples were taken. The temperature in the growth room was then decreased (Table 2) the following day and ROS was measured 30 and 90min into the cold shock at ±17°C and ±8°C respectively. 24 hours later ROS, CF and REC were re-measured and leaves for PA, starch and TSS were selected and freeze dried for subsequent biochemical analysis. The temperature in the growth room was then returned to standard conditions (Table 2) and CF was measured during the recovery period of 6 days.

Table 2. Temperature (°C), photoperiod (h) and duration of the experimental conditions.

Experimental conditions	Temperature (°C) and Photoperiod (h)	Duration
Standard	25°C day, 14°C night and 12h	7 days
Cold shock	5°C day, 5°C night and 12h	24 hours

Simulated frost	+2°C → -6°C at -4°C/h; 1h hold at -6°C; -6°C →+2°C at +4°C/h	5 hours
Recovery period	25°C day, 14°C night and 12h	6 days

2.3. Measurement of reactive oxygen species (extracellular superoxide) levels

The extracellular superoxide production assay was based on the principle of NADPH-mediated oxidation of epinephrine to adrenochrome (Beckett *et al.* 2003, Misra and Fridovich 1972). Electrons donated by $O_2^{\cdot -}$ was sequestered using epinephrine to form adrenochrome which was then estimated spectrophotometrically (Beckett *et al.* 2003, Misra and Fridovich 1972). Two whole leaves that were fully mature were selected from the midsection of the plant after 7 days under standard conditions, 30 and 90min into the cold shock (±17°C and ±8°C respectively) and 24h into the cold shock. The leaves were placed in test tubes filled with 15ml of 1mM epinephrine adjusted to pH 7 with 1M sodium hydroxide. These samples were then incubated at 25°C in the dark on a shaker (Gallenkamp Orbital Incubator, United Kingdom) set at 120rpm for 15min (Beckett *et al.* 2003, Misra and Fridovich 1972). Thereafter, the absorbance of the adrenochrome, representing the extracellular superoxide produced and released into the external environment of the sampled leaves, was determined at 480nm (Helio Thermo Scientific Spectrophotometer, USA) (Beckett *et al.* 2003, Misra and Fridovich 1972). The level of the superoxide produced was calculated by using the adrenochrome molar extinction coefficient of 4020M/cm (Misra and Fridovich 1972). Hence, this measurement of superoxide is representative of the extracellular superoxide concentration released into the external environment of the selected leaves.

2.4. Measurement of total phenolic acid levels

For the extraction of total phenolic acids (PA), the procedure of Tabart *et al*. (2009) was followed. Phenolic acids were extracted by adding 0.1g of the leaf sample (two leaves per plant), ground with a pestle and mortar with liquid nitrogen, into 12.50ml of 95% ethanol. These samples were then placed on a shaker (Labcon, South Africa) at 250rpm in a cold room at 8°C for 3h. Thereafter, 5ml of the extract was centrifuged (Hettich Universal 320, United Kingdom) at 4000rpm for 15min. The resulting filtrate was then used in the colorimetric quantification of total PA.

The procedure for the colorimetric determination of total PA was based on the Folin-Ciocalteau (FC) method with use of a gallic acid equivalent (GAE) standard as detailed by Tabart *et*

al. (2009), Torti *et al.* (1995) and Waterman and Mole (1994). The GAE standard was preferentially selected based on its extensive use for determining total PA in various plant species (Ainsworth and Gillespie 2007, Hou *et al.* 2003, Pennycooke *et al.* 2005, Waterman and Mole 1994), including other *Eucalyptus* species (Vázquez *et al.* 2008). A GAE standard series was firstly established for the range 10-50mg/l at 10mg/l increments. At each standard concentration, triplicates were measured and from each of the three replicates, 1ml of the sample filtrate was used in the assay. To the 1ml of filtrate, 1ml of 95% ethanol, 5ml of distilled water and 0.5ml of 50% FC Reagent was added. The samples were then completely mixed and allowed to incubate for 5min. Thereafter, 1ml of 5% sodium carbonate was added and the samples were mixed on a vortex mixer (MRC Model VM-1000, Israel). The samples were then placed in the dark and allowed to incubate for 1h at room temperature (±24°C). The absorbance of the resultant solution was measured at 760nm (Helio Thermo Scientific Spectrophotometer, USA). Total PA levels were then calculated using the GAE standard and were represented as a GAE in the units of mg GAE/g of leaf on a dry weight basis.

2.5. Measurement of starch and total soluble sugar levels

Starch (*D*-glucose and/or maltodextrins) was extracted according to the methods of Chow and Landhäusser (2004). Two leaves per plant were ground with liquid nitrogen with a pestle and mortar and were then placed in 5ml of 80% ethanol and boiled at 95°C for 10min, and then centrifuged at 2500rpm for 5min. This ethanol extraction and centrifugation was performed twice to ensure sufficient removal of water-alcohol soluble compounds from the leaf tissue to eliminate any dilution of the readings. The centrifugation resulted in a liquid supernatant that contained TSS and a precipitate (pellet) that contained starch. The supernatants from both extractions were used for the TSS analysis and the pellet was used for the starch analysis.

2.5.1. Measurement of starch levels

The Megazyme Total Starch Assay Procedure (Megazyme, Ireland) was used for the colorimetric determination of starch. Starch within the extracted pellet was hydrolysed by adding 3ml thermostable α-amylase and incubated at 100°C for 12min. Incubation involved the mixing of samples on a vortex mixer every four minutes to ensure a homogenous solution. The samples were then transferred to a 50°C water bath and 0.1ml of amyloglucosidase was added. Thereafter, the samples were stirred on a vortex mixer, and were left in a 50°C water bath for 30min. The samples were then transferred to a 10 ml test tube, which was filled to volume with distilled water. The samples were then centrifuged at 3000rpm for 10min and 0.1ml of this extract was combined with 3ml of GOPOD (glucose oxidase plus peroxidase and 4-aminoantipyrine) and was incubated at 50°C in a water bath for 20min. The absorbance of the samples was read at 510nm (Helio Thermo Scientific Spectrophotometer, USA) and the method detailed in the Megazyme Assay Procedure Booklet was

used to calculate the starch content of the samples expressed as mg of starch/g of leaf on a fresh weight basis.

2.5.2. Measurement of total soluble sugar levels

As prescribed by the methods of Chow and Landhäusser (2004), a mixture of glucose (30%), fructose (50%) and galactose (20%) (GFG) was used as a standard for the colorimetric determination of TSS. A GFG dilution series was prepared for the range 25-200mg/l, at 25mg/l increments with triplicates at each of the standard concentrations. The supernatant which was collected during the starch extraction process (detailed above) was used for the determination of TSS. 1ml 2% (w/v) phenol and 2.5ml 95.50% sulphuric acid was added to 1ml of the supernatant and another set of samples were prepared at the same time by adding water instead of phenol; representing samples without phenol. All samples were incubated at room temperature in the dark for 10min and then placed in a 22°C water bath for 30min. The absorbance was read at 490nm (Helio Thermo Scientific Spectrophotometer, USA) and the corrected TSS measures were expressed as mg GFG/g of leaf on a fresh weight basis, using the following equation:

$$[sugar]_{corrected} = \frac{A - A'}{a_s - a'_s}$$

where:

A: absorbance with phenol; A': absorbance without phenol; a_s: absorbance with phenol/TSS with phenol; a'_s: absorbance without phenol/TSS without phenol.

2.6. Measurement of chlorophyll fluorescence

The chlorophyll fluorescence of each eucalypt was measured with an Opti-Sciences OS5 Modulated Fluorometer (USA) using the dark-adapted method (Genty *et al.* 1989). Two whole mature leaves were selected from the midsection of the plant and were dark adapted by placing a clip with a dark screen on the middle of each leaf for one hour (Figure 16).

Chlorophyll fluorescence measurements were taken at the same time every day (10h30 – 11h30) for each eucalypt over the experimental period. The ratio of variable fluorescence (F_v) to maximal fluorescence (F_m) was calculated as follows:

$$F_v/F_m = [(F_m - F_o)/F_m]$$

where:

F_v = variable fluorescence; F_m = maximal fluorescence; F_o = minimal fluorescence.



Figure 16. The clip of the fluorometer attached to the leaf of a eucalypt.

In addition, CF levels of each eucalypt were averaged over the recovery period of 6 days and a ranking of their recovery potential was established, where 1 = highest recovery potential and 10 = lowest recovery potential.

2.7. Exposure of plant material to a simulated frost

In addition to the cold shock at 5°C, leaf samples from each eucalypt were also exposed to a controlled freezing process to simulate a frost event. Four leaves from each plant were selected – two for unfrozen and two for frozen samples. For the samples that were exposed to the simulated frost, two leaf discs (20mm^2) per leaf were cut using a punch and placed into cryovials as prescribed by Raymond *et al.* (1986). The cryovials were transferred to a Planer MRV Kryo 360-1.7 programmable freezer (United Kingdom) which was set to freeze the samples from 2°C to -6°C at a rate of -4°C /h with a hold at -6°C for 1h, and at a thawing rate of +4°C /h from -6°C to 2°C (Figure 17). Thereafter, the samples were placed in 5ml of ultrapure water and left overnight, along with the unfrozen samples that were similarly incubated in 5ml of ultrapure water, under standard growth room conditions of 25°C and 14h of light per day.

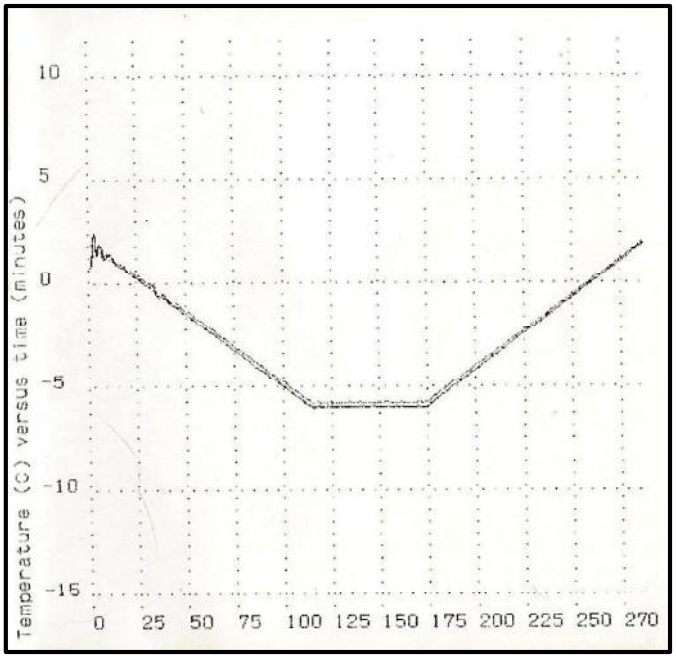

Figure 17. Temperature profile of a simulated frost event (Temperature (°C) vs Time (minutes)) generated with a Planer MRV Kryo 360-1.7 programmable freezer (United Kingdom).

2.8 Measurement of the electrolyte conductance of leaf leachate

Electrolyte conductance readings were taken according to the methods described by Tibbits and Reid (1987) using a Reid and Associates CM 100-2 Multiple Cell Conductivity Meter (South Africa) as follows:

Day 1: One day after freezing, 1ml of the leachate (from the test tubes containing the leaf discs with 5ml of ultrapure water) was pipetted into the electrolyte conductivity machine that measured the specific conductance of the leachate from the unfrozen and frozen samples. The test tubes with the leaf discs and remaining leachate were then boiled at 100°C on a Gemmyco DB-006E dry block heater (Taiwan) for one hour to heat destroy the samples. These samples were then left overnight under standard growth room conditions of 25°C and 14h of light per day.

Day 2: 1ml of the leachate from the denatured samples was pipetted into the electrolyte conductivity machine to measure the specific conductance of the leachate from unfrozen and frozen heat killed samples, representing the total electrolyte conductance of the samples.

Relative electrolyte conductance (%) was calculated under standard and cold shock conditions for both unfrozen and frozen samples as stipulated by Tibbits and Reid (1987) as follows:

$$REC\ (\%) = (EC_i/EC_t) \times 100$$

where:

EC_i = specific electrolyte conductance of the leachate from samples left overnight under standard growth room conditions of 25°C and 14h of light per day;

EC_t = specific electrolyte conductance of the leachate from samples heat-killed at 100°C for one hour and then left overnight under standard growth room conditions of 25°C and 14h of light per day.

In addition, a change in REC (represented as a %) was calculated by determining the difference in REC levels of unfrozen and frozen samples under cold shock conditions. These differences were used to rank the eucalypts in terms of frost tolerance potential where 1 = most tolerant and 10 = least tolerant.

2.9. Data analysis

Descriptive and inferential statistics were performed using STATISTICA version 12.5 (StatSoft Inc. 2015) and R version 3.12 (R Development Core Team 2014). Shapiro-Wilk normality tests were performed across all parameters (ROS, PA, starch, TSS, CF and REC). To test for significant differences in ROS, PA, starch, TSS, CF and REC between the 10 eucalypts under each treatment level for each biochemical (e.g. for ROS under standard conditions, 30min into the cold shock, 90min into the cold shock and 24h into the cold shock), one-way ANOVA tests were performed on data with a normal distribution and, if after applying $\log_{10}$, natural log and Box-Cox transformations data remained not normally distributed, then Kruskal-Wallis tests were performed. To test for significant differences in ROS, CF and REC within each eucalypt, one-way ANOVA tests were performed on data with a normal distribution and, if after applying $\log_{10}$, natural log and Box-Cox transformations data remained not normally distributed, then Kruskal-Wallis tests were performed. To test for significant differences in PA, starch and TSS within each eucalypt between standard and cold shock conditions, Students t-tests were used for normally distributed data and, if after applying $\log_{10}$, natural log and Box-Cox transformations data remained not normally distributed, then Wilcoxon ranked tests were used. A 95% confidence interval was set for all statistical tests.

Principal component analyses (PCA) were also conducted in STATISTICA (StatSoft Inc 2015) for the dataset under standard and cold shock conditions to determine if any of the measured parameters (ROS, PA, starch, TSS, CF and REC) showed a high degree of correlation. A PCA transforms the selected parameters into a number of uncorrelated variables known as ‘principal components’ (Abdi and Williams 2010). The first principal component accounts for the greatest amount of variability in the data, with each succeeding component accounting for the residual variability respectively (Abdi and Williams 2010). The principal components are described as: ‘linear

combinations of the original variables, weighted by their contribution to explaining the variance in a particular orthogonal dimension' (Abdi and Williams 2010).

Factor analyses were then used to assess the variation among the correlated variables and to determine which factors contributed most to the observed variance. The number of factors is equal to the number of variables where each factor accounts for a certain proportion (%) of the overall observed variance (Arena *et al.* 2015). Factor loadings (≥0.7 is considered a heavy loading) were then used to represent the contributions of each measured variable in explaining the variance of the factors.

A component weight plot was then created where the circular unit within each plot provided a visual indication of how well each variable is characterised by the principal components. Each variable (ROS, PA, starch, TSS, CF and REC) is represented by a vector and the angle formed between each variable estimates their level of similarity (or dissimilarity). Angles that are $< 90^0$ indicate a positive relationship; angles that are $> 90^0$ indicate a negative relationship and orthogonal angles (i.e. angles = 90^0) indicate that the variables are linearly independent (i.e. r is close to 0).

Lastly, a case factor coordinate plot was created where each eucalypt was grouped according to Factor 1 and Factor 2 (with each factor created according to the variability contributed by each measured parameter) under standard and cold shock conditions. Eucalypts that are grouped together exhibit similar characteristics and eucalypts that are further away from the groupings may be considered 'unique' according to the PCA.

CHAPTER 3. RESULTS

3.1. Reactive oxygen species levels under standard conditions and 30min, 90min and 24h into the cold shock

The extracellular ROS levels of the eucalypts were variable under standard conditions, 30-90min into the temperature decrease from 25°C to 5°C, and at 24h at 5°C (Figure 18). There was an up-regulation of ROS in *E. grandis*, GN 1, GN 4, and GN 6 30-90min into the cold shock (Figure 18). This up-regulation was seen by a peak in ROS levels 90mins into the cold shock when compared with levels under standard conditions as follows: *E. grandis* increased from 79.73 to 152.03μg adrenochrome/g leaf (FWB); GN 1 increased from 120.96 to 199.40μg adrenochrome/g leaf (FWB); GN 4 increased from 240.04 to 316.89μg adrenochrome/g leaf (FWB); and GN 6 increased from 94.72 to 212.98μg adrenochrome/g leaf (FWB) (Figure 18).This indicated the possibility of ROS signalling in these eucalypts. The changes in ROS levels in *E. grandis*, GN 1 and GN 6 were found to be significant ($p<0.05$) at 30 and/or 90 minutes into the cold shock when compared with levels under standard conditions in each case (see Appendix 21, 22 and 27).

The ROS levels were the highest in *E. nitens* (181.34μg adrenochrome/g leaf (FWB)), GN 3 (294.14μg adrenochrome/g leaf (FWB)), and GN 7 (223.40μg adrenochrome/g leaf (FWB)) only 24h after exposure to the cold shock (Figure 18). This indicated the possibility of a different cold shock signalling mechanism to ROS in these eucalypts. Further, these levels were found to be significantly different ($p<0.05$) when compared with levels under standard conditions and 30 and 90min into the cold shock (see Appendix 24, 28 and 30).

GN 2 and GN 8 elicited an erratic ROS response pattern with initially high ROS levels (175.69 and 216.53μg adrenochrome/g leaf (FWB) respectively) under standard conditions, followed by a significant 74% ($H(3) = 57.751$, $p<0.05$) and 91% ($H(3) = 15.014$, $p<0.05$) decrease in ROS 30mins into the cold shock (Figure 18). At 90min into the cold shock, ROS levels were the highest in these two GNs, however, at 24h exposure to the cold shock levels decreased again (Figure 18).

When comparing ROS levels under standard and cold shock conditions, differences were found to be significant ($p<0.05$) in GN 2, GN 3, GN 6, GN 7 and *E. nitens* (see Appendix 23, 24, 27, 28 and 30). The ROS levels of GN 5 appeared unaffected by the cold shock (Figure 18). It was also noted that absolute ROS levels were generally higher in all of the GNs when compared with the pure species, particularly *E. nitens* (Figure 18). Statistically significant differences of ROS levels between each eucalypt over the experimental period can be found in Appendix 1-4 and within each eucalypt over the experimental period in Appendix 21-30.

