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**THE EVALUATION AND CHARACTERISATION OF
SOUTH AFRICAN WHEAT CULTIVARS FOR
TEMPERATURE STRESS TOLERANCE**

by

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List of symbols and abbreviations

A	= change in substance concentration
ABA	= abscisic acid
ADP	= adenosine phosphate
BMQ	= bread making quality
BSA	= bovine serum albumin
$^{\circ}\text{C}$	= degree celcius
C_1	= initial conductance value for the control
C_2	= final conductance value for the control
Cm	= centimetre
CMS	= cell membrane stability
CV	= coefficient of variance
D	= light path (0.8698 cm)
e	= extinction coefficient NADPH ($5.4099 \text{ mmol}^{-1} \text{ cm}^{-1}$)
EDTA	= ethylenediamine-tetra-acetic acid
F	= dilution factor (4)
FTPs	= frost tolerance proteins
FW	= fresh weight
g	= gram
GK	= glutamyl kinase
GSA	= glutamate semialdehyd
GYP	= Total grain yield per plant
Ha	= hectare
HCL	= hydrochloric acid
HMW	= high molecular weight
HSP	= heat shock proteins
kD	= kilodalton
LMW	= low molecular weight
LSD	= least significant difference
LT50	= lethal temperature of 50%
mg.g^{-1}	= milligrams per gram
mg	= milligram

ml	=	millilitre
mM	=	milimolar
mw	=	molecular weight
NACL	=	sodium chloride
NADPH	=	nicotinamide adenine dinucleotide phosphate
nm	=	nanometre
PKM	=	primary tiller kernel mass
PMSF	=	phenylmethylsulfonyl fluoride
P5C	=	pyrroline – 5- carboxylate
r	=	correlation coefficient
RI	=	relative injury
Rpm	=	revolutions per minute
SE- HPLC	=	size exclusion high performance liquid chromatography
SSS	=	soluble starch synthase
SS	=	sucrose synthesis
S-S	=	disulphide
SST	=	sucrose:sucrose fructosyltransferase
S-H	=	sulphydryl
SPS	=	sucrose phosphate synthesis
SKM	=	secondary tiller kernel mass
SN	=	number of spikes
T ₁	=	initial conductance value for the treatment
T ₂	=	final conductance value for the treatment
TTC	=	2,3,5-triphenyltetrazolium chloride
US	=	Unites States
V	=	volume of sample (ml)

CHAPTER 1

General introduction

Environmental stress is and has always been a big problem in the production of crops in the world and also in South Africa. Many factors limiting yield and quality are environmental. Research on the morphological effects of environmental stress has been done in numerous studies in South Africa (Bartels and Nelson, 1994). It is however, very important to also evaluate the effects of environmental stress on the biochemical processes in wheat plants. Along with a clear understanding of morpho-physiological traits, many researchers believe that the biochemical basis of stress resistance is an essential prerequisite for enhancing crop tolerance to environmental stress (Klueva *et al.*, 2001).

Wheat (*Triticum aestivum* L. and *Triticum turgidum* L.) is the world's leading cereal grain and one of the most important food crops. Its diversity of uses, nutritive content and storage qualities have made wheat a staple food for more than one third of the world's population (Satorre and Slafer, 1999).

High temperature is one of the most prominent abiotic stresses affecting crop productivity (Boyer, 1982). As much as 23% of the earth's land surface shows an annual mean air temperature above 40⁰C. In the temperate zone, soil surface temperatures of 60⁰C have been recorded, and the shoot apices of grasses may be exposed to temperatures around 40⁰C for several hours daily and to 50⁰C for shorter periods. Exposures to heat stress usually occurs on a daily basis (Pollock *et al.*, 1993) and can be accompanied by other stresses, such as water deficits, low night temperatures, and soil metal contamination. It is estimated that heat and drought stress affect 25% of the total arable lands. In the United States average yields of major crops are three to seven times lower than the expected yields. Heat and drought stress cause a major part of these losses (Klueva *et al.*, 2001).

Wheat, in most of the western parts of the Free State experiences high temperature stress in later stages of growth. The possibility of damage is higher when high temperature days are followed by cold temperature nights. Nearly 544 000 ha of wheat were insured against frost damage over the last 10 years in South Africa, of which 47 000 ha were damaged by frost. This led to an average loss of income close to 6.7 million rands annually (Willemse, 1999). This high level of frost damage indicates that wheat cultivars with a high level of tolerance to freezing are needed.

It is estimated that people in developing countries now consume half of the world's wheat and that within 10 years they will consume 60% of all wheat produced. Since the 1960s, wheat consumption has risen almost 5% a year in developing countries, partly due to rising population and partly because as standards of living rise, people tend to eat more wheat in the form of convenience foods (for example, sandwiches). As a result, it is estimated that by 2020 the demand for wheat will be 40% greater than it is today (Pingali, 2000) The growing demand for human food supply makes breeding for high yielding crops with built-in resistance against environmental constraints one of the most important challenges for plant breeders (Zhang et al., 1999). Significant increases in our understanding of the physiological basis of plant stress resistance and the advent of molecular technologies to crop breeding, allow breeders to address these problems much more efficiently than in the past. As a result, in recent years, various abiotic stress tolerance traits were successfully manipulated in several major crop species (reviewed by Klueva et al., 1999).

The objectives of this study were:

1. To evaluate four laboratory tests: triphenyltetrazolium chloride (TTC), cell membrane stability (CMS), proline concentration, and crown survival for their potential use in stress tolerance breeding.
2. To investigate the effect of high and low temperature stress on yield and yield components of wheat.
3. To determine the effect of high and low temperature stress on protein and sugar content in wheat seedlings.

CHAPTER 2

Literature Review

2.1 Wheat

2.1.1 General

Wheat is the number one food directly consumed by humans. More land is devoted worldwide to the production of wheat than any other commercial crop. Wheat is grown all around the world and is the most frequently cultivated of all cereals. A wheat crop is harvested somewhere in the world during every month of the year (Briggle and Curtis, 1987; Cook and Veseth, 1991).

On global basis, wheat provides more nourishment for people than any other food source, and it is the most important carbohydrates food source (Briggles and Curtis, 1987). World production of wheat increases at a significant rate as the nutritional virtues of complex carbohydrates are accepted worldwide. Although wheat is consumed in many forms, including noodles, gruels, cooked cereals, and ready to eat cereals, bread has established itself worldwide as a major convenient and delicious food (Cook and Veseth, 1991).

Wheat is not indigenous to southern and central Africa. It was introduced into the Cape area of South Africa in the late seventeenth century, but it was only in the mid eighteenth century that the production spread and larger quantities of wheat were produced (Anon, 1990). The average size of the annual wheat crop of about 2.2 million metric tons has varied between 1.2 and 3.5 million metric tons over the past years. It is largely determined by the climate and rainfall patterns in the summer rainfall areas of the Free State and Western Cape provinces. The continuous release of varieties adapted to the different conditions will ensure the future of wheat production in South Africa (Randall *et al.*, 1995)

2.1.2 Classification

All wheat belongs to the genus *Triticum*. This genus along with *Horden*, *Secale*, *Bromus* and *Agropyron* make up the most important groups of the grass family *Graminaceae* (*Poaceae*) (Cook and Veseth, 1991). In 1753 Linnaeus proposed the first classification of wheats based on morphological and physiological differences (Bozzini, 1988). According to Orth and Shellenberger (1988) four species of the genus *Triticum* are relevant, namely *T. monococcum* (diploid), *T. turgidum* (tetraploid), *T. timopheevi* (tetraploid) and *T. aestivum* (hexaploid).

T. turgidum (durum or macaroni wheats) and *T. aestivum* (common or bread wheats) are the most widely grown. Bread and durum wheats are allopolyploids. Bread wheat is hexaploid ($2n=6x=42$) and has three genomes (AABBDD). Durum wheat is tetraploid ($2n=4x=24$) and has two genomes (AABB) (Baenzinger *et al.*, 1994). There are also sub classifications of wheat species. Hard red winter wheat and hard red spring (according to when planted) are used for leavened bread production. Soft red winter and common white wheat classes are mainly used for pastries, crackers and cookies. Durum wheat is used for pasta products, like spaghetti and macaroni (Jackel, 1995).

2.1.3 Climate requirements

Wheat is a cool season crop, but it flourishes in many agronomic and climatic zones. Production is concentrated between latitudes 30 to 60⁰N and between 27 to 40⁰S (Nuttonson, 1955). However, it is known that wheat is also grown outside these areas, for example in the Northern hemisphere wheat is cultivated from within the Arctic circle to the equator, provided that it is cultivated at locations of sufficiently high elevation (Briggle and Curtis, 1987). The minimum day temperature for growth is 3-4⁰C, the optimum temperature is 25⁰C and the maximum is 30-32⁰C (Briggle, 1980).

The distribution of wheat and the type of wheat grown in relation to temperature are largely determined by the length of the frost-free period, the minimum winter

temperature, the temperature in relation to the average day-length during the growing season and the maximum temperatures immediately preceding harvest. Cool, moist conditions are needed for satisfactory growth and development of grain, followed by a bright, dry and warm ripening period of 6-8 weeks, with a mean temperature of 18 to 19°C (Nuttonson, 1955).

If the summer temperatures are too low, grains ripen slowly and if the growing season is short, grains are likely to be caught by frost. High temperatures before harvest, especially if accompanied by drying winds, may also be injurious as the development of flowers and filling of the grain might be checked by hot weather (Cook and Veseth, 1991). Leonard and Martin (1963) stated that wheat losses caused by low temperatures are nearly as great as losses brought about by all wheat diseases combined.

2.2 High temperature stress

High temperature stress is prominent among the cardinal ecological factors that determine crop growth and productivity. Habitats of cereals differ dramatically and temperatures above 35°C are common in both temperate and tropical regions. However, cereals that are native to temperate regions usually lack the thermo-tolerance that characterizes most tropical species and are frequently injured during stress (Al-Khatib and Paulsen, 1999).

High temperature stress reduces wheat productivity in many regions and limits response to cultural practices for high yield (Midmore *et al.*, 1984; Shpiler and Blum, 1986). The extent of damage caused by exposure to high temperature differs depending on the crop. Some stages of the plants' life cycle are more heat susceptible than others and heat stress during these stages may be critical for final yield determination, even when the temperature regime of the rest of the growing season is optimum (Abrol *et al.*, 1991).

At whole-plant level, heat stress causes an acceleration of developmental stages, abortion of seed development and impairment of grain filling. At physiological level, high

temperature affects major plant cell functions, including photosynthesis, energy metabolism, and translocation of assimilates (Berry and Bjorkman, 1980; Levitt, 1980 Paulsen, 1994).

Heat stress causes profound modifications of all aspects of cell and whole-plant metabolism. The main sites of injury of plant cells under heat stress are proteins (enzymes) and membranes. These are damaged by the direct effect of heat stress, elevation of intracellular temperature and by secondary stress, oxidative damage and dehydration. The dysfunction of photosynthetic enzymes and membranes leads to inhibition of photosynthesis, which is the major cause of heat stress induced biomass loss in crop plants (Klueva *et al.*, 2001).

Heat stress may interfere with *de novo* protein biosynthesis, inhibit enzyme activity and induce degradation of existing proteins. A heat induced inhibition of constitutive protein synthesis (Nover, 1991), together with an increased enzyme degradation rate, which may deplete the major enzyme pools essential for all functions, occurs during the heat stress and recovery period (Weis, 1981; Rijven, 1986).

2.2.1 High temperature and crop growth and development

Productivity in wheat and other crop species is reduced markedly at high temperatures. All stages of development are sensitive to temperature. It is the main factor controlling the rate of crop development. Development generally accelerates as temperatures increase, a phenomenon that is often described as a linear function of daily average temperature. The growing degree-day concept is a common example of linear model of developmental response to temperature (Abrol *et al.*, 1991).

Wheat development is customarily divided into vegetative and reproductive stages with either ear emergence or anthesis as the event that separates the two stages. These stages are generally based on early-recognised features of the apical meristem. They mark significant changes in morphology or physiology of different crop organs. Number of

leaves and tiller primordia are determined before spikelet initiation, but their subsequent growth and development are controlled by temperature and day length during the differentiation of spikes into spikelets. Similarly, floret number within each spikelet is established by anthesis, at which time the potential grain per spike is established (Abrol *et al.*, 1991).

Several (Asana and Williams, 1965; Abrol *et al.*, 1991; Slafer and Rawson, 1994) experiments have observed the effects of temperature on the duration from sowing or emergence to heading under controlled environmental and field conditions. The major conclusion from their studies was that all genotypes are sensitive to temperature at one stage or another. Temperature sensitivity however, varies greatly with genotypes and the genotype's phenological stages also differ in sensitivity to temperature. The duration of the phase from sowing to first spikelet initiation is less sensitive to change in temperature than are the other phases, although genotypes also differ in thermo-tolerance during this phase. Stages during which environments have the greatest impact on yield, are from the first spikelet initiation or terminal spikelet formation until anthesis. Spikelet number and floral number (potential grain numbers), both dominant yield contributors, are established during these phases. Grain weight, on the other hand, appears to be much less sensitive to heat stress than grain number (Slafer and Rawson, 1994).

2.2.2 High temperature and grain development phase

In experiments under controlled conditions from 25-35⁰C, mean grain weight declined 16% for each 5⁰C increase in temperature (Asana and Williams, 1965). For every 1⁰C rise in temperature there was a depression in grain yield by 8 to 10% mediated through 5 to 6% fewer and 3 to 4% smaller grain weight (Wattal, 1965).

To elucidate the causal factors for reduced grain filling in wheat because of higher temperature, Wardlaw (1974) studied the three main components of the plant system. The three components are: (1) source – flag leaf blade; (2) sink – ear and (3) transport pathway – peduncle. He observed that photosynthesis had a broad temperature optimum

from 20 to 30°C and the rate of ¹⁴C assimilate movement out of the flag leaf and phloem was optimal around 30°C: the rate of ¹⁴C assimilate movement through the stem was independent on temperature range from 1°C to 50°C.

As a result, source-sink relationships altered through grain excision, defoliation, shading treatments and heat stress still reduced grain weight (Wardlaw *et al.*, 1980). These results support earlier findings that temperature effects on grain weight are direct rather than due to assimilate availability (Spiertz, 1974; Bremmer and Rawson, 1978). Furthermore, respiration effects do not appear to be the direct cause of decreased grain size in heat stressed wheat (Wardlaw, 1974).

Reduction of grain weight by heat stress may be explained mostly by effects of temperature on rate and duration of grain growth. As temperature increased from 15/10°C to 21/16°C, duration of grain filling was reduced from 60 days to 36 days and grain growth rate increased from 0.73 to 1.49 mg/grain/day with a result of minimal influence on grain weight at maturity. Further increase in temperature from 21/16°C to 30/20°C resulted in decline in grain filling from 36 to 22 days with a minimal increase in grain growth rate from 1.49 to 1.51 mg/grain/day. Thus mature grain weight was significantly reduced at the highest temperature (Asana and Williams, 1965; Sofield *et al.*, 1977).

2.2.3 The effect of temperature on carbohydrates

Sugars represent the major reserve in seed which are maximally synthesized during germination and mobilized to various tissues, like the stem and internodes in the form of sucrose, glucose and fructose that are readily transportable to sites where they are required for growth and maintain the osmotic regulation of cells. Accumulation of sugars in different parts of the plant is enhanced in response to various environmental stresses (Bewley and Black, 1994).

Gill *et al.*, (2001) reported that a significantly increased total sugar content in sorghum seedlings was found on exposure to high temperature stress. A dramatic change was

observed in the endosperm in response to heat. Fructose content was always higher than the glucose and sucrose content. Sucrose is usually the major carbon source for starch synthesis in most plants and is the form in which carbohydrate is translocated to developing and reserve tissues from the leaves, where it is formed. Donaval *et al.* (1977) reported that the onset of rapid starch synthesis is accompanied by a marked decline in the concentration of sucrose and reducing sugars. Grain sucrose levels are not significantly reduced by moderately high temperatures in any major crops, despite the fact that heat stress increases the rates of grain respiration, leaf senescence and reduces photosynthesis (Klueva *et al.*, 2001).

Starch, along with protein is the major constituent of mature wheat grain. Starch synthesis is highly sensitive to high temperature stress due to the susceptibility of the soluble starch synthase in developing kernels of wheat (Denyer *et al.*, 1994; Jenner 1994). Starch in a wheat grain is deposited in two distinct types of granules: large lenticular (commonly known as A-type) granules, which are initiated early during the development of the endosperm, and smaller spherical (B-type) granules, which are initiated later than the A-type. Environmental conditions during grain development strongly influence the number and the size of the two types of granules. Under conditions of water stress, shading or mineral deficiency, fewer B-type are initiated, and the size of A-type granules but not their number, is reduced. High temperature during grain filling seems to have similar effects to water stress on granule number and size (Hoshikawa, 1962; Moss, 1963).

Bhullar and Jenner (1985) found that warming the plants during the early stages of grain growth significantly reduced the number of starch granules, an effect that was evident only at later stages and which was confined to B-type granules. The contribution of B-granules toward total weight of starch in the grain has been estimated by several techniques and has been shown to be 30 and 50%. It was indicated that the reduction of B-granules accounts for between 30.9 and 48.1%. The number of A-type granules is genetically fixed whereas the number of B-type is dependent on the prevailing conditions during grain growth. Alternatively, the relative stability in the number of A-type granules

to adverse environmental conditions could simply be due to fact that the number of these granules is determined very early in the grain's development and environmental stress did not develop early enough or were not severe enough at the critical stage to reduce the number of A-type granules (Buttrose, 1960).

Reduced incorporation at 35⁰C compared with 30⁰C indicates a comparatively low temperature optimum for the deposition of starch in wheat. High temperature does not reduce the incorporation of 14C into the soluble pool, which provides substrate to the synthesis of starch, so it may be presumed that the temperature response of the incorporation is the reflection of one or more of the partial processes involved in the conversion of sucrose to starch, in wheat (Rijven, 1986; Jenner *et al.*, 1991). Possibilities include the reduced transport of intermediates into the amyloplast due to impairment of the transport function of the amyloplast membrane, or a reduction in the activity of one or more of the enzymes involved in starch biosynthesis. The activity of enzymes involved in the synthesis of starch was reported to be reduced in leaves of grapevine grown at 35/30⁰C and in potatoes grown at 30⁰C. In particular the activity of soluble starch synthase (SSS), with a branching enzyme converting adenosine diphosphate [ADP]-glucose to amylopectic within the amyloplast has been shown to be greatly reduced by temperatures greater than around 30⁰C (Rijven, 1985).

2.2.4 High temperature stress and photosynthesis

Photosynthesis is one of the most heat sensitive functions of plants. Thermal stability of the photosynthetic apparatus differs markedly between species from temperate and tropical environments. Photosynthesis and accumulation of dry matter in temperate cereals were affected adversely by temperature that was near optimum for tropical cereals (Tashiro and Wardlaw, 1989). Exposure of plants to elevated temperatures resulted in a rapid inhibition of photosynthetic CO₂ fixation, oxygen evolution and photophosphorylation (Berry and Bjorkman, 1980). Limitations of CO₂ fixation by high temperature are accompanied by the inactivation of an enzyme rubisco activase, leading

to a decrease in Rubisco activity (Weis 1981; Kobza and Edwards, 1987; Fellar *et al.*, 1988).

High temperature damaged photosynthesis before other processes in sensitive species (Berry and Bjorkman, 1980). In wheat, leaf photosynthesis and Hill reaction activity declined earlier than other important processes such as nitrogen assimilation (Al- Khatib and Paulsen, 1984). Sensitivity of photosynthesis in temperate species to high temperature was attributed to liability of photosystem II (PS II) in thylakoid membranes (Santarius, 1973). Heat inactivation of PS II induces a release of the extrinsic proteins of the oxygen-evolving complex to the thylakoid lumen. Temperatures up to 35°C during maturation of wheat accelerated loss of chloroplast integrity and PS II activity but had little effect on PS I (Xu *et al.*, 1995). Treating wheat seedlings at 42.5°C for 10 minutes irreversibly reduced photosynthetic capacity, Hill reaction activity and membrane stacking (Santarius, 1973).

In an experiment to study the effects of high temperature stress on CO₂ exchange by ears, Wardlaw *et al.* (1980) found that ear photosynthesis decreased if light decreased with temperature and this was paralleled by increasing dark respiration. Thus the reduced net photosynthesis at high temperatures appeared to result from effects of temperature on respiration. These results were also in agreement with assessments made in 1965 by Carr and Wardlaw.

2.2.5 High temperature injury to cell membranes

Recent evidence suggests that biomembranes are the most heat susceptible sides in cells. Semichatova and Egrova (1975) reported that mitochondrial membranes were inactivated by remarkably mild heat treatment. Santarius (1975) showed that thylakoid membranes were much more sensitive to heat than soluble enzymes in chloroplasts. The characteristics feature of the thylakoid membranes is the abundance of polyunsaturated fatty acids that are more prone to oxidative degradation of lipid peroxidation. Vital functions of thylakoids, which are highly sensitive to heat stress, were shown to be

oxygen evolution by water splitting apparatus of photosynthesis and photophosphorylation (Santarius, 1975).

Another important membrane system, by which thermostability can be studied, is photosynthetic cells, the chloroplast. Its two membranes enclose the semi-liquid stroma in which the thylakoid is embedded. The inner of these membranes possesses specific permeability properties. It permits transfer of certain key metabolites while barring macromolecules and most ionic substances from uncontrolled permeation to and from the surrounding cytosol. Breakage of the envelope, or alteration of its properties, could conceivably cause breakdown of normal cell metabolism. Chloroplasts with intact envelopes can be isolated from leaves in a way, which fully preserves the physiological function of these organelles. This allows comparable studies on the effects of heat stress on chloroplast envelopes and thylakoid membranes in cell free preparations of the whole chloroplast (Heber and Kirk, 1975).

The integrity of the envelopes is estimated by measuring light dependent ferricyanide reduction. Since ferricyanide cannot penetrate the envelope, intact chloroplasts are unable to reduce it. The percentage of broken envelope free chloroplast can thus be calculated from the rate of reduction in the test sample and the maximum rate observed after osmotic breakage of the envelopes. Activities of aldose, NAD-dependent malate dehydrogenase, is determined according to Bergmeyer (1970) on the integrity of the chloroplast envelope.

Results have shown that ferricyanide reduction by broken chloroplasts, which contaminated the intact preparation, is progressively inactivated with increasing incubation temperature. The much higher rate measured after heat treatment and subsequent breakage of envelopes, when all chloroplasts can participate in the reaction, also declines, but higher temperatures are needed for the same degree of inactivation. The differences in inactivation are due to the fact that during exposure to heat stress, the envelope-free thylakoids, which are present at a small percentage in the preparation of intact chloroplasts, are in an environment different from that of the thylakoids that are

surrounded by stroma and envelopes. In fact, the heat sensitivity of thylakoid membranes depends on the incubation medium (Santarius, 1973). It was shown that 50% inactivation of ferricyanide reduction and phenazine metosulfate-catalysed cyclic photophosphorylation required considerably lower temperatures when the thylakoids were suspended in water, instead of the sorbitol medium usually applied in experiments. It was shown that those thylakoids that are still enclosed in the envelope are even less heat sensitive than envelope free thylakoids exposed to the incubation medium. This explains the apparent increase in the integrity, as calculated from rates of ferricyanide reduction measured after heat treatment. Furthermore, the rate of determination becomes somewhat inaccurate when the ferricyanide reduction has been largely inactivated by heat. Apart from these limitations, the large differences in the reduction rates of intact and osmotically ruptured chloroplasts observed after heat treatment, clearly indicates that the integrity of the envelopes is little affected, while ferricyanide reduction by the thylakoids becomes inactivated (Krause and Santarius, 1975).

Since changes in the pH of the medium are transmitted to the stroma of isolated chloroplasts, the pH may influence the heat sensitivity of thylakoids even if they are enclosed in the envelope. Results have shown that at higher and lower pH values the heat sensitivity of the thylakoids was much higher. The apparent increase of the integrity due to heating, as seen in the range of pH 6 to 7, is caused by the different heat sensitivity of naked thylakoids and those situated in the stroma of intact chloroplasts. In order to avoid complications, heat treatment is generally carried out at pH 6.7, within the range of the highest thermal stability of the thylakoids (Krause and Santarius, 1975).

2.2.6 High temperature stress and grain lipids

The lipid content of wheat grains is relatively low (2.5 to 3.5% dry weight). Wheat grain lipids have been extensively studied (Morisson, 1979; 1983) and are complex (Hargin and Morisson, 1980). Although changes in lipid composition during development have been reported there have been few studies on wheat grain lipids (Williams *et al.*, 1993). Yet, lipids metabolism in wheat plants is particularly important from several standpoints:

lipids are a major component of membranes and as such they influence the overall growth and development of the plant. They are also an important source of essential fatty acids. Wheat flour is particularly rich in linoleate so that wheat germ oil is an economically viable commodity. Moreover, lipid content and composition profoundly influence the milling properties of flour (Karpati *et al.*, 1990).

Temperature is known to alter the speed of chemical and enzymatic reaction and to have a significant role in physiological effects. As far as lipids are concerned, temperature produces a number of common effects many of which are believed to be related to membrane functions (Harwood *et al.*, 1993). Within a developing seed, the effect on a given storage component (such as lipids) will be influenced by effects on other competing pathway (Bewley and Black, 1994).

Increased (4°C above ambient temperature) growth temperature had general effects of reducing the accumulation of lipid classes. This effect was particularly marked for the non-starch, non-polar lipid fractions. The change on non-starch lipid content was found to be statistically significant (Williams *et al.*, 1994).

Starch lipids were affected by temperature, whereby plants grown at higher growth temperature possessed significantly reduced total amounts of fatty acids/wheat grain. There was a 50% reduction in the amount of the main starch lipid LysoPtdCho, in grains at elevated growth temperature. The relative importance of starch lipids is related to the fact that they are involved in starch biosynthesis in the starchy endosperm (Morisson, 1988b).

Shortening of the growing season, which occurs at higher temperatures, reduces the time available for lipid accumulation in wheat grain. Analysis of lipids in developed grains has supported this theory. Growth at elevated temperatures generally decreases unsaturation while an increase in desaturation at lower temperatures is a protective mechanism (Williams *et al.*, 1994). However, William *et al.* (1995) found that increased temperature

usually increases the apparent conversion of oleate to linoleate (i.e. enhanced 12-desaturation).

Heat stress may be an oxidative stress (Lee *et al.*, 1983). Peroxidation of membranes has been observed at high temperatures (Mishra and Singhal, 1992), which is a symptom of cellular injury. Lipid peroxidation has been defined as oxidative degradation of polyunsaturated lipids containing more than two carbon-carbon double covalent bonding (Girotti, 1990).

2.2.7 High temperature stress and reproduction

In wheat, the number of grains that develop on an ear is dependent on the number of viable florets that are formed and the effective fertilization of these after anthesis (Evans and Wardlaw, 1976). There is evidence of a reduction of grain number per ear associated with high temperature during the stage of booting, i.e. the stage of pollen and embryo sac mother cell meiosis (Saini and Aspinall, 1982; Saini *et al.*, 1983; 1984).

Temperatures higher than 30⁰C seriously reduce grain set during the period between onset of meiosis in the male generative tissue and completion of anthesis (Langer and Olugbemi, 1970). Plants exposed to a temperature of 30⁰C for three days during the reduction of division in the pollen mother cell (PMC) set up to 60% fewer grains than those grown continuously at 20⁰C (Saini and Aspinall, 1982). According to Saini *et al.* (1983), this failure of grain set was found by reciprocal pollinations with unstressed plants, due to induction of both male and female sterility. Pollination of heat stressed pistils with fertile pollen from unstressed plants resulted in a 21% reduction in grain set. Staining of pollen from stressed plants with triphenyl tetrazolium chloride showed that up to 80% of the florets were completely male sterile, while the remainder had a variable degree of infertility.

Saini *et al.* (1983) studied the nature of heat induced female infertility. They specifically looked at ovary anatomy and pollen tube growth exposed to high temperature stress.

They found that high temperature stress appeared to have two major effects on ovule anatomy. One was an effect on the nucellus leading to reduced development and degeneration. The integuments had proliferated to occupy the space that would normally have contained nucellus. The ovary wall appeared to develop normally. The other effect was on the embryo sac, resulting in reduced cellular organisation of curtailed extension. In the most severe cases, nucellus tissue had proliferated into the empty embryo sac cavity.

Heat stress resulted in a reduction in the amount of pollen tube entering the ovary. The wheat ovary contains a single embryo sac, so theoretically only one pollen grain is required for grain set. Only 7.4% of pistils had no pollen tube reaching the ovary, yet female fertility was reduced by 21% (Saini and Aspinall, 1982). Thus, some of the ovaries that attracted pollen tubes were not successfully fertilized, owing to structural abnormalities. There were also fewer stressed than control pistils with more than 20 tube reaching the ovary, indicating a reduced capacity of stressed pistils to support pollen tubes growth which was not due to anatomical abnormality. The poorer pollen tube growth in the experimental ovaries, resulted in shorter tubes, which showed abnormal growth and the deposition of callose, a B-1.3 glucan cell wall component, in the pollen tube and pollen grain. The inhibition in tube growth may be attributable to an effect of the heat stress on pistil development (Saini *et al.*, 1983).

Pollen is one of the only plant organs that is unable to synthesize heat shock proteins (Cooper *et al.*, 1984; Xia and Mascarenhas, 1985), known to protect cells against damage arising from heat and other stress. This may help to explain the particular sensitivity of pollen to high temperatures in a wide range of crop species, including wheat (Saini and Aspinall, 1984). Of the three main processes required for successful fertilization, pollen viability appears to be the most limiting under heat stress. Saini and Aspinall (1982) showed that floret fertility in heat-damaged wheat varied from 20 to 80% by pollinating heat stressed pistils with unstressed pollen.

Pollen viability is particularly sensitive to heat stress in that it has both a relatively low damage threshold and a significant loss in viability once that is surpassed. The critical temperature for pollen viability in wheat is only 30°C (Saini and Aspinall, 1982). This makes grain number susceptible to drastic reductions as a result of the shock of heat stress, especially during pollen mother cell meiosis, when the ear is still within the leaf sheath. As little as three days exposure to 30°C can reduce grain set in wheat by almost 70% (Saini and Aspinall, 1982) and floret fertility of rice by up to 20% when temperature rises above 34°C (Matsui *et al.* 1997).

2.2.8 High temperature stress effect on yield

In wheat, leaf number is relatively insensitive to temperature (Rawson and Zajac, 1993), so that the response of total leaf area to temperature is generally mediated through effects on leaf size and duration. By contrast, most determinants of potential grain number are highly sensitive to temperature and are reduced in proportion to the duration of pre-anthesis development. The number of tillers and consequently ears per plant is reduced by elevated temperature (Rawson, 1986) and both spikelet per ear and number of florets per spikelet tend to decrease as temperature rises above about 15°C in the pre-anthesis period (Fischer and Maurer, 1976; Shpiler and Blum, 1986).

Elevated temperatures significantly reduce the potential number of grains per square meter and this generally results in severe yield losses as the reduction in grain number does not appear to be compensated for by an increase in individual kernel mass (Wardlaw *et al.*, 1989). It is clear from the strong positive relationship between spikelet and floret number and duration of the pre-anthesis period (Shpiler and Blum, 1986) that under elevated temperature, increased rates of organ production are generally not sufficient to compensate for the reduced length of the pre-anthesis period. It should be noted that there is a significant genotypic variation in the response of spikelet and floret number to temperature, with a number of cultivars showing a remarkable lack of temperature sensitivity for these traits (Bagga and Rawson, 1977; Rawson and Zajac, 1993).

For wheat and other winter cereals, heat stress in the pre-anthesis period can manifest itself by changing the rate of development, and this may occur below a threshold as low as 15°C. For summer crops such as maize, reductions in leaf number are not apparent until a maximum of 30°C occur during the vegetative growth stage (Warrington and Kanemasu, 1983) and although effects of temperature on potential grain number are similar to wheat, the threshold tends to be higher in maize than in wheat.

Heat stress frequently reduces the size of organs. In wheat, mature leaf size declined by 50% when temperature was increased from an average of about 15°C to 27°C and leaf area at a given growth stage (Rawson, 1986). Similarly, maize leaf length declined by about 2% per 1°C above 20°C (Ritchie and NeSmith, 1991). Elevated temperatures reduce plant height of wheat (Fischer and Maurer, 1976).

2.2.9 High temperature stress effect on quality

Grain quality is a simple term that describes the complex balance of grain constituents. It may be thought of a grain as being made up of a vast array of ingredients, each of which has different properties. If yield is the sum of these ingredients, quality is the result of their relative proportions. Most significant heat-induced alterations of grain composition are qualitative changes in the protein complement leading to deterioration of quality (Klueva *et al.*, 2001). According to MacRitchie (1984) and Wrigley (1996), grain protein percentage is fundamental to the quality of wheat grown, as it is the overriding determinant of dough strength, the property for which wheat grain is most valued (Stone, 2001).

Protein complements of wheat grain include specific storage proteins, glutenins that are classified into high molecular weight glutenins (HMW), low molecular weight glutenins (LMW) and gliadins (MacRitchie, 1984). Glutenins form high molecular mass polymers stabilized by intermolecular disulphide bonds, while gliadins are monomers. Breadmaking quality (BMQ) is a quality of wheat flour to form good loaves of bread and it is mainly determined by high molecular weight glutenins (MacRitchie, 1984). When

heat stress is imposed on developing wheat seeds, reduced starch accumulation results in decreased kernel weight and increased relative protein content in the grain. Relative proportions of high molecular weight glutenins, low molecular weight glutenins and gliadins change, leading to an end product (flour) with changed qualities (Stone, 2001).

The early fieldwork of Finney and Fryer (1958) showed that temperature above a certain (32°C) threshold during the grainfilling period of wheat resulted in a breakdown in the usually positive relationship between flour protein percentage and grain quality. Specifically as the number of hours above 30°C in the last 15 days of grainfilling period increased, loaf volume and dough mixing time fell below that which would have been predicted on the basis of cultivar and the protein percentage of unstressed grain.

For many years, there was no further examinations of the effects of heat stress on grain quality, until a controlled environment studied by Randall and Moss (1990) showed that exposure to as little as three days of very high temperature ($35/30^{\circ}\text{C}$) during grainfilling substantially reduced dough strength, although loaf volume was not responsive to heat stress. Subsequent field based studies by Blumenthal *et al.*, (1991) showed that maximum dough resistance and loaf volume declined as the number of hours above 35°C increased, while dough extensibility tended to increase with heat stress. They used farinograph testing that showed that heat stress during mid-grainfilling produced dough with a longer development time but slower breakdown.

Heat-induced accumulation of gliadins (Blumenthal *et al.*, 1990) was hypothesised to lead to a dough weakening effect. Heat-shock element-like sequences were discovered in the promoters of gliadins genes. Blumenthal *et al.* (1990a;b; 1994) demonstrated that the ratio of glutenin to gliadin (polymer to monomer) decreased as a result of heat stress because gliadin synthesis continued during heat stress while there was a greatly decreased synthesis of glutenin proteins. In these studies, glutenin (> 100 kD) and gliadin (< 100 kD) protein were quantified using size-exclusion (SE) HPLC of an unreduced extract of grain protein (Blumenthal *et al.*, 1993; 1994).

A number of studies (Nicolas, 1994; Blumenthal *et al.*, 1990; Maestri *et al.*, 2002) have focused on the ratio between gliadin and glutenin content as a potential indication of heat stress on dough properties. Stone and Nicolas (1994) reported on two extreme genotypes from a survey of a large number of wheat cultivars that had been heat-stressed in the glasshouse. Cultivar Ospray, showed a dramatic decrease in glutenin to gliadin ratio, and little change for cultivar Ergret.

According to Maestri *et al.* (2002) there is no significant evidence to date that stress induced gliadin accumulation in grains developing under heat stress, and thus increased proportion of gliadins is unlikely to account for decreased BMQ. Treglia *et al.*, (1999) was also in support of these findings. They did not observe an increase of gliadin expression at the level of mRNA accumulation in the seeds developing under heat stress. Expression of HMW glutenin sub-units was reduced only at the late stage of grain filling. Bernadin *et al.* (1995) reported no significant difference in glutenin-to-gliadin ratios for five US wheat varieties as a result of many days of heat stress at 40⁰C. They did, however detect considerable increase due to heat stress in protein associated with heat shock response.

Blumenthal *et al.* (1990) studied some glasshouse grown samples. They studied the gliadin content in samples to determine whether changes in dough properties might be related to an increased rate of gliadin synthesis during the heat stress. HPLC analysis on these samples indicated that there was a higher proportion of monomeric (gliadin) protein in grain samples that had been stressed.

2.2.10 Heat shock response

Exposure to very high temperature induces heat shock response in all known living organisms. This response to heat stress is complex but is generally characterized by selective transcription and translation of heat shock mRNA, at the expense of “normal” mRNA (Ballinger and Pardue, 1982; Hickey and Weber, 1982), leading to a simultaneous reduction of “normal” protein synthesis and the stimulation of synthesis of heat shock

proteins (HSP) (Hendershot *et al.*, 1992). This putatively required a thermo-tolerance or enhanced the ability of heat-shocked organism to tolerate and recover from heat stress (Henle and Dethlefsen, 1978; Lindquist *et al.* 1982). Sensitivity of small subunit mRNA to heat shock suggests that decreased synthesis of chloroplast proteins produced in the cytoplasm may be an important causal factor of heat damage to plants (Vierling and Nguyen, 1992).

Heat shock proteins are generally synthesized at temperatures 8⁰C to 10⁰C above normal growing temperatures. For many plant species temperatures of 35⁰C and above are considered as temperatures that can induce the synthesis of HSP. Plants in their natural habitats frequently experience these temperatures. The appearance of HSP in plants has been correlated with the acquisition of heat resistance (Lindquist, 1986). The acquired thermo-tolerance afforded by the heat shock response has been shown to be a potentially important means of reducing the effects of heat stress on plants in general (Sachs and Ho, 1996) and wheat in particular (Blumenthal *et al.*, 1990; Vierling and Nguyen, 1992). It is generally thought that some HSPs play an important role in the development of heat tolerance by acting as molecular chaperones. Genotypic differences in the level of HSP synthesis was reported in grain sorghum and these were related to heat tolerance in some lines during germination. It was suggested that genetic differences in high temperature susceptibility might be correlated with variations in the temporal development of the capacity to synthesise HSP and acquire thermal tolerance (Klueva *et al.*, 2001).

The development of thermal-tolerance in a crop cultivar was best demonstrated in soybean seedling "Wayne", by Lin *et al.* (1984). They showed that the growth of seedlings was protected during a subsequent incubation at 45⁰C for 2h. This has led to the suggestion that HSP provide a foundation for thermal protection, which is an important component of overall cellular thermal tolerance. Wheat leaf tissue exhibited an alteration in the pattern of protein synthesis when heat shocked either at 34⁰C or 37⁰C for 2h. The appearance of HSP (13) in wheat leaf tissue was observed by one-dimensional SDS-PAGE analysis after a heat shock of 34⁰C for 2h. Significant quantitative differences between cultivars Mustang and Sturdy were observed in the HSPs 22, 26, 33 and 42.

Synthesis of the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) was reduced at 34°C in the cultivar Sturdy compared to Mustang. No significant differences were observed in the polypeptide pattern of the high molecular weight HSP at 34°C.

Quantitative differences were observed in the synthesis of HSP 22 and more subtle differences in the synthesis of major HSP16 and 17. Increased synthesis of HSP 33, 42 and 62 was observed in the heat tolerant Mustang. Several high molecular weight HSP (83, 85, 92, 74 KD) were synthesized at elevated levels in the susceptible cultivar, Sturdy. Results with Mustang and Sturdy suggested that the level of low molecular weight HSP is positively correlated to genetic differences in thermal tolerance. Data did not allow a conclusion on cause and effect relationship, but supported the hypothesis that low molecular weight HSP play an adaptive role in thermal tolerance (Krishnan *et al.*, 1989)

2.3 Freezing stress

2.3.1 The freezing process in plants

Generally, freezing in plants consists of conversion of liquids in cells to a solid state, which is accompanied by loss of heat. There are two types of freezing in plants, first is vitrification, which is the solidification of cellular components into crystals (amorphous), vitrification of liquids in cells is a result of rapid freezing more than 30°C/min of plant tissues to a very low temperature. It is more enhanced by hardening of plants to survive temperatures closed to absolute zero. Secondly is crystallization, which is the arrangement of liquid molecules in orderly structures. Crystallization (ice formation) is a very common phenomenon in nature. At sub-zero temperatures, ice forms in the intracellular spaces where water is the purest (Idle, 1966).

If the speed of cooling is slow enough (in nature the cooling rate seldom exceeds 1°C/h), cells freeze extracellular, causing cell dehydration of cytoplasmic solutes and reduction in

cell volume and surface area, all factors that can potentially damage cells irreversibly. It has been shown that ice normally crystallizes first in the large vessels in the case of leaves. Freezing proceeds along the vessels from a few nucleation points and all parts of the shoots at a relatively high velocity proportional to the supercooling. Freezing in the vessels is to be expected, since their large diameter does not favour supercooling and their dilute sap has the highest freezing point of any of the plants' water. Once ice forms in the vessels, it will spread throughout the plant body (Idle, 1966; Olien *et al.*, 1968).

Ice is partitioned from the protoplasm by the cell wall and plasma membrane. Since no ice forms inside the protoplast, the cell fluids remain in a liquid state. Not all the water in a cell freezes at once, due to dissolved cell solutes and its interaction with cellular components (Glasstone, 1964; Gusta *et al.*, 1975). Prior to freezing the freezing point depression of cells is generally in the range of -1 to -1.5°C (0.5- 0.7 osmolal). More than 60% of the crown tissue water is frozen at -4°C , which results in an increase in the cellular fluids to approximately 5 osmolal. This increased solute concentration prevents the interior of the cell from freezing. At -10°C , over 90% of the total freezable water is frozen and the cell becomes severely dehydrated. At -20°C , nearly all the freezable water is frozen except for the small fraction that is held tightly by cellular compounds (it is not available for freezing even at -40°C). The quantity of water that migrates from the cell to the growing ice crystal is therefore a function of the temperature (Gusta *et al.*, 1975).

During freeze-induced dehydration, the plasma membrane remains attached to the cell wall, causing the cell to collapse (Levitt, 1956; Salcheva and Samygin, 1963). If the extracellular spaces are too small to accommodate the growing ice crystal, the cells are crushed, ruptured or separated by the splitting of the cell wall along the middle lamella (Levitt, 1956). As the cell wall collapses, the protoplasm is pushed against the outer cell wall in the form of a ring and the plasmatic strains break. Upon thawing the cells become rehydrated and expand back to their original volume. If the cell wall shrinks in size, this phenomenon has been termed pseudo or frost plasmolysis. If the cells have been injured by freezing, their membranes leak and they are unable to re-gain full turgor (Levitt, 1956; Stepomkus 1984).

The reduced semi-permeability properties of the cell membranes due to freeze injuries have led many researches to suggest that the membrane is the primary site of injury. Cellular compounds start to leak from the tissue immediately upon thawing with no measurable lag period. Depending on how homogeneous the tissue is, there is little or no leakage prior to exposure to killing temperatures. Results based on microscopic observation, fluorescent changes in all cells under frozen conditions and nuclear magnetic resonance studies suggest that freezing injury occurs during the process of freezing upon cooling below the frost killing temperature (Salcheva and Samygin, 1963).

Intracellular ice formation is invariable lethal. Therefore, the freezing process within the living organisms that tolerate the presence of ice within their tissues involves the formation of extracellular ice at a high subzero temperature. During freezing, as much as 70 to 80 % of the liquid water in the tissue is frozen as extracellular ice, resulting in cellular dehydration (Levitt, 1980). The ability of organisms to modify the growth of ice and to withstand extensive dehydration is a key element of survival. In some cases, extracellular freezing is accompanied by a second mechanism known as deep supercooling, in which water is maintained in the liquid state, even at temperatures far below the heterogeneous nucleation point (Burke *et al.*, 1976).

2.3.2 Low temperature stress and development

Temperature stress inhibits growth, development and thus yields of wheat in at least three ways. Firstly, development from the emergence through tillering, stem elongation, flowering and grain filling is driven by growing degree-days or accumulated head units. Secondly, wheat requires a certain minimum time within a favourable temperature range to go from seed to seed. The ideal temperature for growth and development of wheat is between 10 and 24⁰C, provided that no other limiting factors such as too much or too little water or light influence the normal plant development. Accumulation of growing degree-days within this temperature range leads to large, well-tillered plants with wide leaves and big heads. Thirdly, wheat plants are sensitive to temperature extremes during critical stages of development. Results of these extremes include frost injury to

internodes and the florets, winterkill, frozen leaves or root and damage (Cook and Veseth, 1991).

Certain stages of plant growth and development are more sensitive to low temperatures than other phases (Levitt, 1980), with dormancy generally representing the most tolerant stage. Temperature is one of the most important factors controlling inhibition of developmental changes such as flowering, although the basic mechanisms of temperature effects are not addressed in most studies. Generally, reproductive organs are sensitive to chilling and freezing stress. The blossoms of many herbaceous and woody plants of temperate origin freeze between -2 and -5°C . Sex expression of flowers is also influenced by temperature. Usually low temperature favours femaleness (Henslop- Harrison, 1972) or promotes male sterility.

Wheat usually flowers one week after the heads appear. The flowering stage is the most sensitive stage in wheat. Small differences in temperature duration of exposure or other conditions can cause large differences in the amount of injury. Exposure to freezing temperatures at the flowering stage kills the male parts of the flowers and causes male sterility. Floral parts are extremely sensitive to freezing and once frost reaches them, ice spreads readily through anthers, filaments, ovules and stigmas. Glumes, lemmas and paleas are much less tolerant of freezing than are leaves, but on occasion suffer extracellular freezing without obvious symptoms developing. However, no grain has been found to develop in a floret where the lemma or palea has been visibly damaged, indicating lack of any restriction of ice propagation from these structures to floral organs. After freezing the anthers are white and desiccated or shrivelled instead of their normal yellow colour (Cook and Veseth, 1991).

2.3.3 Freezing stress effects on the cell membrane

Membrane injury is a universal manifestation of freezing injury to biological systems and many of the methods commonly used to quantitate freezing injury are based on this fact. As early as 1912, Maximov concluded that freezing damage was the result of the freeze-induced removal of water from the surface of the plasma membrane. It can only be said that freezing results in membrane rupture or loss of semi-permeability. Researchers (Dowgert and Steponkus, 1984) indicated that both intracellular and extracellular ice formation results in injury specifically to the plasma membrane. Moreover, the extracellular ice formation results in dehydration and plasmolysis and subsequent deplasmolysis *per se* was not the injurious event. Rather, ruptures of the membrane occurred upon deplasmolysis. Freezing injury is typically related to -

- loss of semipermeability,
- loss of active transport ions,
- degradation of phospholipids,
- redistribution of proteins due to lateral displacement,
- dehydration induced phase transition in biological membranes such as lamellar to the hexagonal II phase transitions (Sakai and Larcher, 1987).

Freezing injury is apparent at plant level as the inability of the plant to initiate re-growth of leaves and roots, and this has been correlated with injury of specific group of cells in the basal region of the crown. At cellular and sub-cellular level, degree of injury is related to membrane degradation apparent as increased leakage of electrolytes and reduced recovery of the membrane proteins and lipids from freeze-thaw injured wheat seedlings (Levitt, 1980; Borochoy *et al.*, 1987). Alterations in the lipid phase properties of membranes have been reported using freeze-fracture electron microscopy (Gordon-Kamm and Steponkus, 1984). It has been suggested that this membrane injury, in at least non-acclimated wheat seedlings, may be mediated by oxygen free radicals produced during freeze-thaw cycle. However there are many other possible mechanisms of freezing injury to plant tissue (Levitt, 1980; Steponkus, 1984).

Although the plasma membrane is recognized as the primary site of freezing injury, the nature of the injury has been controversial even to this day. Part of controversy is because hypotheses have been too simplistic and attempted (or required) to explain all forms of freezing injury. Theories that involve single mechanisms of freezing injury are too restrictive (Levitt, 1980). Steponkus (1984) has advocated that freeze-thaw injury is multifaceted and that cold injury is a combination of many different changes, all of which may limit tolerance under a given set of circumstances.

2.3.3.1 Levitt's hypothesis

It has been proposed that freezing injury is caused by aggregation of proteins as a result of the formation of –SS (disulphide) bonds from –SH (sulphydryl) groups in adjoining protein molecules as they approach one another as the protoplasm is freeze-dehydrated. Protein molecules thus undergo a conformational change, which results in destruction either of enzyme activity or membrane integrity. Low temperature tolerance can therefore be attributed to prevention of disulphide bonding. The sulphydryl hypothesis is based on the observation that an increase in the number of protein disulphide groups accompanies freezing but does not occur when freezing does not lead to injury. Furthermore, there is a correlation between the SH content of proteins in a tissue homogenate and frost tolerance. Proteins from frost tolerant plants have fewer –SH groups and they are less readily oxidized than those of tender plants (Levitt, 1980).

The hypothesis explains a number of puzzling features of frost tolerance, one being the relationship between growth and sensitivity to low temperature. Rapid growth is associated with high –SH content and also with sensitivity to frost damage. Naturally, the larger the number of –SH groups per unit protein, the more difficult it must be to prevent their oxidation to intermolecular –SS- bonds on freezing. The hypothesis has been tested in a model system with a thiolated form of gelatin, thiogel. The melting point of a gel made from thiogel rises with an increase in number of –SS- bonds so this can be used as an indication of –SS- bond formation. When the gel is freeze-hydrated its melting point

rises much more rapidly than when kept unfrozen. Because thiogel is a denatured protein it cannot be used to test this hypothesis (Steponkus, 1984).

Experiments with native protein, bovine serum albumin (BSA), has confirmed that native –SS- protein do not aggregate freeze dehydration but the –SH- protein does. On the basis of experiments with model proteins, Levitt (1980) suggested that frost injury results from the following chain of event. Firstly low temperature denaturation of proteins reversibly unmasking active –SH groups. Secondly freeze dehydration removing vacuolar water resulting in cell contraction which applies stress of protoplasmic proteins activating their –SH bonds and removing protoplasmic water. Thirdly decreasing the distance between the reversibly denatured protein molecules and intermolecular bonding due to the –SH oxidation forming –SS- bonds aggregates proteins irreversibly, killing the cell (Levitt, 1980).

Efforts by Siminovitch *et al.* (1967) were concerned with localization of injury and resistance in cellular membrane and indicated that resistance is an intimate property of the components of the plasma membrane rather than some property arising from purely colligative action of solutes. Evidence for an increase in membrane structure during cold acclimation was presented and termed augmentation. Pomeroy and Siminovitch (1971) provided electron microscopic evidence that the process of augmentation was manifested by marked invagination, in the plasma membrane with some folding appearing to be in process of pinching off into vesicles in the peripheral cytoplasm.

Steponkus (1984) has presented evidence that cold acclimation is a result of combined forces of both membrane alteration and increase in soluble cytoplasmic components, especially sugars, with end results being a membrane more resistant to the rigors of freezing. Evidence (Hincha *et al.*, 1986) supports this concept in that a difference in freezing resistance of chloroplast thylakoids isolated from the acclimation and non-acclimated tissue could be demonstrated only in the presence of sucrose.

2.3.4 Active transport and freezing injury

Palta *et al.* (1977) indicated that the semi-permeable properties of the membrane are injured by dehydration whereas freezing stress damages the ion and sugar transport mechanisms. Heber (1967) reported that freezing of chloroplast membranes uncouples photophosphorylation from the electron transport and inactivates light dependent ATPases. It was pointed out that the primary injury is most likely to affect active transport mechanism. This follows from the post-thaw recovery of injury, which led to disappearance of the infiltration of the tissue and marked reduction in the effuse of ion and sugar from the cells. The reason for this absorption of water by cells is due to the influx of ion and sugars from the extracellular solution. Since this influx is against the concentration gradient it must be active in nature (Steponkus and Wiest, 1978).

Membrane located ATPases are believed to constitute ion pumps and several such ATPases have been found to be involved in ion uptake by plant cells. It may be suggested that mechanisms for active sugar transport may conceivably be the loci of the initial freezing injury. The hypothesis would also explain the high potassium and sugar concentration in effuse from frozen and thawed, but still living cells (Steponkus and Wiest, 1978). If freezing damaged the potassium activated ATPases and mechanisms for active sugar transport could be visualised as sites for facilitated effusion. This would result in a net efflux of potassium and sugars. Furthermore, repair of damage can be visualized as a reversible injury to these active transport pumps which temporarily get inactivated and following recovery are able to plumb back the potassium and sugar in the vacuole resulting in disappearance of the infiltration of tissue. Irreversible damage to the pumps will lead to the continued increased flux of ion and sugars as was observed (Steponkus and Wiest, 1978).

2.3.5 Low temperature and metabolic changes

During acclimation to freezing, the plant's metabolism changes dramatically and consequently metabolic levels and enzyme activity change. A common hypothesis has

been that freezing tolerance is associated with the accumulation of specific metabolites termed as “cryoprotectants” that protect the cell during freeze-thaw cycle (Levitt, 1980). But it has proven difficult to determine which of these metabolic changes are critical to the acquisition of freezing tolerance, which are simply just adaptation to growth at low temperature. Most studies (Siminovitch *et al.*, 1967; Levitt, 1980; Tognetti *et al.*, 1990; Olien and Clark, 1995) have attempted to correlate acclimation with metabolic changes. Osmotic concentration increases with acclimation although this depresses the freezing point of ice nucleation point. It is insufficient to account for changes in freezing tolerance. The major changes to osmotic potential are due to changes in sugars and there is a good correlation between sugar content and freezing tolerance. Sugars may suppress the freezing point of the tissue and act as a cryoprotectant to preserve protein structure (Levitt, 1980)

The water content of the tissue is inversely related to freezing tolerance. Acclimation promotes both water loss from the tissue and accumulation of starch and protein, which are not osmotically active. The loss of water has obvious adaptive advantages because there would be less water to freeze, less ice to accommodate. Lipids also accumulate in many species during acclimation and fatty acids tend to be more saturated. In many cases increase in lipids is associated with a proliferation of cellular membranes. There is a close correlation between soluble protein content and freezing tolerance. This is associated with an increase in the amount of tRNA required for protein synthesis (Siminovitch *et al.*, 1967; Levitt, 1980).

As the level of growth regulators change, abscisic acid (ABA) commonly increases and gibberellin decreases. The growth promoting effect of gibberellins is negatively associated with freezing tolerance, whereas the quiescence or dormancy caused by ABA is positively associated with tolerance. An inverse relationship between rate of respiration (growth) and freezing tolerance is usually observed. However this may be independent on the stage of acclimation and seems to be a complex relationship. Low temperature acclimation of seedling and plants requires photosynthesis and therefore light and carbon dioxide presumably to support the accumulation of sugars, proteins and other solutes.

However, like respiration the relationship with freezing tolerance is complex depending on species and stage of development (Levitt, 1980).