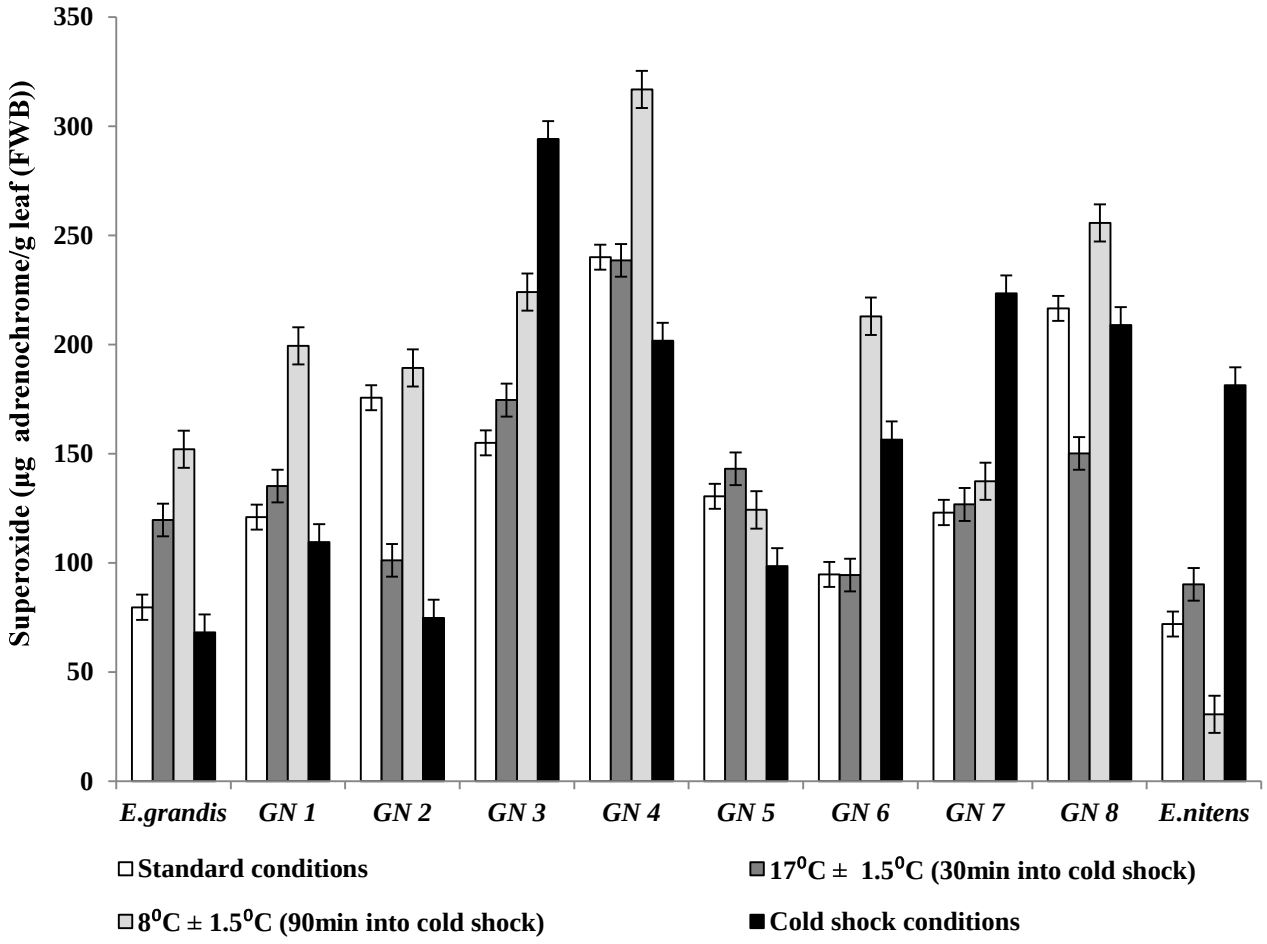

Figure 18. Extracellular reactive oxygen species (ROS) (superoxide: µg adrenochrome/g leaf (FWB)) levels of each eucalypt over the experimental period. Data represented are means ± standard error. Standard conditions: 7 days 25°C /14°C day/night and 12h photoperiod; Cold shock conditions: 24hrs 5°C /5°C day/night and 12h photoperiod.

3.2. Total phenolic acid levels under standard and cold shock conditions

Two trends were observed in the total PA levels amongst the cold shocked eucalypts where levels either noticeably decreased or increased. Total PA levels of *E. grandis* and GN 3 decreased by a significant 22% ($V=791$, $p<0.05$) from 7.32 to 5.73mg GAE/0.05g leaf (DWB) and 18% ($V=528$, $p<0.05$) from 8.22 to 6.74mg GAE/0.05g leaf (DWB) respectively under cold shock conditions (Figure 19). Conversely, total PA levels of *E. nitens*, GN 4, GN 5 and GN 7 increased by 26%, 58%, 24% and 20% to 8.01, 6.15, 6.78 and 7.60mg GAE/0.05g leaf (DWB) respectively under cold shock conditions (Figure 19). These increases were also found to be significant ($p<0.05$, Appendix 31). It was also noted that the increase in total PA levels of GN 4 from 3.88 to 6.15mg GAE/g leaf was correlated with a marked decrease in ROS levels under cold shock conditions in this GN (Figures 18 and 19). Total PA levels of GN 2, GN 6 and GN 8, on the other hand, were not significantly altered by the cold shock ($p>0.05$, Appendix 31, Figure 19). Statistically significant differences in PA levels between the eucalypts under standard and cold shock conditions can be found in Appendix 5 and 6.

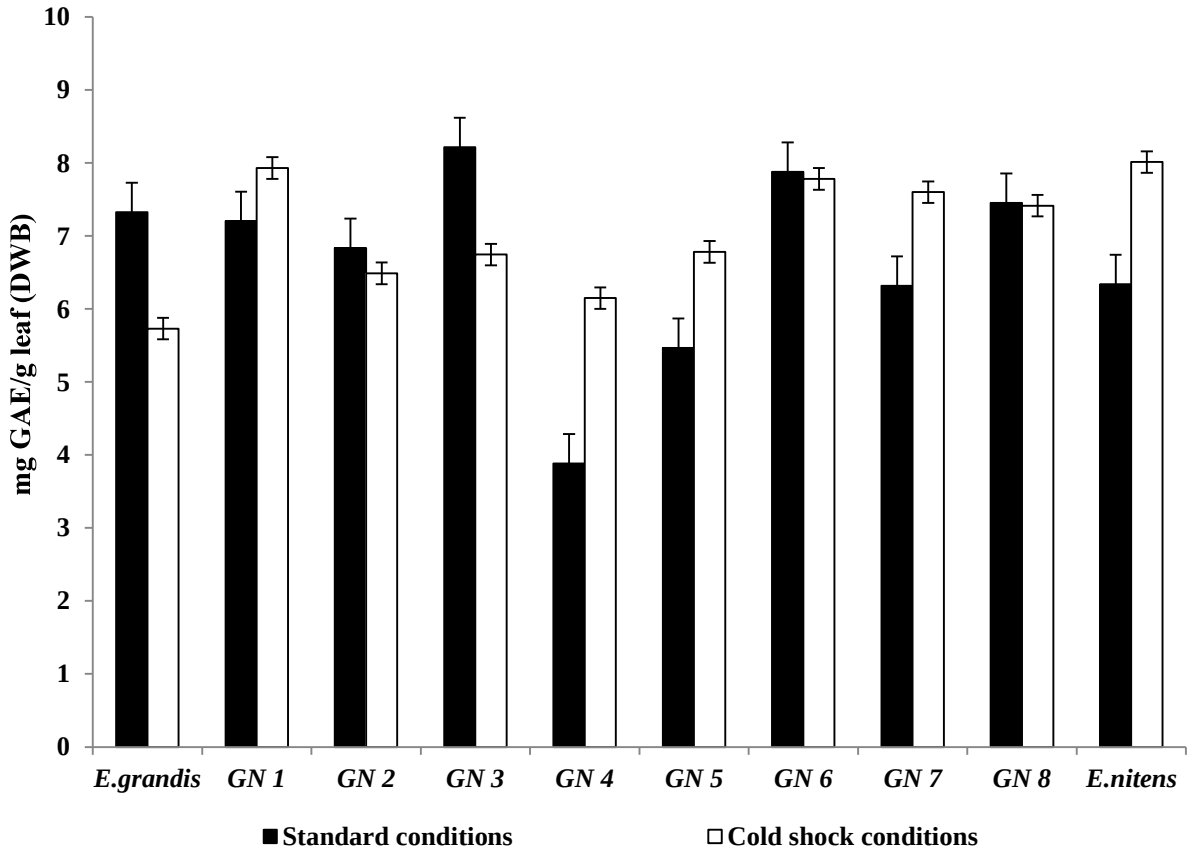

Figure 19. Total phenolic acid (PA) (mg GAE/g leaf (DWB)) levels of each eucalypt over the experimental period. Data represented are means ± standard error. Standard conditions: 7 days 25°C /14°C day/night and 12h photoperiod; Cold shock conditions: 24hrs 5°C /5°C day/night and 12h photoperiod.

3.3. Starch levels under standard and cold shock conditions

Starch levels of the eucalypts varied only slightly between standard and cold shock conditions (Figure 20). Although not statistically significant ($p>0.05$), marked differences in starch levels were observed in GN 3, GN 6 and GN 7 where levels: decreased by 22% from 23.67 to 18.46mg starch/g leaf (FWB), increased by 33% from 11.26 to 15.02mg starch/g leaf (FWB) and decreased by 25% from 20.86 to 15.74mg starch/g leaf (FWB) respectively under cold shock conditions (Figure 20, Appendix 32). It was also noted that absolute levels of starch were lowest in *E. nitens* under both standard (3.22mg starch/g leaf (FWB)) and cold shock conditions (4.03mg starch/g leaf (FWB)) when compared with the other eucalypts (Figure 20). Statistical differences in the starch levels between the eucalypts and within each eucalypt under standard and cold shock conditions can be found in Appendix 7 and 8.

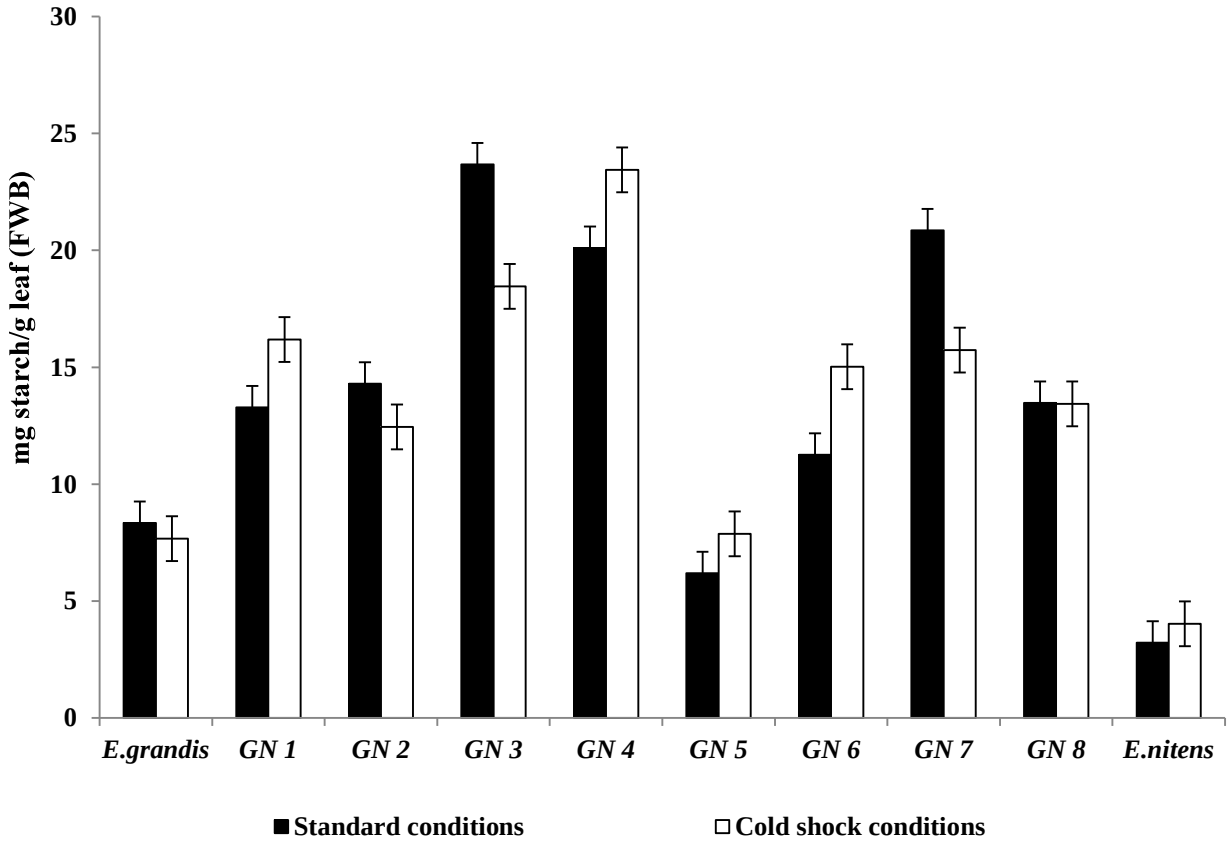

Figure 20. Starch (mg starch/g leaf (FWB)) levels of each eucalypt over the experimental period. Data represented are means ± standard error. Standard conditions: 7 days 25°C /14°C day/night and 12h photoperiod; Cold shock conditions: 24hrs 5°C /5°C day/night and 12h photoperiod.

3.4. Total soluble sugar levels under standard and cold shock conditions

The trends in TSS levels differed considerably among the control (standard conditions) and experimental (cold shock) eucalypts, with *E. grandis* and 3 GNs eliciting the greatest change in TSS levels under cold shock conditions (Figure 21). Total soluble sugar levels of *E. grandis* increased significantly ($Z=0$, $p<0.05$) by 201% from 16.09 under standard conditions to 48.41mg TSS/g leaf (FWB) under cold shock conditions (Figure 21). Similarly, TSS levels of GN 6 increased substantially ($Z=17$, $p<0.05$) by 409% from 4.10 under standard conditions to 20.87mg TSS/g leaf (FWB) under cold shock conditions (Figure 21). Conversely, TSS levels of GN 2 and GN 3 decreased by 41% from 43.41 under standard conditions to 25.66 mg TSS/g leaf (FWB) under cold shock conditions, and 76% from 33.47 to 7.87mg TSS/g leaf (FWB) under cold shock conditions respectively (Figure 21). The 76% decrease in GN 3 was found to be statistically significant ($Z=75$, $p<0.05$). The absolute TSS levels of *E. nitens* were much lower under both standard and cold shock conditions (11.41 and 8.22mg TSS/g leaf (FWB) respectively) when compared with the other eucalypts (Figure 21). Statistical differences in the TSS levels between the eucalypts and within each eucalypt under standard and cold shock conditions can be found in Appendix 9 and 10.

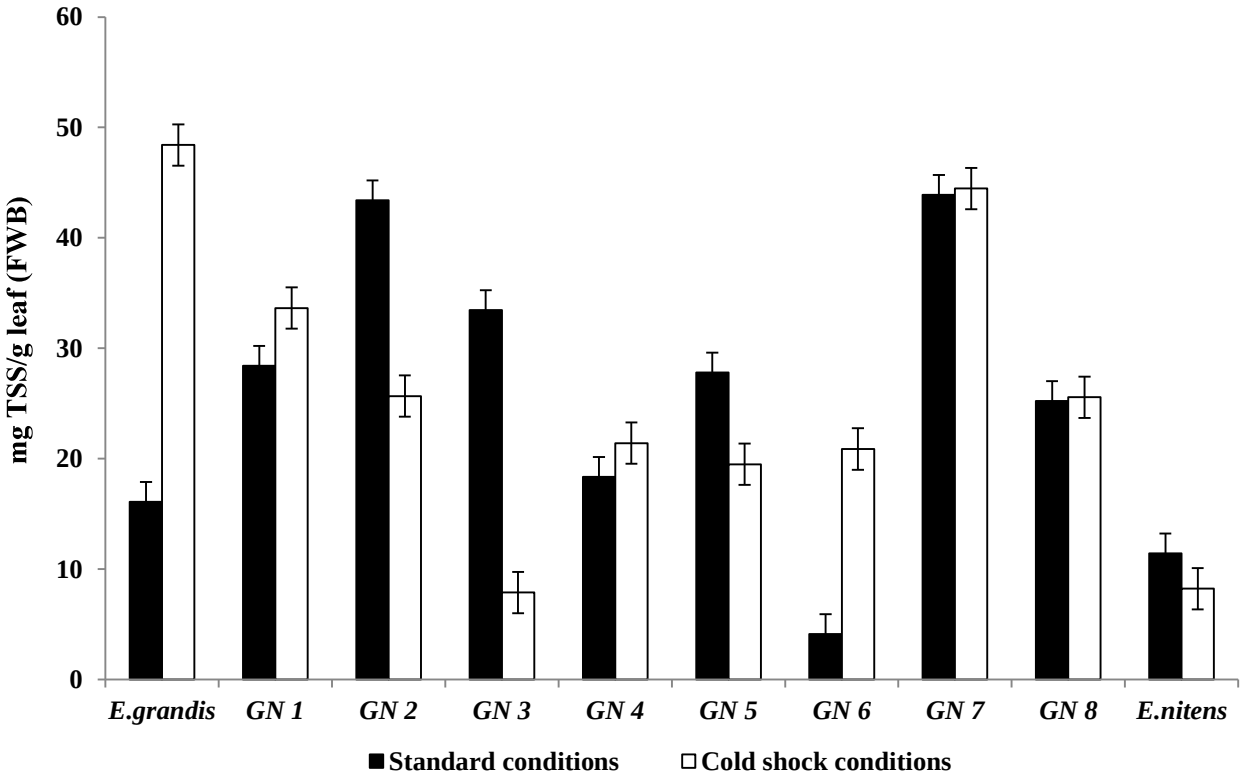

Figure 21. Total soluble sugar (TSS) (mg TSS/g leaf (FWB)) levels of each eucalypt over the experimental period. Data represented are means ± standard error. Standard conditions: 7 days 25°C /14°C day/night and 12h photoperiod; Cold shock conditions: 24hrs 5°C /5°C day/night and 12h photoperiod.

3.5. Chlorophyll fluorescence levels under standard and cold shock conditions and during the recovery period

The photosystems of half of the eucalypts were unaffected by the cold shock when considering CF (*Fv/Fm*) levels, with *E. nitens* and 4 GNs displaying notably altered CF levels (Table 3). The photosystems of *E. nitens*, GN 3 and GN 7 appeared to have been the most affected by the cold shock, with CF levels decreasing to 0.57, 0.55, and 0.70 respectively under these conditions (Table 3), although, the decrease in CF levels only of GN 3 was found to be statistically significant ($H(5) = 56.111$, $p<0.05$). Interestingly, CF levels of GN 8 increased significantly ($H(5) = 56.111$, $p<0.05$) from 0.47 ± 0.43 under standard conditions to 0.87 ± 0.17 under cold shock conditions (Table 3).

The photosystems of *E. nitens* and GN 7 appeared to recover from the cold shock, with CF increasing to levels greater than 0.80 during the recovery period (Table 3). On the other hand, CF levels of GN 3 were found to be less than 0.80 during the recovery period, indicating that the photosystems of this GN were unable to recover from the cold shock optimally (Table 3). During the recovery period, the photosystems of GN 2 and GN 6 appeared most unstable with CF levels decreasing to lows of 0.63 and 0.37 respectively on day 2 (Table 3). This indicated that these two GNs may have been affected by the cold shock but were capable of eliciting a delayed response.

Further, under standard conditions, the average CF levels of all of the tested GNs (0.76 ± 0.13) was not markedly different to either parent species. However, under cold shock conditions, the average CF levels of the GNs (0.77 ± 0.10) was closer to the CF levels of *E.grandis* (0.86 ± 0.05) compared with *E.nitens* (0.57 ± 0.34) (Table 3). Statistically significant differences in CF levels between each eucalypt under standard conditions, cold shock conditions and during the recovery period can be found in Appendix 11 – 16 and within each eucalypt under standard conditions, cold shock conditions and during the recovery period in Appendix 34 – 43.

Table 3. Chlorophyll fluorescence (CF) (*Fv/Fm*) levels of each eucalypt over the experimental period. Data represented are means ± standard deviation. Standard conditions: 7 days 25°C /14°C day/night and 12h photoperiod; Cold shock conditions: 24hrs 5°C /5°C day/night and 12h photoperiod; Recovery period: 6 days 25°C /14°C day/night and 12h photoperiod. *indicates that CF levels under cold shock conditions were statistically significantly different to CF levels under standard conditions.

Eucalypt	Standard conditions	Cold shock conditions	Recovery period			
			Day 1	Day 2	Day 5	Day 6
E. grandis	0.85 ± 0.13	0.86 ± 0.05	0.88 ± 0.05	0.82 ± 0.20	0.79 ± 0.26	0.90 ± 0.27
GN 1	0.78 ± 0.25	0.86 ± 0.10	0.86 ± 0.06	0.68 ± 0.35	0.87 ± 0.14	0.88 ± 0.04
GN 2	0.78 ± 0.17	0.80 ± 0.21	0.82 ± 0.14	0.63 ± 0.35	0.84 ± 0.14	0.64 ± 0.30
GN 3	0.84 ± 0.16	0.55 ± 0.32*	0.90 ± 0.02	0.76 ± 0.21	0.73 ± 0.12	0.75 ± 0.06
GN 4	0.85 ± 0.06	0.78 ± 0.28	0.87 ± 0.04	0.87 ± 0.15	0.87 ± 0.02	0.86 ± 0.05
GN 5	0.80 ± 0.12	0.79 ± 0.10	0.83 ± 0.14	0.85 ± 0.14	0.86 ± 0.07	0.87 ± 0.04
GN 6	0.69 ± 0.33	0.77 ± 0.25	0.81 ± 0.09	0.37 ± 0.37	0.85 ± 0.09	0.73 ± 0.31
GN 7	0.84 ± 0.05	0.70 ± 0.31	0.85 ± 0.05	0.83 ± 0.05	0.84 ± 0.04	0.84 ± 0.12
GN 8	0.47 ± 0.43	0.87 ± 0.17*	0.86 ± 0.05	0.61 ± 0.38	0.86 ± 0.07	0.90 ± 0.03
E. nitens	0.80 ± 0.16	0.57 ± 0.34	0.80 ± 0.06	0.89 ± 0.13	0.88 ± 0.05	0.88 ± 0.17

3.5.1. Recovery potential of the eucalypts based on chlorophyll fluorescence

In terms of recovery potential from the cold shock, the mean CF values of the eucalypts were calculated over the recovery period and were used to rank each eucalypt in order of descending recovery potential (Table 4). It was found that GN 4, *E. nitens* and GN 5 displayed the highest recovery potential; and GN 2, GN 3 and GN 6 the lowest recovery potential. GN 6 in particular had the lowest mean CF level of 0.69 ± 0.22 during the recovery period (Table 4). Overall, the majority of the eucalypts, including *E. grandis*, maintained optimal CF levels during the recovery period (Table 4).

Table 4. Chlorophyll fluorescence (CF) (*Fv/Fm)* levels of each eucalypt averaged during the recovery period of 6 days at 25°C /14°C day/night and 12h photoperiod with corresponding recovery potential rankings. Data represented are means ± standard deviation and rankings are in descending order of recovery potential where 1 = highest recovery potential and 10 = lowest recovery potential.

Eucalypt	CF	Recovery potential ranking
E. grandis	0.85 ± 0.05	4
GN 1	0.83 ± 0.09	6
GN 2	0.73 ± 0.11	9
GN 3	0.79 ± 0.08	8
GN 4	0.87 ± 0.00	1
GN 5	0.85 ± 0.02	3
GN 6	0.69 ± 0.22	10
GN 7	0.84 ± 0.01	5
GN 8	0.81 ± 0.13	7
E. nitens	0.86 ± 0.04	2

3.6. Relative electrolyte conductance levels of unfrozen and frozen samples under standard and cold shock conditions

Under standard conditions, REC levels of all the eucalypts varied only slightly when exposed to the simulated frost (Std frozen), with the exception of GN 1, GN 2, GN 4 and GN 8. The REC levels of frozen GN 1 and GN 8 samples were recorded as 24.76 and 28.27% lower under these conditions and the REC levels of frozen GN 2 and GN 4 samples increased by 24.06% and 28.90% respectively under these conditions (Figure 22). When comparing the REC levels from unfrozen eucalypts under standard (Std unfrozen) and cold shock conditions (CS unfrozen), a notable difference was only displayed by GN 1 and GN8 where the levels were significantly lower ($H(3) = 11.608$, $p<0.05$ and $H(3) = 9.273$, $p<0.05$ respectively) in both cases (Figure 22, Appendix 45 and 52).

Under cold shock and simulated frost conditions (CS frozen), *E. grandis* and 2 GNs were most affected (Figure 22). The membrane integrity of *E. grandis*, GN 1 and GN 3 was disrupted the most by these conditions; as displayed by the 67.86, 90.11 and 43.73% increase in REC levels in these

eucalypts (Figure 22). These increases were also found to be significant (H (3) = 8.509, p<0.05; H (3) = 11.608, p<0.05; and H (3) = 23.138, p<0.05 respectively, Appendix 44, 45 and 47). The REC levels of the remaining eucalypts displayed only minor changes over the experimental period (Figure 22). Statistically significant differences in REC levels between the eucalypts of unfrozen and frozen samples under standard and cold shock conditions can be found in Appendix 17 – 20 and within each eucalypt in Appendix 44 – 53.

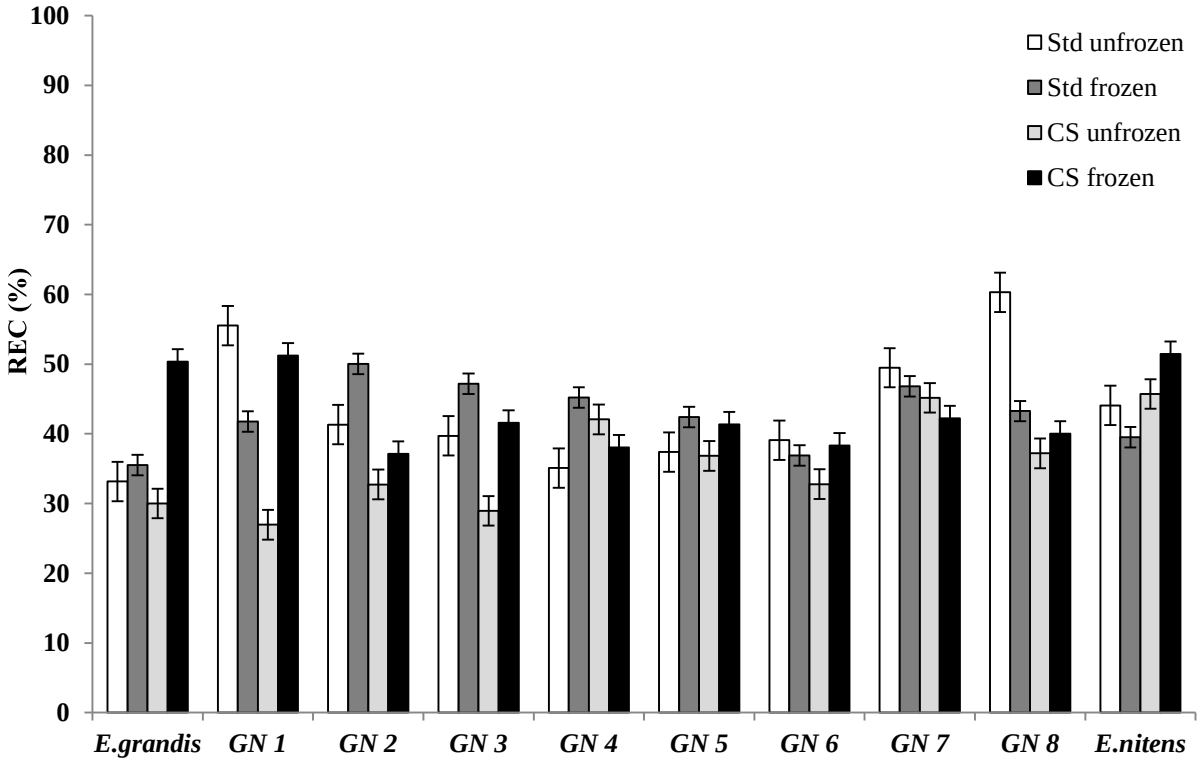

Figure 22. Relative electrolyte conductance (REC) (%) levels of each eucalypt over the experimental period. Std unfrozen = unfrozen samples under standard conditions: 7 days 25°C /14°C ^{0}C day/night and 12h photoperiod; Std frozen = frozen samples under standard conditions: 7 days 25°C/14°C day/night and 12h photoperiod; CS unfrozen = unfrozen samples under cold shock conditions: 24hrs 5°C/5°C day/night and 12h photoperiod; CS frozen = frozen samples under cold shock conditions: 24hrs 5°C/5°C day/night and 12h photoperiod.

3.6.1. Change in relative electrolyte conductance of unfrozen and frozen samples under cold shock conditions as a proxy for frost tolerance ranking of the eucalypts

It was apparent that GN 1, *E. grandis* and GN 3 had the largest increase in REC levels (90.10, 67.84 and 43.73% respectively) between unfrozen and frozen samples under cold shock conditions and were subsequently ranked as the three least tolerant eucalypts (Table 5). On the other hand, GN 4, GN 7 and GN 8 appeared the most tolerant with REC levels of frozen samples recorded as 9.56 and

6.49% lower compared with unfrozen samples in the case of GN 4 and GN 7. In the case of GN 8, REC levels increased the least by 7.65% (Table 5).

Table 5. The change in relative electrolyte conductance (REC) (%) levels of unfrozen and frozen samples of the eucalypts under cold shock conditions with corresponding frost tolerance rankings. The rankings are in order of decreasing tolerance; i.e. 1 = most tolerant and 10 = least tolerant. Negative REC indicates a decrease in REC.

Eucalypt	**Change in REC (%)**	**Tolerance ranking**
E. grandis	67.84	9
GN 1	90.10	10
GN 2	13.47	6
GN 3	43.73	8
GN 4	-9.56	1
GN 5	12.31	4
GN 6	16.89	7
GN 7	-6.49	2
GN 8	7.65	3
E. nitens	12.54	5

3.7. Principal component analyses of the eucalypts and measured variables under standard and cold shock conditions

The PCAs provided three important outputs that were considered under standard and cold shock conditions: 1) factor loadings on the measured variables; 2) a plot with the component weights of each variable; and 3) a plot with the case factor coordinates of each eucalypt. It was decided that Factor 1 and Factor 2 provided sufficient explanation of the total variability under both standard and cold shock conditions, therefore, analysis of the PCAs only considered Factor 1 and Factor 2 in each instance.

3.7.1. Principal component analysis under standard conditions

Under standard conditions, Factor 1 and Factor 2 accounted for a total of 65.30% of the variability (Factor 1: 44.18% + Factor 2: 21.12% = 65.30% total variability). The factor loading analysis revealed that REC of frozen samples, ROS and starch with high factor loadings of +0.97, +0.82 and +0.76 respectively had the strongest associations to Factor 1under standard conditions (Figure 23). The strongest associations with Factor 2 under standard conditions were that of REC of unfrozen samples (-0.74), CF (-0.62) and ROS (+0.51) (Figure 23).

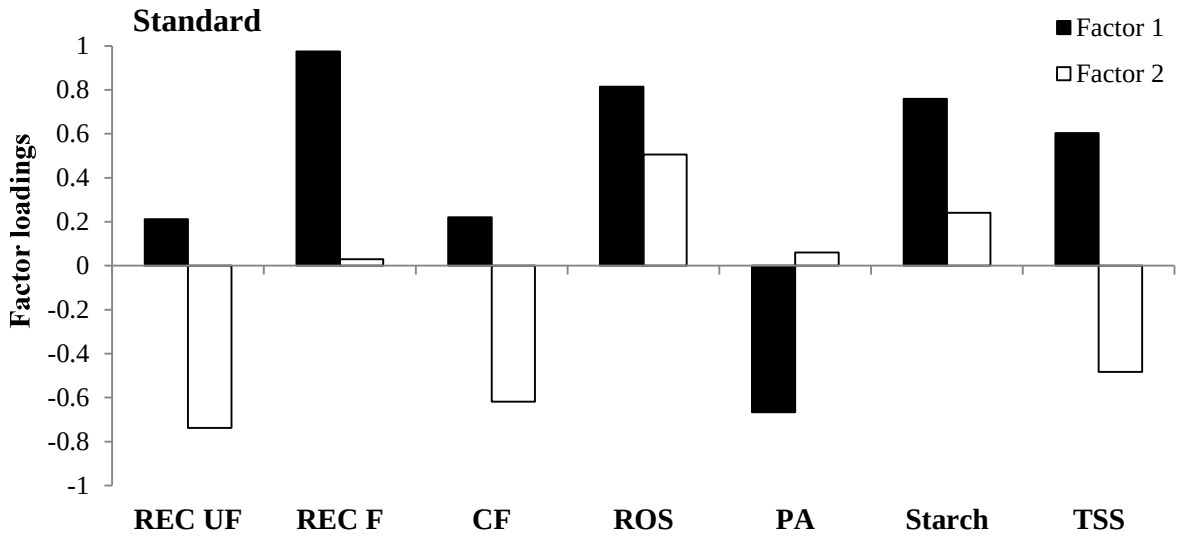

Figure 23. Factor loadings on the measured variables of the eucalypts under standard conditions. REC UF = relative electrolyte conductance of unfrozen samples; REC F = relative electrolyte conductance of frozen samples; CF = chlorophyll fluorescence; ROS = reactive oxygen species; PA = phenolic acids; TSS = total soluble sugars.

The projection of the variables on the factor plane (Factor 1 x Factor 2, Figure 24a) revealed that CF and REC of unfrozen samples were the most strongly positively correlated variables. Moderate positive correlations were also observed between ROS, starch and REC of frozen samples (Figure 24a). In contrast, PA appeared to have a negative relationship with all of the other variables, especially REC of frozen samples (Figure 24a).

Three groupings were formed by the projection of the eucalypts on the factor plane (Factor 1 x Factor 2, Figure 24b). Interestingly, *E. grandis* and *E. nitens* were grouped together under standard conditions, along with GN 6 (Figure 24b). The other two groupings comprised of GN 1 and GN 5 (group 2, Figure 24b), and GN 3 and GN 2 (group 3, Figure 24b) respectively. In addition, group 1 and 2 were separated from group 3, GN 7 and GN 4 by Factor 2 (Figure 24b). Factor 1 also largely separated GN 7 from GN 8 and GN 4 (Figure 24b).

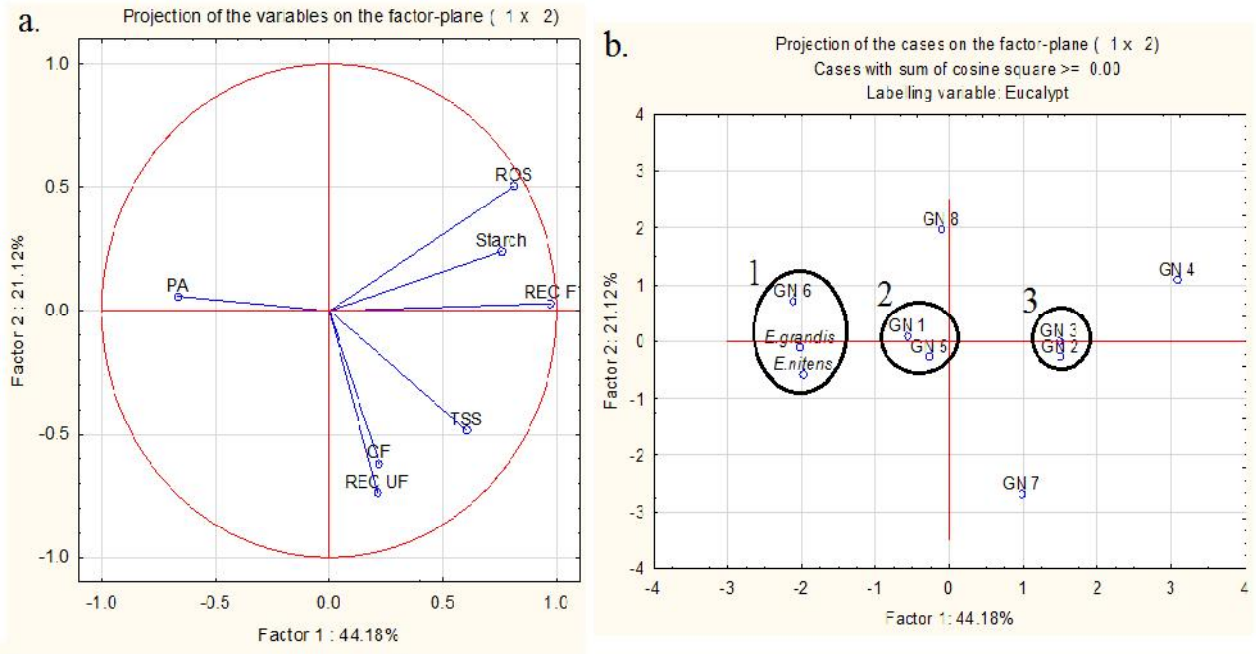

Figure 24. Principal component analysis with a.) a projection of the variables investigated on the factor plane; and b.) a projection of each eucalypt investigated on the factor plane under standard conditions (1 week at 25°C/14°C and 12h). Factor 1 and Factor 2 were used for both projections. REC UF = relative electrolyte conductance of unfrozen samples; REC F = relative electrolyte conductance of frozen samples; CF = chlorophyll fluorescence; ROS = reactive oxygen species; PA = phenolic acids; TSS = total soluble sugars.

3.7.2. Principal component analysis under cold shock conditions

Under cold shock conditions, Factor 1 and Factor 2 accounted for a total of 61.89% of the variability (Factor 1: 37.80% + Factor 2: 24.09% = 61.89% total variability). The top 3 factor loadings were displayed by ROS (-0.84), CF (+0.76) and TSS (+0.75) for Factor 1 and REC of unfrozen samples (+0.71), PA (-0.65) and starch (+0.62) for Factor 2 under cold shock conditions (Figure 25).