2.3.5.1 Low temperature effect on carbohydrates

In early fall, all-perennial plants accumulate carbohydrate reserves in the form of starch or fructan. However a subsequent conversion of carbohydrate polymer reserves into soluble sugars is observed at the beginning of a cold period (Levitt, 1980). The principal forms of sugars that appear are oligosaccharides such as raffinose, and stachytose (Tognetti *et al.*, 1990)

The accumulation of soluble sugars is concomitant with an increase in several enzymatic activities involved in carbohydrate metabolism: amylases, sucrose synthase, sucrose phosphate synthase, fructose-1, 6- biphosphatase, sedohptulose-1, 7-biphosphatase and rubisco. The increase in some enzymytic activities, sucrose synthase, sucrose phosphate synthase, and amylase, is related to the appearance of new, cold-specific isoenzymes. It appears that the accumulation of free sugars involves an enhancement of all photosynthetic pathways. Although the capacity to accumulate soluble sugars is commonly considered an important factor in freezing tolerance, it is difficult to determine to what extent this mechanism is responsible for plant winter hardiness. Varietal or interspecific differences in soluble sugar contents are often positively correlated with the level of freezing tolerance (Tognetti *et al.*, 1990; Olien and Clark, 1995). One explanation for the variable correlation between freezing tolerance and soluble sugar contents lies in the different capacities of plants to photosynthesise at cold temperature (Griffith and Ferullo, 2001).

In a comparison of winter and spring cultivars of rape and wheat, Hurry *et al.* (1995) demonstrated that only winter cultivars are capable of improving their photosynthetic efficiency at low temperatures, leading to higher production of sugars during cold acclimation. Griffith and McIntyre (1993) proposed that degree of freezing tolerance is related not only to photosynthetic capacity in overwintering annuals such as winter rye

but also to the partitioning of photoassimilates between processes involved in growth and freezing tolerance.

Although sugars are usually involved in metabolic adjustments within the cell, Olien (1984) proposed that sugars accumulate in the apoplast following a freeze-thaw cycle and that these sugars prevent the adhesion of ice to critical tissues within the crowns of winter rye. Antikainen and Griffith (1997) confirmed that sugars accumulate in the apoplast of the leaves of rye, wheat and barley during cold acclimation and hypothesized that sugars may play a role complementary to antifreeze proteins modifying the growth of ice. The mechanism for sugar accumulation has been examined in winter oat during the second phase of hardening. After oat plants are exposed to sub-zero temperatures, activities of carbohydrate- degrading enzymes invertase and fructan exohydrolase increase in the apoplast of crowns. Soluble sugars also accumulate in the apoplast. These sugars not only include glucose, fructose, and sucrose but also fructans with degrees of polymerisation ranging from three to greater than seven (Livingston and Henson, 1998). Because the level of apoplastic sugars is too small to lower the freezing point of apoplastic fluids, they are envisioned to interact with ice and prevent adhesions. These sugars could also protect cell walls and membranes during freeze-thaw cycles (Livingston and Henson, 1998).

In addition to their role in osmoregulation, soluble sugars are suspected to have cryoprotective effects on biomembranes and proteins. For example, it was shown that interactions between sugars and hydrophilic extremities of phospholipids modify phase transition of lipid bilayers (Strauss and Hauser, 1986).

During the first stage of hardening, several metabolic events were shown to occur in cabbage and winter rape plants, e.g. hydrolysis of starch and accumulation of reducing sugars and soluble proteins (Levitt, 1980; Griffith and Ferullo, 2001). These events are commonly known to occur in other plants, including wheat. Soluble sugar composition and starch reserves are significantly altered during the cold acclimation period. In one example the sugar content of cabbage leaves has been positively correlated with freezing tolerance. Cabbage seedlings when exposed to acclimating temperature (5°C) acquired

freezing tolerance down to -6°C . Sucrose, glucose, raffinose and fructose increased gradually during cold acclimation such that their level was positively correlated with the degree of freezing tolerance. The same sugars were found to increase in wheat under low temperatures. It is well known that sucrose is a free sugar commonly accumulated in response to low temperature. Myo-inositol and starch did not show any accumulation. Freezing tolerance was lost in only one day of de-acclimation at control temperatures and this was associated with a large reduction in sugar content (Kacperska-Palacz, 1978).

Exposure of grass from temperate cool climate zones to chilling temperature leads to accumulation of fructan in the aerial parts (Eagles, 1967), which is protected by an increase in the level of sucrose in the plant cell (Pontis, 1990). The increase in the content of fructan is thought to be connected to the acclimation process of the plant to low temperatures or it just may be a consequence of low demand for photosynthates at chilling temperatures (Pollock *et al.*, 1989). Tognetti *et al.* (1989) studied fructan metabolism in young wheat leaves during cold acclimation and deacclimation period. Moreover, Tognetti *et al.* (1990) used four different wheat varieties cultivated in climates ranging from subtropics to North Patagonia in Argentina to study sucrose and fructan metabolism in leaves exposed to low temperatures. It was found that the most cold tolerant cultivars showed higher levels of soluble sugars as well as enzyme activities. Metabolic changes observed were not due to anatomical or morphological differences produced during growth at 4°C .

In winter cereal, fructans are the principal storage carbohydrate in the crown (Archbold, 1940; Olien and Clark, 1995). It is distributed throughout the plant and accumulates when photosynthesis exceeds utilization, such as during the chilling acclimation period (Jeong and Housley, 1990; Olien and Clark, 1995). Fructans seem to act as a short-term storage form of carbohydrate, regulate sucrose levels and provide osmoregulation of cellular activity (Nelson and Smith, 1986). Winter cereals exposed to freezing temperatures of -3°C convert fructan to cryoprotective sugars (Olien and Clark, 1993) such as fructose and sucrose. These are deposited in the intracellular liquid. The concentration of carbohydrates in the apoplast is increased. Fructan content and metabolism has been

shown to be closely related to freezing tolerance in cereals (Tognetti *et al.*, 1990; Santoniani *et al.*, 1993). Fructan content has been associated with the overwintering ability of wheat in regions of Japan, characterised by a deep persistent snow cover throughout winter (Yukuwa and Watanabe, 1991)

It has been shown (Tognetti *et al.*, 1989) that the rate of fructan accumulation at low temperature correlates with gene expression of enzymes in fructan metabolism, which involves several enzymes. A temporary increase in activity of the enzymes for fructan synthesis, sucrose sucrosefructosyltransferase (SST) at temperatures above 0°C and a decrease in the temperature above 0°C have been reported in winter wheat (Jeong and Housley, 1990).

Since initiation of fructan synthesis is related to sucrose concentration, activities of sucrose metabolising enzymes during the acclimation period were determined. Sucrose synthesis (SS) and sucrose-phosphate-synthesis (SPS) activities in roots increased during the cold period. The rise in SS activity may be related to the unloading of sucrose translocated from the aerial parts as it has been suggested that this enzyme activity may be associated with sink demand. The increase in SPS activity, on the other hand, may be related to sucrose re-synthesis, either because it was cleaved during unloading or due to the utilization of the glucose formed in sucrose sucrose fructosyl transferase (SST) catalysed reaction (synthesis of fructosyl sucrose). Sucrose exported from leaves provides roots with the carbon skeleton for growth and respiration (Pontis, 1989).

2.3.5.2 Low temperature effect on lipids

The other metabolic changes, which were shown to be a specific response of the plant tissue to lowering environmental temperatures, are lipid and the phospho-lipid transformation. Low, but positive temperatures have been demonstrated to increase unsaturation of fatty acids. A positive correlation between lipid content and hardiness was often noted in the early literature (Levitt, 1980; Palta *et al.*, 1993). The lipid components of plant cells change dramatically as the plant acclimates to freezing stress.

Early work has shown that lipids became more unsaturated with acclimation. Due to the importance of the plasma membrane in freezing tolerance, improvements of the isolation of purified membrane fraction have characterized changes in the lipid composition of the plasma lemma in detail (Steponkus, 1984; Lynch and Steponkus, 1987).

Most studies have been conducted on the degree of lipid unsaturation and the fluidity of membranes since they are the primary sites of injury. Marked growth temperature dependent alterations in fatty acid composition and the unsaturation of mitochondrial phospho-lipids were observed during hardening of four wheat cultivars. Structural transitions occurred at lower temperatures in cold grown material and were quantitatively greater in winter hardy cultivars. Farkas *et al.* (1975) concluded that the degree of lipid unsaturation was correlated with cultivar hardiness. De La Roche *et al.* (1975) found that the change in lipid unsaturation of membranes was the same in all four wheat cultivars differing in cold hardiness. The increased unsaturation of lipids at hardening temperature may be more related to vernalization than cold hardening. But Thompson and Zalik (1973) in opposition to this interpretation found that, both spring and winter rye seedlings showed this increased unsaturation in the first four weeks of vernalization.

Uemura and Yoshida (1984) analysed a plasma membrane enriched fraction from cold hardened winter rye seedlings and found that the degree of fatty acid unsaturation and proportion of phospholipid classes changed only slightly during hardening. These changes suggested that fatty acids changes may not be as dramatic as once thought for cold hardening in winter cereals. The contents of phospholipids and some phospholipid fractions, phosphatidyl choline and phosphatidyl ethanolamine were found to increase in cold hardened wheat.

Phosphatidyl-ethanolamine, phosphatidyl-choline and an unidentified component increased markedly in autumn in winter wheat then decreased towards spring. Phosphatidyl-glycerol accumulated in late autumn and seemed to be transformed to the other three substances under conditions both artificial and natural hardening. In wheat, however, the phospholipid concentration was unchanged indicating no preferential

synthesis of individual phospholipid. It has, in fact been suggested that increased phospholipid synthesis may not be a prerequisite to hardening of winter wheat, but may be required to maintain resistance. In contrast to these results a decrease in temperature caused a considerable increase in total content of phospholipid in winter wheat mainly due to phosphatidyl-choline and phosphatidyl-ethanolamine, less to phosphatidyl-inositol and phosphatidyl-glycerol (Yoshida and Sakai, 1967).

Other lipids have also been found to increase during hardening of some plants. In wheat seedlings, sterols actually decreased during the initial stages of hardening, but increased later in the root to a final value equal to or greater than the initial value (Davis and Finkner, 1973). It has long been known that low temperature increases the degree of unsaturation of fatty acids. An increase in unsaturation of fatty acids occurred during the hardening of the seedlings of 10 cereal varieties at 2°C. Spin labelling showed that the phase transition of lipids occurred at a lower temperature in the cold grown seedlings (Miller *et al.*, 1974). The more resistant the variety, the greater the change. This change in unsaturation, however, occurred to the same degree in two hardier varieties of wheat and was not pronounced in the roots. On the other hand, a much larger increase in content of linolic acid occurred at 2°C in a mutant winter wheat genotype than present in a spring genotype (ref).

In support of a role for unsaturation of fatty acids in freezing tolerance, treatment of 12-day old winter wheat plants BASF 13-38, 36 hours before frost hardening, simultaneously and completely inhibited both the hardening and accumulation of linolic acid in the roots during hardening (Willemot *et al.*, 1977).

2.3.5.3 Low temperature effect on proteins

In recent years it has become apparent that plants respond to adverse environmental stress conditions through alterations in protein synthesis patterns. In general, when the environment becomes unsuitable for optimal growth and development of the plant, stress related proteins are synthesized. Newly synthesized proteins appear to be more or less

specific to a given environmental stress i.e., anaerobic proteins for anoxia and anaerobiosis and heat shock proteins for super-optimal temperatures (Key *et al.*, 1981).

Cold acclimation results in altered gene expression leading to synthesis of specific proteins and certain enzymes, which are responsible for development of freezing tolerance. Several pre-existing proteins abundant in tissues of plants grown under normal temperatures declined on exposure to low temperature. However many new transcripts and polyploids are synthesized, which appear to play a major role in the acclimation of plants to freezing temperatures (Griffith *et al.*, 1997).

Uemura and Yoshida (1984) while studying cold acclimation in winter rye seedlings observed that more than 20 proteins disappeared in the plasma membrane during acclimation and the concentration of 11 proteins increased whereas 26 new proteins were synthesized. Protein synthesis playing a major role in cell regulation logically should be involved in a metabolic process such as development of cold hardiness in living plant cell. Numerous observations have been reported relative to changes in protein concentrations, patterns, synthesis and degradation during cold hardening. As early as 1927, Doyle and Clynch suggested that protein synthesis involvement in cold hardening of conifer needles through observations of changes in major hydrolytic enzyme activities. Siminovitch and Briggs (1949) observed an increase in soluble protein concentrations during cold hardening of black locust (*Robinia pseudoacacia*). They found that soluble proteins in black locust bark cells increased in fall and declined in spring and that this changes closely paralleled changes in tissue freezing tolerance. Accumulation of soluble proteins in cold acclimating tissue, while not universal, is generally accepted as an important step in the development of freezing tolerance, since many researchers have reported similar responses during cold acclimation (Levitt, 1980).

Increases in soluble proteins have been found during hardening of grains. Glycoproteins have been reported in wheat and rye, during hardening. Cytoplasmic tests indicated an increase in both cytoplasmic and basic nuclear proteins (Levitt, 1980). Heber (1959) found that the protein content of chloroplasts increased during hardening of wheat.

Pulse-labelling studies of cold tolerant and cold sensitive wheat failed to reveal changes in the chloroplast or membrane proteins (Rochat and Therrien, 1975), but during cold acclimation, cold tolerant Kharkov wheat synthesized two soluble proteins that cold-sensitive Selkirk wheat did not synthesize. The two proteins had very high molecular weights and appeared to be hydrophilic. Cloutir (1983) observed quantitative changes in proteins but did not detect the appearance of any new polypeptide resulting from cold acclimation of wheat.

In all these studies exposure to low temperature caused a change in protein synthesis pattern when compared to the pattern of synthesis at warm temperature. Changes included the appearance of new polypeptide bands and either diminished concentration or disappearance of a few bands present in warm growth plants (Key et al., 1981; Perras and Sarhan, 1989; Griffith, 1997).

Perras and Sarhan (1989) studied protein synthesis in leaves, crown and roots during hardening of freezing tolerant winter wheat (*T. aestivum* L. cv Frederick and cv Norstar) and freezing sensitive spring wheat (*T. aestivum* L. cv Glenlea). The steady state and newly synthesized proteins labelled with [³⁵S] methionine were resolved by one and two-dimensional polyacrylamide gels. Results showed that cold hardening induced important changes in the soluble protein pattern upon the tissue and cultivar freezing tolerance. Analysis of the protein pattern revealed the accumulation of high molecular weight protein that appeared to be induced by the low temperature hardening. This band was particularly evident in the two cold tolerant cultivars Norstar and Frederick, but less in the cold-sensitive cultivar Glenlae. In addition, eight protein bands increased in intensity tissues subjected to hardening conditions, while the content of three other bands (157, 42 and 34 kD) decreased. The 157 kD protein almost disappeared at the end of hardening period (40 days), particularly in two resistant cultivars.

The induction of the 200 kD protein in the early stages of cold hardening and its accumulation throughout the whole hardening process, suggest that this low temperature induced protein may be required for the acquisition, development and/or maintenance of

the increased freezing resistance in wheat. The induction of this 200 kD protein as well as the increased synthesis of 75 kD peptide in all tissue suggested that part of genetic regulation associated with increased freezing tolerance took place at whole plant level. Analysis of *in vivo* protein synthesis revealed that a number of proteins were newly expressed or repressed while others were differently regulated by the cold hardening conditions. The appearance of eight new polypeptides in the protein synthesis patterns of hardened wheat tissues suggested that cold acclimation induced modification at gene level (Perras and Sarhan, 1989).

Among the polypeptides induced by hardening conditions, some were synthesised at a higher rate in two cold tolerant than in cold sensitive one, indicating a correlation between the degree of expression of this gene and the degree of tolerance reached by the different genotypes. These proteins (200 kD in three tissues, 36 kD in the leaves and 64 kD and 52 kD in roots) were therefore considered as part of the potential family of frost tolerance proteins (FTP) e.g. proteins strongly associated with the development and maintenance of increased freezing resistance (Perras and Sarhan, 1989).

2.3.5.4 Low temperature effect on abscisic acid (ABA)

The phytohormone abscisic acid (ABA) plays a regulatory role in many physiological processes in plants under different stress conditions such as drought, cold, light and temperature. Plants in these stress conditions result in increased amounts of ABA. Abscisic acid is defined as a plant hormone that mainly acts to inhibit growth, promotes dormancy and help the plant to tolerate stressful conditions. The plant growth slows down and then it will assume a dormant state. This is the complete opposite of what auxin, gibberellins and cytokines, the other plant hormones will do to the plant. This hormone inhibits cell division in the vascular cambium, also for winter, by suspending primary and secondary growth. The most impressive effect of abscisic acid is the inhibition of growth and the maintenance done on the dormancy of buds. Also this hormone acts as a stress hormone helping plants to cope with the adverse conditions that the plant might come across. ABA slows seed germination and improves wheat tolerance

to cold and drought stress (Dorffling *et al.*, 1990). It has been suggested that ABA may trigger some of the freezing tolerance proteins (Dorffling and Askman, 1989) and the changes in lipid composition of membranes (Farkas *et al.*, 1985). In an earlier study with only two winter wheat varieties (Amanda and Holme) Lalk and Dorffling (1985) found a steady increase in ABA levels in both varieties during a hardening programme lasting five weeks.

Increases in ABA levels in wheat plants in response to cold hardening under growth chambers conditions have been reported by Machakova *et al.* (1989) and by Taylor *et al.* (1988). In field grown winter wheat, Taylor *et al.* (1988) found a maximum in the ABA level in November and December at the same time that freezing resistance reached its maximum. Chen and Gusta (1983) support the hypothesis that ABA is intimately involved in the process, which are responsible for freezing tolerance. Exogenous ABA improves freezing tolerance in cell cultures and the whole plants. There are two mechanisms in which ABA would confer freezing tolerance in plants, maintenance of favourable water balance and induction of specific sets of genes whose products protect against freezing. High levels of ABA also increase during hardening showing a peak at the time when the second decrease in lethal temperature of 50% leaves (LT50) takes place (Dorffling *et al.*, 1990). Machackova *et al.* (1989) concluded from studies with three wheat varieties and four putative markers that ABA levels were the best markers for frost hardiness followed by proline.

2.3.5.5 Low temperature effect on amino acids

The osmotic adjustment of dehydrated cells is not completely explained by the accumulation of soluble sugars. Other osmolytes, such as free amino acids, glycinebetaine (N-N-N trimethylglycine) and polyamines, are thought to contribute to lowering water potential within the protoplast during freeze-induced desiccation. This assumption is reinforced by the fact that these compounds often accumulate in response to water and osmotic stresses. An increase in proline content during cold acclimation has been found in a number of species (Koster and Lynch, 1992) and in some cases, the level

of proline has been positively correlated with freezing tolerance. In higher plants, proline accumulates under stress and shows an association with stress adaptation (Paleg and Aspinall, 1981; Lalk and Dorffling, 1985). Proline also accumulates in algae under stress (Bartels and Nelson, 1994).

Igarashi and colleagues (1997) showed that the mRNA encoding 1-pyrroline-5-carboxylate synthetase, an enzyme involved in the proline biosynthesis pathway, was induced by cold in *Oryza sativa*. Although rice is freezing sensitive, this observation suggests that proline production during cold acclimation could be controlled by transcriptional regulation of 1-pyrroline-5-carboxylate synthetase. It has been shown in higher plants that proline is synthesised from either the glutamate pathway or ornithine pathway. The glutamate pathway is initiated by phosphorylating L-glutamate to L-glutamyl-g-phosphate by g-glutamyl kinase (g-GK is converted to glutamate g-semialdehyde by GSA dehydrogenase (GSAAd; EC 1.4.1.3). Finally GSA spontaneously forms D¹-pyrroline-5-carboxylate (P5C), and P5C is reduced to proline by P5C reductase (P5CR; EC 1.5.1.2). In the ornithine d-aminotransferase (d-OAT; EC 2.6.11.3) and then follows the same route to form P5C as in the glutamate pathway (Delauney and Verma, 1993).

A stimulation of synthesis is a factor contributing to stress-induced proline accumulation, but which pathway becomes responsible for proline synthesis is dependent on the plant system involved or the kind of stress. Under osmotic stress the glutamate pathway is generally considered the primary route for proline synthesis. However in the case of the NaCl-tolerant *Brassica juncea* L., the NaCl-induced proline synthesis is suggested to be derived from the ornithine pathway with dOAT as the principle enzyme in addition, the excess nitrogen supply switches the synthesis of proline from the glutamate to the ornithine pathway (Delauney and Verma, 1993).

Changes in amino acids and glycine butane content in response to cold stress in wheat seedlings were investigated (Dorffling *et al.*, 1990). Accumulation of proline in plants during cold stress is well established, however, other amino acids have also been reported

to accumulate in various plants during cold stress. The total amino acid content of cold-stressed wheat leaves increased 3.7 fold on a dry weight basis. Threonine, glycine, valine and isoleucine made minor contributions to this increase. Moderate to high contributions to total amino acid accumulation came from aspartic acid, asparagine, glutamine, alanine, 4-aino butyric acid and proline. However proline concentration in plants at low temperature increased more that 52-fold over the control proline concentration (Dorffling and Askman, 1989).

Several researchers had shown that applied proline acts as a cryoprotectant (Withers and King, 1979; Van Swaaij *et al.*, 1987; Koster and Lynch, 1992) and treatments, which increased the endogenous level of proline, also increased the resistance to chilling of freezing stress (Bornman and Jansson, 1980). Varieties with higher freezing resistance accumulated proline faster and reached higher levels than less freezing resistant genotypes. Kaldy and Freyman (1984) were among those who proposed to use the increase in proline during cold hardening for the detection of cold hardiness in winter wheat breeding programmes. Proline is being used as a chemical marker for winter hardiness in barley (Dobslaw and Bielka, 1988).

The conclusion that a causal relationship exists between proline levels and freezing resistance lead to the idea that breeding for higher freezing resistance could be achieved by selection and breeding for high proline levels. Successful attempts have been reported where, in potato, rich cell lines could be selected after selection of hydroxyproline containing media (Van Swaaij *et al.*, 1987). Plants that regenerated from these cells showed an increased freezing tolerance and had achieved high proline levels.

2.4 Plant viability screening techniques

2.4.1 Cell Membrane Stability

Cold, drought and heat stress damage and injury occur in plant cells where leakage of electrolytes takes place. This, unavoidably, has a negative effect on the electron transport

system of the plant. Electrolyte leakage is a measurement of membrane stability. Cell membranes perform a vital role of regulating the passage of materials into and out of the cell. The technique of using cell membrane stability for screening plant material for potential anti-oxidant activity (stress tolerance) is based on an indirect monitoring of cell membrane intactness after a stress treatment, measuring K^+ leakage with an atomic absorption spectrophotometer or a conductivity meter. Membrane leakage is measured to determine oxidant damage. The anti-oxidant system in plants acts as important stress tolerance mechanisms by protecting membranes against damage caused by toxic oxygen species, superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) produced under environmental and xenobiotic stress conditions. Because of the chloroplast/chlorophyll protection action of a high anti-oxidant activity in tolerant plants compared to sensitive plants, better yields can be obtained in field grown plants subjected to stress, including environmental and chemical stresses. Cell membrane stability is estimated by the relative rate of electrolyte leakage from the leaf tissue samples after being subjected to stress. Electrolyte leakage is estimated by measuring the electrical conductivity of the medium with which the leaf sample is equilibrated (Ruter, 1993)

2.4.2 2.3.5-Triphenyltetrazolium Chloride (TTC)

Environmental stress tolerance is manifested by the relative ability of the plant to sustain a smaller reduction on physiological or metabolic activity as its water potential decreases. During stress, damage and injury occurs to plant cells where leakage of electrolytes takes place. This unavoidably has a negative effect on the electron transport system of plants (Laurie, 1999). Vital staining with TTC is used as a method of viability measurement and can provide information about whether individual cells are functioning or not (De Ronde and Van Der Mescht, 1997).

In TTC staining, 2,3,5-triphenyltetrazolium salt (TTC) is cleaved to formazan by the succinate-tetrazolium reductase system that belongs to the respiratory chain of the mitochondrion (Steponkus and Lanphear, 1967) and is active only in viable cells. TTC reduction occurs in the mitochondria by the tetrazolium salt accepting electrons from the

electron transport via the dehydrogenase pathway, where formazan is formed (Nachlas *et al.*, 1960; Berridge *et al.*, 1996). Therefore the amount of formazan formed, correlates directly to number of metabolically active cells in the piece of tissue. Formazan has a red colour, which can be monitored spectrophotometrically.

The ability of viable cells to reduce tetrazolium salt appears to be a superior measure of environmental stress tolerance for both experimental and genotype selection (Chen *et al.*, 1982). Plant mitochondria contain a branched mitochondrial electron transport chain where electrons reduce oxygen in a reaction catalysed by cytochrome oxidase. Succinic dehydrogenase provides plenty of electrons to the electron transport chain and its activity indicates the metabolic state of the organ (Aithol and Ramasarma, 1969). Plants with a tolerance to environmental stress produce a higher activity of succinic dehydrogenase and have the ability to reduce more of the TTC into formazan.

2.4.3 Proline content

When plants are subjected to environmental stress they respond by altering the physiological and metabolic activities as a way of acclimation to stress. An increase in proline content by environmental stress has been reported and has been suggested as a test for tolerance to water stress (Sigh *et al.*, 1974). In 1984, Kaldy and Frey also proposed to use the increase of proline content during cold hardening for the detection of cold hardiness in winter wheat breeding programmes. Proline is been used as a chemical marker for winter hardiness in barley (Dobslaw and Bielka, 1988).

Van Heerden and De Villers (1996) observed a higher proline accumulation during drought stress in drought tolerant spring wheat cultivars than in more sensitive cultivars. Karamanos *et al.* (1983) also noticed that more proline accumulated with increased water stress before heading than after ear emergence in the leaves, stems and roots of wheat varieties. Results indicate that proline accumulation during drought stress may be a potential indicator of drought tolerance in spring wheat cultivars.

2.4.4 Crown survival

There are many stress factors that influence survival of plants and the interrelation among these factors can be extremely complex (Olein, 1971). Under natural conditions a confounding of the effects of these factors and a lack of opportunity to replicated experiments over time has severely handicapped plant breeders in their efforts to produce artificial tests to simulate natural factors. Many artificial freezing tests have been developed and to a limited extent, have been employed in the evaluation of cold hardiness in cereals. Results of these tests have been found to correlate well under natural conditions when genotypes having a wide range of frost hardiness are studied (Dexter, 1956).

Field survival is the most commonly used method of evaluating winter cereal plants for winter hardiness. According to Fowler *et al.* (1981), this method is simple, requires minimum labour and is the ultimate test of cultivars' winter hardiness. Field evaluation also allows for large scale, inexpensive characterization of breeding material against the full range of factors affecting winter survival. Unfortunately, results of field survival trials are often inconclusive due to either complete death or complete survival of all of most plants within a trial (Martin, 1927; Quisenberry and Clark, 1929; Fowler and Gusta, 1979). Even when differential death does occur, it is often irregular with large variations accruing within very short distances. This inability to control stress levels leads to low precision and large experimental errors, which prevent the detection of small, but important differences among genotypes or treatments (Martin, 1927; Quisenberry and Clark, 1929; Fowler and Gusta, 1979).

The inherent difficulties with field trials have stimulated interest in the development of cold hardiness prediction tests to complement field screening. Already in 1956, Dexter concluded that results of such tests were generally well correlated with field assessments of winter hardiness and recent methodological refinements have improved the correlation. On the other hand, Fowler *et al.* (1983) concluded that, although controlled environments should allow more rigid results control of freezing conditions, comparative

studies suggested that field trials usually provide more repeatable results and have lower experimental errors. The decision on which method to apply should largely depend on how frequently field-testing results in good differentiation and on equipment available for screening under artificial conditions. Wherever possible, both methods should be applied.