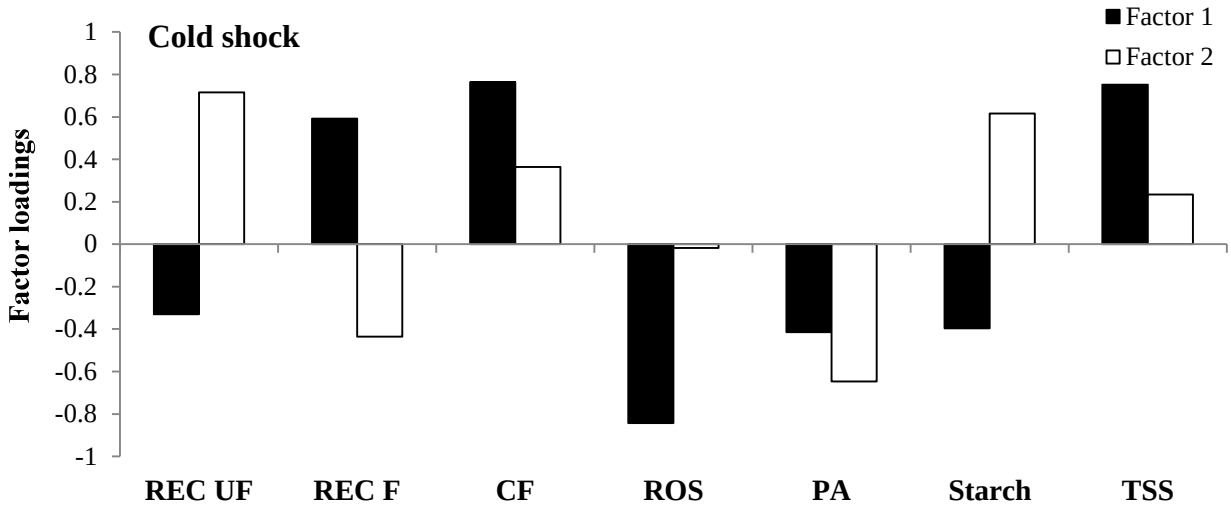

Figure 25. Factor loadings on the measured variables of the eucalypts under cold shock conditions. REC UF = relative electrolyte conductance of unfrozen samples; REC F = relative electrolyte conductance of frozen samples; CF = chlorophyll fluorescence; ROS = reactive oxygen species; PA = phenolic acids; TSS = total soluble sugars.

The projection of the variables on the factor plane (Factor 1 x Factor 2, Figure 26a) revealed that starch and REC of unfrozen samples and CF and TSS were the two sets of variables that showed the strongest positive correlation. Moderate correlations were also seen between ROS and PA and TSS and REC of frozen samples (Figure 26a). Negative correlations were displayed again by PA in relation to starch, CF, TSS and REC of unfrozen samples in particular (Figure 26a). Relative electrolyte conductance of frozen samples was also strongly negatively correlated with ROS, starch and REC of unfrozen samples (Figure 26a).

One major grouping was formed by the projection of the eucalypts on the factor plane (Factor 1 x Factor 2, Figure 26b). This grouping included GN 2, GN 5, GN 6, GN 7 and GN 8 (Figure 26b). The remaining eucalypts appeared widely spread over the factor plane, indicating their strong independence from the other eucalypts (Figure 26b). GN 4 and *E. grandis* in particular were the greatest outliers (Figure 26b). Furthermore, Factor 1 largely influenced the separation of GN 4 from GN 1, GN 3 and *E. nitens*, whereas Factor 2 largely influenced the separation of GN 3, GN 4 and *E. nitens* from GN 1 and *E. grandis* (Figure 26b).

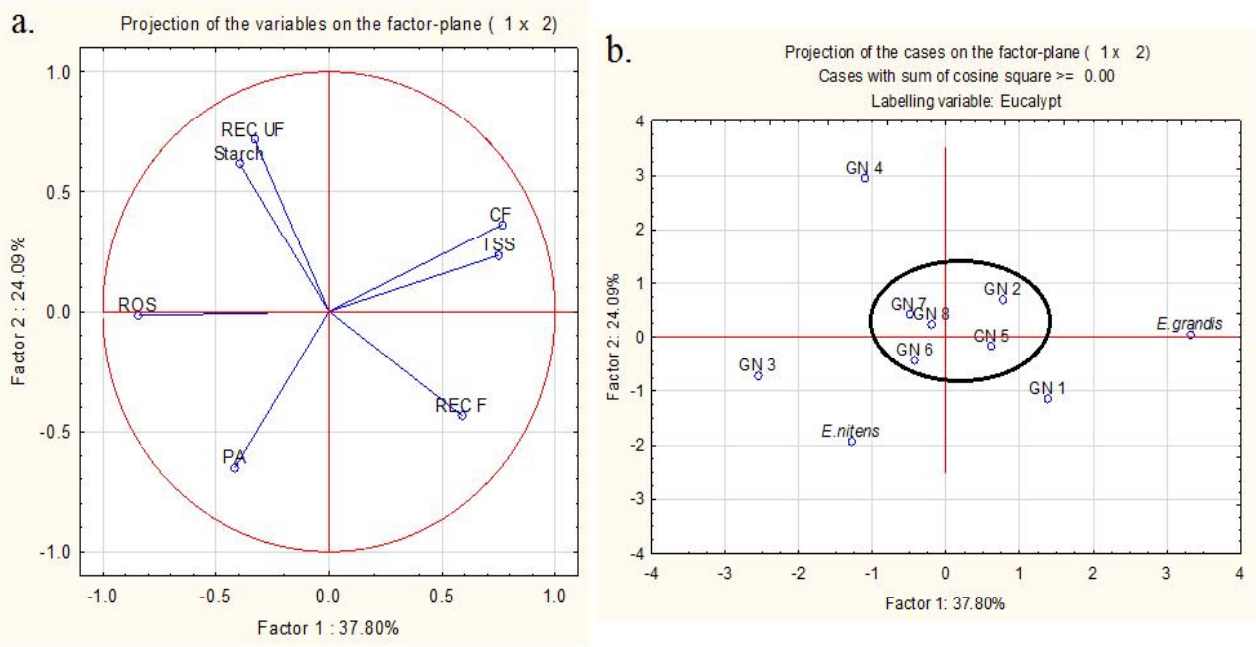

Figure 26. Principal component analysis with a.) a projection of the variables investigated on the factor plane; and b.) a projection of each eucalypt investigated on the factor plane under cold shock conditions (24h at 5°C/5°C and 12h). Factor 1 and Factor 2 were used for both projections. REC UF = relative electrolyte conductance of unfrozen samples; REC F = relative electrolyte conductance of frozen samples; CF = chlorophyll fluorescence; ROS = reactive oxygen species; PA = phenolic acids; TSS = total soluble sugars.

CHAPTER 4. DISCUSSION

4.1. Reactive oxygen species were produced in three distinct patterns under standard conditions and 30min, 90min and 24h into the cold shock

Levels of ROS, even under standard conditions, are generally very variable among plant species and individuals as they are dependent on a multitude of interacting factors (Apel and Hirt 2004, Beckett *et al.* 2005, Desikan *et al.* 2005). This variability was demonstrated in this study in particular where ROS was found to be one of the main measured variables (other variables: PA, starch, TSS, CF and REC) strongly influencing the separation of the eucalypts under both standard and cold shock conditions according to the PCAs (Figures 23-26). Furthermore, the exact role of ROS in plants under stressed conditions is also dependent on whether ROS is transiently or persistently produced, the exact chemical identity of the ROS, the site of ROS production, the plant's history of stress encounters and the developmental stage of the plant at the time of the stress application (Apel and Hirt *et al.* 2004, Desikan *et al.* 2005, Gechev *et al.* 2006).

It has been recognized that even a small change in ROS levels have the ability to effect signal transduction and influence downstream responses (Verslues and Zhu 2005). It is also important to note that ROS levels are seldom investigated in isolation and an integrated approach is often applied where the downstream effects of ROS are concurrently considered through the determination of various biochemical and physiological responses. In terms of ROS effects in *Eucalyptus*, research has focussed primarily on the role of ROS from the genomic perspective of low temperature and/or frost responses in *E. grandis*, *E. nitens* and various *Eucalyptus* hybrids (Byrne *et al.* 1997, Cao *et al.* 2015, Tibbits and Hodge 2003). Only a few studies have explicitly investigated the ROS content of eucalypts in low temperature experiments (e Silva *et al.* 2008, Liu *et al.* 2014).

In this study, extracellular ROS was produced in three distinct patterns in the eucalypts over the experimental period, and certain GNs elicited ROS patterns similar to that of *E. grandis* or *E. nitens* (Figure 18). For instance, the pattern of ROS production in GN 1, GN 4, and GN 6 was also elicited by the cold sensitive *E. grandis* where there was a distinct oxidative burst 30-90mins into the cold shock, followed by a decrease in ROS levels after 24h exposure to the cold shock (Figure 18). The decrease in ROS levels after 24h exposure to the cold shock was particularly interesting, given the fact that *E. grandis* is cold sensitive and ROS levels did not persist to a damaging stage as would be expected in a sensitive species (Hasanuzzaman *et al.* 2013, Wohlgemuth *et al.* 2002). Therefore, according to this study, *E.grandis* may have the ability to control ROS levels under cold shock conditions.

In contrast, GN 3 and GN 7 elicited a ROS pattern similar to that of *E. nitens* where levels were highest (294.14 and 223.40μg adrenochrome/g leaf (FWB) respectively) after 24h exposure to the cold shock (Figure 18). Since *E. nitens* is cold tolerant, it is possible that an oxidative burst was not necessary early on (30-90min) into the cold shock to activate downstream stress tolerance response mechanisms. Liu *et al.* (2014) conducted a similar experiment in which they investigated the H_2O_2 levels (H_2O_2 being a type of ROS) in *E. dunnii*, a moderately cold tolerant eucalypt species, under a low temperature treatment of 4^0C over a 48h period. They found that the H_2O_2 levels in *E. dunnii* increased by 76% at 24h exposure to the low temperature treatment (Liu *et al.* 2014), similar to that of the response of *E. nitens*, GN 3 and GN 7 in the present investigation, where ROS levels were highest at 24h under cold shock conditions (181.34, 294.14 and 223.40μg adrenochrome/g leaf (FWB) respectively, Figure 18).

It is also known that in certain higher plants, abiotically-induced ROS accumulation renders plants vulnerable to fungi and bacteria (Beckett *et al.* 2005, Murphy *et al.* 1998). In plants that are sensitive to pathogens in particular, the extracellular production of ROS may be used as a defense mechanism, with ROS being directly toxic to pathogens or resulting in the activation of 'systematic acquired resistance' defence mechanisms within the plant (Beckett *et al*. 2005). Since *E. nitens* is sensitive to *Mycosphaerella* leaf blotch disease, it may be speculated that the up-regulation of ROS after the 24h cold shock exposure may have been a defense mechanism against the possibility of exposure to this pathogen, where high ROS levels would be used to aid in defense against it. In addition, it has been identified that one of the side effects of activating photoprotective processes includes a controlled increase in ROS levels (Anderson *et al.* 1997, Apel and Hirt 2004, Edreva 2005). However, it is also known that *Mycosphaerella* generally occurs in *E. nitens* at lower altitudes and warmer temperatures; therefore it is not likely that a pathogen defense was activated in this situation (Hunter *et al.* 2004). In this regard, it was then proposed that activation of photoprotective processes may have occurred in *E. nitens* as evidenced by CF levels during the recovery period (Table 3), resulting in a peak in ROS levels under cold shock conditions (Figure 18) in this study. However, to confirm whether the spike in ROS 24h into the cold shock occurred as a result of a pathogen defense mechanism or activation of photoprotective processes in *E. nitens*, further studies aimed at determining these variables exclusively is required.

Under standard conditions, ROS levels of GN 2 and GN 8 were also high compared with most of the other eucalypts, followed by an erratic change in ROS levels once exposed to the cold shock (Figure 18). The decrease in ROS levels at 30min into the cold shock in these GNs indicates the possible use of basal ROS, resulting in the elimination of ROS as temperatures were detected to be decreasing (Figure 18). This drop was followed by a transient increase in ROS at 90min into the cold shock which may be interpreted as a second ROS signal (Figure 18). This observation was similar to a

study by Desikan *et al.* (2005), who posited that the second peak in ROS served to intensify the initial ROS signal if the second peak is carefully controlled. In this case, these two GNs did manage to eliminate ROS effectively as levels were found to be much lower at 24h into the cold shock, compared with levels at 90min into the cold shock (Figure 18).

Overall, the levels of ROS in the eucalypts investigated in this study are thought to have induced protective mechanisms and acclimation responses to the cold shock rather than effecting destruction. This was inferred from the plants' survival to the cold shock during the recovery period. In addition, it is also difficult to explicitly state whether the extracellular ROS levels measured in this study were involved in intracellular signal transduction as genomic responses were not investigated in conjunction. It is therefore suggested that future studies incorporate this aspect into investigations where possible.

4.2. Total phenolic acid levels increased in certain eucalypts under the cold shock

Like many other biochemicals, the composition of the soluble PA is unique in each plant, with differences in concentration depending on the genetic composition of the plant, the level of available carbohydrates, the type of PA present and the localization of the PA (Briggs and Schultz 1990). In this study, total soluble PA of the leaves was determined but the specificity in terms of the type of PA and the exact site of production were not investigated. Phenolic acids are generally present in low concentrations during 'standard' growth and development processes in plants, however, under abiotic stress conditions, the concentration of PA increases (Bartwal *et al*. 2012). The accumulation of PA in plants under stressful conditions serves as an antioxidant to prevent the build-up of damaging compounds that could lead to plant damage and cell death (Close *et al*. 2003). The primary importance of PA however, lies in their ability to strictly control ROS levels to ensure continued functioning in signal transduction and prevention of possible toxicity to the plant (Gechev *et al.* 2006). Therefore, it is essential to also consider PA levels in conjunction with ROS levels and to determine whether the PA may have served as an antioxidant.

Under standard conditions, the PA levels were the highest recorded in this series of experiments (6.780 – 8.216mg GAE/g leaf (DWB)) in all the tested eucalypts, except GN 4 (Figure 19). A study by Chapuis-Lardy *et al.* (2002) also found similar levels of PA (gallic acid = 5.97 – 10.53mg.g^{-1} dry leaf) in the leaves of *Eucalyptus uropellita*, *Eucalyptus urograndis* and the hybrid, 'E. PF1' (*Eucalyptus alba* x an undetermined parent with dominance of *Eucalyptus urophylla*). The Chapuis-Lardy *et al.* (2002) study was conducted in the Pointe Noire region of the Congo where the mean temperature at the time of PA measurement was 25°C, comparable to the 'standard conditions' in this study when PA was also measured. This supports the assertion that the basal levels of foliar PA measured in this study were within the usual range thus far measured in certain eucalypts.

It was also noted that PA levels of *E. nitens* increased by 26% from 6.34 under standard conditions to 8.012mg GAE/g leaf (DWB) when exposed to the cold shock conditions in this study (Figure 19). Furthermore, GN 4, GN 5 and GN 7 also elicited a similar response, with a 58%, 24% and 20% increase in their PA levels to 6.15, 6.78 and 7.60mg GAE/g leaf (DWB) respectively under cold shock conditions (Figure 19). In comparison, a study by Ntiyantiya (2004) that investigated the concentration of soluble PA in the leaves of various *Eucalyptus* species in the Draycott area of KwaZulu-Natal during February, April and July of 2004 concluded that an increase in PA levels is a characteristic response in selected cold tolerant eucalypts when exposed to lower temperatures. That study focussed on eucalypts that were part of a tree breeding trial established in 2000; the trees were thus, two years of age at the time of the experiment (Ntiyantiya 2004). Included in the study were *E. benthamii*, *Eucalyptus macarthurii* and *Eucalyptus fastigata* (Ntiyantiya 2004). *Eucalyptus benthamii* and *E. macarthurii* possess a high degree of cold hardiness (Hart and Nutter 2012, Swain and Gardner 2004) and *E. fastigata* a moderate degree of cold hardiness (Menzies *et al.* 1981). The levels of PA of these species during February and July of 2002 were thus comparable with the PA levels of the cold tolerant *E. nitens* under standard and cold shock conditions respectively in this study. The PA levels of *E. benthamii*, *E. macarthurii* and *E. fastigata* were reported as ±5.00, ±8.00 and ±6.00mg gallic acid/g leaf (DW) respectively during February (late summer) (Figure 27, Ntiyantiya 2004); similar to that of the PA levels of *E. nitens* recorded as 6.34mg GAE/g leaf (DWB) under standard conditions in this study. Thereafter, a substantial increase in PA levels can be seen in *E. benthamii*, *E. macarthurii* and *E. fastigata* during July (mid-winter) where levels in these eucalypts increased to ±8.00, ±11.00 and ±12mg gallic acid/g leaf (DW) respectively (Figure 27, Ntiyantiya 2004); similar to that of *E. nitens* in this study where PA levels were recorded as 8.01mg GAE/g leaf (DWB) under cold shock conditions. Therefore, it may be deduced that an increase in PA levels in *E. nitens* in this study, consistent with the results from the study by Ntiyantiya (2004), may be interpreted as a characteristic response in selected cold tolerant eucalypts when exposed to low temperatures.

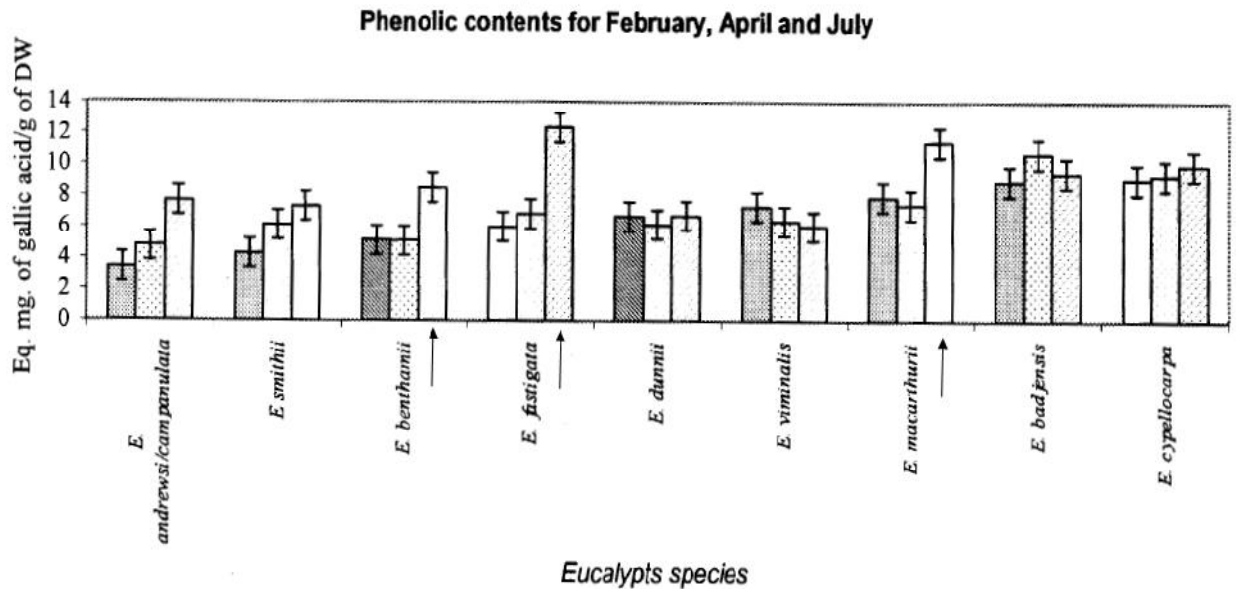

Figure 27. The concentration of total soluble phenolic acids (mg of gallic acid/g of DW leaf) of nine *Eucalyptus* species during February, April and July of 2002 respectively (Adapted from Ntiyantiya 2004).