Controlled freeze tests that employ a single medium temperature (Wiebel and Quisenberry, 1941; Roberts and Grant, 1968; Fowler *et al.*, 1973), prolonged freezing (Thomas *et al.*, 1988), or a range of temperatures to measure lethal dose temperature (Fowler *et al.*, 1981) have been extensively done to identify differences in plant cold hardiness. Controlled freeze tests have the advantage over field testing in that they are faster, give greater control of environmental conditions and provide the opportunity for replication over time (Salmon, 1933; Pomeroy and Fowler, 1973). However, difficulties in estimating critical killing temperatures, large experimental errors, their destructive nature and the need for large populations limit the use of these tests to homogenous plant populations.

The limitations of field survival trials and controlled freeze tests have resulted in an ongoing search for a rapid screening test that can bypass the freezing and recovery process. Ideally such a test must be highly correlated with field survival, simple, repeatable, rapid, nondestructive and require only a simple plant for analysis (Fowler *et al.*, 1981). There are several options for exposing wheat plants to low temperatures. Often boxes or pots in which wheat has been planted are placed directly in freezing chambers. This has the advantage of not disturbing the plants before stress exposure but requires larger freezing cabinets and a longer exposure period, due to thermal inertia of the large soil amount. In many cases, sowing is done in boxes, flats or pots, which makes it easy to handle, regardless of weather conditions, but is relatively laborious. The other choice is to pick up plants from breeding plots in the field. This approach is more economical, but picking plants from the field is more dependent on the weather conditions and can be hampered by snow cover or frozen soil (Fowler *et al.*, 1983; 1986; Larsson, 1986; Fowler *et al.*, 1993).

The crown is the most critical region of winter cereal, since destruction of these tissues results in death of the plant. It can be concluded that winter survival is determined by the ability of the crown tissues to sustain freezing and subsequently recover from intact apical meristems and lateral promeristems. The lower peripheral crown meristem from which new roots arise, is the most critical tissue of the crown. Crowns are prepared by trimming the upper part of the plant 2-3 cm above the crown, and roots to 0.5-1cm. Crowns are then put into plastic bags, vials tubes, moist sand, etc. (Gusta *et al.*, 1978; Fowler *et al.*, 1981). Other methods expose to plants to freezing stress by transplanting from the fields into small boxes, trays or some type of supporting device (e.g. Roottrainers) with a small amount of moist sand or soil (Poltarev, 1990; O'Connor *et al.*, 1993; Ryabchun, *et al.*, 1995). Using young seedling has the advantage of reducing test duration and the amount of soils needed but as differential survival is more difficult to obtain, evaluation is usually based on leaf damage. Larsson (1986) found a very good correlation between seedling leaf damage and field winter hardiness.

Many plants acquire freezing tolerance by exposure to temperatures just above freezing (cold-hardening). Under field conditions in the fall winter cereals cold acclimate when exposed to crown temperatures below 10⁰C. The cold hardiness level of different plant parts, such as leaves, crowns and roots is dependent upon the temperature to which each part has been exposed. Because the crown contains tissues that are necessary for plant survival, it is the soil temperature at crown depth that determines critical cold acclimation rates.

According to Gusta *et al.* (1982) acquired low temperature tolerance is rapidly lost when cereals are exposed to crown temperatures above 10⁰C. The warmer the crown temperature between 10 and 18⁰C, the more rapidly the low temperature tolerance is lost. Another variable that is important in controlled-freeze test procedures is time, because prolonged exposure of winter cereal crowns also leads to a reduction of low temperature tolerance. Improving freezing tolerance of winter cereals is an important aspect of crop improvement, but confounding environmental and genetic aspects associated with winter hardiness interact to produce a complex system. This has made it difficult for breeders to

improve winter hardiness by trying to select freezing tolerant genotypes under field conditions (Grafius, 1981).

Temperature stress is a huge problem in crop production worldwide as it reduces yield and quality. It is of significance to understand the physiological basis of plant stress tolerance and the advent of molecular technologies to crop breeding. This study focuses on the physiology of stress tolerance in South African wheat cultivars. Different screening techniques are evaluated to determine their efficiency in breeding for temperature stress tolerance. The changes in the biochemical contents were also determined so as to give a better understanding of the physiology.

CHAPTER 3

Influence of high and low temperature stress on yield and yield components

3.1 Introduction

Temperature effects on yield are complex. Crop responses to a change in temperature depend on the temperature optima for photosynthesis, growth and yield, all of which may differ. When temperature is below the optimum for photosynthesis, a small increase in temperature can greatly stimulate crop growth. The opposite is true when temperature is near maximum for yield (Baker and Allen, 1993). Temperature exerts a fundamental influence on crop development as evidenced by the widespread use of thermal time as a predictor of the duration of the developmental phase. Basically, rate of development increases curvilinear with temperature (Slafer and Rawson, 1994) and as a result the duration of each development phase declines as temperature rises. Wheat is vulnerable to high temperature during reproductive stages, as kernel number and kernel weight or both can be diminished (Wiegand and Cuellar, 1981).

Over 7 million hectares of wheat worldwide is subjected to continual heat stress with mean daily temperatures exceeding 17.2°C in the coolest month of the year (Zhong-Hu and Rajaram, 1994; Guha Sarkar *et al.*, 2001). Cultivation of wheat is limited by high temperature stress at both ends of the cropping season and high temperature stress has an adverse effect on wheat productivity. Comparisons between favorable and high temperature field environments showed a larger than fourfold difference in wheat yield (Midmore *et al.*, 1984; Shpler and Blum, 1986; Zhong-Hu and Rajaram, 1994). There is ample evidence of a reduction in grain number per ear associated with high temperature stress during booting stage, i.e. the stage of pollen and embryo sac mother cell meiosis (Saini and Aspinall, 1982; Saini *et al.*, 1983, 1984).

Banath and Single (1976) have estimated that more than 50% of potential yields had been lost as a result of frost. Temperatures of -3°C and below can be lethal to wheat ears in the heading stages. After emergence of the flag leaf sheath, the wheat ear is highly susceptible to damage by frost. When a fully developed ear is injured by frost, sterility may be seen in one, a few, or all spikelets and in severe cases the glumes are killed. Components of wheat yield that can be influenced by environmental factors after ear emergence are the number of grains within a spikelet and the weight of the mature grains (ref). Final weight per grain is the function of the rate and duration of dry matter accumulation (Brocklehurst, 1977) and both these components are sensitive to temperature.

Plant species can only grow between specie-specific minimum and maximum temperatures. In between there is an optimal temperature at which growth is at its maximum. Above maximum temperature the specie productivity will be reduced by heat stress or high temperature stress and below the minimum temperature it will be reduced by low temperature stress (McDaniel, 1982).

This study was undertaken to study the influence of temperature stress on the yield components of wheat.

3.2 Materials and Methods

The experiment was conducted in a greenhouse at the University of the Free State. The 10 entries (Table 3.1) were grown in pots containing 3 kg of soil. A completely randomized block design with six replications was used. Plants were irrigated as necessary and Chemicult liquid fertilizer was administered two times a week. When the primary spike was 20% into anthesis, temperature stress was imposed. For the high temperature treatment, plants were placed in a heated growth cabinet at 30°C for three days. For the low temperature treatment, plants were placed in a cold room at -7°C for three days, the same temperature was set for both night and day. After the treatments, the pots were transferred back to the glasshouse. At maturity individual plants were

harvested and measurements were done on the following characteristics at both temperature levels:

Total grain yield per plant (GYP): total mass of the kernels of primary and secondary tillers.

Primary tiller kernel mass (PKM): kernel mass of the kernels from the primary tiller spike.

Secondary tiller kernel mass (SKM): total kernel number of all kernels from the secondary tiller spike.

Kernel number (KN): total kernel number of both primary and secondary tillers.

Number of spikes per plant (SN): number of productive spikes per plant.

Spikelet number (SNP): number of spikelets on the primary spike of each plant.

All statistical analyses were done with Agrobase (Agrobase, 2000).

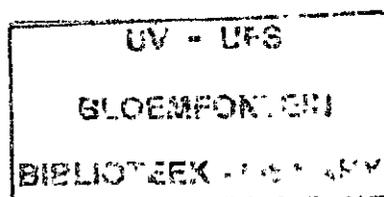


Table 3.1 Cultivars screened for temperature tolerance

Entry	Cultivar	Growth habit
1	Betta Dn	Winter
2	Gariep	Facultative
3	Elands	Winter
4	Hugenoot	Winter
5	Caledon	Winter
6	Pan 3211	Facultative
7	Pan 3232	Winter
8	Pan 3235	Winter
9	SST 124	Facultative
10	Tugela Dn	Winter

3.3 Results

Affected tissues showed signs of chlorosis several days after low- and high temperature stress. As ears developed, sterility of some florets, whole spikelets or whole ears became apparent. Sterility was usually first seen as shriveling of anthers, that occurred in florets which otherwise appeared normal. The pattern of damage to ears was generally irregular, some cultivars had a tendency to remain fertile but with poor seed setting in the case of the low temperature treatment, with freezing resulting in shrunken or shriveled seeds. Mean squares were significant for all characteristics at both treatments, except for SNP at the high temperature treatment (Table 3.2), which indicated that entries varied significantly for the measured characteristics.

Table 3.2 Mean squares for measured yield components at both treatments

	SN	SNP	KN	PKM (g)	SKM (g)	GYP (g)
High temperature treatment						
Block	10.040	88.227	887.320	0.112	0.206	0.347
Entry	122.556**	258.037	13912.296**	0.907**	1.560**	2.273**
Low temperature treatment						
Block	1.040	118.587	82.587	0.017	0.008	0.019
Entry	5.993*	333.030**	1294.933**	0.078**	0.047**	0.151**

** $p \leq 0.01$, * $p \leq 0.05$

SN= number of spikes per plant, SNP = spikelet number, KN = kernel number, PKM = primary tiller kernel mass, SKM = secondary tiller kernel mass, GYP = total grain yield

Table 3.3 Mean values of measured yield components at low and high temperatures

Cultivar	SN		SNP		KN		PKM (g)		SKM (g)		GYP (g)	
	LT	HT	LT	HT	LT	HT	LT	HT	LT	HT	LT	HT
Betta Dn	4	11	39	24	26	52	0.23	0.28	0.01	0.63	0.24	0.92
Hugenoot	2	7	16	25	4	56	0.0012	0.27	0	0.26	0.0012	0.53
Gariep	2	3	15	17	9	46	0.32	1.24	0.022	0.21	0.34	1.45
Elands	2	6	17	34	1	60	0.01	1.17	0	0.37	0.01	1.54
Pan 3211	3	10	22	38	4	68	0.01	0.96	0.03	0.63	0.03	1.59
Pan 3232	3	9	24	27	16	75	0.08	0.81	0.04	0.55	0.12	1.36
Caledon	3	5	16	19	20	52	0.05	0.25	0.022	0.18	0.07	0.43
SST 124	5	17	14	20	46	200	0.014	0.49	0.029	1.8	0.43	2.3
Tugela Dn	3	3	21	23	2	29	0	0.77	0.02	0.04	0.02	0.81
Pan 3235	3	4	17	23	0	42	0	0.29	0	0.14	0	0.42
Mean value	2.800	7.500	22.867	24.833	13.317	68.000	0.081	0.653	0.039	0.479	0.119	1.133
LSD (0.05)	0.992	2.35	6.533	7.846	5.762	22.69	0.088	0.291	0.075	0.267	0.123	0.437

SN= number of spikes per plant, SNP = spikelet number, KN = kernel number, PKM = primary tiller kernel mass, SKM = secondary tiller kernel mass, GYP = total grain yield, LT= low temperature, HT = high temperature, LSD = least significant differences.

3.3.1 The low temperature treatment

SST 124 and Betta Dn had significantly more productive spikes per plant than the other entries; they ranked first and second respectively (Table 3.3 and Figure 3.1). Hugenoet, Elands and Gariiep ranked eighth, ninth and tenth as they had the lowest numbers of productive spikes per plant.

Number of spikelets on the primary spike of the plant (SNP)

Betta Dn ranked first, and it had a significantly higher number of spikelets on the primary spike per plant than all other entries (Figure 3.2). PAN 3232, PAN 3211 and Tugela Dn ranked second, third and fourth respectively. Entries like Gariiep and SST 124 had a low number of spikelets on the primary spike and ranked ninth and tenth respectively.

Total number of kernels of both primary and secondary spikes (KN)

SST 124 had a significantly higher kernel number than all other entries (Figure 3.3). Ranking second, third and fourth were Betta Dn, Caledon and PAN 3232 respectively. Tugela Dn and Elands exhibited a very low number of kernels, ranking eighth and ninth. PAN 3235 ranked in the last position with no kernels at all.

Primary tiller kernel mass (PKM)

Primary tiller kernel mass varied from 0.32g to zero (Figure 3.4). Gariiep had a significantly higher PKM than all other entries. Betta Dn and SST 124 ranked second and third respectively. Hugenoet and Tugela Dn ranked last with no primary kernels.

Secondary tiller kernel mass (SKM)

Secondary tiller kernel mass varied between 0.25g and zero. SST 124 ranked first with significantly higher secondary tiller kernel mass than all other entries (Figure 3.5). PAN 3232 and PAN 3211 ranked second and third respectively with no significant difference between them. Ranking in the last three positions were Hugenoet, Elands and Pan 3235 with a mass of zero.

Grain yield per plant (GYP)

SST 124 had a higher grain yield than all other entries except for Gariiep (Figure 3.6). Gariiep and Betta Dn ranked second and third. Hugenoot and Pan 3235 ranked in the last two positions, as they yielded no grain.

3.3.2 The high temperature treatment

Number of productive spikes per plant (SN)

SST 124 had a significantly higher SN than all other entries (Table 3.3 and Figure 3.1). Beta Dn and Pan 3211 ranked second and third respectively. Entries such as PAN 3235, Tugela Dn and Gariiep had the lowest number of productive spikes per plant.

Number of spikelets on the primary spike of the plant (SNP)

PAN 3211 had a significantly higher SNP than all other entries, except for Elands (Figure 3.2). Caledon and Gariiep had the lowest number on spikelets on the primary spike per plant and ranked ninth and tenth respectively.

Total kernel number of both primary and secondary spikes (KN)

SST 124 had a significantly higher number of kernels than the other entries (Figure 3.3). PAN 3232 and PAN 3211 ranked second and third respectively. Gariiep, PAN 3235 and Tugela Dn had the lowest kernel numbers and ranked in the last three positions respectively.

Primary tiller kernel mass (PKM)

Gariiep had a significantly higher PKM than all entries except for Elands and PAN 3211 (Figure 3.4). Hugenoot and Caledon had the lowest rankings indicating a low mass for the primary tiller kernels.

Secondary tiller kernel mass (SKM)

SST 124 had a significantly higher secondary tiller kernel mass than the rest of the entries (Figure 3.5). Betta Dn and PAN 3211 ranked second and third respectively. Caledon,

PAN 3235 and Tugela Dn had a low secondary tiller kernel mass, ranking in the last three positions.

Grain yield per plant (GYP)

SST 124 yielded significantly more than all other entries. PAN 3211, Elands and PAN 3232 ranked second, third and fourth, and significantly outyielded the remaining entries (Figure 3.6). Hugenoet, Caledon and PAN 3234 had the lowest yield

3.4 Phenotypic Correlations

3.4.1 Correlations at the low temperature treatment

Total grain yield was positively and highly significantly correlated with all the other measured characteristics, except for the number of spikelets on the primary spike (Table 3.4). Primary tiller kernel mass was positively and significantly correlated with total kernel number. Secondary tiller kernel mass was positively correlated with number of productive spikes ($r = 0.6598$) and total kernel number ($r = 0.7715$). There was a positive and highly significant correlation between kernel number and number of productive spikes ($r = 0.6380$). Number of productive spikes per plant correlated positively to all the characteristics. The correlation was highly significant to kernel number ($r = 0.6380$), secondary tiller kernel mass ($r = 0.6598$) and grain yields ($r = 0.4703$).

Table 3.4 Phenotypic correlations for characteristics measured for the low temperature treatment

	SN	SNP	KN	PKM	SKM
SNP	0.0873				
KN	0.6380 **	0.0032			
PKM	0.1247	-0.0407	0.3669 *		
SKM	0.6598 **	-0.1033	0.7715 **	0.1519	
GYP	0.4703 **	-0.0891	0.7137 **	0.8232 **	0.6862 **

** $p \leq 0.01$, * $p \leq 0.05$

SN= number of spikes per plant, SNP = spikelet number, KN = kernel number, PKM = primary tiller kernel mass, SKM = secondary tiller kernel mass, GYP = total grain yield

3.4.2 High temperature treatment correlations

Grain yield was positively and significantly correlated with all the measured characters except for number of spikelets on the primary spike per plant (Table 3.5). The correlation of grain yield and number of spikelets on the primary spike was weak in comparison to the other characters. Secondary tiller kernel mass had a positive and highly significant correlation with number of productive spikes ($r = 0.7724$) and the total number of kernels ($r = 0.8056$). Primary tiller kernel mass was positively correlated to all the characters and the correlation was significant for grain yield ($r = 0.6672$) and number of spikelets on the primary spike ($r = 0.3347$).

Kernel number was positively correlated with all the characters but the correlation was only highly significant between number of productive spikes ($r = 0.7445$), secondary tiller kernel mass ($r = 0.8056$) and grain yield ($r = 0.6843$).

Table 3.5 Phenotypic correlations for characteristics measured for the high temperature treatment

	SN	SNP	KN	PKM	SKM
SNP	0.2879 *				
KN	0.7445 **	0.1179			
PKM	0.0067	0.3347 *	0.1303		
SKM	0.7724 **	0.0548	0.8056 **	0.0324	
GYP	0.5798 **	0.2559 *	0.6843 **	0.6672 **	0.7661 **

** $p \leq 0.01$, * $p \leq 0.05$

SN= number of spikes per plant, SNP = spikelet number, KN = kernel number, PKM = primary tiller kernel mass, SKM = secondary tiller kernel mass, GYP = total grain yield

3.5 Discussion

SST 124 performed very well under both high- and low temperature stress in terms of yield and related characteristics (SN, KN and SKM). PAN 3235 was very sensitive to low temperatures, and failed to give any seed after treatment, but at the same time it also had the lowest yield after the high temperature treatment. It therefore seems to be very sensitive to temperature extremes.

Betta Dn performed poorly under high temperature stress for yield. In terms of the yield characteristics it only performed well for SN and SKM, but it yielded well (ranking 3rd) after the low temperature treatment. Gariep was the 2nd best yielder after low temperature stress, but it did not perform well in terms of yield characteristic except for PKM, where it had the highest value. Seemingly, PKM contributed positively toward its total grain yield.

Elands was the 3rd lowest yielder after the low temperature treatment and it showed a poor performance in terms of the yield characteristics. Seemingly it is sensitive to low temperatures although it performed well under high temperature stress (ranking 3rd). It had high SNP and PKM values for high temperature stress. Hugenoot was sensitive to both temperature treatments as it gave low yields. It had the 2nd lowest PKM and had average performance on other yield characteristics after high temperature stress. It performed poorly for all the yield characteristics after low temperature stress. It therefore seems to be sensitive to temperature extremes.

PAN 3211 performed well under high temperature stress in terms of yield and related characteristics and it had the highest SNP. But after low temperature stress it performed poorly and yielded average. It seems to be sensitive to low temperature stress but tolerant to high temperature stress.

Significant and positive correlation was seen between kernel number and total grain yield for both temperature treatments. This is in agreement with reports by Shipler and Blum (1991), who found that kernel number per spike was the most important component affecting yield variation among wheat cultivars under temperature stress. Rawson (1986) stated that kernel number is the most important yield component affecting heat tolerance for yield. Marcellos and Single (1984) found that with lower temperature, grain set was markedly reduced so that by -5°C ears were virtually sterile, resulting in very low kernel numbers. Water in tissues of wheat supercools readily at temperatures in the range likely to be encountered in the field so that freezing is initiated by the development of frost crystals. It was proposed that freezing might commence in florets of the emerged ear as a result of the spread of crystallization from the peduncle or spikelet structures (Marcellos and Single, 1976; 1979).

Shipler and Blum (1986) concluded that reduction in kernel numbers resulted from a reduced number of spikelets per plant. In regards with high temperature, this study was in contrast with their conclusion, because a non-significant correlation between kernel

number and a number of primary spikelets was found in this study but a highly significant and positive correlation was found under freezing stress.

Moustafa *et al.* (1996), working under drought stress conditions, showed that low number of spikelets per spike compensated for the higher number of spikes per plant of a genotype. Oosterhuis and Cartwright (1983) found that the development of the wheat spike is extremely sensitive to stress applied either at the late vegetative and transition phases or during the late internode immediately prior to spike emergence.

In discussing heat tolerance, Blum (1988) noted that high temperature, particularly from the onset of spike initiation to anthesis, accelerated plant development and resulted in smaller spikes and lower yields. This study found a positive and significant correlation between spike number and total grain yield for both temperature treatments, showing that it is also an important component that can affect total yield negatively or positively, which is in agreement with findings by Blum (1988). In contrast Rawson (1986) and Shipler and Blum (1986) reported that early heat (emergence to double ridge) had a slightly negative effect on spike number, but spike number was not a limitation to yield under hot conditions. Shipler and Blum (1986) defined heat tolerance by relative reductions in grain yield from normal winter conditions to the hot summer under full irrigation. They found that under cool conditions spike number was the most important yield component affecting the variation in grain yield among cultivars.

Oosterhuis and Cartwright (1983), working with moisture stress, noted that secondary and tertiary tiller kernel mass were reduced within the spikelet in comparison with the primary tiller kernel mass. They found that it was because the kernels of the secondary tiller filled later than that of the primary tiller. Florets and spikelets tended to die at the terminal ends of the spikes. Labuschagne (1989) was also in agreement with their findings, stating that the last formed spikes were most sensitive to stress. Results of this study also supported these findings, as they showed a positive and highly significant correlation between secondary spike yield and total grain yield. This indicates that secondary spike yield is one of the important yield components affecting grain yield.

Significant correlation between primary spike yield and yield was seen, and it was suggested that selection for this character under heat stress would increase yield. Under high temperature stress, this study revealed a non-significant correlation between primary spike yield and yield. Low primary tiller kernel mass and the non-significant correlation observed in this study, might be attributed to failure of fertilization, because plants were exposed to stress conditions when the anthers were extruding and still yellow, shedding pollen. Many researchers have stated that for successful seed establishment, pollen must remain viable and the stigma receptive and pollen tubes must grow properly into the ovules. Fertilization should succeed and embryo and endosperm should develop properly. Pollen viability and growth is one of the most heat sensitive of these developmental stages in cereals (Saini and Aspinall, 1982; Stone, 2001).

Results of this study revealed that there are positively correlated with all other yield characteristics for both high and low temperature stresses. This means that by selecting for all or one of these characteristics, which indirectly will give rise to high grain yield. It can be suggested that SST 124 tolerant to both temperature extremes and so can be planted at any environment. In contrast PAN 3235 indicated sensitivity to Both temperature extremes. Betta Dn exhibited high temperature sensitivity so, can be planted at certain environments.

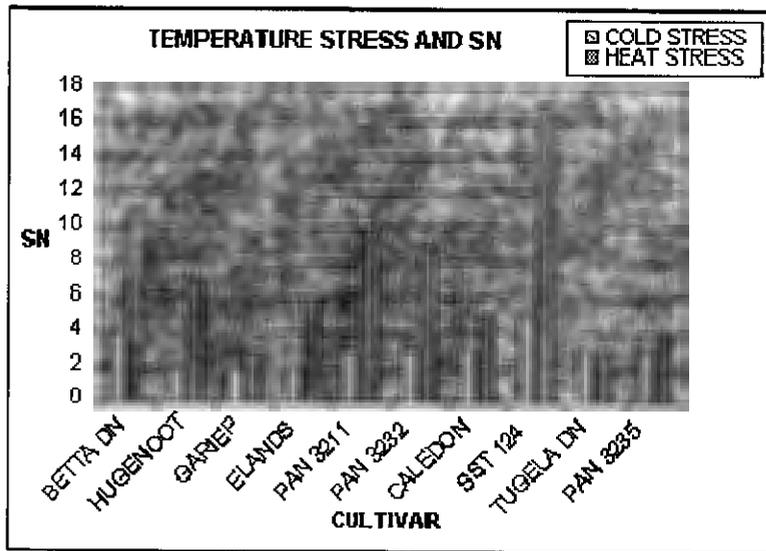


Fig. 3.1 The effect of temperature stress on number of spikes per plant

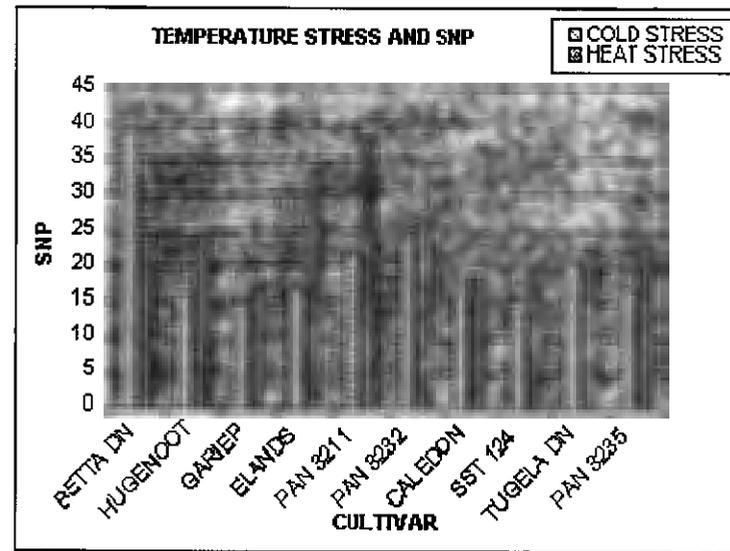


Fig. 3.2 The effect of temperature stress on a number of spikelets on the primary spike per plant

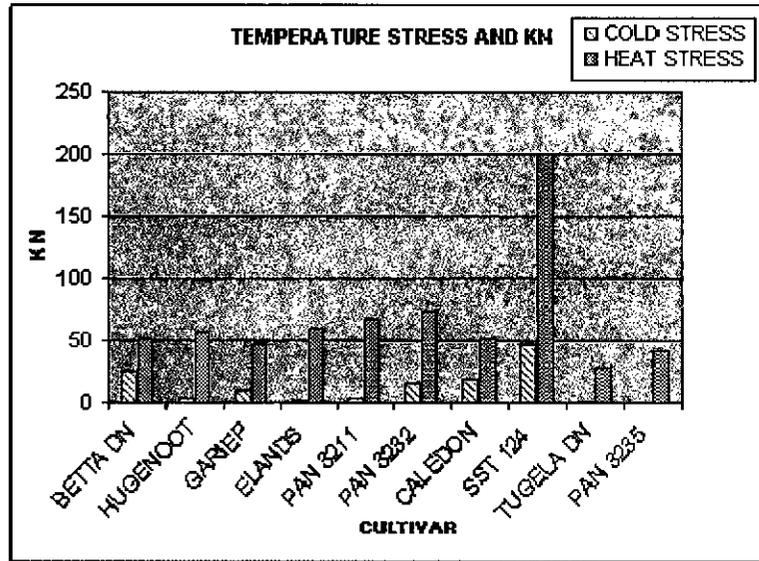


Fig 3.3 The effect of temperature stress on number of kernels per plant

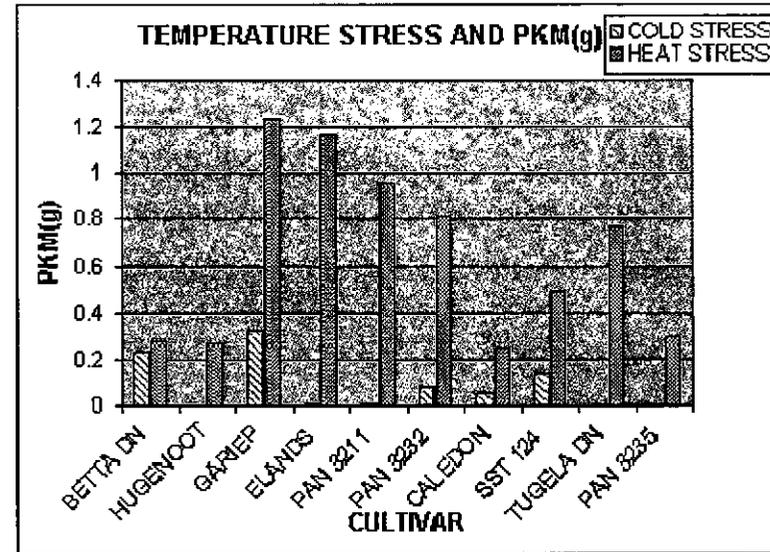


Fig 3.4 the effect of temperature stress on the primary kernel mass per plant

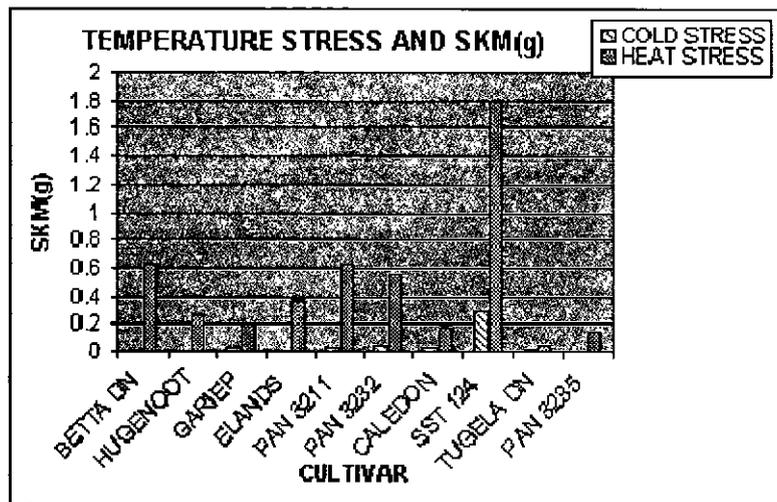


Fig 3.5 The effect of temperature stress on secondary kernel mass per plant

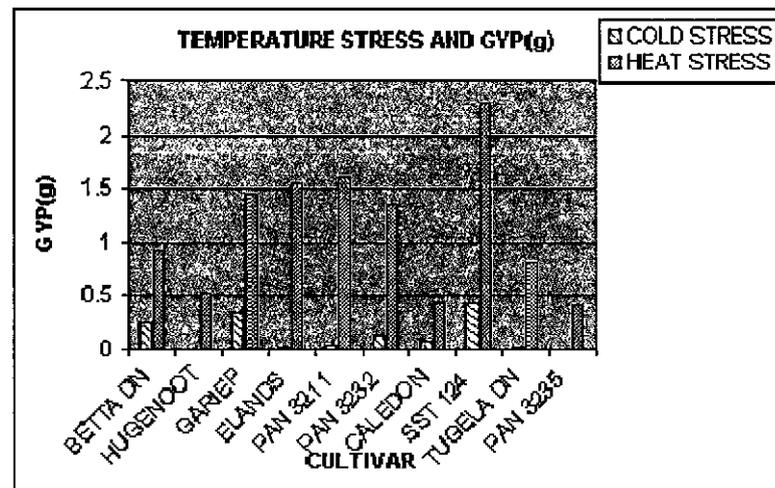


Fig 3.6 The effect of temperature stress on total grain yield per plant.

CHAPTER 4

The evaluation of metabolites of wheat seedlings under high and low temperature stress

4.1 Introduction

Plants resort to many adaptive strategies in response to different abiotic stresses such as high salt, temperature and water stress, which ultimately affect the plant growth and productivity (Epstein *et al.*, 1980; Yancey *et al.*, 1982). Plants use different mechanisms to protect from these stresses, including change in morphological and developmental pattern as well as physiological and biochemical processes. Adaptation to all these stresses is associated with metabolic adjustment that leads to accumulation of several organic solutes like sugars, polyols, betaines, proline and proteins (Flowers *et al.*, 1977; Greenway and Munns, 1980; Yancey *et al.*, 1982). Carbohydrate changes are of particular importance, because of their direct relationship with such physiological processes as photosynthesis, translocation and respiration. Among the soluble carbohydrates, sucrose and fructans have a potential role in adaptation to these stresses (Williams *et al.*, 1992; Housley and Pollock, 1993; McKersie and Leshem, 1994). Sucrose can act in water replacement to maintain membrane phospholipids in the crystalline phase and to prevent structural changes in soluble proteins. Glucose participates in cross-linking with proteins by a complex glycosylation reaction between amino and carbonyl groups known as the Millard reaction (Koster and Leopold, 1988). As respiratory substrates, monosaccharides promote respiration and mitochondrial electron transport, which would seem to oppose the onset of quiescence and favour metabolism, energy production and formation of oxygen radicals (Leprince *et al.*, 1993). Numerous studies have demonstrated quantitative and qualitative changes in the free saccharide content of plants exposed to low temperatures. Many researchers have suggested that accumulation of soluble sugars under low temperatures is an important

factor in freezing tolerance (Levit, 1980; Bhullar and Jenner, 1983; Williams *et al.*, 1992).

Thornley (1971) stated that high temperatures lead to a shortage of carbohydrates for grain growth, due to the fact that high temperature stress increases the rates of grain respiration (Chowdhury and Wardlaw, 1978) and leaf senescence (Kuroyanagi and Paulsen, 1985). However, Bhullar and Jenner (1983) reported a significant reduction of sucrose and other soluble sugars in the ears of wheat after exposure to high temperature.

A protein is a primary product of a structural gene and therefore serves as a marker for that particular gene. Genes are coupled into genetic systems and because of this, proteins may also serve as markers for such systems, including chromosomes and the genome as a whole. Because of this, the totality of protein markers gives considerable insight into genome or genotype structure and could be used to resolve breeding problems. Protein biosynthesis is one of the temperature labile processes of metabolism (Bernstam, 1978).

Low temperatures result in altered gene expression, leading to synthesis of specific proteins and certain enzymes, which are responsible for the development of freezing tolerance. Several pre-existing proteins, abundant in tissues of plants grown under normal temperatures, declined on exposure to low temperatures. However, many new transcripts and polyploids are synthesized, which appear to play a major role in the acclimation of plants to freezing temperatures (Griffith *et al.*, 1997).

A critical site of high temperature stress in plant cells, is proteins, in particular enzymes. High temperature stress may interfere with the *de novo* protein biosynthesis, inhibit enzyme activity and induce degradation of existing proteins. Under high temperature stress, thermo-tolerant cells should be able to maintain enzymes and structural proteins in the functional state and be able to recover from any stress-induced inhibition of the functions upon termination of heating. Thermo-tolerant plants possess thermo-stable enzymes and structural proteins (Klueva *et al.*, 2001).

The aim of this study was to evaluate the effect of temperature stress (freezing and high temperature) on the metabolite levels of wheat.

4.2 Materials and methods

4.2.1 Growing conditions

The experiment was conducted in a greenhouse at the University of the Free State. The 15 entries (listed in Table 4.1) were grown in pots containing 3 kg of soil; five plants were planted per pot. An optimum temperature was maintained until the plants reached four to five leaf stages.

Control temperature treatment

Plants were left in the glasshouse under control temperatures (25°C) for the duration of the low- and high temperature treatment, receiving water as necessary. Chemicult liquid fertilizer was administered twice a week. Extraction of sugars and proteins were done on fresh leaf material.

Low temperature treatment

Plants were transferred to a cold room (2°C) for acclimation and kept there for three weeks, receiving water as necessary to avoid desiccation; temperature was kept constant night and day. Chemicult liquid fertilizer was administered twice a week. After three weeks, freezing treatment was conducted in a way to avoid supercooling. The temperature was reduced 1°C an hour until -7°C and kept there for an hour. The temperature was then increased at 1°C an hour until 2°C followed by extraction of sugars and proteins on fresh leaf material.

High temperature treatment

Plants were transferred straight from the glasshouse to the heat cabinet at 35°C and kept there for three days; temperature was kept constant night and day. During the three days they were watered as necessary. Chemicult liquid fertilizer was administered twice a week. After the treatment, carbohydrate and protein extractions were done on fresh leaf material.

Table 4.1 Cultivars screened for temperature tolerance

Entry	Cultivar	Growth habit
1	Betta Dn	Winter
2	Caledon	Winter
3	Elands	Winter
4	Gariep	Facultative
5	Hugenoot	Winter
6	Kariega	Spring
7	Molen	Winter
8	PAN 3211	Facultative
9	PAN 3232	Winter
10	PAN 3235	Winter
11	PAN 3349	Winter
12	PAN 3377	Winter
13	SST 399	Winter
14	SST 124	Facultative
15	Tugela Dn	Winter

where c = concentration mg per fresh gram of fresh weight, V = final volume (ml), molecular weight of the substance, ΔA = change in substance concentration, F = dilution factor (4), e = extinction coefficient of NADPH ($5.4099 \text{ mmol}^{-1} \text{ cm}^{-1}$), d = light path (0.8698 cm), v = sample volume (ml).

Total protein determination

The extraction buffer consisted of 12.5 mM TRIS (hydroxymethyl) aminomethane, 2 mM Ethylene-diamine-tetra-acetic acetic (EDTA), 10 mM Mercapto-ethanol (added just before use), 2 mM Phenylmethylsulfonyl fluoride (PMSF), titrated to pH 6.8 with hydrochloric acid (HCl), which was made up to 200 ml with distilled water.

Extraction of proteins was carried out according to the method by Pretorius and Small (1991). A gram of fresh material (leaves) was ground in liquid nitrogen and homogenized by addition of 6 ml protein extraction buffer (the buffer was added in increments). The homogenate was transferred to a clean Eppendorf vial and centrifuged at 12000 rpm for 10 minutes at room temperature. The supernatant was then transferred into a clean Eppendorf vial and kept on ice until the protein content was determined. Determination of the protein content was done using the Biorad method (Bradford, 1976) and spectrophotometer readings by means of the micro-plate reader. An Elisa-plate with four replicates of the blank and four replicates of a standard protein (bovine γ -globulin) was used. The absorbance was read at 595nm.

4.3 Results

The mean squares for the entries for sucrose, glucose and proteins for all three temperature treatments were highly significant, indicating that there were large differences between the entries for these characteristics (Table 4.2).

Table 4.2 Mean squares for total sugars, sucrose, glucose and proteins for three temperature treatments

	Control		Low temperature		High temperature	
	Block	Entry	Block	Entry	Block	Entry
Total sugars	28.124	78732.825**	22.015	2100.503**	10.605	98053.250**
Glucose	4.746	800.570**	0.298	889.387**	4.544	28317.330**
Sucrose	24.781	65275.647**	19.248	1071.821**	8.450	24255.654**
Protein	2305.211	510794.039**	448.82	302462.145**	2664.936	117858.918**

** $p \leq 0.01$

Table 4.3. Mean values of sucrose and glucose at three temperature treatments

Entry	Control			Low temperature			High temperature		
	Glucose	Sucrose	Total sugars	Glucose	Sucrose	Total sugars	Glucose	Sucrose	Total sugars
Betta Dn	51.90	349.00	400.90	24.00	40.73	64.73	17.30	16.57	33.87
Gariep	41.03	409.91	450.94	31.81	23.93	55.74	22.88	127.77	150.65
Elands	51.35	336.03	387.38	10.05	44.64	54.69	12.28	19.53	31.81
Molen	7.31	5.95	13.26	25.11	52.45	77.56	33.93	145.07	179.00
Caledon	26.90	3.40	30.30	7.81	50.59	58.40	160.17	164.32	324.49
Tugela Dn	23.16	6.32	29.48	44.09	43.89	87.98	11.72	17.48	29.20
Kariega	8.37	6.70	15.07	25.11	11.72	36.83	46.88	29.57	76.45
Hugenoot	15.84	16.74	32.58	65.30	43.80	109.10	396.25	343.14	739.39
SST 124	3.72	10.88	14.60	13.95	82.58	96.53	52.45	101.02	153.47
SST 399	11.91	8.10	20.01	2.79	46.50	49.29	78.13	34.03	112.16
Pan 3211	7.07	13.95	21.02	5.58	5.95	11.53	41.86	45.75	87.61
Pan 3232	5.58	23.71	29.29	12.84	38.50	51.34	48.55	18.41	66.96
Pan 3235	8.93	12.55	21.48	28.65	41.29	69.94	64.74	25.11	89.85
Pan 3349	13.38	6.70	20.08	6.70	41.85	48.55	71.44	81.29	152.73
Pan 3377	11.53	1.40	12.93	5.02	15.06	20.08	20.84	1.46	22.30
Mean	19.232	81.60	100.83	20.59	38.90	59.49	71.96	78.24	150.20
LSD (0.05)	1.72	5.23	5.32	5.94	5.49	4.1462	1.45	3.99	8.78
CV (%)	8.34	5.97	4.92	6.58	9.59	6.50	7.71	6.54	5.46

LSD = least significant differences, CV = coefficient of variance

Glucose and sucrose content

Averaged over genotypes, the glucose content was 19.232 mg.g⁻¹ fresh weight (FW) at the control temperature treatment, 20.588 mg.g⁻¹ FW under the low temperature and 71.961 mg.g⁻¹ FW under the high temperature treatment (Table 4.3). There was a higher increase of glucose content under the high temperature than under low temperature treatment. A similar pattern was observed with sucrose content. More sucrose accumulated under the high temperature than low temperature treatment. Averaged over genotypes, sucrose content was 81.596 mg.g⁻¹ FW at control temperature, 38.898 mg.g⁻¹ FW under low temperature and 78.235 mg.g⁻¹ FW under high temperature treatment.

Control temperature treatment

Total sugar content

Gariiep, Betta Dn and Elands ranked first, second and third respectively, they had significantly higher carbohydrate content than all other entries. Kariega, SST 124 and Molen had low content of carbohydrates consequently ranked thirteenth, fourteenth and fifteenth, respectively.

Glucose content

Betta Dn, Elands and Gariiep had significantly higher glucose content than all other entries and they ranked first, second and third respectively. PAN 3211 and PAN 3232 ranked thirteenth, fourteenth exhibiting low glucose content. SST 124 had the lowest glucose content of all entries.

Sucrose content

Gariiep, Betta Dn and Elands ranked first, second and third respectively. They had significantly higher sucrose content than all other entries. Tugela Dn and Molen had low sucrose content and consequently ranked thirteenth, fourteenth respectively. Caledon had the lowest sucrose content.

Low temperature treatment

Total sugar content

Hugenoot had the highest total carbohydrate content of all other entries. Ranking second and third respectively were SST 124 and Tugela Dn. Kariega, PAN 3377 and PAN 3211 exhibited very low carbohydrates content and ranked thirteenth, fourteenth and fifteenth respectively.

Glucose content

Hugenoot had significantly higher glucose content than all the other entries. Tugela Dn and Gariiep ranked second and third respectively. Entries like PAN 3211 and PAN 3377 exhibited low glucose content and ranked thirteenth, fourteenth respectively. SST 399 had a lower glucose content than all other entries.

Sucrose content

SST 124, Molen and Caledon ranked first, second and third respectively; SST 124 had the highest sucrose content. PAN 3377 and Kariega ranked thirteenth, fourteenth respectively. PAN 3211 had the lowest sucrose content.

High temperature treatment

Total sugar content

Total carbohydrate content varied from 739.39 mg.g⁻¹ FW to 22.30 mg.g FW⁻¹. Hugenoot had the highest total carbohydrate content. Ranking second and third were Caledon and Molen. Betta Dn, Elands, Tugela Dn and PAN 3377, exhibited a very low content of total carbohydrate and consequently ranked twelfth, thirteenth, fourteenth and fifteenth, respectively.

Glucose content

Hugenoot and Caledon ranked first and second respectively, they exhibited significantly higher glucose content than all other entries. SST 399 and PAN 3349 ranked third and fourth respectively. Betta Dn, Elands and Tugela Dn had low glucose content and they ranked thirteenth, fourteenth and fifteenth, respectively.

Sucrose content

Hugenoot had significantly higher sucrose content than all other entries. Ranking second, third and fourth were Caledon, Molen and Gariep respectively. Tugela Dn and Betta Dn ranked thirteenth, fourteenth, showing a low sucrose content. PAN 3377 ranked last.

Protein content

Averaged across genotypes, protein content was 1835.97mg.g⁻¹ FW at the control temperature, 1881.85 mg.g⁻¹ FW at the high temperature treatment and 2761.46 mg.g⁻¹ FW at the low temperature treatment (Table 4.4). It was evident that there was a significant increase of protein level under low temperature treatment in all entries compared to the high temperature treatment.

Table 4.4. Mean values of proteins (mg.g⁻¹ FW) for three temperature treatments

Entry	Temperature treatments		
	Control	Low	High
Betta Dn	1324.56	2704.98	1610.92
Gariep	1897.56	2688.85	2155.20
Elands	1461.59	2489.87	1750.10
Molen	1462.97	2378.56	1515.33
Caledon	1565.39	3003.84	1753.67
Tugela Dn	1551.55	3518.19	1963.07
Kariega	1447.75	2823.13	2195.17
Hugenoot	1564.01	2641.47	1769.47
SST 124	1922.71	2522.53	1911.87
SST 399	1829.80	2412.41	2092.30
PAN 3211	2444.29	3091.68	1811.10
PAN 3232	2321.10	2677.57	1759.23
PAN 3235	1778.54	3165.02	2040.43
PAN 3349	25.31.68	2492.52	2041.37
PAN 3377	2435.99	2811.85	1858.47
Mean	1835.966	2761.459	1881.846
LSD (0.05)	43.69	125.98	61.12
CV (%)	2.22	4.26	3.03

LSD = least significant differences, CV = coefficient of variance

Control temperature treatment

PAN 3349, PAN 3211 and PAN 3232 ranked first, second and third respectively, they had higher protein content than all the other entries. Ranking thirteenth, fourteenth and fifteenth, were Elands, Kariega and Betta Dn respectively as they had low protein content.

Low temperature treatment

Tugela Dn had the highest protein content. Ranking second, third and fourth respectively were PAN 3235, PAN 3211 and Caledon. Elands, SST 399 and Molen ranked last as they exhibited a low protein content.

High temperature treatment

Kariega, Gariep and SST 399 had higher protein content than all the other entries, they ranked first, second and third respectively. Eland, Betta Dn and Molen ranked thirteenth, fourteenth and fifteenth respectively.

4.4 Discussion

Molen and Caledon performed well under both high and low temperature stress in terms of total sugar content and sucrose content. They both exhibited an increase of glucose content under high temperature but Caledon had a decrease of glucose content under low temperature treatment. Hugenoet and SST 124 are among entries that performed well, as they had an increase of glucose and total sugars under both high and low temperature treatment.

Meanwhile entries like Betta Dn, Gariep, Elands and PAN 3377 were negatively affected by temperature extremes. PAN 3377 had a decrease in sucrose content but its glucose content increased under low temperature treatment. Betta Dn, Gariep, and Elands performed poorly under both high and low temperature treatments. They failed to accumulate more glucose and sucrose, resulting in decreased total sugar content.

Numerous studies have demonstrated quantitative and qualitative changes in free saccharide content of plants exposed to low temperatures. Levitt (1980) proposed that metabolites

accumulate during cold acclimation and carbon assimilation proceeds efficiently as temperature drops. He suggested that resulting metabolic changes lead to plant adaptation to freezing stress. In this study, it was found that more sucrose than glucose accumulated and this is in agreement with a report by Guy (1990) who stated that sucrose is the main free sugar that is commonly accumulated in response to low temperatures. According to Levitt (1980) the accumulation of sucrose may be ascribed to a decreased demand for photosynthates, as well as to a modification of activities of enzymes that regulate sucrose concentration, as there was an increase in total carbohydrate content following low temperature exposure in certain cultivars.

Most of the sucrose present in mesophyll cells, which accumulate sucrose, is thought to be contained within the vacuole (Huber 1989). However, during cold acclimation most monosaccharides, sucrose and raffinose accumulate in extracellular space, where they can contribute to cryoprotection (Koster and Lynch, 1992).

Sucrose is not the only carbohydrate that accumulates at low temperatures. Lesser amounts of glucose and fructose are frequently accumulated. This study also showed some increase in glucose content, though it was less relative to sucrose content increase. Chatterton *et al.* (1987) also reported slight increase of glucose concentrations in roots, leaves and leaf bases of wheatgrass and redtop, at low temperatures.

Although the capacity to accumulate soluble sugars is commonly considered an important factor in freezing tolerance, it is difficult to determine to what extent this mechanism is responsible for winter hardiness. In some cases, a positive correlation was found between freezing tolerance and accumulation of soluble sugars (Holaday *et al.*, 1992). Tognetti *et al.* (1990) also found that most cold tolerant cultivars showed higher levels of soluble sugars and enzyme activities. Dionne *et al.* (2001) reported that sucrose levels were not related to the differential freezing tolerance among the three annual bluegrass ecotypes tested in their study. Instead, they found that cold sensitive ecotypes accumulated significantly higher levels of sucrose, glucose and fructose than the cold tolerant ecotypes. Their results were in agreement with Castonguay *et al.* (1995) who reported that levels of sucrose in fully acclimated alfalfa (*Medicago sativa* L) were poorly related to the differences in freezing tolerance between cultivars.

Gill *et al.* (2001) reported a significant increase in total sugar content in sorghum seedlings on exposure to high temperature stress. In this study, an increase in sugar content of most entries was also observed on exposure to the high temperature treatment. Many researchers agreed that grain sucrose levels are not significantly reduced by moderately high temperature in any of the major crops, despite the fact that high temperature stress increased the rate of grain respiration leaf senescence and reduced the rate of photosynthesis (Ford *et al.*, 1976; Chowdhury and Wardlaw, 1978; Kuroyangi and Paulsen, 1985; Bhullar and Jenner, 1986; Singletary *et al.*, 1994).

Photosynthesis is extremely sensitive to supra-optimal temperature and is often the first metabolic process that is damaged (Paulsen, 1994). The optimum temperature for photosynthesis is lower than that of respiration (Nilsen and Orcutt, 1996). Therefore, high temperature may cause an imbalance between photosynthesis and respiration processes and carbohydrate depletion. This was noticed from some entries in this study, as their carbohydrate levels declined on exposure to high temperature treatment. In their study, Xu and Huang (2000), working on temperature effects on carbohydrate metabolism in creeping bentgrass, found that carbon imbalance and limited carbohydrate accumulation in shoots were more severe for heat sensitive cultivars than for heat tolerant ones. In the study of the effect of heat stress and changes in carbohydrate concentration in the developing anthers of tomato, Pressma *et al.*, (2002) reported that during anther development, the concentration of total soluble sugars gradually increased in the anther walls and pollen grains (but not in the locular fluid), reaching a maximum at anthesis. Continuous exposure of plants to high temperatures prevented the transient increase in starch concentration and led to decreased concentration of soluble sugars in the anther walls and pollen grain. In the locular fluid, however, a higher soluble sugar concentration was detected under high temperature regimes throughout anther development.

Total protein content

PAN 3349 exhibited sensitivity to both high and low temperature treatments. The protein content dropped after treatments, but this reduction was more evident under high temperature treatment than low temperature treatment. PAN 3211, PAN 3377 and PAN 3232 had a drastic reduction of protein content under high temperature treatment, but at the same time they exhibited an accumulation of protein content after

low temperature treatment. Therefore, they seem to be sensitive to high temperatures but tolerant to low temperatures in terms of protein content accumulation.

Under high temperature treatment, SST 124 had a slight reduction of protein content but had high protein content after low temperature treatment. Tugela Dn performed well under both high and low temperature treatments. Its protein content increased, particularly after low temperature treatment. Another entry that performed well under both temperature treatments was PAN 3235, as its protein content increased.

Siminovitch and Briggs (1949) observed increased insoluble protein concentration during cold hardening of black locust (*Robinia psuedoacacia*). They found that soluble protein in the black locust bark increased in the fall and declined in the spring, and that these changes closely paralleled changes in tissue freezing tolerance. In the present study, the material tested exhibited either a reduction or an increase of protein content. This is in agreement with many other reports, because in all studies conducted, exposure to low temperature caused a change in the protein synthesis pattern when compared to the pattern of synthesis at warm temperatures. The changes included the appearance of new polypeptides bands and either diminished or disappearance of a few bands. But Cloutir (1983) observed quantitative changes in protein but did not detect the appearance of any new polypeptide resulting from cold acclimation of wheat.

Pulse labelling studies of cold tolerant and cold sensitive wheat failed to measure changes in the chloroplast or membrane protein (Rochat and Therrien, 1975) but during cold acclimation cold tolerant Kharkov wheat synthesized two soluble proteins that cold sensitive Selkirk wheat did not synthesize. According to Levitt (1980) accumulation of soluble proteins in cold acclimation tissues, while not universal, is generally accepted as an important step in the development of freezing tolerance.

Protein synthesis was studied by Perras and Sarhan (1989) in leaves, coleoptiles and roots during cold hardening of freezing tolerant winter wheat and freezing sensitive spring wheat. Results showed that cold hardening induces important changes in soluble protein patterns, depending upon the tissue and the variety's freezing tolerance. Among proteins induced or increased in hardened tissues, some were

expressed at higher level in freezing tolerant varieties than sensitive ones, indicating a correlation between the synthesis and accumulation of these proteins and the degree of freezing tolerance. These proteins suggested that freezing tolerance proteins might have an important role in the cellular adaptation to freezing.

Protein synthesis in general, is a very thermosensitive metabolic process and change in temperature can affect both the amount of and polypeptides produced. If the growth temperature of the plant is increased beyond a particular point, a sudden decline in the ability to synthesize proteins occurs concomitantly when seedling growth ceases. Viswanathan and Khanna-Chopra (2001) reported an increase in protein synthesis when the temperature was increased from 15 to 25⁰C, but significantly decreased by 32% at 35⁰C. Results of this study indicated an increase in protein content in all entries except PAN 3349, PAN 3211, PAN 3377, PAN 3232 and SST 124. They had the highest protein content under control temperatures but on exposure to high temperatures their protein content declined.

Valluri *et al.* (1988) studied drought related protein synthesis by evaluating the uptake and incorporation of amino acids, determined by scintillation counts. They showed that the efficiency of protein synthesis decreased with increasing concentration of mannitol. The effect of temperature on protein synthesis of cotton leaf tissue was compared for different heat treatments. Results showed that protein synthesis increased from control treatment to moderate stress and then decreased during a more severe stress. They found that there was a significant difference between the moderate stress treatment (37⁰C) and the rest of the treatments. This was due to the fact that the heat shock proteins (HSP) were also being synthesized, but at 40⁰C the normal protein synthesis declined. The results of this study also showed an increase in protein content of most entries from control treatment to a high temperature treatment (35⁰C).

The data from this study indicates that there is a change of biochemical composition that occurs under stress conditions. Whether they can be used as a measure of temperature stress tolerance is questionable. The aim of this study was to determine the effect of temperature stress on carbohydrates and proteins however a most of the cultivars indicated an increase in their carbohydrate and protein concentraion, which according to the literature is as indication of tolerance.