Corresponding to the 58% and 24% respective increase in PA levels of GN 4 and GN 5 under the cold shock conditions (Figure 19) was the decrease in ROS levels in these two GNs (Figure 18). This was an indication of the possible use of PA in an antioxidant capacity since ROS levels were especially high 90min into the cold shock treatment in GN 4 (316.89µg adrenochrome/g leaf (FWB), Figure 18). These ROS levels then decreased by 36% while PA levels increased by 58% under cold shock conditions. A number of studies have identified the antioxidant role of ROS-scavenging PA under various environmental conditions (amongst others, Bartwal *et al.* 2012). For example, Dai and Mumper (2010) provided an account of numerous instances of PA acting as antioxidants in various plant species (in experiments investigating the use of PA in cancer treatments). Rivero *et al.* (2001) also demonstrated that the accumulation of PA in tomato plants (*Lycopersicon esculentum*) inhibited oxidation under cold stress. Further, Kirakosyan *et al.* (2003) found that cold stress yielded higher levels of PA compared with the control unstressed plants in two species of hawthorn; *Crataegus laevigata* and *Crataegus monogyna*. These PA levels were also strongly correlated to high antioxidant capacity in these two species (Kirakosyan *et al.* 2003). In light of the evidence in the literature, it is possible that the high PA levels in GN 4 under cold shock conditions were involved in antioxidant activities in this GN, resulting in correspondingly low ROS levels.

4.3. Starch levels were only moderately affected by the cold shock, with the exception of three GNs

It has been shown that leaf starch content responds variably to low temperature stress, either transiently accumulating or degrading (Keller *et al.* 2013, Lu and Sharkey 2006). In this regard, Keller *et al.* (2013) identified the typical genes activated in plants under low temperatures that play a role in cell protection. It was suggested that one of the molecular functions of cryoprotection (protection from freezing) activated by BMY8 in chloroplasts included the degradation of starch into maltose and glucose (Keller *et al.* 2013, Lu and Sharkey 2006). Under low temperatures, maltose is involved in the protection of proteins and the photosynthetic electron transport chain and glucose aids in membrane stability (Kaplan and Guy 2005, Lu and Sharkey 2006). Peng *et al.* (2015) also found that a decrease in starch levels during the early stages of cold stress is either a result of chilling injury or cold adaptation.

In the present study, clear changes in starch levels were observed in GN 3 and GN 7, where levels decreased by 22% and 25% respectively under cold shock conditions; a possible intensified response similar to *E. grandis* where levels also decreased, but only by 8% (Figure 20). The decrease in starch levels under low temperatures may also be attributed to the repressed processes of photosynthesis, resulting in a lower rate of starch synthesis in the chloroplast (Figure 20, Peng *et al.* 2015). As such, coinciding with the decrease in starch levels under cold shock conditions in GN 3 (Figure 20) was the significant change in CF levels (Table 3). Chlorophyll fluorescence levels decreased from 0.84 ± 0.16 under standard conditions to 0.55 ± 0.32 under cold shock conditions in this particular GN (Table 3). This indicated that the synthesis of starch in GN 3 was most likely affected because of the suppression of photosynthesis under the cold shock conditions (Peng *et al.* 2015). In addition, it has also been established that suppressed photosynthesis under low temperatures may result in the generation of ROS that is also destructive to photosynthetic apparatus (Peng *et al.* 2015, Takami *et al*. 2013). In line with this explanation was the 90% increase in ROS levels between standard and cold shock conditions in GN 3 (Figure 18). Therefore, it seems that the decrease in starch levels in GN 3 under cold shock conditions may have occurred as a result of suppressed starch synthesis on account of chilling injury in the form of photosynthetic apparatus damage and/or high ROS levels. On the other hand, since CF levels of GN 7 were not affected significantly by the cold shock, it is likely that the 25% decrease in starch levels of this GN may have been a form of a cold adaptive response rather than chilling injury as noted by Peng *et al.* (2015). In the same way, starch accumulation in cold-stressed *Chlamydomonas reinhardtii* was classified by Valledor *et al*. (2013) as an adaptive mechanism rather than a result of chilling injury.

Conversely, the only marked increase in starch levels was displayed by GN 6 where levels increased by 33% under the cold shock conditions, similar to *E. nitens* where levels also increased by

25% (Figure 20). Corresponding to the increase in starch levels of GN 6 was the substantial 409% increase in TSS levels in this GN. This indicated that starch, which is classified as a storage compound, could have been mobilized into TSS and not restored due to the cold conditions, characteristic of a cold tolerant response where TSS serves as a cryoprotectant (Keller *et al.* 2013, Lu and Sharkey 2006, Oliveira and Peñuelas 2004). Similarly, Peng *et al.* (2015) found that starch and soluble sugars accumulated in paper mulberry when exposed to 4°C over a 0-72h period where levels were significantly high, even after 24h. It has also been recognized that starch and TSS accumulation may occur simultaneously during the early stages of low temperature stress and/or cold acclimation as a cryoprotective response (Peng *et al.* 2015, Yuanyuan *et al.* 2009). Further, it is also possible that cold-tolerant Calvin-cycle enzymes were activated by GN 6, resulting in the production of large amounts of soluble sugars, as Strand *et al.* (1999) explains in the case of Arabidopsis (Colombia ecotype) plants. In that study, when the Arabidopsis plants were suddenly exposed to 5°C after being grown at 23°C, an escalation in the activity of numerous Calvin-cycle enzymes and essential enzymes for sucrose biosynthesis was found (Strand *et al.* 1999). In the same way, it is therefore possible that the 33% increase in starch under cold shock conditions may have been used to produce additional TSS to aid in low temperature tolerance in GN 6, particularly at 5°C.

Overall, GN 2, GN 3 and GN 7 elicited responses similar to the cold sensitive *E. grandis* with starch levels decreasing under cold shock conditions; and GN 1, GN 4, GN 5 and GN 6 elicited responses similar to the cold tolerant *E. nitens* with starch levels increasing under cold shock conditions (Figure 20). Additionally, in both instances, pronounced changes in starch levels were displayed only by GN 3, GN 7 and GN 6. The responses of these three eucalypts were characterized as: cold sensitive for GN 3; cold adaptive for GN 7; and cold tolerant for GN 6.

4.4. Total soluble sugar levels in four eucalypts were markedly affected by the cold shock

One of the recorded physiological responses to cold stress includes an increased concentration of soluble sugars and it is suggested that these carbohydrates are involved in ensuring tolerance to low temperatures by maintaining membrane integrity (Close *et al.* 2003, Ögren *et al.* 1997, Yuanyuan *et al.* 2009). Different classes of sugars perform specific functions in attaining low temperature tolerance in plants, however, this study considered total soluble sugars as they have been the most commonly detected in a number of species of land plants when subjected to low temperatures (e.g. Yuanyuan *et al.* 2009).

The TSS levels of *E. grandis* and GN 6 increased by 201% and 409% respectively under the cold shock conditions (Figure 21). Since *E. grandis* is a cold sensitive species, it is possible that the large accumulation of TSS from 19.01 to 48.41mg TSS/g leaf (FWB) may have been a protective response. In the same way, Peng *et al.* (2015) found that within a 24h exposure to 4°C, soluble sugar

levels of the cold-sensitive paper mulberry increased from 19.3 to 40.5mg soluble sugar/g leaf (FWB). Moreover, the increase in TSS levels of *E. grandis* in this study being classified as a protective response is supported by the relatively unaffected CF levels of this eucalypt under cold shock conditions (0.86 ± 0.05, Table 3) and during the recovery period (0.85 ± 0.05, Table 4). In the case of GN 6, the increase in TSS levels may have also been a protective response, as CF levels increased from 0.69 ± 0.33 under standard conditions to 0.77 ± 0.25 under cold shock conditions (Table 3). However, GN 6 was also classified as having the lowest recovery potential among the eucalypts, with CF levels averaging 0.69 ± 0.22 during the recovery period (Table 4). Therefore, it is possible that the increase in TSS levels in GN 6 may have served a transiently protective role, rather than a long term (i.e. 6 day) protective role as was the case with *E. grandis*. The positive relationship between TSS and CF in this study was supported by the PCA where a strong correlation between TSS and CF was displayed under cold shock conditions (Figure 26a).

On the contrary, the TSS levels of GN 2 and GN 3 decreased by 41% and 76% respectively under the cold shock conditions (Figure 21). Corresponding with these decreases were a 13% and 22% decrease in starch levels in these GNs (Figure 20). Also coinciding with the responses in starch and TSS in these two GNs, were affected CF levels where GN 2 was ranked 9/10 and GN 3 8/10 in terms of recovery potential (1 = highest recovery potential, 10 = lowest recovery potential, Table 4). It is known that leaf starch is converted into sugars under low temperatures by the process of starch metabolism (Ashworth *et al.* 1993, Bornke and Sonnewald 2011, Yuanyuan *et al.* 2009). Consistent with this is the results from a study conducted by Oliveira and Peñuelas (2004) where it was suggested that a decrease in starch and soluble sugars in *Cistus albidus* grown at 5°C was associated with the consumption of stored carbohydrates. Since CF was affected by the cold shock in these two GNs, it is likely that the stored carbohydrates were used to compensate for the decreased production of starch as a result of reduced photosynthesis. This explanation is supported by Peng *et al.* (2015) where it was found that a reduction in energy capture during photosynthesis under cold stress decreases the rate at which starch is synthesized in the chloroplast (Figure 11).

Interestingly, the TSS levels of *E. nitens* appeared unaffected and remained low (8.22mg TSS/g leaf (DWB)) after the 24h cold shock when compared with some of the other eucalypts (Figure 21). Since *E. nitens* is cold tolerant, the 24h cold shock may not have been perceived as a ‘stress’ *per se* for this species. However, it is probable that should the cold shock have persisted for over 24h, an accumulation of TSS would have been likely.

4.5. Photosystem efficiency by measure of chlorophyll fluorescence was generally unaffected by the cold shock, with most eucalypts displaying a high recovery potential

The *Fv/Fm* ratio is an indication of the functional state of photosynthetic apparatus (i.e. the energy capturing reactions of photosynthesis) in plants where, values of 0.85 are considered optimal in leaves of healthy plants in most species, values close or equal to 0.90 imply a very high efficiency and values lower than 0.80 indicate some degree of photosynthetic impairment (Björkman and Demmig 1987, DeEll *et al.* 1999). Photosystem functionality and efficiency is limited by low temperatures as photosynthetic apparatus is damaged, energy capture and use is affected, or the accumulation of oxidative pressure (e.g. by ROS accumulation) results in photodamage (Close 2012). A persistent decrease in CF levels post-stress removal may be interpreted as an indication of dysfunctional photosystems and a temporary decrease in CF levels may be an indication of the down-regulation of photosynthesis as a protective mechanism (Anderson *et al.* 1997).

In this study, the lowest CF levels were recorded in GN 3 and *E. nitens* under cold shock conditions (0.55 ± 0.32 and 0.57 ± 0.34 respectively, Table 3). Since *E. nitens* is cold tolerant, it is possible that this species may have temporarily initiated photoprotective processes upon perception of the sudden decrease in temperature (Adams III *et al.* 2006). In support of this explanation, rather than the possibility of damage to the photosystems of *E. nitens* by the cold shock, is the normalization of CF levels during the recovery period in this species (0.86 ± 0.04, Table 4) and being ranked 2/10 among the eucalypts in terms of recovery potential (1 = highest recovery potential, 10 = lowest recovery potential). In the same way, CF levels of GN 3 increased to moderate levels during the recovery period (0.79 ± 0.08, Table 4) which was also ranked 8/10 among the eucalypts in terms of recovery potential (1 = highest recovery potential, 10 = lowest recovery potential). If the photosystems of these two eucalypts had been irrecoverably damaged under the cold shock conditions, particularly low CF levels would have persisted during the recovery period. Therefore, this type of temporary reduction in energy capturing reactions of photosynthesis is considered a photochemical adjustment to allow for the discharge of excess energy that would otherwise be very harmful to the plants (Anderson *et al.* 1997, Huner *et al.* 1993). A study by Ball *et al.* (1991) also found that cold-induced reduction in energy capturing reactions in juvenile *Eucalyptus pauciflora* plants occurred as a protective release of absorbed light energy rather than as a result of photosystem II damage. Likewise, Anderson *et al.* (1997) suggested that temporary reductions in photosynthesis are, more often than not, a protective strategy rather than a damaging process. In addition, Anderson *et al.* (1997) also stated that reduced energy capturing reactions may lead to an increase in ROS as a side-effect under sustained light exposure. This was similarly exhibited in *E. nitens* and GN 3 in this study where ROS levels were the highest (181.34 and 294.14μg adrenochrome/g leaf (FWB) respectively, Figure 18) under cold shock conditions. Nevertheless, it appears that these levels of ROS

were not detrimental in *E. nitens* in particular as CF levels were averaged at 0.86 ± 0.04 during the recovery period (Table 4). This also resulted in *E. nitens* being ranked 2/10 in terms of recovery potential (1 = highest recovery potential, 10 = lowest recovery potential), as expected since this species is cold tolerant.

Contrary to what was anticipated, CF levels of the cold sensitive *E. grandis* were unaffected by the cold shock (Tables 3 and 4). Gusta and Wisniewski (2013) explain that sugars play a role in the resiliency of photosynthesis under low temperatures. This may serve to elucidate the lack of notable changes of CF in *E. grandis*, paralleled by high TSS levels under cold shock conditions (Table 3 and Figure 21). Genga *et al*. (2011) noted that sugars are used in stabilizing the photosystem II complex under low temperatures and Kaplan and Guy (2005) also found that an increase in soluble sugars contributed to the protection of the photosynthetic electron transport chain during low temperature stress. Therefore, it is possible that the accumulation of TSS under cold shock conditions could have enabled the photosystems of *E. grandis* to withstand the cold shock conditions. This is supported by the CF levels of *E. grandis* during the recovery period, which averaged 0.85 ± 0.05, also resulting in *E. grandis* being ranked 4/10 among the eucalypts in terms of recovery potential (1 = highest recovery potential, 10 = lowest recovery potential, Table 4).

Interestingly, GN 2 appeared to display a delayed response to the cold shock as CF levels were cyclically low and high during the recovery period (range of 0.63 to 0.84, Table 4), also resulting in GN 2 being ranked 9/10 among the eucalypts in terms of recovery potential (1 = highest recovery potential, 10 = lowest recovery potential, Table 4). Analogous to this, TSS levels in GN 2 decreased by 41% under cold shock conditions when compared with levels under standard conditions (Figure 21). Kaplan and Guy (2005) also found that *Arabidopsis thaliana* plants with reduced soluble sugars also exhibited diminished CF levels after 6h exposure to a 4°C cold shock. It is, therefore, possible that the high-low cycling of CF levels exhibited by GN 2 could be attributed to the 41% decrease in TSS levels in this GN under cold shock conditions. Furthermore, this change in CF levels was confirmed to be a consequence of the cold shock rather than the natural variation of CF levels in this GN by measurement of CF levels over a 5 day period under standard conditions, where levels averaged 0.81 ± 0.02.

4.6. Relative electrolyte conductance was notably affected by the cold shock and/or simulated frost in three eucalypts

Plant membranes are susceptible to the effects of temperature fluctuations, where low temperatures largely disturb membrane fluidity (Los and Murata 2004). Membrane fluidity can be reversibly or permanently altered in response to temperature changes, depending on the severity and the duration of exposure to the altered temperature (Lichtenthaler 1996, Sangwan *et al.* 2002).

Generally, the REC method does not account for whether high REC is related to irreversible or reversible damage (Hodge *et al.* 2012) and consequently, REC levels were not used as an indication of cellular death but rather as a proxy for assessing changes in membrane integrity and fluidity in this study (Boorse *et al.* 1998).

The REC of all the eucalypts, except GN 1 and GN 8, varied only slightly between standard and cold shock conditions (Std unfrozen and CS unfrozen, Figure 22). Similarly, a study by Peng *et al.* (2015) also found that the REC of paper mulberry in response to a cold shock of 4°C over a period of 0-24h varied only marginally. In comparison, REC levels in GN 1 and GN 8 in this study actually decreased considerably under cold shock conditions compared with REC levels under standard conditions (Std unfrozen versus CS unfrozen, Figure 22). This implies that the membrane integrity of these eucalypts may not have been compromised by a 24h cold shock of 5°C. In addition, the elevated REC of GN 8 under standard conditions (60%, Figure 22) implies that the membrane constitution in this GN might have been particularly leaky under normal growing conditions. However, when exposed to freezing temperatures, REC decreased by 28% (Std frozen, Figure 22). Thereafter, REC remained relatively constant and unaffected by the cold shock and/or the simulated frost (CS unfrozen and CS frozen, Figure 22). Thus, it appears that phase transitions of the cellular membrane lipids in GN 8 might have occurred upon perception of low temperatures, resulting in the stabilization of membrane fluidity as an adaptive response under cold shock and/or simulated frost conditions (Los and Murata 2004).

The eucalypts that appeared most affected by the simulated frost under cold shock conditions, however, were *E. grandis*, GN 1 and GN 3. Under cold shock conditions, there was a respective difference of 68, 90 and 44% in the REC levels between unfrozen and frozen samples in these three eucalypts (Figure 22). Since *E. grandis* is a cold sensitive species, the response elicited under the cold shock and simulated frost conditions was expected. In this regard, it would be interesting to ascertain the difference in membrane lipid composition between *E. grandis*, *E. nitens* and the GNs. It is also worth noting that although corresponding TSS levels in *E. grandis* were highest under cold shock conditions, it is possible that these sugars did not function efficiently enough under freezing conditions to sufficiently protect the cell membranes, or, electrolyte leakage may have occurred from cell types that did not effectively accumulate TSS, as suggested by Kaplan and Guy (2005). Alternatively, the TSS accumulated may have been used to maintain some degree of membrane integrity in *E. grandis*. It is possible that the REC levels of *E. grandis* may have vastly exceeded 50% under cold shock conditions if the TSS levels of this eucalypt did not increase by 201%. The latter explanation is probably more likely since soluble sugar accumulation has been linked to the maintenance of membrane integrity, and consequently, decreased electrolyte conductance levels in numerous studies (Ashworth *et al*. 1993, Travert *et al.* 1997, Xin and Browse 2000). For example, a

study by Travert *et al*. (1997) investigated the enrichment of *E. gunnii* x *E. globulus* and *E. cypellocarpa* x *E. globulus* hybrids with specific soluble sugars to determine the effect on their frost tolerance. It was found that certain soluble sugars were linked to the maintenance of membrane integrity and served as osmoprotectants (Travert *et al.* 1997). As a result, it is possible that the TSS accumulated in *E. grandis* in this study may have served – to some extent – as an osmoportectant to aid in membrane integrity.

Interestingly, the REC levels of the cold tolerant *E. nitens* were the highest compared with the other eucalypts under cold shock and simulated frost conditions (51%, CS frozen, Figure 22). However, Boorse *et al.* (1998) and Zhang *et al.* (1987) also emphasize that vital cellular solutes are induced under low temperature and freezing conditions in certain plants. These solutes have the ability to leak from the plasma membrane and increase REC levels (Boorse *et al.* 1998, Zhang *et al.* 1987). Consequently, since *E. nitens* is tolerant to low temperatures, it is likely that this species may constitutively produce solutes to protect the cellular membranes, thereby allowing it to tolerate the simulated frost conditions and resulting in high REC levels under cold shock and simulated frost conditions. Alternatively, the species tolerates colder temperatures and cold shocks using other mechanisms.