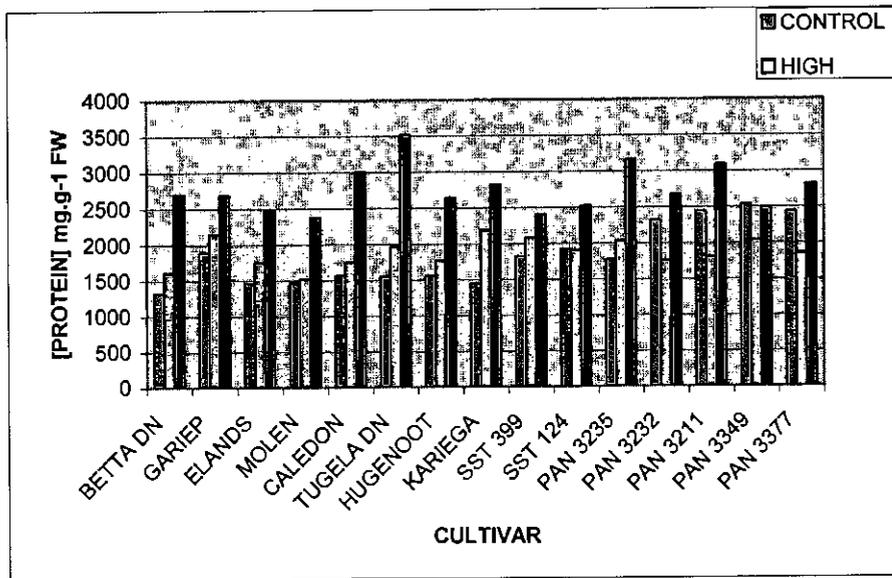


Fig. 4.1 The effect of temperature stress on leaf protein content in wheat seedlings

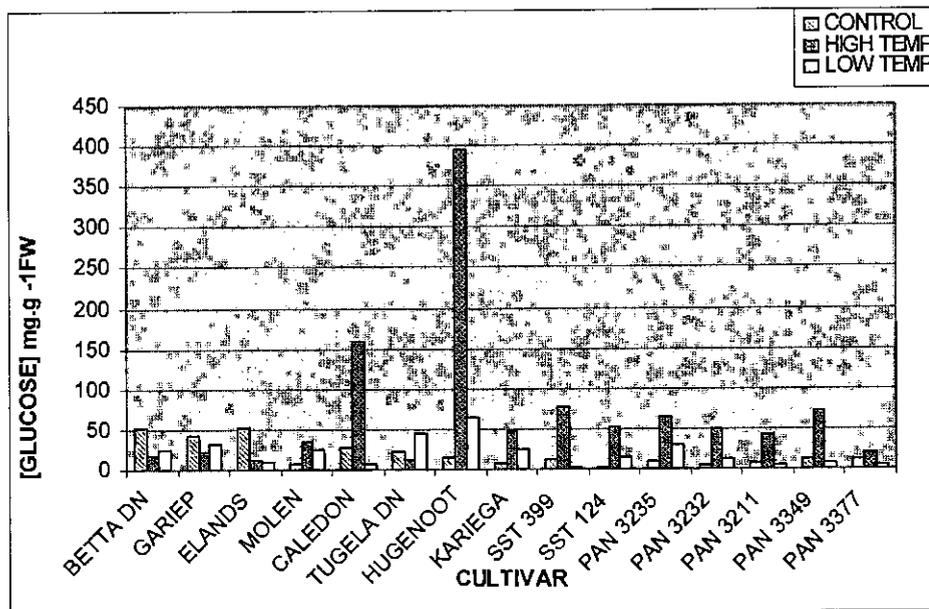


Fig. 4.2 Temperature stress effect on glucose level

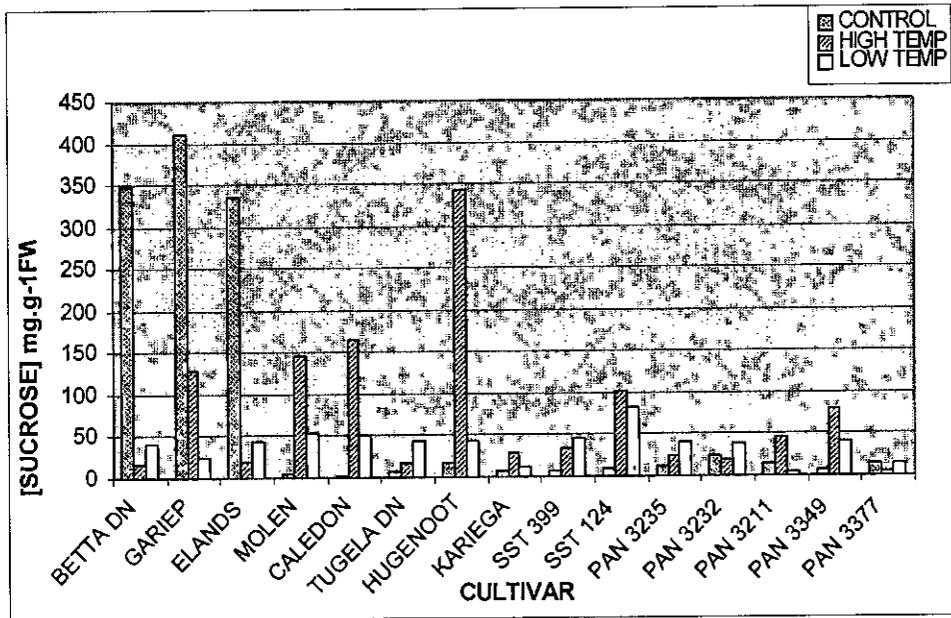


Fig. 4.3 Temperature effect on sucrose level

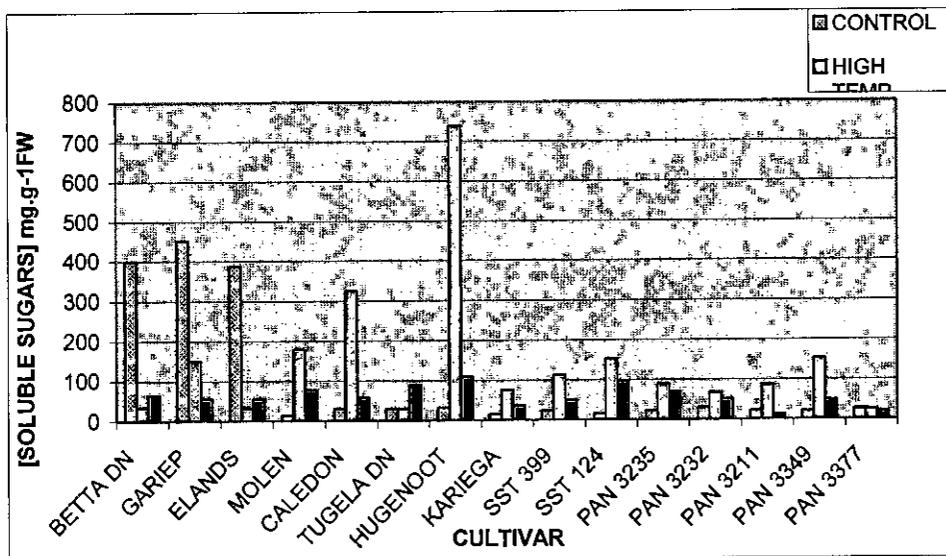


Fig. 4.4. Temperature effect on total carbohydrate level

CHAPTER 5

Plant viability as a function of temperature stress

5.1 Introduction

Extreme temperatures often limit plant productivity through damage to living tissues. It is not only temperature but also the rate of temperature change (Steffen *et al.*, 1989) and the duration of exposure (Rajashekar *et al.*, 1983) that determine the amount of injury. Furthermore, plant susceptibility to such damage is genetically determined and is a function of precondition to treatment and growth stage as well as physiological state. To quantify the plant injury response and to be able to express differences between plant material and treatments, much research has been devoted to finding absolute measures of plant injury resulting from temperature stress (Levitt, 1980). The most common methods of assessing tissue viability following stress are re-growth (Ishikawa *et al.*, 1995), triphenyl tetrazolium chloride reduction (TTC) (Steponkus and Lamphearl, 1967; Towill and Mazur, 1975; De Ronde *et al.*, 1995; Vratsanos and Rossouw, 1991), vital staining (Sugawara and Sakai, 1978; Chen *et al.*, 1982), plasmolysis (Palta *et al.*, 1977), leakage of ions (Sukumaran *et al.*, 1972) or measurement to ultra violet absorbing compounds (Wiest *et al.*, 1976) and proline content change (ref).

Proline accumulation in plants under stress conditions shows adaptation and association with stress adaptation. It is thought to play a protective role in plants subjected to several kinds of stress. Accumulation of free proline may also contribute to scavenging of active oxygen species that accumulate during water deficit conditions, by enhancing photochemical electron transport activities (Alia *et al.*, 1991). Considerable amounts of free proline accumulate in leaves and shoots during cold hardening. Proline accumulation is positively correlated to genotype-specific frost tolerance (Dorffling *et al.*, 1990). Measuring proline content after temperature stress can therefore provide vital information about potential temperature tolerance of cultivars.

One of the earliest and most universal measures of plant temperature injury has been electrolyte leakage. Increased loss of electrolytes, presumably diffusible components of cell sap, has been documented across an array of tissues. Electrolyte leakage of sugars, amino acids, organic acids, proteins and other solutes, characterizes cellular injury due to high temperature, chilling or freezing stress. The consensus is that electrolyte leakage reflects damage to cellular membranes. Membrane composition and structure is of primary importance for functioning of plant cells during high temperature stress. Membrane function is as important for such cell processes as photosynthesis and respiration. Disruption and damage to membranes would detrimentally alter their permeability and result in loss of solutes (Steponkus, 1984).

The ability of viable cells to reduce various tetrazolium salts, has been reported for a variety of organisms (Vratsanos and Rossouw, 1991). TTC reduction occurs in the mitochondria by the tetrazolium salt accepting electrons from the electron transport chain via the dehydrogenase pathway (Nachlas et al., 1960). Plant mitochondria contain a branched mitochondrial electron transport chain where electrons reduce oxygen in a reaction catalysed by cytochrome oxidase. Cytochrome oxidase in a high enough concentration is not rate limiting. The limiting enzyme in the sequence of electron transport in this system is known to be succinic dehydrogenase. Succinic dehydrogenase provides plenty of electrons to the electron transport chain and its activity indicates the metabolic state of the organ (Aithol and Ramasarma, 1969). Plants with tolerance to environmental stress produce a higher activity of succinic dehydrogenase and have the ability to reduce more of the TTC into formazan (De Ronde *et al.*, 1995; Van Der Merwe *et al.*, 1998).

It has been reported that most important for plant survival, the crown meristem must be able to produce new roots and tillers, so some methods expose just the crowns to freezing temperatures. The crown is the most critical region of winter cereals, since destruction of these tissues results in death of the plant. It can be concluded that winter survival is determined by the ability of the crown tissues to tolerate freezing and subsequently recover from intact apical meristems and lateral promeristems. The lower peripheral crown meristem from which new roots arises, is the most critical tissue of the crown (Gusta *et al.*, 1978; Fowler *et al.*, 1981).

There are many stress factors that influence survival of plants, and the interrelation among these factors can be extremely complex (Olein, 1971). Under natural conditions, a confounding of the effects of these factors, and a lack of opportunity to replicate experiments over time, has severely handicapped plant breeders in their efforts to produce artificial tests to simulate natural factors (Larsson, 1986). The aim of this study was to evaluate the use of 2,3,5-triphenyl tetrazolium chloride reduction, cell membrane stability and proline content as a measurement of high temperature tolerance in wheat seedlings and crown survival and proline content as the measure of freezing tolerance.

5.2 Material and Methods

Growth conditions

The experiment was conducted in a greenhouse at the University of the Free State. The 15 entries (listed in Table 5.1) were grown in pots containing 3 kg of soil; five plants were planted per pot. An optimum temperature (25⁰C) was maintained until the plants reached four to five leaf stages.

5.2.1 Proline extraction

The method of Bates et al. (1973) was used. Fresh leaf samples (0.1g) were crushed in liquid nitrogen before adding 10 ml 3% sulphosalicylic acid. The supernatant was collected and vacuum filtrated through a Buchner funnel. Two ml acid ninhydrin and 2 ml acetic acid were combined with 2 ml of the filtrate. Samples were incubated for one hour in a boiling waterbath, and thereafter the reaction was terminated on ice. Free proline was extracted by the addition of 4 ml toluene. The reaction mixture was mixed vigorously with a vortex for approximately 15 seconds and allowed to warm at room temperature. Absorbance of the chromophore containing toluene phase was read at 520 nm. The concentration of free proline was calculated from a standard curve using the following equation: $[(\mu\text{g proline/ml} \times \text{ml toluene})/115.5 \mu\text{g}/\mu\text{mole}]/[(\text{g sample})/5] = \mu\text{moles proline/g dry weight}$.

Preparation of the standard curve

A dilution of known proline concentrations was made with distilled water. The reaction was then followed as in the proline extraction procedure, but using raw proline instead of the leaf material. The absorbance was measured as in the extraction procedure and then a scatter plot graph was drawn with the Sigma Plot programme. The concentration of proline was then extrapolated from the graph using the equation of the straight-line.

5.2.2 Cell membrane stability test

Leaf samples of about 10 mm in diameter were taken from fully expanded young leaves with the tips cut off. Five samples were taken from two or three leaves per genotype. Samples were kept in an airtight test tube, wetted with a drop of water and transferred to the laboratory within an hour.

The method of Sullivan (1972) was followed. Samples were washed with three changes of distilled water to remove surface-adhered electrolytes. After the final rinsing, 1 ml of distilled water was added to all the vials (control and treatment) and covered with parafilm. Heat treatment vials were incubated in a water bath at 50°C for an hour, while the control vials were maintained in a water bath at 25°C during the same period. After the treatment period, 9 ml of distilled water was added to both heat-treated and control vials. The vials were kept at 10°C for 24 h, to allow diffusion of electrolytes from the plant material. Vials were then brought to 25°C and shaken, to mix the contents. An initial conductance of vial contents was determined with an electrical conductivity meter. After the reading, the vials were placed in an autoclave held at a pressure of 0.10 MPa for 15 min to kill the plant tissue and release all the electrolytes. The vials were cooled to 25°C, contents were mixed and a final conductance measurement made. Membrane thermostability was expressed as relative injury (RI) from the following calculation:

$$RI (\%) = 1 - \{ [1 - (T_1/T_2)] / [1 - (C_1/C_2)] \} \times 100$$

Where T and C refer to conductance values for treatment and control vials respectively and subscripts 1 and 2 refer to initial and final conductance readings, respectively.

5.2.3 Triphenyl tetrazolium chloride (TTC) test

Leaves were collected from the top half of the plant excluding the growth tips. Thirty leaf discs of 7 mm in diameter were used for the control treatment in a water bath at 29⁰C and incubated in 3 ml of 0.2 M sodium phosphate buffer (McKenzie, 1996). Leaf discs in the acclimation treatment were submitted to a moderate stress of 40⁰C for three hours, followed by a lethal heat of 50⁰C. Leaf discs exposed to lethal temperatures, were sampled over a period of 180 minutes, at intervals of 30 min. Leaf discs were submerged in 3 ml of 0.8% TTC solution. The samples were vacuum filtrated to ensure that the TTC solution penetrated the tissue (Chen *et al.*, 1982). Discs were incubated overnight to allow the reduction of TTC to formazan. After 18 hours, discs were washed with distilled water and transferred to 3 ml of 95 % ethanol. The samples were boiled, causing the cells to burst and spill the cell contents and then dried. Samples were re-suspended in 3 ml of 95% ethanol. Five replicates of each time interval were measured. The formazan concentration was estimated spectrophotometrically at 485 nm (De Ronde *et al.*, 1995).

5.2.4 Crown survival test

5.2.4.1 Wooden box screening

Wooden boxes of 130 cm length, 65 cm width and 15 cm depth made of 2.5 cm thick planks were kept on benches in a rain-protected glasshouse. The box was lined with polythene sheets and filled with soil to a 12 cm depth leaving 3 cm space at the top for watering. The polythene lining along the sides and bottom of the box ensured even distribution of water in the boxes. Equidistant holes were made in straight rows to plant each genotype. Boxes were watered as necessary and chemicult liquid fertilizer was administered three times a week. Low temperature treatment started when the plants reached three to four leaf stages. The box was transferred to a cold room at 2⁰C for the acclimation period of three weeks. The temperature was reduced 1⁰C an hour

until -7°C and kept there for an hour. The temperature was then increased with 1°C an hour until 2°C and then the box was transferred back to the glasshouse and survival and recovery percentages were counted over two weeks.

5.2.4.2 Crown survival test

Plants were transferred to a cold room (2°C) for acclimation and kept there for three weeks, receiving water as necessary to avoid desiccation. Chemicult liquid fertilizer was administered twice a week. Plants were dug out and the roots were shaken free of loose soil. Crowns were prepared for freezing by removing all plant parts more than 4 cm above and 1 cm below the crown. Freezing treatment was conducted in a way to avoid supercooling. Prepared crowns were washed and placed in plastic freezer bags. The temperature was reduced 1°C an hour until -7°C and kept there for an hour. The temperature was then increased a 1°C an hour. Crowns were re-painted in pots and left to grow in the glasshouse under optimum growth temperature. After three weeks the leaflength measurements in centimeters were done three times at 7 days intervals.

Table 5.1 Cultivars screened for temperature tolerance

Entry	Cultivar	Growth habit
1	Betta Dn	Winter
2	Caledon	Winter
3	Elands	Winter
4	Gariep	Facultative
5	Hugenoot	Winter
6	Kariega	Spring
7	Molen	Winter
8	PAN 3211	Facultative
9	PAN 3232	Winter
10	PAN 3235	Winter
11	PAN 3349	Winter
12	PAN 3377	Winter
13	SST 399	Winter
14	SST 124	Facultative
15	Tugela Dn	Winter

5.2.5 Statistical analysis

Data of cell membrane stability, proline and crown survival test was subjected to analysis of variance using the Agrobase (2000) statistical software programme.

5.3 Results

The mean squares of screening methods were all highly significant for both high and low temperature treatment (Table 5.2).

Table 5.2 Mean squares for the screening methods studied at three temperature treatments

	Control		Low		High	
	Block	Entry	Block	Entry	Block	Entry
CMS (%)					1.904	120.567**
Proline concentration	0.000	2.955**	0.000	0.492**	0.000	0.063**
Crown survival test	0.528	65.448**	12.230	111.942**		

** $p \leq 0.01$

CMS = cell membrane stability

Table 5.3 Mean values of proline ($\mu\text{moles proline/g dry weight}$) for three temperature treatments

Entry	Temperature treatments		
	Control	Low	High
Betta Dn	1.85	0.38	0.53
Gariep	0.23	0.09	0.56
Elands	0.22	0.18	0.33
Molen	0.24	0.3	0.78
Caledon	2.99	0.27	0.62
Tugela Dn	0.74	0.15	0.49
Hugenoot	0.17	0.18	0.15
Kariega	0.26	0.32	1.32
SST 399	0.04	0.09	0.36
ST 124	2.9	0.03	0.43
PAN 3211	0.92	0.26	0.13
PAN 3232	0.27	0.6	0.43
PAN 3235	0.29	0.14	0.36
PAN 3349	0.19	0.38	0.31
PAN 3377	0.21	0.26	1.6
Mean	0.768	0.561	0.242
LSD (0.05)	0.0243	0.0125	0.0105
CV (%)	2.95	2.07	4.05

LSD = least significant differences, CV = coefficient of variance

5.3.1 Proline test

Control temperature treatment

Caledon, SST 124 and Betta Dn ranked first, second and third respectively. They had significantly higher proline content than all other entries. PAN 3349 and Hugenoet ranked thirteenth and fourteenth respectively, exhibiting low proline content. SST 399 had the lowest proline content (Table 5.3) (Fig. 5.1)

High temperature treatment

PAN 3232, Betta Dn and PAN 3349 ranked first, second and third respectively, indicating high proline content. Gariiep, SST 399 and SST 124 had low proline content and consequently ranked thirteenth, fourteenth and fifteenth respectively (Table 5.3) (Fig. 5.1)

Low temperature treatment

PAN 3377 and Kariega had significantly higher proline content than the other entries and they ranked first and second respectively. Molen and Caledon ranked third and fourth respectively. PAN 3349, Hugenoet and PAN 3211 ranked thirteenth, fourteenth and fifteenth respectively; they had lower proline content than all other entries (Table 5.3) (Fig. 5.1)

5.3.1.1 Discussion

PAN 3211, PAN 3349, PAN 3377, SST 399, Kariega and Molen showed an increase of proline content under both high and low temperature treatment, so it can be concluded that they are low and high temperature tolerant. Hugenoet only exhibited an increase of proline content under high temperatures, whereas it declined under low temperatures. It can therefore be concluded that it is low temperature sensitive.

Elands, Gariiep and PAN 3235 failed to increase their proline content under the high temperature treatment, indicating sensitivity to high temperatures. Entries like Betta Dn, Caledon, Tugela Dn, PAN 3211 and SST 124 did not have an increase of proline content under either temperature treatments, instead, their proline content declined, so it can therefore be concluded that they are sensitive to temperature extremes.

The accumulation of proline in higher plants under stress is associated with stress adaptation (Paleg and Aspinall, 1981; Lalk and Dorffling, 1985). Plants synthesize proline in response to osmotic stress. In 1996, Van Heerden and De Villers observed a higher proline accumulation during drought stress in drought tolerant spring wheat cultivars than in more sensitive cultivars. These findings lead them to conclude that cultivars that produced high proline content under drought and control conditions could be drought tolerant. In the present study, cultivars responded differently to temperature extremes. Some cultivars exhibited an increase of proline content under both high and low temperature treatments. Other cultivars showed an increase for the high temperature treatment, but not for low temperature treatment. Similarly some cultivars showed an increase of proline content only under the low temperature treatment, but not for high temperature treatment. Chang and Lee (1999) reported an increase of free proline accumulation in marine red macroalge after exposure to high temperature. Free proline accumulation was also reported by De Ronde *et al.* (2000) in cotton under high temperature stress. They suggested that an increase in proline is the result of heat tolerance. It was previously found that proline levels increased in leaves as a result of heat stress but a study on desert plants could not link the concentration increase to the leaf temperature (Laurie *et al.*, 1994).

Proline is also known to accumulate in plants during low temperature stress. Several researchers have found that applied proline acts as a cryoprotectant (Withers and King, 1979; Bornman and Jansson, 1980; Dobslaw and Bielka, 1988) and that treatments which increased endogenous level of proline also increased the resistance to chilling and freezing stress (Bornman and Jansson, 1980; Dobslaw and Bielka (1988) found that proline levels are closely related to freezing tolerance in winter barley and are used as chemical markers in breeding programmes for this cereal crop. In 1984, Kaldy and Freyman were the first who proposed the use of free proline increase during hardening for the detection of cold hardiness in winter wheat breeding programmes. Free proline was also reported by Yelenosky (1979) in citrus. Higher proline was significantly correlated with the ability to test plants for surviving freeze damage. In contrast to these results, Gusta *et al.* (1982) found no correlation between proline levels and field survival in 14 winter wheat varieties.

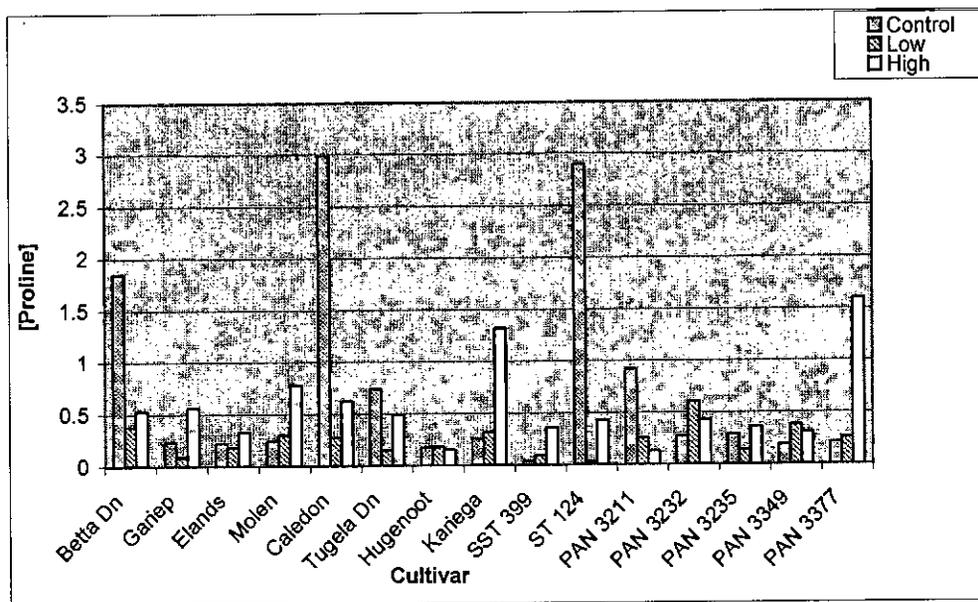


Fig.5.1 The effect of temperature stress on proline concentration

5.3.2 Cell membrane stability results

Table 5.4 Mean values for cell membrane stability for the tested material

Entries	Injury (%)
Betta Dn	95.49
Gariep	92.16
Elands	95.11
Molen	95.07
Caledon	83.54
Tugela Dn	94.38
Hugenoot	93.07
Kariega	95.58
SST 124	93.41
SST 399	93.84
PAN 3235	93.55
PAN 3232	93.13
PAN 3211	34.37
PAN 3349	94.31
PAN 3377	85.11
Mean	92.807
LSD (0.05)	1.99
CV (%)	2.46

LSD = least significant differences, CV = coefficient of variance

Results indicated a highly significant variation between the entries. Kariega, Betta DN and Elands ranked first, second and third, respectively. They had high cell membrane injury. Gariep, PAN 3377 and PAN 3211 had less injury so they ranked thirteenth, fourteenth and fifteenth respectively (Table 5.4) (Fig. 5.2).

5.3.2.1 Discussion

The use of the cell membrane stability to evaluate tolerance under stress conditions, and its significant contribution as a screening method, was reported in different crops including wheat (Blum and Ebercon, 1981; Mark *et al.*, 1991). They reported that genotypes that exhibited a low percentage injury could be considered as tolerant. The rate of injury to cell membranes is commonly used as a measure of tolerance to plant

stresses such as freezing and heat. Bewley (1979) also reported the critical role of cell membrane stability under conditions of stress as a major component of tolerance. Results of this study showed that cultivars differed in injury level, some had high percentage of injury and those can be classified as heat sensitive, whereas some had low injury percentage and can be classed as heat tolerant. Shanahan *et al.* (1990) obtained a significant increase in yield of spring wheat in hot locations by selection of membrane-thermostable lines, as determined by measurements of flag leaves at anthesis. By applying the cell membrane injury test on winter wheat seedlings, Saadalla *et al.* (1990) found a high correlation in membrane themostability between seedlings and flag leaves at anthesis of genotypes grown under controlled environmental conditions. Reynolds *et al.* (1994) reported that variation in membrane-thermostability of both field acclimated flag leaves and seedlings grown in controlled conditions, was associated with heat tolerance in warm wheat-growing regions.