Since the REC levels of unfrozen cold shocked and frozen cold shocked samples were not drastically higher than REC levels of standard unfrozen samples, with the exception of *E. grandis*, GN 1 and GN 3 according to frost tolerance rankings (Table 5), it may be inferred that the membrane integrity in all the eucalypts tested were not irrecoverably compromised by the cold shock and simulated frost. Similarly, Peng *et al.* (2015) found that the cell wall architecture of paper mulberry plants, including the cell membranes, reconfigured to adapt to a cold stress of 4°C during a 24h exposure period. According to literature, this type of reconfiguration typically also includes an increase in fatty acids, resulting in a more rigid membrane constitution that also affects the structure of the cell wall (Los and Murata 2004, Welti *et al.* 2002). Consequently, it is possible that these eucalypts, except *E. grandis*, GN 1 and GN 3, were able to tolerate the cold shock and simulated frost by reconfiguration of their cell membranes, as indicated by REC levels. The mechanisms through which this occurred, either through the use of soluble sugars or fatty acid unsaturation is unclear and would require microscopy analyses and experimental quantification of fatty acids.

4.7. Reactive oxygen species, chlorophyll fluorescence and total soluble sugars led to one main grouping of the GNs under cold shock conditions

The PCAs revealed that under both standard and cold shock conditions, ROS had the highest factor loading on Factor 1 (Figures 23a and 25a). This implied that regardless of the growing conditions, ROS was the most important physiological characteristic driving variability among the

eucalypts. This corresponds with the literature which shows that ROS is a very specific measure that is dependent on a number of factors, with each plant exhibiting unique ROS patterns (Apel and Hirt 2004, Beckett *et al*. 2005, Suzuki and Mittler 2006). The other two measured variables with high factor loadings for Factor 1 under standard conditions included starch and REC of frozen samples. However, under cold shock conditions, the top three measured variables driving variation among the eucalypts included CF and TSS, in addition to ROS (Figure 25a). It is known that TSS are accumulated and/or used when plants are exposed to low temperatures (Xin and Browse 2000, Yuanyuan *et al*. 2009) and that the photosystems of plants are affected by temperature changes, particularly abrupt ones (Binder and Fielder 1996, Rizza *et al.* 2001, Roháček *et al.* 2008). As a result, it was not surprising that these two physiological measures were found to be two of the main drivers of variation among the eucalypts tested in this study according to the PCA under cold shock conditions.

Under standard conditions, ROS, starch and REC of the frozen material separated the eucalypts into three main groups, with a few outliers (Figure 24b). The most interesting observation was that of *E. grandis* and *E. nitens* being grouped together under standard conditions even though these two species are opposite in terms of low temperature tolerance. In this regard, it is possible that *E. nitens* does not display its low temperature tolerance characteristics under standard conditions or the mechanisms for such tolerance were not here measured. Also, none of the GNs were grouped closer to either of their parent species under standard conditions (Figure 24b). However, under cold shock conditions where ROS, CF and TSS were the drivers of variation, only one main grouping was formed that included the majority of the GNs. Again, none of the GNs grouped closely with either *E. grandis* or *E. nitens* under the cold shock (Figure 26b).

Stelkens and Seehausen (2009) explain that hybrids usually resemble strong characteristics of one of their parents, or, exhibit intermediate traits that lie between their parental means. The latter was seen in GN 1, which appeared in the middle of *E. grandis* and *E. nitens* on the PCA factor plane (Figure 26b). However, for the majority of the GNs under cold shock conditions, a large grouping was formed just outside the intermediate range of *E. grandis* and *E. nitens* on the PCA factor plane (Figure 26b). Stelkens and Seehausen (2009) further noted that exhibition of parental or intermediate traits may not always be the case due to 'transgressive segregation'. This occurrence results in the expression of traits outside of the phenotypic range of the plant's parent species (Stelkens and Seehausen 2009). This was clearly the case for the majority the GNs under cold shock conditions, particularly GN 4 which was a strong outlier (Figure 26b). Grattapaglia and Kirst (2008) also noted that transgressive segregation is particularly useful in the commercial *Eucalyptus* industry and is often used to identify hybrid individuals with traits superior to that of their parent species. However, to

confirm whether this was the case in this study, genetic linkage map analysis would simultaneously need to be conducted.

Another explanation that could clarify the large separation of the GNs from their parent species is the concept of 'environmentally-dependent hybrid superiority' that arises through a number of genetic processes, including epistasis, trait complementarity and hybrid vigour (Potts and Dungey 2004). These processes have all been widely displayed by *Eucalyptus* hybrids and are further explored by Potts and Dungey (2004). Griffin (1988) explains that 'complementarity occurs through additive effects and results from synergy among independent traits in specific environments where both parent species are not as adapted as their hybrid'. Such an example is displayed by *E. urophylla* x *E. grandis* hybrids in the Congo (Vigneron *et al.* 2000). Additionally, additive inheritance of a single adaptive trait occurs when a hybrid outperforms its parent species at an intermediate position along an environmental gradient, as seen in KwaZulu-Natal, South Africa, where GNs are preferred over *E. nitens* only in certain environments (Potts and Dungey 2004). On the other hand, hybrid vigour has been reported by a number of researchers investigating eucalypts (Gwaze *et al.* 2000, Madhibha *et al.* 2013, Potts and Dungey 2004, Vigneron *et al.* 2000) where hybrids strongly outperform their parent species.

In this study, it was found that most of the GNs possessed higher levels of certain biochemicals compared with their parent species under standard conditions, and more so under cold shock conditions in certain instances. For example, in terms of ROS levels, some of the GNs had up to four times more ROS when compared with *E. grandis* and *E. nitens* under standard conditions (Figure 18). This was also the case with starch and TSS levels, with *E. nitens* in particular displaying low levels of these biochemicals under both standard and cold shock conditions (Figures 20 and 21). The high levels of measured biochemicals in some of these GNs could account for their separation from their parent species by virtue of hybrid superiority. However, identification of the presence of hybrid superiority in the GNs by expression of either of the above-mentioned phenomena requires vast genetic and phenotypic analysis, combined with field analysis at selected environmental gradients.

4.8. Which eucalypts displayed low temperature and frost tolerance potential?

Different genotypes are expected to respond differently to low temperatures with hybrid genotypes eliciting an even higher degree of low temperature and/or frost tolerance trait variability. For example, Tibbits *et al.* (2006) highlighted the considerable variation in frost tolerance traits within *E. globulus* families. This was also displayed by the GNs in this study where physiological and statistical analysis revealed a high degree of variability in the responses of each GN under standard conditions, and even more so under cold shock conditions.

Chlorophyll fluorescence during the recovery period was used as a proxy for low temperature tolerance at the functional physiological level and REC was used as an indication of frost tolerance at the biochemical level. To determine which eucalypts displayed potential tolerance to low temperatures, their CF levels during the recovery period was assessed and a ranking of their recovery potential to the cold shock was established. To determine which eucalypts displayed frost tolerance potential, the difference in the REC levels of unfrozen and frozen samples was determined under cold shock conditions and a ranking of their frost tolerance was established. It was found that all the eucalypts, except GN 6, GN 2 and GN3, displayed a high recovery potential to the cold shock (Table 4). On the other hand, the eucalypts that ranked the most frost tolerant were GN 4, GN 7 and GN 8; and the least frost tolerant were GN 1, *E. grandis* and GN 3 (Table 5). Interestingly, the latter eucalypts (i.e., GN 1, GN 3 and *E. grandis)* were also classified as outliers in the PCA under cold shock conditions (Figure 26b). However, caution is advised when considering the frost tolerance rankings in isolation as a large difference in REC levels between unfrozen and frozen samples may not necessarily be associated with a low tolerance and other factors such as the possible accumulation of low temperature tolerance solutes should also be reviewed (Boorse *et al.* 1998).

4.9. Comparing the laboratory experiments with Sappi field trials

Comparing laboratory results with field results may provide insight into how plants respond in an uncontrolled environment (Hodge *et al.* 2012) and could validate or refute certain claims about the data. In this case, it was useful to be able to compare laboratory results with field trials that were conducted by the Sappi Shaw Research Centre during 2013 and 2014, where a number of GNs were established during January each year in areas prone to frost events. A ranking of frost tolerance for each GN was established based on a frost damage assessment post-exposure to frost events when temperatures were recorded between -5°C and -9°C during mid-June (Sappi 2015). A subjective scoring system was used to assess the phenotypic frost damage with ascending scores corresponding with increasing frost damage (see Appendix 54 for scoring system used). The eucalypts were an average of 39cm tall at the time of assessment (Maritz and Brink 2015).

There was considerable variability in the frost tolerance rankings when comparing 2013 and 2014 field trials, with the exception of GN 1 and GN 2 (Table 6, Maritz and Brink 2015). These two GNs were respectively ranked as the most frost tolerant among the GNs for both field trials (Table 6, Maritz and Brink 2015). On the other hand, the GNs that were most damaged by the frost, and consequently classified as the most frost sensitive, included GN 5, GN 7 and GN 8 for the 2013 field trial and GN 8, GN 3 and GN 5 for the 2014 field trial (Table 6, Maritz and Brink 2015). These inconsistencies were speculatively attributed to the differing intensities in the frost events each year (Maritz and Brink 2015).

Table 6. Frost tolerance rankings of the GNs from the 2014 and 2013 field trials (Adapted from Maritz and Brink 2015).

GN	2014 rank	2013 rank
GN 1	1	1
GN 2	2	2
GN 3	7	4
GN 4	4	3
GN 5	8	6
GN 6	3	5
GN 7	5	7
GN 8	6	8

Despite the varying frost intensities during the 2013 and 2014 field trials, GN 1 and GN 2 were consistently ranked the most phenotypically frost tolerant when compared with the other GNs. As a result, it is worth noting the responses of these two GNs in terms of their low temperature performance under laboratory conditions. Laboratory analysis of these GNs revealed a peak in ROS 90min into the cold shock and marginal changes in PA and starch levels (Figures 19 and 20). Most intriguing was the cyclically high and low CF levels of GN 2 during the recovery period, coupled with a 41% decrease in TSS under cold shock conditions and the marginal fluctuation of REC levels between frozen and unfrozen samples under cold shock conditions (Table 3 and Figures 21 and 22). There was a 90% difference in the REC levels between unfrozen and frozen samples of GN 1 under cold shock conditions, resulting in this GN being ranked the least frost tolerant (Figure 22 and Table 5) according to this measure of sensitivity. Since this GN was identified as the most phenotypically frost tolerant according to the field trials, it is possible that REC levels of frozen cold shocked samples were recorded high due to the possible production and accumulation of low temperature tolerance solutes, or perhaps as a consequence of rapid and drastic alteration of physiological activity in an attempt to maintain membrane fluidity under lower temperatures.

GN 8 appeared as one of the three most frost sensitive GNs in both 2013 and 2014 frost trials (Table 6), therefore, it was essential to also note the response of this GN under laboratory conditions. The levels of PA, starch and TSS were unaffected by the cold shock and the difference in REC levels

of unfrozen and frozen samples was negligible under cold shock conditions in this GN. It is possible that since this GN is frost sensitive in the field, the lack of changes in biochemical activity observed during the laboratory experiments serves as a possible explanation for the field result. In other words, it is possible that the sensing and response mechanisms in this GN are not well established, hence the lack of discernible changes in stress biomolecules in the experiments conducted. However, it is also worth noting that the PA and starch profiles of GN8 were in the same range as GN 1 and GN 2.

In comparison, a study by Hodge *et al.* (2012) which also compared field and laboratory results of frost tolerant pines showed that frost tolerant rankings from laboratory experiments corresponded well with field rankings. However, that study involved the investigation of the responses of whole plants and non-subjective measurements of relative conductivity, an approach not possible in the present study due to the unavailability of appropriate equipment for whole plant freezing at the time of the investigations. Furthermore, the analysis conducted on the Sappi field trials was more subjective, having been only based on phenotypic observations. In comparison, the present study included more detailed laboratory analysis which made interpretations more complex. Still, it is recommended that future studies include the freezing of whole plants as this would allow for a more reliable comparison with field trials. It is also suggested that a common assessment for frost tolerance be applied for both field trials and laboratory experiments (e.g. consider REC or CF for both field trial and laboratory experiments). Hence, if laboratory experiments are optimized to yield results that correspond well with field results, the efficiency of determining the frost tolerance potential of the eucalypts under laboratory conditions could be achieved more effectively.

CHAPTER 5. RECOMMENDATIONS AND CONCLUSIONS

None of the techniques to assess abiotic stress responses, alone, or in combination, appear to be able to identify cold/frost tolerant/sensitive plants that explicitly correlate with the field trial results. However, the results generated are valuable and there are a number of factors that should be considered and are recommended for future studies. These factors include 1) identification of the environmental conditions around the seed source and explicit consideration of the pedigree information of the eucalypts under investigation; and 2) common assessment criteria for field and laboratory trials. In addition, the mechanisms of cold and frost tolerance are multifaceted and occur in conjunction with other biological mechanisms to maintain normal physiological functions under the stressed conditions. Therefore, determining frost tolerance potential is an ongoing process and it is suggested that, where possible, a systems-approach be applied where physiology and the 'omics' (genomics, proteomics, transcriptomics and metabolomics) are investigated at the whole plant level. Furthermore, explicit attention is also recommended for the GNs that displayed possible transgressive segregation characteristics or hybrid superiority, as identified by the PCA and their high levels of biochemicals under cold shock conditions. These GNs may possess traits outside of the phenotypic mean of *E. grandis* and/or *E. nitens*, resulting in a more superior eucalypt with a greater degree of low temperature and/or frost tolerance than either of their parents, and are worth investigating. After all, for commercial forestry purposes, it is not the mean hybrid performance but the performance of the best clones that is of interest.

In summary, according to the laboratory results, it was found that all the eucalypts, except GN 6, GN 2 and GN 3 were tolerant to the cold shock by displaying a high recovery potential according to CF levels and the eucalypts that were ranked the most frost tolerant according to REC levels were GN 4, GN 7 and GN 8. In contrast, the least frost tolerant eucalypts were GN 1, *E. grandis* and GN 3. It may also be concluded that all of the eucalypts investigated, in this study, apart from *E. grandis*, GN 1 and GN 3, may be suitable for plantation establishment in areas prone to frost in South Africa.

It is perhaps also worth mentioning in conclusion, that the detailed biochemical and physiological information gleaned from the cold shock and simulated frost responses of the GNs and their parent species is potentially very valuable for the advancement of eucalypt breeding programmes. It is thus, recommended that these results be taken into consideration when allocating these GNs for field establishment in different climatic regions, particularly those regions susceptible to low temperatures and frost events.

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CHAPTER 7. APPENDIX

1. Between the eucalypts

Appendix 1. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of ROS of the eucalypts under standard conditions; H (df=9, N=556)=131.9673.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		0.268856	**0.000002**	**0.001984**	**0.000000**	**0.040406**	1.000000	1.000000	**0.000000**	1.000000
GN 1	0.268856		0.243169	1.000000	**0.000161**	1.000000	1.000000	1.000000	**0.001969**	**0.037954**
GN 2	**0.000002**	0.243169		1.000000	1.000000	1.000000	**0.001580**	**0.014773**	1.000000	**0.000000**
GN 3	**0.001984**	1.000000	1.000000		1.000000	1.000000	0.136040	0.539406	1.000000	**0.000224**
GN 4	**0.000000**	**0.000161**	1.000000	1.000000		**0.002184**	**0.000000**	**0.000002**	1.000000	**0.000000**
GN 5	**0.040406**	1.000000	1.000000	1.000000	**0.002184**		1.000000	1.000000	**0.016848**	**0.004159**
GN 6	1.000000	1.000000	**0.001580**	0.136040	**0.000000**	1.000000		1.000000	**0.000004**	1.000000
GN 7	1.000000	1.000000	**0.014773**	0.539406	**0.000002**	1.000000	1.000000		**0.000056**	0.535971
GN 8	**0.000000**	**0.001969**	1.000000	1.000000	1.000000	**0.016848**	**0.000004**	**0.000056**		**0.000000**
E. nitens	1.000000	**0.037954**	**0.000000**	**0.000224**	**0.000000**	**0.004159**	1.000000	0.535971	**0.000000**	

Appendix 2. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of ROS of the eucalypts 30min into the cold shock; H (df=9, N=556)=44.18641.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	1.000000	0.157359	1.000000	1.000000	1.000000	1.000000	1.000000	0.767157
GN 1	1.000000		1.000000	0.727456	1.000000	1.000000	0.731051	1.000000	1.000000	**0.007068**
GN 2	1.000000	1.000000		**0.018082**	1.000000	0.591868	1.000000	1.000000	0.586676	1.000000
GN 3	0.157359	0.727456	**0.018082**		1.000000	1.000000	**0.004419**	0.210673	1.000000	**0.000051**
GN 4	1.000000	1.000000	1.000000	1.000000		1.000000	0.314169	1.000000	1.000000	**0.004518**
GN 5	1.000000	1.000000	0.591868	1.000000	1.000000		0.163223	1.000000	1.000000	**0.001853**
GN 6	1.000000	0.731051	1.000000	**0.004419**	0.314169	0.163223		1.000000	0.180404	1.000000
GN 7	1.000000	1.000000	1.000000	0.210673	1.000000	1.000000	1.000000		1.000000	0.561198
GN 8	1.000000	1.000000	0.586676	1.000000	1.000000	1.000000	0.180404	1.000000		**0.003089**
E. nitens	0.767157	**0.007068**	1.000000	**0.000051**	**0.004518**	**0.001853**	1.000000	0.561198	**0.003089**	

Appendix 3. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of ROS of the eucalypts 90min into the cold shock; H (df=9, N=556)=170.7088.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		**0.001450**	1.000000	1.000000	1.000000	1.000000	0.354250	1.000000	0.208480	**0.000000**
GN 1	**0.001450**		**0.000066**	**0.000001**	**0.000006**	0.081646	**0.000000**	0.090389	**0.000000**	1.000000
GN 2	1.000000	**0.000066**		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	**0.000000**
GN 3	1.000000	**0.000001**	1.000000		1.000000	**0.041920**	1.000000	**0.037629**	1.000000	**0.000000**
GN 4	1.000000	**0.000006**	1.000000	1.000000		0.386776	1.000000	0.346840	1.000000	**0.000000**

GN 5	1.000000	0.081646	1.000000	**0.041920**	0.386776		**0.003686**	1.000000	**0.002747**	**0.000000**
GN 6	0.354250	**0.000000**	1.000000	1.000000	1.000000	**0.003686**		**0.003155**	1.000000	**0.000000**
GN 7	1.000000	0.090389	1.000000	**0.037629**	0.346840	1.000000	**0.003155**		**0.002376**	**0.000000**
GN 8	0.208480	**0.000000**	1.000000	1.000000	1.000000	**0.002747**	1.000000	**0.002376**		**0.000000**
E. nitens	**0.000000**	1.000000	**0.000000**	**0.000000**	**0.000000**	**0.000000**	**0.000000**	**0.000000**	**0.000000**	

Appendix 4. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of ROS of the eucalypts 24h into the cold shock; H (df=9, N=556)=159.2153.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		**0.001224**	1.000000	**0.000000**	**0.000000**	0.153572	**0.000000**	**0.000000**	**0.000000**	0.113834
GN 1	**0.001224**		0.050283	**0.005468**	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
GN 2	1.000000	0.050283		**0.000000**	**0.000000**	1.000000	**0.000225**	**0.000009**	**0.000000**	1.000000
GN 3	**0.000000**	**0.005468**	**0.000000**		1.000000	**0.000000**	**0.003608**	**0.028977**	1.000000	**0.000000**
GN 4	**0.000000**	1.000000	**0.000000**	1.000000		**0.000047**	1.000000	1.000000	1.000000	**0.000074**
GN 5	0.153572	1.000000	1.000000	**0.000000**	**0.000047**		0.219242	**0.023749**	**0.000093**	1.000000
GN 6	**0.000000**	1.000000	**0.000225**	**0.003608**	1.000000	0.219242		1.000000	1.000000	0.290682
GN 7	**0.000000**	1.000000	**0.000009**	**0.028977**	1.000000	**0.023749**	1.000000		1.000000	**0.033292**
GN 8	**0.000000**	1.000000	**0.000000**	1.000000	1.000000	**0.000093**	1.000000	1.000000		**0.000142**
E. nitens	0.113834	1.000000	1.000000	**0.000000**	**0.000074**	1.000000	0.290682	**0.033292**	**0.000142**	

Appendix 5. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of phenolic acids of the eucalypts under standard conditions; H (df=9, N=408)=167.5376.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	1.000000	**0.000106**	**0.000000**	**0.000321**	1.000000	1.000000	1.000000	1.000000
GN 1	1.000000		1.000000	**0.010159**	**0.000000**	**0.000017**	1.000000	1.000000	1.000000	1.000000
GN 2	1.000000	1.000000		**0.000050**	**0.000000**	**0.000146**	1.000000	1.000000	1.000000	1.000000
GN 3	**0.000106**	**0.010159**	**0.000050**		**0.000000**	**0.000000**	1.000000	**0.000000**	**0.023525**	**0.000001**
GN 4	**0.000000**	**0.000000**	**0.000000**	**0.000000**		1.000000	**0.000000**	**0.000031**	**0.000000**	**0.000016**
GN 5	**0.000321**	**0.000017**	**0.000146**	**0.000000**	1.000000		**0.000000**	**0.014035**	**0.000000**	**0.007754**
GN 6	1.000000	1.000000	1.000000	1.000000	**0.000000**	**0.000000**		0.066792	1.000000	0.153822
GN 7	1.000000	1.000000	1.000000	**0.000000**	**0.000031**	**0.014035**	0.066792		0.675879	1.000000
GN 8	1.000000	1.000000	1.000000	**0.023525**	**0.000000**	**0.000000**	1.000000	0.675879		1.000000
E. nitens	1.000000	1.000000	1.000000	**0.000001**	**0.000016**	**0.007754**	0.153822	1.000000	1.000000	

Appendix 6. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of phenolic acids of the eucalypts under cold shock conditions; H (df=9, N=408)=150.2681.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		**0.000000**	**0.034921**	1.000000	1.000000	0.102390	**0.000001**	**0.000000**	**0.000000**	**0.000000**
GN 1	**0.000000**		**0.003978**	**0.000261**	**0.000000**	**0.000651**	1.000000	1.000000	1.000000	1.000000
GN 2	**0.034921**	**0.003978**		1.000000	0.268144	1.000000	0.285095	**0.001210**	0.089547	**0.000329**

GN 3	1.000000	**0.000261**	1.000000		1.000000	1.000000	**0.028065**	**0.000069**	**0.006723**	**0.000018**
GN 4	1.000000	**0.000000**	0.268144	1.000000		0.706989	**0.000013**	**0.000000**	**0.000000**	**0.000000**
GN 5	0.102390	**0.000651**	1.000000	1.000000	0.706989		0.084722	**0.000130**	**0.019076**	**0.000032**
GN 6	**0.000001**	1.000000	0.285095	**0.028065**	**0.000013**	0.084722		1.000000	1.000000	1.000000
GN 7	**0.000000**	1.000000	**0.001210**	**0.000069**	**0.000000**	**0.000130**	1.000000		1.000000	1.000000
GN 8	**0.000000**	1.000000	0.089547	**0.006723**	**0.000000**	**0.019076**	1.000000	1.000000		1.000000
E. nitens	**0.000000**	1.000000	**0.000329**	**0.000018**	**0.000000**	**0.000032**	1.000000	1.000000	1.000000	

Appendix 7. P-values from the ANOVA test (2-tailed) of starch of the eucalypts under standard conditions; F stat (df=9, F=19.464).

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		0.260753	0.080227	**0.000000**	**0.000001**	0.982891	0.907538	**0.000173**	0.054215	0.317777
GN 1	0.260753		0.999951	**0.000029**	**0.023286**	**0.015178**	0.991203	0.741463	1.000000	**0.000268**
GN 2	0.080227	0.999951		**0.000238**	0.098003	**0.002657**	0.884195	0.978228	0.999999	**0.000038**
GN 3	**0.000000**	**0.000029**	**0.000238**		0.712618	**0.000000**	**0.000001**	**0.000305**	**0.000003**	**0.000000**
GN 4	**0.000001**	**0.023286**	0.098003	0.712618		**0.000000**	**0.001186**	0.277002	**0.010203**	**0.000000**
GN 5	0.982891	**0.015178**	0.002657	**0.000000**	**0.000000**		0.272726	**0.000001**	**0.000834**	0.920618
GN 6	0.907538	0.991203	0.884195	**0.000001**	**0.001186**	0.272726		0.117720	0.919500	**0.012383**
GN 7	**0.000173**	0.741463	0.978228	**0.000305**	0.277002	**0.000001**	0.117720		0.677455	**0.000000**
GN 8	0.054215	1.000000	0.999999	**0.000003**	**0.010203**	**0.000834**	0.919500	0.677455		**0.000008**
E. nitens	0.317777	**0.000268**	**0.000038**	**0.000000**	**0.000000**	0.920618	**0.012383**	**0.000000**	**0.000008**	

Appendix 8. P-values from the ANOVA test (2-tailed) of starch of the eucalypts under cold shock conditions; F stat (df=9, F=7.9509).

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		0.098827	0.771618	**0.026548**	**0.000035**	1.000000	0.275406	0.140544	**0.007369**	0.960208
GN 1	0.098827		0.247706	0.116225	0.999982	1.000000	0.598477	**0.009660**	0.810135	0.171618
GN 2	0.771618	0.247706		0.994273	0.965685	0.802849	**0.031755**	0.975974	0.993549	**0.001866**
GN 3	**0.026548**	0.116225	0.994273		**0.000046**	0.132215	0.182463	0.310492	0.163453	**0.000002**
GN 4	**0.000035**	0.999982	0.965685	**0.000046**		1.000000	0.117595	0.859211	0.678528	0.945825
GN 5	1.000000	1.000000	0.802849	0.132215	1.000000		0.999911	0.255328	0.866138	**0.029697**
GN 6	0.275406	0.598477	**0.031755**	0.182463	0.117595	0.999911		0.898751	0.671657	**0.011386**
GN 7	0.140544	**0.009660**	0.975974	0.310492	0.859211	0.255328	0.898751		0.567451	0.929348
GN 8	**0.007369**	0.810135	0.993549	0.163453	0.678528	0.866138	0.671657	0.567451		0.998132
E. nitens	0.960208	0.171618	**0.001866**	**0.000002**	0.945825	**0.029697**	**0.011386**	0.929348	0.998132	

Appendix 9. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of total soluble sugars of the eucalypts under standard conditions; H (df=9, N=146)=54.81883.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	**0.019449**	0.278225	1.000000	1.000000	1.000000	**0.029136**	1.000000	1.000000
GN 1	1.000000		1.000000	1.000000	1.000000	1.000000	**0.033303**	1.000000	1.000000	0.932643
GN 2	**0.019449**	1.000000		1.000000	**0.043811**	1.000000	**0.000007**	1.000000	1.000000	**0.001791**
GN 3	0.278225	1.000000	1.000000		0.533546	1.000000	**0.000316**	1.000000	1.000000	**0.032038**
GN 4	1.000000	1.000000	**0.043811**	0.533546		1.000000	1.000000	0.064162	1.000000	1.000000
GN 5	1.000000	1.000000	1.000000	1.000000	1.000000		**0.013644**	1.000000	1.000000	0.497017
GN 6	1.000000	**0.033303**	**0.000007**	**0.000316**	1.000000	**0.013644**		**0.000012**	0.257816	1.000000
GN 7	**0.029136**	1.000000	1.000000	1.000000	0.064162	1.000000	**0.000012**		1.000000	**0.002756**
GN 8	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	0.257816	1.000000		1.000000
E. nitens	1.000000	0.932643	**0.001791**	**0.032038**	1.000000	0.497017	1.000000	**0.002756**	1.000000	

Appendix 10. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of total soluble sugars of the eucalypts under cold shock conditions; H (df=9, N=146)=52.14199.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	**0.040237**	**0.000033**	0.063254	**0.012071**	**0.012081**	1.000000	1.000000	**0.000011**
GN 1	1.000000		1.000000	0.058815	1.000000	1.000000	1.000000	1.000000	1.000000	**0.017672**
GN 2	**0.040237**	1.000000		1.000000	1.000000	1.000000	1.000000	0.342684	1.000000	1.000000
GN 3	**0.000033**	0.058815	1.000000		1.000000	1.000000	1.000000	**0.000763**	1.000000	1.000000
GN 4	0.063254	1.000000	1.000000	1.000000		1.000000	1.000000	0.433474	1.000000	1.000000
GN 5	**0.012071**	1.000000	1.000000	1.000000	1.000000		1.000000	0.125140	1.000000	1.000000
GN 6	**0.012081**	1.000000	1.000000	1.000000	1.000000	1.000000		0.116436	1.000000	1.000000
GN 7	1.000000	1.000000	0.342684	**0.000763**	0.433474	0.125140	0.116436		1.000000	**0.000240**
GN 8	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000		0.786039
E. nitens	**0.000011**	**0.017672**	1.000000	1.000000	1.000000	1.000000	1.000000	**0.000240**	0.786039	

Appendix 11. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence of the eucalypts under standard conditions; H (df=9, N=294)=29.30547.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	0.795853	1.000000	1.000000	**0.047053**	0.396498	1.000000	0.086273	0.341640
GN 1	1.000000		1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
GN 2	0.795853	1.000000		0.237014	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
GN 3	1.000000	1.000000	0.237014		1.000000	**0.023191**	0.125187	0.502151	**0.029835**	0.109320
GN 4	1.000000	1.000000	1.000000	1.000000		0.705093	1.000000	1.000000	0.713728	1.000000
GN 5	**0.047053**	1.000000	1.000000	**0.023191**	0.705093		1.000000	1.000000	1.000000	1.000000
GN 6	0.396498	1.000000	1.000000	0.125187	1.000000	1.000000		1.000000	1.000000	1.000000
GN 7	1.000000	1.000000	1.000000	0.502151	1.000000	1.000000	1.000000		1.000000	1.000000
GN 8	0.086273	1.000000	1.000000	**0.029835**	0.713728	1.000000	1.000000	1.000000		1.000000

E. nitens	0.341640	1.000000	1.000000	0.109320	1.000000	1.000000	1.000000	1.000000	1.000000	

Appendix 12. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence of the eucalypts under cold shock conditions; H (df=9, N= 294)=83.16322.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	1.000000	**0.000045**	1.000000	0.369869	1.000000	1.000000	1.000000	**0.000611**
GN 1	1.000000		0.997194	**0.000000**	1.000000	**0.008967**	1.000000	0.420958	1.000000	**0.000003**
GN 2	1.000000	0.997194		**0.005755**	1.000000	1.000000	1.000000	1.000000	0.073849	0.102343
GN 3	**0.000045**	**0.000000**	**0.005755**		**0.000007**	0.281403	**0.001790**	**0.011820**	**0.000000**	1.000000
GN 4	1.000000	1.000000	1.000000	**0.000007**		0.095129	1.000000	1.000000	1.000000	**0.000078**
GN 5	0.369869	**0.008967**	1.000000	0.281403	0.095129		1.000000	1.000000	**0.000329**	1.000000
GN 6	1.000000	1.000000	1.000000	**0.001790**	1.000000	1.000000		1.000000	0.154714	**0.031300**
GN 7	1.000000	0.420958	1.000000	**0.011820**	1.000000	1.000000	1.000000		**0.025577**	0.210173
GN 8	1.000000	1.000000	0.073849	**0.000000**	1.000000	**0.000329**	0.154714	**0.025577**		**0.000000**
E. nitens	**0.000611**	**0.000003**	0.102343	1.000000	**0.000078**	1.000000	**0.031300**	0.210173	**0.000000**	

Appendix 13. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence of the eucalypts on recovery day 1; H (df=9, N=294)=50.01806.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	0.050917	1.000000	1.000000	1.000000	**0.000627**	0.740100	1.000000	1.000000
GN 1	1.000000		0.654651	0.152006	1.000000	1.000000	**0.019475**	1.000000	1.000000	1.000000
GN 2	0.050917	0.654651		**0.000042**	1.000000	0.449107	1.000000	1.000000	1.000000	1.000000
GN 3	1.000000	0.152006	**0.000042**		0.085756	0.215684	**0.000000**	**0.001121**	0.085937	**0.003539**
GN 4	1.000000	1.000000	1.000000	0.085756		1.000000	**0.042469**	1.000000	1.000000	1.000000
GN 5	1.000000	1.000000	0.449107	0.215684	1.000000		**0.011595**	1.000000	1.000000	1.000000
GN 6	**0.000627**	**0.019475**	1.000000	**0.000000**	**0.042469**	**0.011595**		1.000000	0.183655	1.000000
GN 7	0.740100	1.000000	1.000000	**0.001121**	1.000000	1.000000	1.000000		1.000000	1.000000
GN 8	1.000000	1.000000	1.000000	0.085937	1.000000	1.000000	0.183655	1.000000		1.000000
E. nitens	1.000000	1.000000	1.000000	**0.003539**	1.000000	1.000000	1.000000	1.000000	1.000000	

Appendix 14. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence of the eucalypts on recovery day 2; H (df=9, N= 294)=81.15617.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	**0.017591**	0.555897	0.553774	1.000000	**0.000054**	1.000000	0.597651	1.000000
GN 1	1.000000		0.416632	1.000000	**0.024071**	1.000000	**0.004394**	1.000000	1.000000	1.000000
GN 2	**0.017591**	0.416632		1.000000	**0.000000**	**0.015755**	1.000000	1.000000	1.000000	**0.025984**
GN 3	0.555897	1.000000	1.000000		**0.000246**	0.519311	1.000000	1.000000	1.000000	0.707207
GN 4	0.553774	**0.024071**	**0.000000**	**0.000246**		0.601518	**0.000000**	**0.000264**	**0.000074**	0.408504
GN 5	1.000000	1.000000	**0.015755**	0.519311	0.601518		**0.000046**	1.000000	0.553583	1.000000
GN 6	**0.000054**	**0.004394**	1.000000	1.000000	**0.000000**	**0.000046**		0.210656	1.000000	**0.000092**

GN 7	1.000000	1.000000	1.000000	1.000000	**0.000264**	1.000000	0.210656		1.000000	1.000000
GN 8	0.597651	1.000000	1.000000	1.000000	**0.000074**	0.553583	1.000000	1.000000		0.783032
E. nitens	1.000000	1.000000	**0.025984**	0.707207	0.408504	1.000000	**0.000092**	1.000000	0.783032	

Appendix 15. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence of the eucalypts on recovery day 5; H (df=9, N=294)=57.85731.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	1.000000	**0.000005**	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
GN 1	1.000000		**0.047498**	**0.000000**	0.088834	0.928732	0.053282	0.937802	1.000000	1.000000
GN 2	1.000000	**0.047498**		**0.002708**	1.000000	1.000000	1.000000	1.000000	1.000000	0.283415
GN 3	**0.000005**	**0.000000**	**0.002708**		**0.000968**	**0.000047**	**0.001379**	**0.000046**	**0.000122**	**0.000000**
GN 4	1.000000	0.088834	1.000000	**0.000968**		1.000000	1.000000	1.000000	1.000000	0.496468
GN 5	1.000000	0.928732	1.000000	**0.000047**	1.000000		1.000000	1.000000	1.000000	1.000000
GN 6	1.000000	0.053282	1.000000	**0.001379**	1.000000	1.000000		1.000000	1.000000	0.325386
GN 7	1.000000	0.937802	1.000000	**0.000046**	1.000000	1.000000	1.000000		1.000000	1.000000
GN 8	1.000000	1.000000	1.000000	**0.000122**	1.000000	1.000000	1.000000	1.000000		1.000000
E. nitens	1.000000	1.000000	0.283415	**0.000000**	0.496468	1.000000	0.325386	1.000000	1.000000	

Appendix 16. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence of the eucalypts on recovery day 6; H (df=9, N=294)=93.77447.

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		1.000000	**0.000000**	**0.000000**	0.324402	1.000000	**0.010276**	**0.000002**	1.000000	**0.000008**
GN 1	1.000000		**0.000153**	**0.000040**	1.000000	1.000000	0.656868	**0.000907**	1.000000	**0.003236**
GN 2	**0.000000**	**0.000153**		1.000000	0.056822	**0.007728**	1.000000	1.000000	**0.000009**	1.000000
GN 3	**0.000000**	**0.000040**	1.000000		**0.008353**	**0.001311**	0.156169	1.000000	**0.000003**	1.000000
GN 4	0.324402	1.000000	0.056822	**0.008353**		1.000000	1.000000	0.216534	1.000000	0.519113
GN 5	1.000000	1.000000	**0.007728**	**0.001311**	1.000000		1.000000	**0.034924**	1.000000	0.097022
GN 6	**0.010276**	0.656868	1.000000	0.156169	1.000000	1.000000		1.000000	0.070843	1.000000
GN 7	**0.000002**	**0.000907**	1.000000	1.000000	0.216534	**0.034924**	1.000000		**0.000057**	1.000000
GN 8	1.000000	1.000000	**0.000009**	**0.000003**	1.000000	1.000000	0.070843	**0.000057**		**0.000214**
E. nitens	**0.000008**	**0.003236**	1.000000	1.000000	0.519113	0.097022	1.000000	1.000000	**0.000214**	

Appendix 17. P-values from the ANOVA test (2-tailed) of relative electrolyte conductance of unfrozen samples of the eucalypts under standard conditions; F stat (df=9, F=4.0381).

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		0.887942	0.755097	**0.000347**	0.998773	0.997339	0.862393	0.117555	0.807115	0.820413
GN 1	0.887942		0.999999	**0.034790**	0.397679	0.999747	1.000000	0.936378	0.999999	1.000000
GN 2	0.755097	0.999999		0.064808	0.246675	0.996305	1.000000	0.983664	1.000000	1.000000
GN 3	**0.000347**	**0.034790**	0.064808		**0.000018**	**0.005895**	**0.040326**	0.463243	0.109035	**0.049585**
GN 4	0.998773	0.397679	0.246675	**0.000018**		0.823312	0.359701	**0.011110**	0.328085	0.308493

GN 5	0.997339	0.999747	0.996305	**0.005895**	0.823312		0.999480	0.592461	0.997026	0.998703
GN 6	0.862393	1.000000	1.000000	**0.040326**	0.359701	0.999480		0.951475	1.000000	1.000000
GN 7	0.117555	0.936378	0.983664	0.463243	**0.011110**	0.592461	0.951475		0.992980	0.968395
GN 8	0.807115	0.999999	1.000000	0.109035	0.328085	0.997026	1.000000	0.992980		1.000000
E. nitens	0.820413	1.000000	1.000000	**0.049585**	0.308493	0.998703	1.000000	0.968395	1.000000	

Appendix 18. P-values from the ANOVA test (2-tailed) of relative electrolyte conductance of frozen samples of the eucalypts under standard conditions; F stat (df=9, F=2.0005).