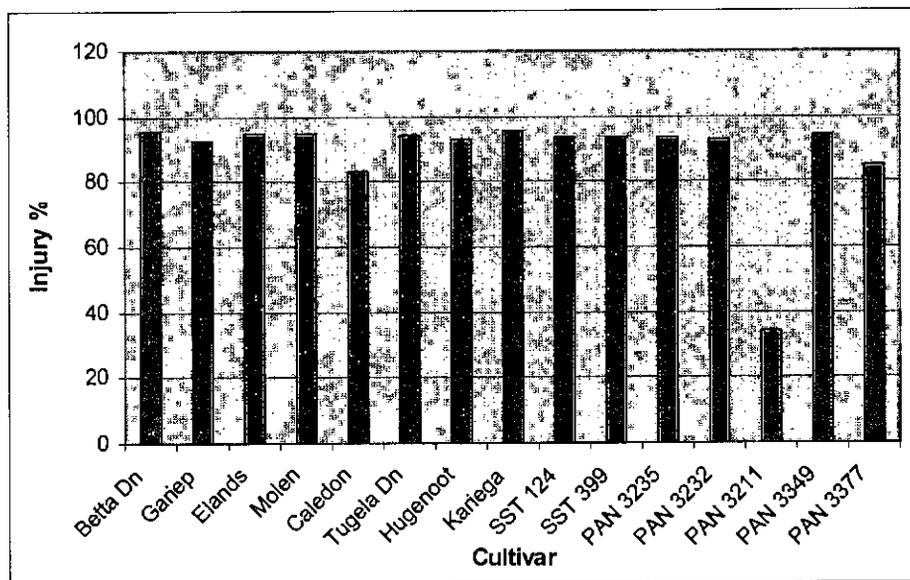


Fig. 5.2 Cell membrane high temperature stress injury

5.3.3 TTC results

Table 5.5 Mean values for TTC at two temperature levels

Cultivar	Control temperature treatment						High temperature treatment					
	Time Interval						Time Interval					
	A	B	C	D	E	F	A	B	C	D	E	F
Betta Dn	0.04677	0.04913	0.0059	-0.0712	-0.1421	0.01513	0.0237	0.0359	0.0388	0.0299	0.0288	0.0291
Gariép	0.0155	-0.0018	-0.2800	0.0665	-0.0488	0.0081	0.0211	0.4777	-0.0036	0.0263	-0.01119	0.0429
Elands	0.0163	0.0273	-0.0887	-0.0585	0.0103	0.0074	0.0183	-0.0085	0.0028	-0.0169	0.0171	-0.0046
Molen	-0.2535	-0.25	-0.05416	0.0068	-0.173	-0.0264	0.0221	-0.1772	0.0146	0.0057	-0.0009	0.0167
Caledon	0.0203	0.1824	-0.0159	-0.1944	0.0554	0.0248	0.0111	-0.128	-0.0715	0.0106	0.0016	0.0667
Tugela Dn	-0.2392	0.0426	-0.0064	-0.0091	0.2731	-0.1557	0.0207	-0.0003	0.0186	0.0329	-0.0134	-0.0122
Hugenoot	-0.0296	0.0126	-0.2038	0.0197	0.0090	0.0108	0.0877	0.0128	0.0199	0.026	-0.2775	0.0057
Kariega	0.0052	0.0282	0.0091	-0.2874	0.0097	-0.0441	-0.2855	0.0310	0.0137	-0.1749	0.0061	0.0051
SST 124	0.0115	0.0258	-0.2037	0.0115	0.0257	0.0085	0.0296	0.0167	-0.2368	0.0068	0.0143	0.0778
SST 399	0.0134	0.0205	-0.0118	0.0051	-0.0044	0.0165	-0.1089	-0.2622	-0.0375	-0.1173	-0.0456	0.0157
PAN 3211	-0.04235	0.0094	-0.1226	0.0071	-0.1176	0.0168	0.0210	-0.0686	-0.0526	-0.1117	0.0731	-0.2393
PAN 3232	-0.1994	-0.0816	0.0237	-0.0013	0.01834	-0.0057	0.0311	-0.0384	0.0324	-0.1408	-0.1071	0.0180
PAN 3235	-0.1064	-0.1398	-0.2841	-0.0276	-0.0599	-0.0805	-0.0643	-0.2121	-0.1605	0.0137	-0.0112	-0.1417
PAN 3349	-0.0176	-0.0085	-0.0065	-0.0137	-0.0031	-0.0083	-0.0403	-0.0758	-0.0026	-0.0155	0.0025	-0.0104
PAN 3377	-0.0049	-0.0135	-0.0356	-0.0161	-0.0091	-0.0309	-0.0146	-0.0093	-0.0229	-0.0155	0.0299	-0.0096

Absorbance values of the stress treatment showed that Betta Dn (Fig. 5.3), Gariep (Fig. 5.4), Elands (Fig. 5.5) and Molen (Fig. 5.6) performed better over time, since the absorbance values of the control treatment were lower than under stress conditions. This response indicated that these cultivars are heat tolerant. The absorbance values of SST 399 (Fig. 5.12), PAN 3235 (Fig. 5.15), and PAN 3349 (Fig. 5.16) were low under stress conditions for most of the time compared to control conditions. It can therefore be concluded that these cultivars are heat sensitive. Other cultivars indicated a fluctuation of absorbance values over time, an indication of tolerance for only short periods of stress (Table 5.5) (Fig. 5.3 – 5.17).

5.3.3.1 Discussion

The analysis of heat tolerance is based on the overall response of a plant when subjected to high temperatures. A precise evaluation can be difficult to obtain because heat tolerance has a low heritability and repeatability. De Ronde and Van Der Mescht (1997) used TTC reduction as a measure of drought tolerance and heat tolerance in cotton. They postulated that formazan production would be lower in the stress treatment than in control sample in sensitive cultivars. This would be due to the fact that moderate stress would be experienced as severe stress in sensitive cultivars and the plant would not adapt to drought stress and subsequently die. Tolerant cultivars, on the other hand, would have the ability to withstand an otherwise lethal stress when preheated with moderate stress. This would result in higher formazan production in the stressed sample than in the control sample. Van Der Merwe *et al.* (1998) reported that after two hours of prolonged heat stress, formazan production decreased and the absorbance values of the stress treatment were lower than the control treatment for heat sensitive plants in cotton

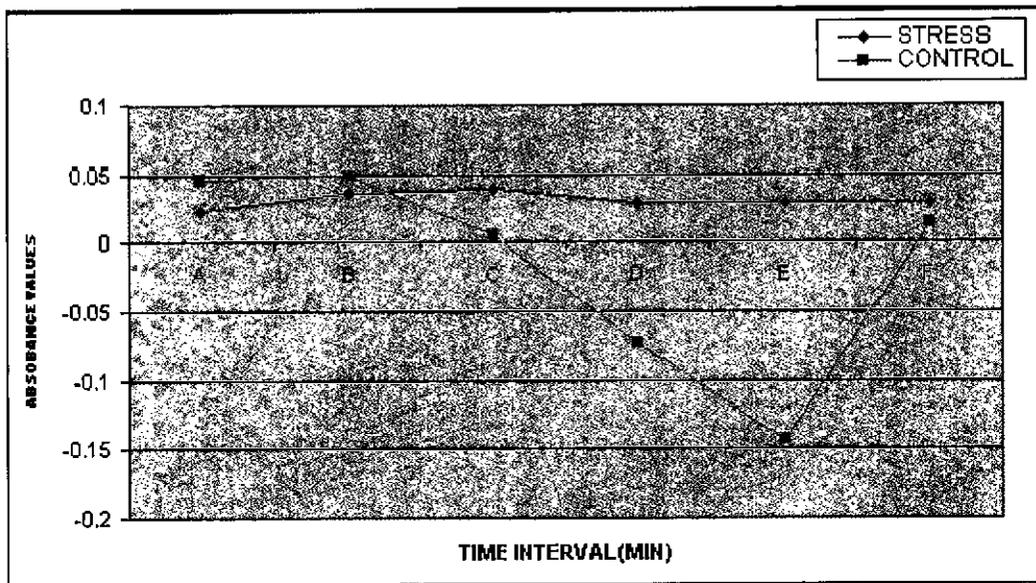


Fig. 5.3 Triphenyltetrazolium chloride assay of Beta Dn subjected to high temperature stress over time

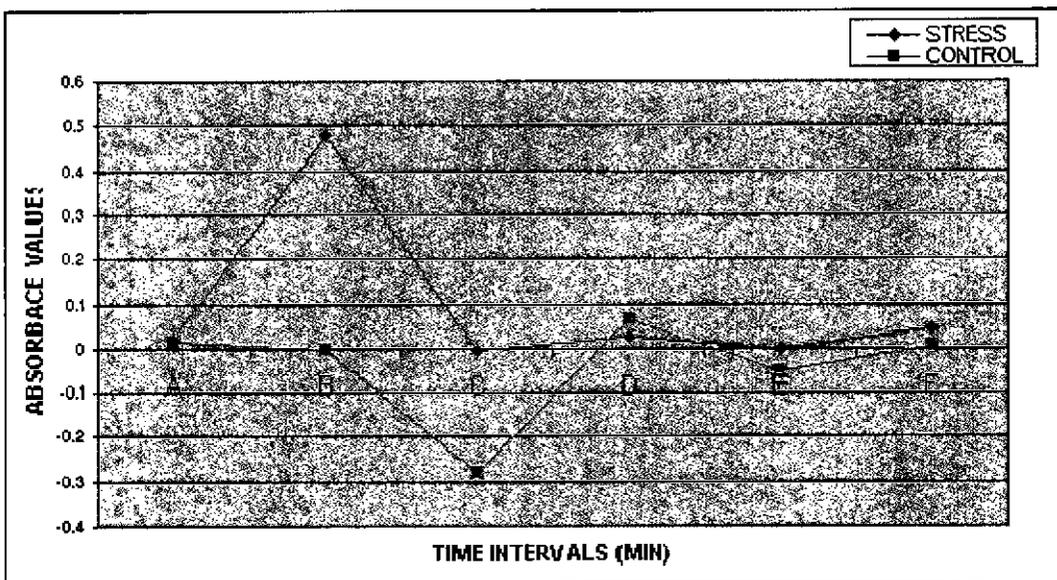


Fig. 5.4 Triphenyltetrazolium chloride assay of Gariep subjected to high temperature stress over time

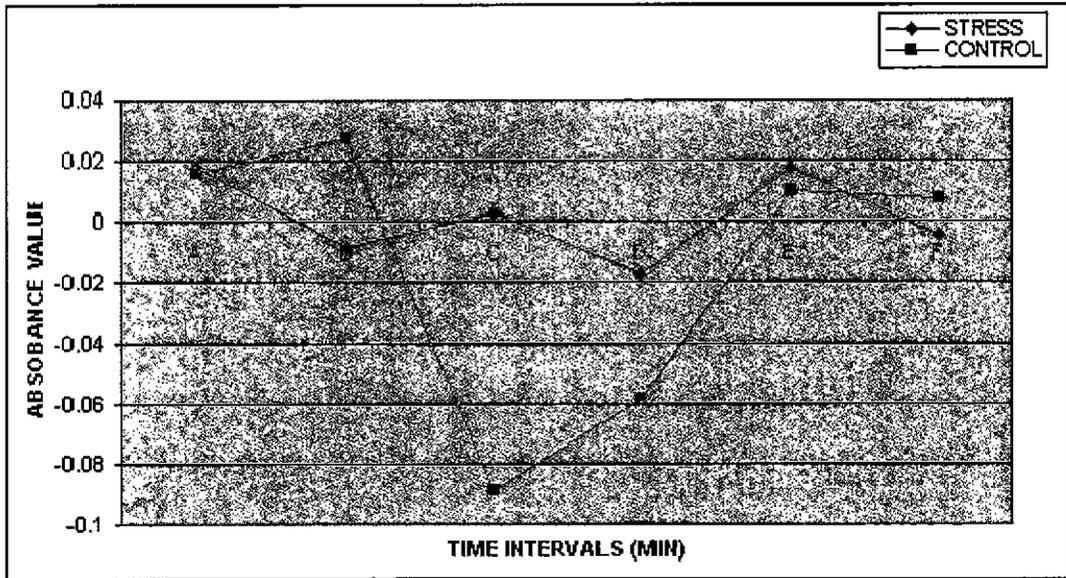


Fig. 5.5 Triphenyltetrazolium chloride assay of Elands subjected to high temperature stress over time

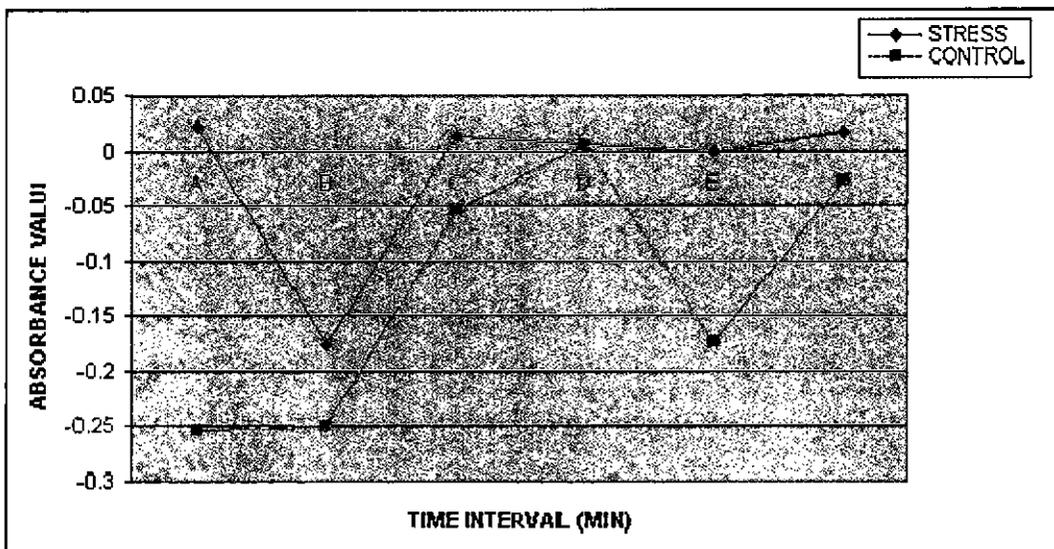


Fig. 5.6 Triphenyltetrazolium chloride assay of Molen subjected to high temperature stress over time

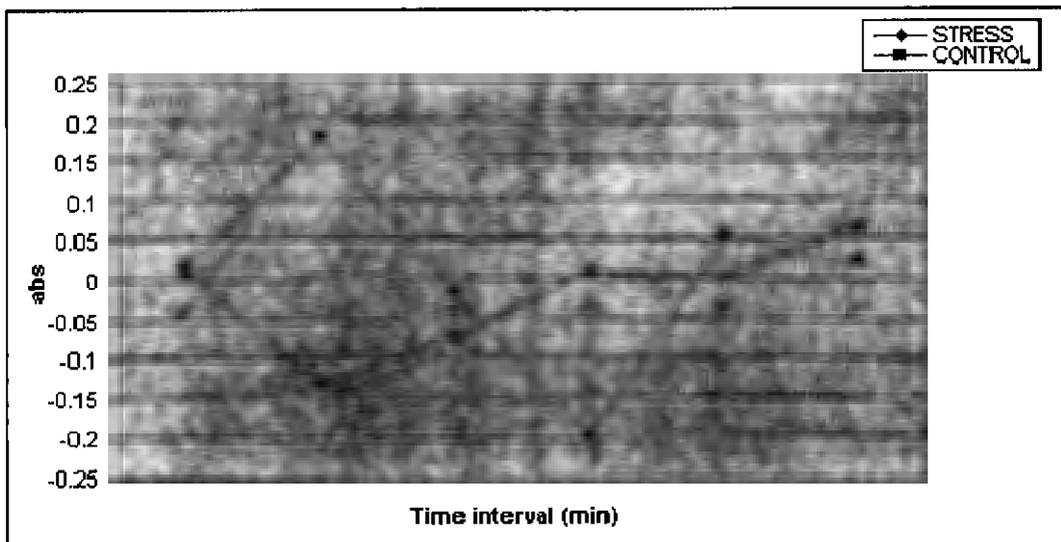


Fig. 5.7 Triphenyltetrazolium chloride assay of Caledon subjected to high temperature stress over time

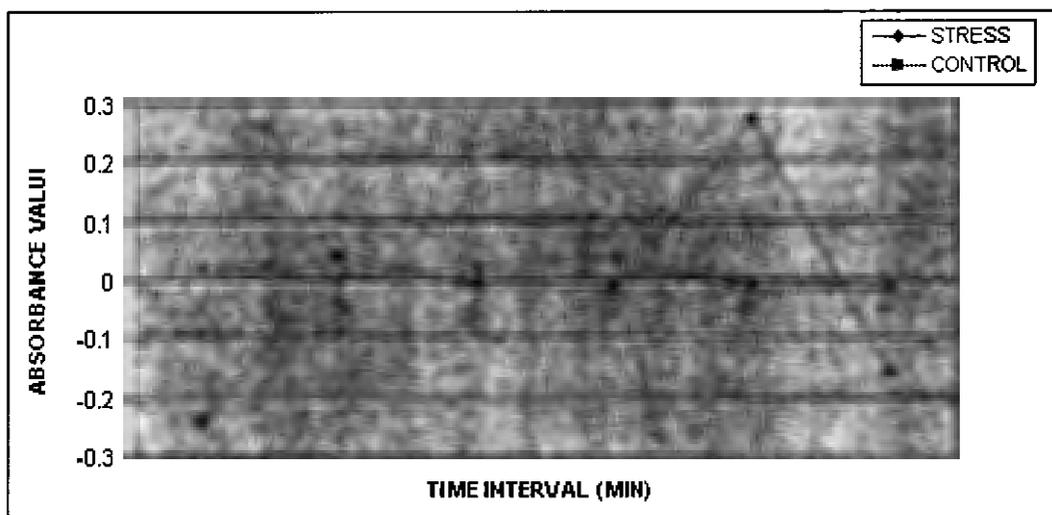


Fig. 5.8 Triphenyltetrazolium chloride assay of Tugela Dn subjected to high temperature stress over time

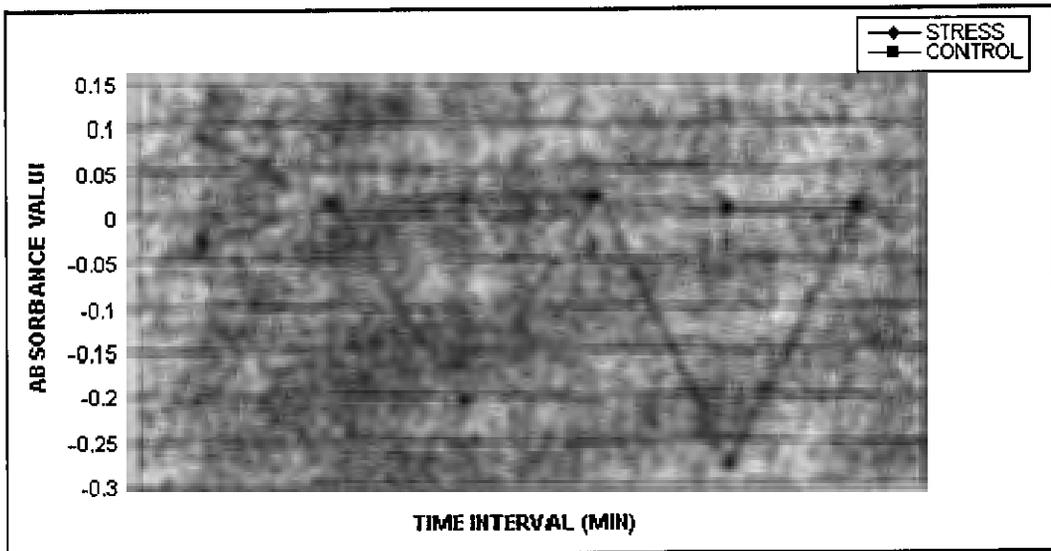


Fig. 5.9 Triphenyltetrazolium chloride assay of Hugonoot subjected to high temperature stress over time

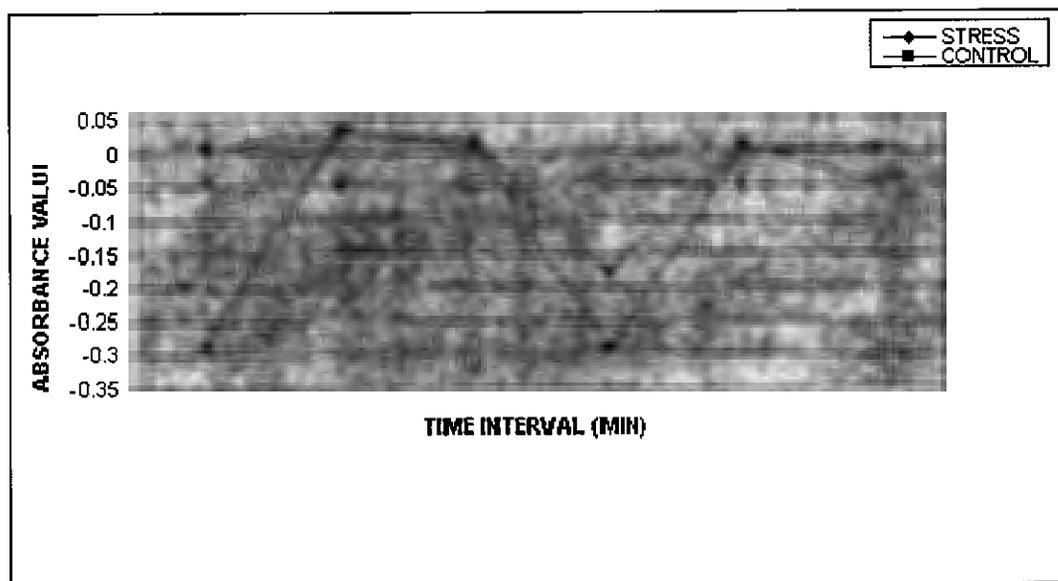


Fig. 5.10 Triphenyltetrazolium chloride assay of Kariaga subjected to high temperature stress over time

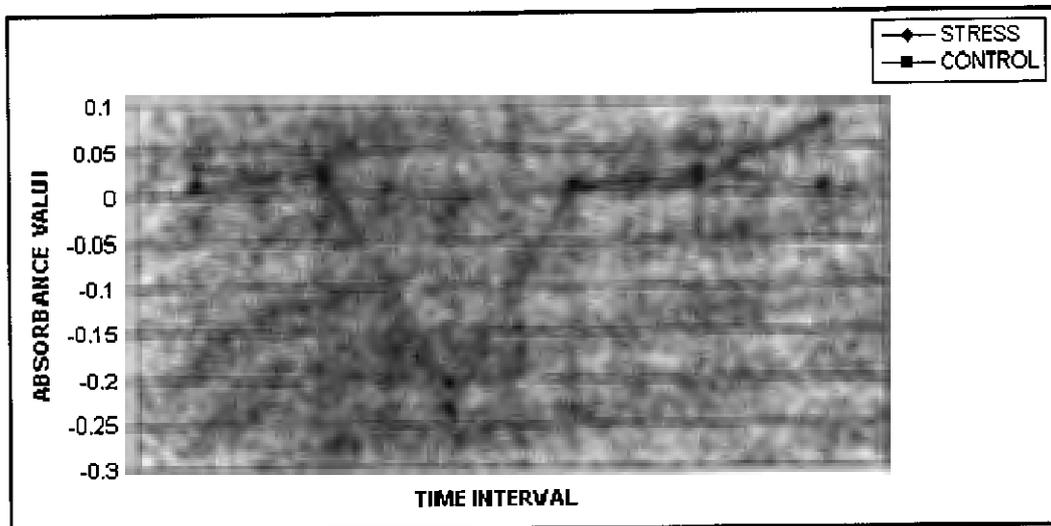


Fig. 5.11 Triphenyltetrazolium chloride assay of SST 124 subjected to high temperature stress over time

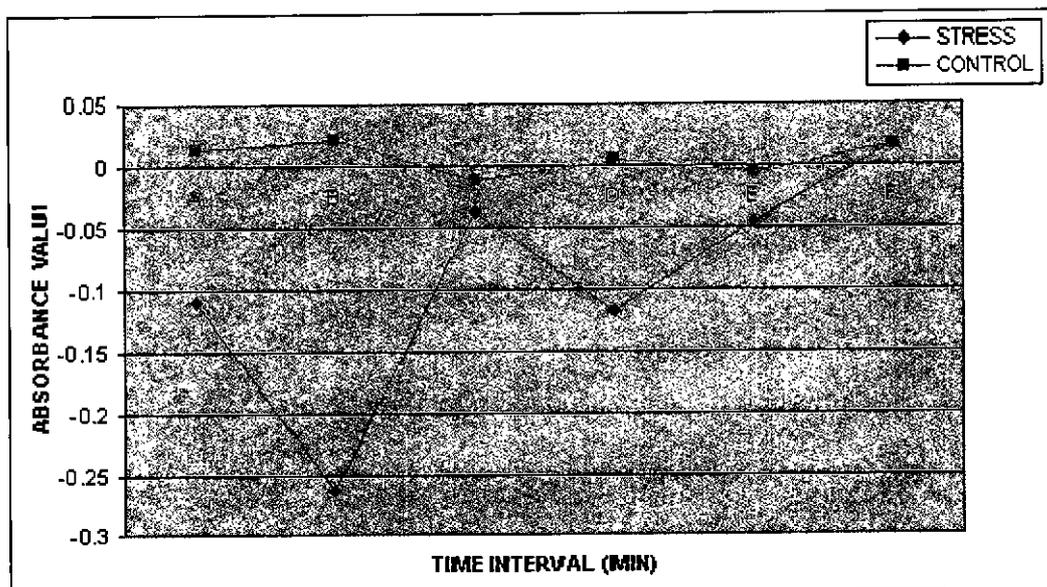


Fig. 5.12 Triphenyltetrazolium chloride assay of SST 399 subjected to high temperature stress over time

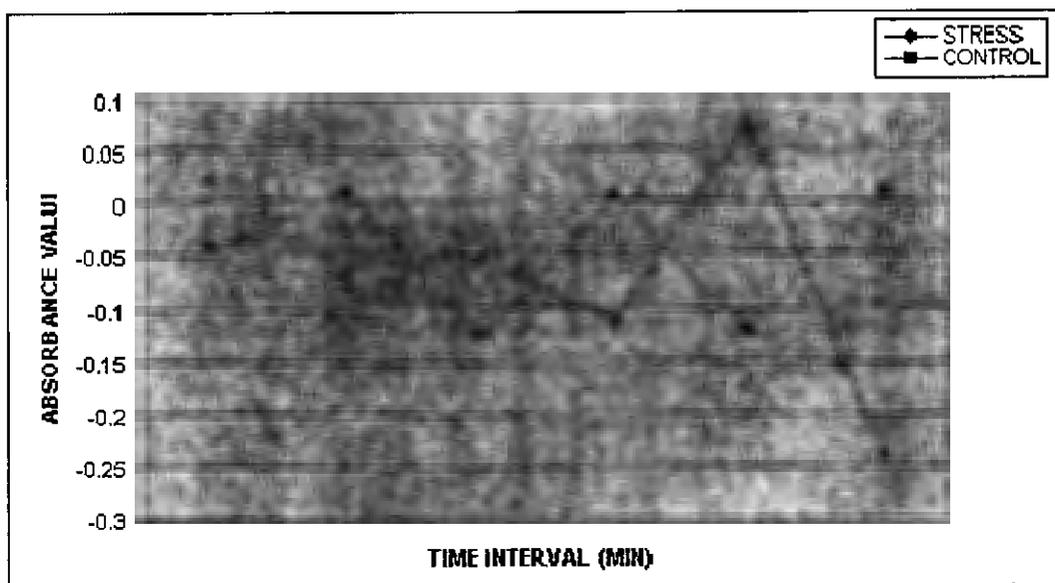


Fig. 5.13 Triphenyltetrazolium chloride assay of Pan 3211 subjected to high temperature stress over time