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		0.998928	0.999983	0.080360	0.379177	0.582374	0.866218	0.990266	0.217520	0.662612
GN 1	0.998928		0.244047	0.641271	0.866193	0.984952	0.999916	0.496976	0.899499	1.000000
GN 2	0.999983	0.244047		1.000000	0.992325	0.906153	0.605823	0.999994	0.996766	0.424710
GN 3	0.080360	0.641271	1.000000		0.999662	0.989010	0.899183	1.000000	0.999852	0.799460
GN 4	0.379177	0.866193	0.992325	0.999662		0.999983	0.992357	0.999885	1.000000	0.962460
GN 5	0.582374	0.984952	0.906153	0.989010	0.999983		0.999950	0.988697	0.999983	0.998690
GN 6	0.866218	0.999916	0.605823	0.899183	0.992357	0.999950		0.856245	0.994292	1.000000
GN 7	0.990266	0.496976	0.999994	1.000000	0.999885	0.988697	0.856245		0.999966	0.708610
GN 8	0.217520	0.899499	0.996766	0.999852	1.000000	0.999983	0.994292	0.999966		0.972411
E. nitens	0.662612	1.000000	0.424710	0.799460	0.962460	0.998690	1.000000	0.708610	0.972411	

Appendix 19. P-values from the ANOVA test (2-tailed) of relative electrolyte conductance of unfrozen samples of the eucalypts under cold shock conditions; F stat (df=9, F=10.701).

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis		0.729949	0.970203	0.999969	**0.00457**	0.289414	0.939378	**0.000007**	0.168341	**0.000029**
GN 1	0.729949		0.080805	0.993403	**0.000001**	**0.001059**	0.053900	**0.000000**	**0.000638**	**0.000000**
GN 2	0.970203	0.080805		0.895673	0.181288	0.961665	1.000000	**0.001758**	0.844949	**0.005223**
GN 3	0.999969	0.993403	0.895673		**0.009995**	0.263882	0.843904	**0.000076**	0.157203	**0.000218**
GN 4	**0.004570**	**0.000001**	0.181288	**0.009995**		0.915388	0.247384	0.907029	0.996717	0.975597
GN 5	0.289414	**0.001059**	0.961665	0.263882	0.915388		0.982959	0.112293	0.99999	0.218022
GN 6	0.939378	0.053900	1.000000	0.843904	0.247384	0.982959		1.000000	1.00000	**0.008816**
GN 7	**0.000007**	**0.000000**	**0.001758**	**0.000076**	0.907029	0.112293	**0.003092**		0.432544	0.999999
GN 8	0.168341	**0.000638**	0.844949	0.157203	0.996717	0.99999	0.902455	0.432544		0.616234
E. nitens	**0.000029**	**0.000000**	**0.005223**	**0.000218**	0.975597	0.218022	**0.008816**	0.999999	0.616234	

Appendix 20. P-values from the ANOVA test (2-tailed) of relative electrolyte conductance of frozen samples of the eucalypts under cold shock conditions; F stat (df=9, F=2.070).

	E. grandis	**GN 1**	**GN 2**	**GN 3**	**GN 4**	**GN 5**	**GN 6**	**GN 7**	**GN 8**	***E. nitens***
E. grandis	1.000000	0.999983	0.868378	0.920817	0.999757	1.000000	0.999015	0.999997	0.773455	0.999999
GN 1	0.999983	1.000000	0.998704	0.999521	1.000000	1.000000	1.000000	1.000000	0.999939	0.999757
GN 2	0.868378	0.998704	1.000000	0.999999	0.981950	0.880064	0.992681	0.868378	1.000000	0.474074

GN 3	0.920817	0.999521	0.999999	1.000000	0.999521	0.999631	0.999991	0.998112	1.000000	0.889004
GN 4	0.999757	1.000000	0.981950	0.999521	1.000000	0.999829	1.000000	0.999362	0.608437	0.512116
GN 5	1.000000	1.000000	0.880064	0.999631	0.999829	1.000000	0.998869	1.000000	0.596654	0.924559
GN 6	0.999015	1.000000	0.992681	0.999991	1.000000	0.998869	1.000000	0.995650	0.654973	0.550542
GN 7	0.999997	1.000000	0.868378	0.998112	0.999362	1.000000	0.995650	1.000000	0.525537	0.947068
GN 8	0.773455	0.999939	1.000000	1.000000	0.608437	0.596654	0.654973	0.525537	1.000000	0.732489
E. nitens	0.999999	0.999757	0.474074	0.889004	0.512116	0.924559	0.550542	0.947068	0.732489	1.000000

2. Within each eucalypt

Appendix 21. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of *E. grandis* over the experimental period; H (df=3, N=256)=52.16993.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		**0.025456**	**0.000000**	1.000000
30 min into cold shock	**0.025456**		**0.041507**	**0.000813**
90 min into cold shock	**0.000000**	**0.041507**		**0.000000**
Cold shock	1.000000	**0.000813**	**0.000000**	

Appendix 22. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 1 over the experimental period; H (df=3, N=256)=13.23147.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		1.000000	**0.038792**	1.000000
30 min into cold shock	1.000000		**0.002648**	1.000000
90 min into cold shock	**0.038792**	**0.002648**		**0.019063**
Cold shock	1.000000	1.000000	**0.019063**	

Appendix 23. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 2 over the experimental period; H (df=3, N=256)=57.75124.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		**0.000036**	1.000000	**0.000000**
30 min into cold shock	**0.000036**		**0.000061**	0.696705
90 min into cold shock	1.000000	**0.000061**		**0.000000**
Cold shock	**0.000000**	0.696705	**0.000000**	

Appendix 24. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 3 over the experimental period; H (df=3, N=256)=22.30559.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		1.000000	0.170992	**0.000047**
30 min into cold shock	1.000000		1.000000	**0.002886**
90 min into cold shock	0.170992	1.000000		0.135171
Cold shock	**0.000047**	**0.002886**	0.135171	

Appendix 25. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 4 over the experimental period; H (df=3, N=256)=5.212467.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		0.183797	1.000000	1.000000
30 min into cold shock	0.183797		0.516362	1.000000
90 min into cold shock	1.000000	0.516362		1.000000
Cold shock	1.000000	1.000000	1.000000	

Appendix 26. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 5 over the experimental period; H (df=3, N=256)=10.23351.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		1.000000	1.000000	0.278813
30 min into cold shock	1.000000		1.000000	**0.017968**
90 min into cold shock	1.000000	1.000000		0.074414
Cold shock	0.278813	**0.017968**	0.074414	

Appendix 27. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 6 over the experimental period; H (df=3, N=256)=56.48599.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		1.000000	**0.000000**	**0.006490**
30 min into cold shock	1.000000		**0.000000**	**0.001056**
90 min into cold shock	**0.000000**	**0.000000**		**0.029451**
Cold shock	**0.006490**	**0.001056**	**0.029451**	

Appendix 28. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 7 over the experimental period; H (df=3, N=256)=12.56445.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		1.000000	0.999050	**0.005938**
30 min into cold shock	1.000000		1.000000	**0.033083**
90 min into cold shock	0.999050	1.000000		0.336848
Cold shock	**0.005938**	**0.033083**	0.336848	

Appendix 29. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of GN 8 over the experimental period; H (df=3, N=256)=15.01376.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		**0.020194**	1.000000	1.000000
30 min into cold shock	**0.020194**		**0.001904**	**0.047733**
90 min into cold shock	1.000000	**0.001904**		1.000000
Cold shock	1.000000	**0.047733**	1.000000	

Appendix 30. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of reactive oxygen species of *E. nitens* over the experimental period; H (df=3, N=256)=23.74374.

	Standard conditions	**30 min into cold shock**	**90 min into cold shock**	**Cold shock**
Standard conditions		1.000000	0.220895	0.052360
30 min into cold shock	1.000000		**0.006855**	0.869836
90 min into cold shock	0.220895	**0.006855**		**0.000015**
Cold shock	0.052360	0.869836	**0.000015**	

Appendix 31. P-values and V-statistics from Wilcoxon ranked tests showing significant differences in phenolic acid levels of each eucalypt under standard and cold shock conditions.

Eucalypt	**p-value**	**V-stat**
E. grandis	**3.631e-10**	791
GN 1	**0.0232**	119
GN 2	0.4582	650
GN 3	**6.493e-12**	528
GN 4	**7.562e-06**	30
GN 5	**4.054e-06**	170
GN 6	0.2446	270
GN 7	**8.525e-06**	87

GN 8	0.0652	419
E. nitens	**5.657e-05**	193

Appendix 32. P-values and t-statistics from independent-samples t-tests showing significant differences in starch levels of each eucalypt under standard and cold shock conditions; df=14.

Eucalypt	p-value	|t-stat|
E. grandis	0.7249	0.361
GN 1	0.3174	-1.037
GN 2	0.3214	1.028
GN 3	0.1082	1.817
GN 4	0.4847	-0.726
GN 5	0.0547	-1.458
GN 6	0.2200	-1.305
GN 7	0.6043	2.776
GN 8	0.0583	-0.436
E. nitens	0.3584	-0.974

Appendix 33. P-values and V-statistics from Wilcoxon ranked tests showing significant differences in total soluble sugar levels of each eucalypt under standard and cold shock conditions.

Eucalypt	p-value	V-stat
E. grandis	**3.327e-09**	0
GN 1	0.562	56
GN 2	0.093	101
GN 3	**1.056e-05**	75
GN 4	0.495	54
GN 5	0.159	96
GN 6	**0.0250**	17
GN 7	0.782	62
GN 8	0.641	14
E. nitens	0.424	50

Appendix 34. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of *E. grandis* over the experimental period; H (df=5, N=192)=19.16271.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		1.000000	1.000000	1.000000	1.000000	**0.019837**
Cold shock	1.000000		1.000000	1.000000	1.000000	**0.003529**
Recovery Day 1	1.000000	1.000000		1.000000	1.000000	0.065112
Recovery Day 2	1.000000	1.000000	1.000000		1.000000	**0.003035**
Recovery Day 5	1.000000	1.000000	1.000000	1.000000		0.161713

Recovery Day 6	**0.019837**	**0.003529**	0.065112	**0.003035**	0.161713

Appendix 35. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 1 over the experimental period; H (df=5, N=192)=19.16271.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		1.000000	1.000000	1.000000	**0.014450**	0.445500
Cold shock	1.000000		1.000000	1.000000	1.000000	1.000000
Recovery Day 1	1.000000	1.000000		1.000000	**0.039513**	0.900561
Recovery Day 2	1.000000	1.000000	1.000000		**0.013333**	0.420775
Recovery Day 5	**0.014450**	1.000000	**0.039513**	**0.013333**		1.000000
Recovery Day 6	0.445500	1.000000	0.900561	0.420775	1.000000	

Appendix 36. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 2 over the experimental period; H (df=5, N=192)=14.96140.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		1.000000	1.000000	1.000000	1.000000	1.000000
Cold shock	1.000000		1.000000	0.050874	1.000000	0.101427
Recovery Day 1	1.000000	1.000000		1.000000	1.000000	1.000000
Recovery Day 2	1.000000	0.050874	1.000000		0.090780	1.000000
Recovery Day 5	1.000000	1.000000	1.000000	0.090780		0.174751
Recovery Day 6	1.000000	0.101427	1.000000	1.000000	0.174751	

Appendix 37. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 3 over the experimental period; H (df=5, N=192)=56.11138.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		**0.000006**	1.000000	0.805669	**0.011419**	**0.004202**
Cold shock	**0.000006**		**0.000000**	**0.024964**	1.000000	1.000000
Recovery Day 1	1.000000	**0.000000**		**0.027218**	**0.000078**	**0.000021**
Recovery Day 2	0.805669	**0.024964**	**0.027218**		1.000000	1.000000
Recovery Day 5	**0.011419**	1.000000	**0.000078**	1.000000		1.000000
Recovery Day 6	**0.004202**	1.000000	**0.000021**	1.000000	1.000000	

Appendix 38. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 4 over the experimental period; H (df=5, N=192)=16.05214.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		1.000000	1.000000	**0.018124**	1.000000	1.000000
Cold shock	1.000000		1.000000	0.829504	1.000000	1.000000
Recovery Day 1	1.000000	1.000000		**0.036550**	1.000000	1.000000
Recovery Day 2	**0.018124**	0.829504	**0.036550**		**0.014220**	0.091586
Recovery Day 5	1.000000	1.000000	1.000000	**0.014220**		1.000000
Recovery Day 6	1.000000	1.000000	1.000000	0.091586	1.000000	

Appendix 39. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 5 over the experimental period; H (df=5, N=192)=17.99526.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		1.000000	0.926124	1.000000	0.907474	0.536147
Cold shock	1.000000		**0.036960**	**0.046278**	**0.035875**	**0.016746**
Recovery Day 1	0.926124	**0.036960**		1.000000	1.000000	1.000000
Recovery Day 2	1.000000	**0.046278**	1.000000		1.000000	1.000000
Recovery Day 5	0.907474	**0.035875**	1.000000	1.000000		1.000000
Recovery Day 6	0.536147	**0.016746**	1.000000	1.000000	1.000000	

Appendix 40. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 6 over the experimental period; H (df=5, N=192)=32.28593.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		1.000000	1.000000	**0.014049**	1.000000	1.000000
Cold shock	1.000000		0.579113	**0.000149**	1.000000	1.000000
Recovery Day 1	1.000000	0.579113		0.281111	0.169708	1.000000
Recovery Day 2	**0.014049**	**0.000149**	0.281111		**0.000016**	**0.000563**
Recovery Day 5	1.000000	1.000000	0.169708	**0.000016**		1.000000
Recovery Day 6	1.000000	1.000000	1.000000	**0.000563**	1.000000	

Appendix 41. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 7 over the experimental period; H (df=5, N=192)=17.64936.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		1.000000	1.000000	1.000000	0.184988	1.000000
Cold shock	1.000000		1.000000	1.000000	0.325535	0.993913

Recovery Day 1	1.000000	1.000000		1.000000	0.902861	0.364078
Recovery Day 2	1.000000	1.000000	1.000000		0.234731	1.000000
Recovery Day 5	0.184988	0.325535	0.902861	0.234731		**0.000539**
Recovery Day 6	1.000000	0.993913	0.364078	1.000000	**0.000539**	

Appendix 42. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of GN 8 over the experimental period; H (df=5, N=192)=35.16090.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		**0.004005**	1.000000	1.000000	0.882805	**0.000289**
Cold shock	**0.004005**		0.329737	**0.000985**	1.000000	1.000000
Recovery Day 1	1.000000	0.329737		1.000000	1.000000	0.052724
Recovery Day 2	1.000000	**0.000985**	1.000000		0.380958	**0.000058**
Recovery Day 5	0.882805	1.000000	1.000000	0.380958		0.256816
Recovery Day 6	**0.000289**	1.000000	0.052724	**0.000058**	0.256816	

Appendix 43. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of chlorophyll fluorescence levels of *E. nitens* over the experimental period; H (df=5, N=192)= 50.84140.

	Standard conditions	**Cold shock**	**Recovery Day 1**	**Recovery Day 2**	**Recovery Day 5**	**Recovery Day 6**
Standard conditions		0.239857	1.000000	0.517142	**0.000750**	1.000000
Cold shock	0.239857		**0.001580**	**0.000091**	**0.000000**	0.855583
Recovery Day 1	1.000000	**0.001580**		1.000000	0.145292	0.724032
Recovery Day 2	0.517142	**0.000091**	1.000000		0.783414	0.131671
Recovery Day 5	**0.000750**	**0.000000**	0.145292	0.783414		**0.000076**
Recovery Day 6	1.000000	0.855583	0.724032	0.131671	**0.000076**	

Appendix 44. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of *E. grandis* over the experimental period; H (df=3, N=128)= 8.509.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.998512	0.753083	0.150531
Standard frozen	0.998512		0.864110	0.219578
Cold shock unfrozen	0.753083	0.864110		**0.036480**
Cold shock frozen	0.150531	0.219578	**0.036480**	

Appendix 45. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 1 over the experimental period; H (df=3, N=128)=11.608.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.953409	**0.014047**	0.989791
Standard frozen	0.953409		0.105352	0.887113
Cold shock unfrozen	**0.014047**	0.105352		**0.023892**
Cold shock frozen	0.989791	0.887113	**0.023892**	

Appendix 46. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 2 over the experimental period; H (df=3, N=128)=13.247.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.427083	0.087140	0.419055
Standard frozen	0.427083		**0.007991**	0.060350
Cold shock unfrozen	0.087140	**0.007991**		0.993229
Cold shock frozen	0.419055	0.060350	0.993229	

Appendix 47. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 3 over the experimental period; H (df=3, N=128)=23.138.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.098287	**0.000100**	0.175125
Standard frozen	0.098287		**0.008436**	0.287240
Cold shock unfrozen	**0.000100**	**0.008436**		0.550693
Cold shock frozen	0.175125	0.287240	0.550693	

Appendix 48. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 4 over the experimental period; H (df=3, N=128)=7.331.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.168398	0.112044	0.112044
Standard frozen	0.168398		0.999977	0.977900
Cold shock unfrozen	0.112044	0.999977		0.949994
Cold shock frozen	0.112044	0.977900	0.949994	

Appendix 49. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 5 over the experimental period; H (df=3, N=128)=1.894.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.892527	0.981892	0.578556
Standard frozen	0.892527		0.970953	0.858031
Cold shock unfrozen	0.981892	0.970953		0.832466
Cold shock frozen	0.578556	0.858031	0.832466	

Appendix 50. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 6 over the experimental period; H (df=3, N=128)=9.210.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.663894	**0.024854**	0.595750
Standard frozen	0.663894		0.126411	0.912785
Cold shock unfrozen	**0.024854**	0.126411		0.411087
Cold shock frozen	0.595750	0.912785	0.411087	

Appendix 51. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 7 over the experimental period; H (df=3, N=128)=1.917.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.998111	0.839039	0.930765
Standard frozen	0.998111		0.858031	0.902937
Cold shock unfrozen	0.839039	0.858031		0.569957
Cold shock frozen	0.930765	0.902937	0.569957	

Appendix 52. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of GN 8 over the experimental period; H (df=3, N=128)=9.273.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.999799	0.589943	**0.032944**
Standard frozen	0.999799		0.811818	0.116031
Cold shock unfrozen	0.589943	0.811818		0.195773
Cold shock frozen	**0.032944**	0.116031	0.195773	

Appendix 53. P-values from the Kruskal-Wallis multiple comparisons test (2-tailed) of relative electrolyte conductance levels of *E. nitens* over the experimental period; H (df=3, N=128)=5.126.

	Standard unfrozen	**Standard frozen**	**Cold shock unfrozen**	**Cold shock frozen**
Standard unfrozen		0.981892	0.845493	0.595750
Standard frozen	0.981892		0.261020	0.214035
Cold shock unfrozen	0.845493	0.261020		0.688909
Cold shock frozen	0.595750	0.214035	0.688909	

Appendix 54. Subjective scoring system used for the frost damage assessment for the Sappi field trials (Adapted from Maritz and Brink 2015).

Score	**Phenotype**
0	No evidence of damage
1	Slight scorching on some tips
2	Moderate scorching on all tips
3	Severe scorching on most leaves
4	Extremely severe scorching on all leaves