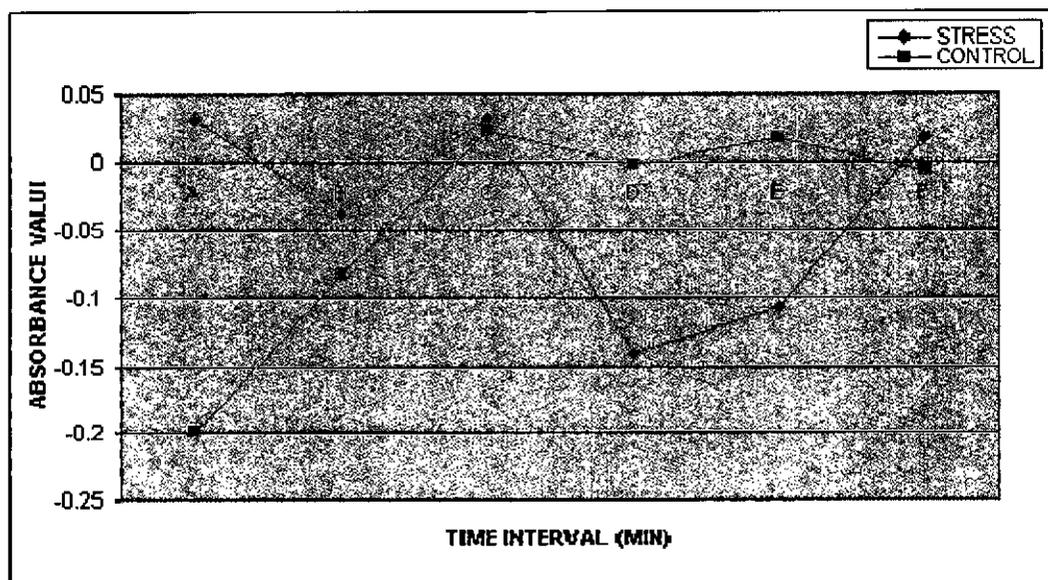


Fig. 5.14 Triphenyltetrazolium chloride assay of Pan 3232 subjected to high temperature stress over time

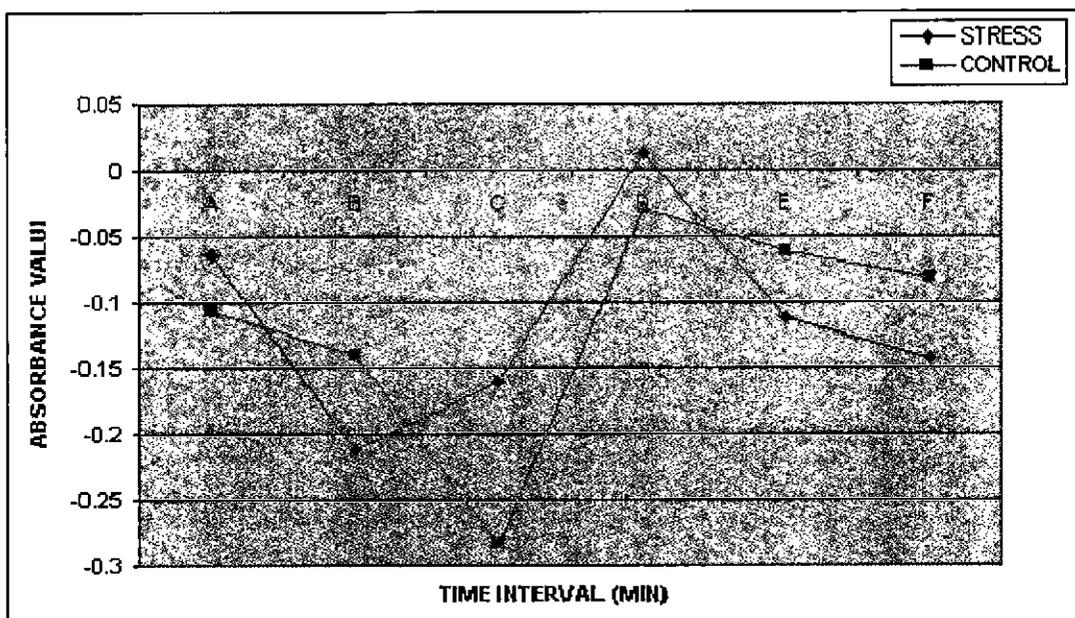


Fig. 5.15 Triphenyltetrazolium chloride assay of Pan 3235 subjected to high temperature stress over time

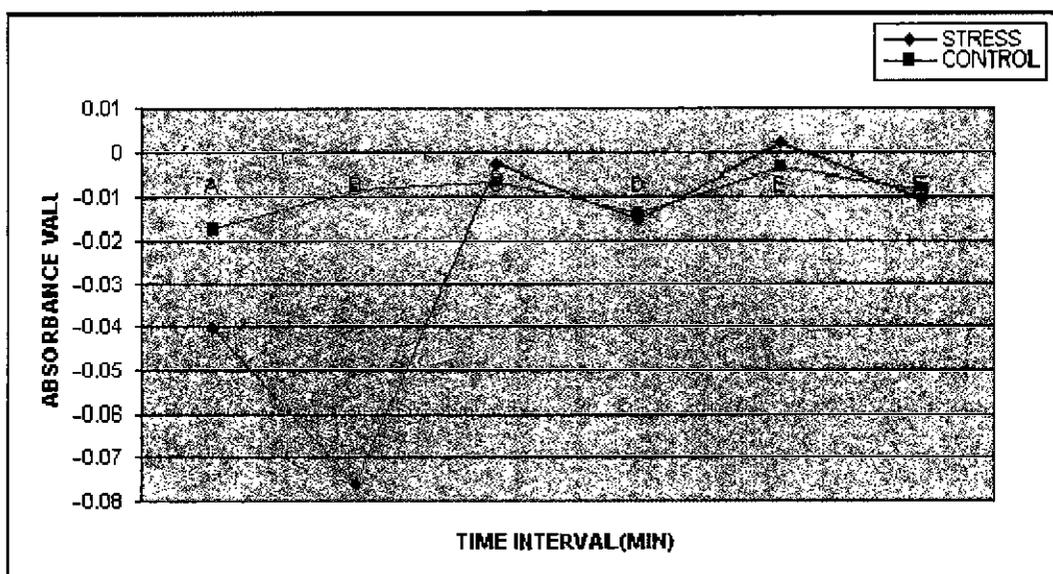


Fig. 5.16 Triphenyltetrazolium chloride assay of Pan 3349 subjected to high temperature stress over time

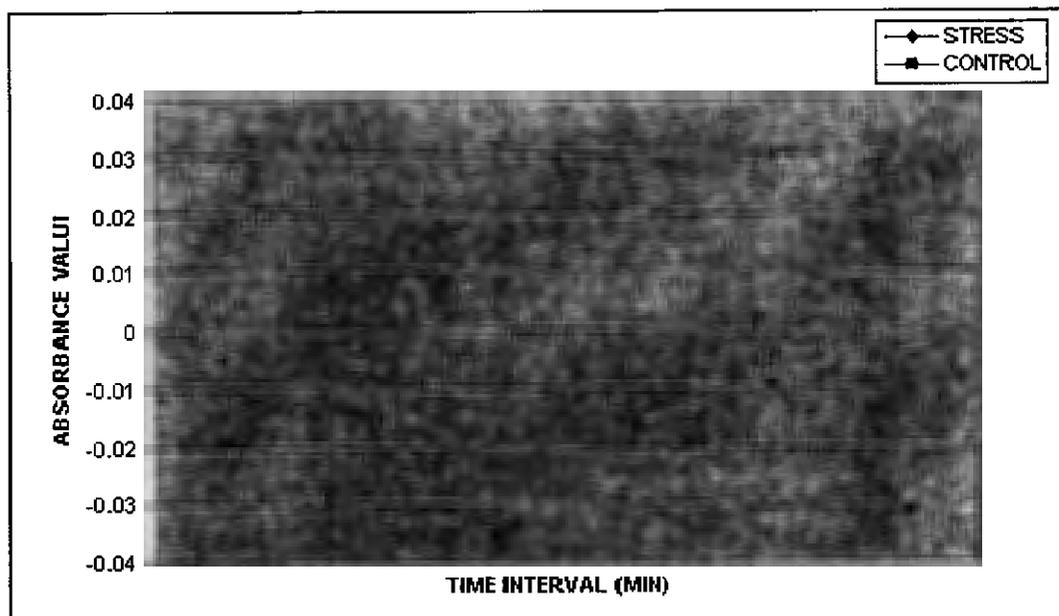


Fig. 5.17 Triphenyltetrazolium chloride assay of Pan 3377 subjected to high temperature stress over time

5.3.4.1 Wooden box test

Cultivars responded differently to freezing stress with the lowest percentage of survival at 10% and the highest survival percentage of 100%. Plants that were so damaged during freezing that they eventually succumbed were eliminated from the surviving class (Table 5.6). (Fig. 5.19).

Table 5.6 Survival and recovery percentage of the tested entries

Entry	0 Day	7 Days	14 Days
Betta Dn	20	20	40
Gariep	20	20	30
Elands	40	40	50
Molen	100	100	100
Caledon	50	50	60
Hugenoot	50	50	50
Kariega	20	20	20
SST 124	30	30	30
SST 399	100	100	100
PAN 3235	50	50	80
PAN 3232	30	30	30
PAN 3211	70	70	80
PAN 3349	20	20	20
PAN 3377	10	10	10

Day 0 (survival %)

Molen and SST 399 had a 100% survival rate. PAN 3211 followed with 70% survival, Caledon, Hugenoot and PAN 3235 had 50% plants still living after the cold stress. The lowest percentage survival was observed in PAN 3377 (10%). Betta Dn and Gariep both had 20% survival.

Day 7 (recovery %)

No changes were observed compared to day 0.

Day 14 (recovery %)

Recovery percentage varied from 10% to 30%, but other cultivars had 0% recovery, they remained the same as in the 0 day results, meaning that they are freezing stress sensitive. PAN 3235 showed a 30% recovery, bringing the total number of plants classed as alive up to 80%. PAN 3232 showed 10% recovery, so the total number of plants alive was 80%. Other cultivars that also showed 10% recovery were Caledon, Elands and Gariiep, bringing the total number of plants alive up to 60%, 50% and 30% respectively. Betta Dn exhibited 20% recovery, bringing the total number of plants alive, up to 40%.

5.3.4.2 Leaf length

There was a highly significant variation for leaf length at both control and low temperature treatments. The leaf length at the control temperature varied from 24.40 cm (Tugela Dn) to 12.0 cm (Kariega) and 28.40 cm (Tugela Dn) to 7.20 cm (PAN 3232) after low temperature treatment.

Control temperature treatment

The leaves of Tugela Dn, ranking first were significantly longer than the rest of the entries. Betta Dn and Caledon ranked second and third respectively. PAN 3232 and Kariega exhibited the weakest leaf re-growth with shortest leaf length in comparison to other entries and they ranked low.

Low temperature treatment

Tugela Dn had significantly longer leaves than other entries. SST 124 and PAN 3211 followed, ranking second and third respectively. Betta Dn and PAN 3377 ranked thirteenth and fourteenth respectively with short leaves. PAN 3232 exhibited the weakest leaf re-growth having the shortest leaves.

5.3.4.3 Discussion

Molen and SST 399 were not affected by low temperature stress. They maintained 100% plant survival after treatment. Entries like PAN 3377, PAN 3349, PAN 3232, SST 124, Betta Dn and Gariép had less than 50% plant survival, indicating low temperature sensitivity. Other entries had survival percentage above 50%. It can therefore be concluded that they are low temperature tolerant.

Leaf length of entries such as Betta Dn, PAN 3235, PAN 3377 and PAN 3232 was shorter at the low temperature treatment, showing weak leaf regrowth and it can be concluded that they are low temperature sensitive. Entries like Tugela Dn and PAN 3211, that exhibited longer leaves, indicating a strong leaf re-growth, can be referred to as low temperature tolerant.

The majority of work reported on artificial tests for frost hardiness in cereals has dealt almost exclusively with plant survival. In this study both plant survival and leaf re-growth as a frost hardiness variable, were evaluated. Marcellos and Burke (1979) demonstrated that leaves of several unhardened spring wheat cultivars have the ability to tolerate temperature as low as -7 to -9°C . Leaves of spring wheat Kite, Manitou and Oxly in the hardened state, tolerated -9°C to -10°C before injury became apparent. Veiz and Sutka (1993) used leaf re-growth to measure cold tolerance of some Chinese Spring detelosomics. After freezing, the leaves were cut off with scissors, a few centimetres above the soil, so that re-growth could be evaluated. After 16 days the plants were rated on a zero to five scale. Those who died were scored zero, while the well developed tillering plants scored five points. In this study, all the entries showed 100% leaf re-growth but with different lengths. Some entries had shorter leaf length than others, indicating a weak leaf re-growth, while others had longer leaves, showing a tolerance to low temperature. Hundred percent leaf re-growth of all the entries in this study may be attributed to the test temperature. Maybe it was not low enough to completely kill the entries sensitive to low temperature treatment.

Controlled freezing tests on plants that have been cold acclimated in artificial environments, are routinely used to measure low temperature tolerance in physiological and genetic studies (Fowler *et al.*, 1981). The cold hardiness level of

different plants parts such as leaves, crowns and roots are dependent upon the temperature to which each part has been exposed. Because the crown contains tissues that are necessary for plant survival, it is the soil temperature at crown depth that determines critical cold acclimation rates. Death of the crown tissue will result if the soil temperature falls below the plant's minimum survival temperature that is 2 to 3⁰C warmer than their minimum survival temperature that will cause immediate damage and reduction in cold hardiness (Fowler *et al.*, 1999).

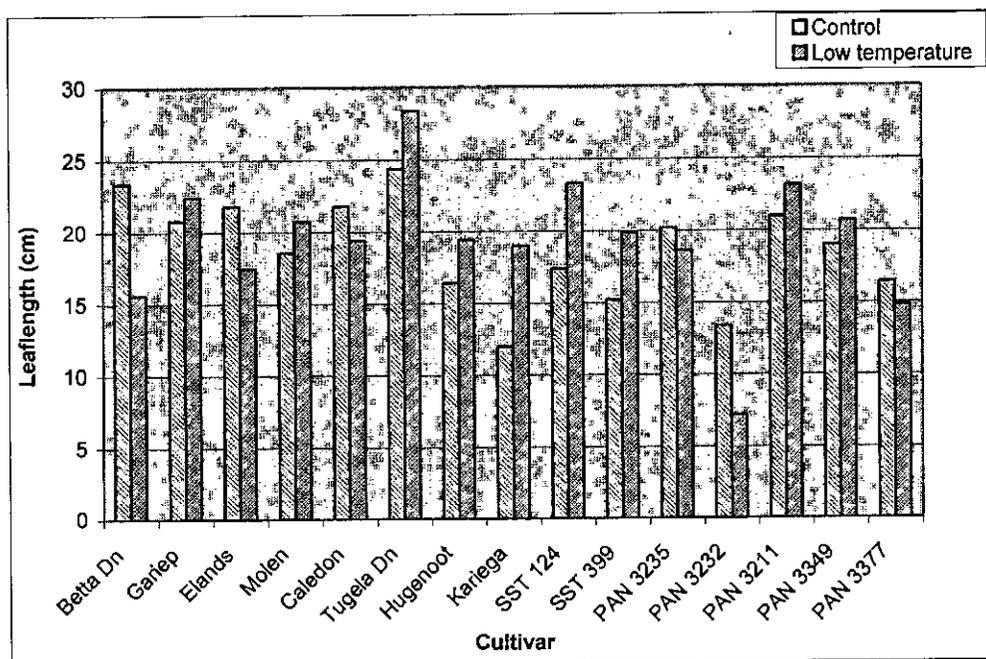


Fig. 5.18 The effect of low temperature on leaf-length of wheat cultivars

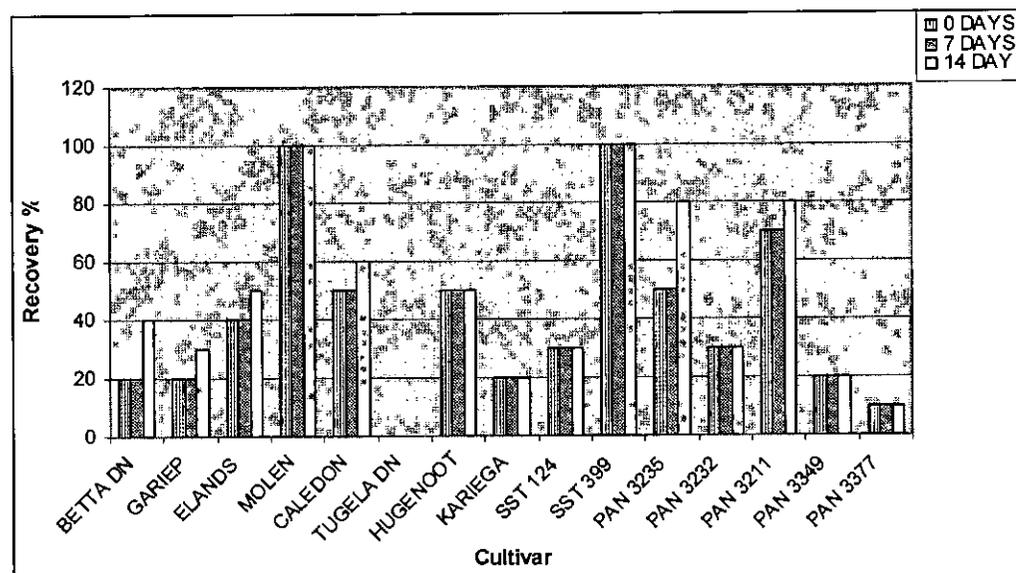


Fig. 5.19 Recovery percentage of wheat cultivars after low temperature stress

CHAPTER 6

General conclusions

Grain yield was found to be highly and positively correlated with other yield components for both high and low temperature stress, except for number of spikelets on the primary spike under high temperature stress. SST 124 and Gariép were tolerant under both high and low temperature stress as they yielded significantly higher than the other cultivars. Betta Dn and PAN 3232 were found to be only low temperature tolerant but high temperature sensitive, as they yielded better under low temperatures but gave poor yield under high temperature stress. On the other hand PAN 3211 and Elands yielded better under high temperature stress but poor under low temperature stress. Based on this performance, it is concluded that they are high temperature tolerant but low temperature sensitive. PAN 3235 and Hugenoet yielded poorly under both temperature extremes, so they are temperature stress sensitive.

Extensive research has shown that an increase in proline content in higher plants under stress conditions is a stress tolerance reaction. Kaldy and Freyman (1984) were the first who proposed the use of increased proline during cold hardening for detection of cold hardiness in winter wheat breeding programmes. Dobslaw and Bielka (1988) observed positive correlation between proline accumulation and freezing resistance in winter barley and they used proline as a chemical marker for freezing resistance in breeding programmes for winter hardiness in this crop.

Results of this study showed an increase of proline content under temperature stress in certain cultivars. PAN 3377, PAN 3349, SST 399, Molen and Kariéga had an increase of proline content under both high and low temperature stress treatments. According to literature, they can be regarded as temperature stress tolerant (Kaldy and Freyman, 1984; Dobslaw and Bielka, 1988). Some cultivars reacted as either low or high temperature stress tolerant or sensitive. There were other cultivars that were temperature stress

sensitive, as they showed no increase of proline content under either high or low temperature stress.

Cell membrane stability was another test used as a measure of high temperature stress tolerance. Cell membranes perform a vital role of regulating the passage of materials into and out of the cell. Its stability is estimated by the relative rate of electrolyte leakage from the leaf tissue samples after being subjected to stress. Electrolyte leakage is a measure of membrane stability (Ruter, 1993). It has been reported that genotypes, which exhibited a low percentage of cell membrane injury, could be considered tolerant. According to the results of this study, PAN 3211 was the cultivar with the lowest percentage injury and therefore the highest temperature tolerance. Overall, the results from this particular study are not satisfactory, because the percentage of injury of most cultivars was too high to make a valid statement.

Vital staining with TTC is used as a method of viability measurement and can provide information about whether individual cells are functioning or not (De Ronde and Van Der Mescht, 1997). TTC reduction was used as a measure of heat tolerance in this study. Cultivars responded significantly different from each other. Betta Dn, Gariép, Elands and Molen reacted heat tolerant, as their formazan production was higher after high temperature stress treatment than the control treatment. In contrast, other cultivars like SST 399, PAN 3235 and PAN 3349 reacted heat sensitive, because their formazan production was lower after the treatment than the control treatment. The other cultivars showed tolerance for only short periods of stress. This can be seen as heat avoidance, but it can render poor yields if the stress is experienced in the early stages of grain development.

Marcellos and Burke (1979) found that leaves of several spring wheat cultivars were able to tolerate temperatures as low as -7 to -9°C . The leaves of the wheat cultivar Norstar can tolerate -18°C (Marcellos and Burke, 1979; Chen and Gusta, 1983; Gusta and Chen, 1987). Gusta and Chen (1987) observed that leaves of Columbus and Neepawa wheat survived temperatures as low as -8°C . Veisz and Sutka (1993) used leaf re-growth to

measure cold tolerance of some Chinese Spring ditelosomics. The cultivars in this study showed a high level of freezing tolerance, as they all were able to recover after exposure to freezing stress. The leaves of Tugela Dn, SST 124 and PAN 3211 grew the longest, an indication of a high level of freezing tolerance. Betta Dn, PAN 3377 and PAN 3232 had the shortest leaves after freezing exposure, which indicates a low level of freezing tolerance. Other cultivars had only intermediate levels of freezing tolerance.

In 1989, Perras and Sarhan studied protein synthesis in leaves, coleoptiles and roots during cold hardening of freezing tolerant winter wheat and freezing sensitive spring wheat. They observed that cold hardening induces important changes in the soluble protein pattern, depending upon the tissue and variety freezing tolerance. Of the proteins induced or increased in hardened tissues, some were expressed at a higher level in the freezing tolerant varieties, than in the sensitive ones, indicating a correlation between the synthesis and accumulation of these proteins and the degree of freezing tolerance. These proteins proved to be freezing tolerance proteins which may have an important role in the cellular adaptation to freezing. They also reported changes in the soluble protein patterns of cold hardened etiolated wheat seedlings.

Accumulation of sugars in different parts of the plant is enhanced in response to various environmental stresses (Bewley and Black, 1994). Gill *et al.* (2001) reported that significantly increased total sugar content in sorghum seedlings was found on exposure to high temperature stress. Moreover Tognetti *et al.* (1990) used four different wheat varieties cultivated in climates ranging from the subtropics to North Pantagonia in Argentina, to study sucrose and fructan metabolism in leaves exposed to low temperatures. It was found that the most cold tolerant cultivars showed higher levels of soluble sugars and enzyme activities.

A high degree of temperature tolerance was observed in the cultivars by use of these biochemical changes (carbohydrate and protein level) induced by exposure to temperature stress. All cultivars except PAN 3349 exhibited a significant increase in protein content under low temperature stress, which can be regarded as low temperature

tolerance. Most of these cultivars also showed an increase of protein content under high temperature stress, except PAN 3349, SST 124, PAN 3377, PAN 3211 and PAN 3232, which can be classed as high temperature sensitive.

There was also an increase of carbohydrate level observed from the cultivars after exposure to temperature stress. Gariep, Elands and Betta Dn behaved differently from the other cultivars as they failed to increase their carbohydrate level, but instead it declined after treatment.

Comparing all the results from this study, it thus can be concluded that a reasonable level of tolerance to high and low temperature stress does exist in South African wheat cultivars. However, it was noted that the cultivars exhibited different levels of tolerance to the temperature treatments. In his study Jacobs (1999) observed that growth habit of cultivars has an influence on their reaction to freezing temperatures, with winter types being more tolerant than the spring types. In contrast, the results from this study suggest that growth habit of the cultivar does not have an influence on freezing tolerance.

Cultivars like PAN 3232, PAN 3377 and SST 124 exhibited tolerance to low temperatures in most of the tests, so it can be concluded that they are freezing tolerant. Elands exhibited sensitivity to low temperatures in most tests. Gariep, SST 399 and Kariega were the only cultivars that exhibited high temperature tolerance in most tests, therefore it can be concluded they are high temperature tolerant.

The data presented in this study indicated differences between cultivars in response to the two temperature extremes. Certain cultivars reacted either tolerant or sensitive to both high and low temperature stress, whereas there were some that reacted as either tolerant or sensitive to high temperature stress but reacted the opposite to low temperature stress. Cultivars also had different reactions to different screening techniques. In this study, the cell membrane stability test gave inconclusive results in comparison to the other tests, so therefore based on these findings, it is concluded that the method is not accurate and reliable. In conclusion the other screening methods are advantageous as they are neither laborious nor time consuming. More reliable and sensitive test results may be achieved

by increasing test period and the use of multiple test temperatures rather than a single test temperature.

CHAPTER 7

Summary

Extreme temperatures are detrimental to plant growth and development and thus affect the productivity of various crops around the world. In South Africa, nearly 544 000 hectares of wheat were insured against frost damage over the last 15 years. Wheat in most of the western parts of Free State, experiences high temperature stress in later stages of growth. In this study, 15 cultivars were evaluated to test their response to high and low temperature stress.

Ten of the cultivars were tested to determine the influence of high and low temperature stress on yield and yield components. There was a high and positive correlation between total grain yield and other yield components. Some cultivars performed well and gave good yields under both high and low temperature stress, indicating stress tolerance. Other cultivars showed temperature stress sensitivity, as they yielded poorly. Low temperature stress proved to be the most devastating as some cultivars failed to produce any seed and some had poor and shrunken seeds.

Accumulation of metabolites during stress has been correlated with the level of stress tolerance. A study to evaluate the effect of high and low temperature stress on proteins and carbohydrate (glucose and sucrose) content in seedlings was done. There was a significant difference in the increase of protein content between high and low temperature stress. Protein content increased significantly more under low temperature stress than high temperature stress. All cultivars except PAN 3349 had increased protein content under low temperature stress but a few cultivars exhibited high temperature sensitivity as they failed to increase their protein content. Changes were also observed on carbohydrate level. There was a notable increase in glucose and sucrose content in certain cultivars. It was also noted that sucrose content increased significantly more than the glucose content and that not all the cultivars that exhibited increased sucrose content necessarily had a glucose content increase as well.

Different screening methods were evaluated for their efficiency as measure of stress tolerance. Results from the cell membrane stability test from this study were inconclusive, which raises questions on the reliability and efficiency of this method as a measure of high temperature tolerance. Another test that was conducted is TTC, which gave good and reliable results. It showed that there are definite high temperature tolerance differences in South African cultivars. Some cultivars proved to be high temperature tolerant as they had high TTC reduction under stress temperatures, whereas some cultivars showed sensitivity with low TTC reduction. It was also found that some cultivars are able to avoid stress, whereby they tolerate stress for only short periods.

A crown survival study was also done and leaf-length and percentage survival were studied as measures of freezing tolerance. Results indicated a high level of freezing tolerance in cultivars, as they remained viable and were able to grow new leaves after exposure to stress. The cultivars in this study can be grouped as tolerant, intermediate and sensitive. On the basis of this study the tests were reliable and can be utilised as a measure for freezing tolerance.

The use of proline content as indication of tolerance to stress has been established and in use for a long time. The effect of high and low temperature stress was studied with the aim of evaluating proline content as a measure of temperature stress. Results showed increased levels of proline after temperature treatments in comparison to a control treatment in some cultivars. Some cultivars exhibited an increase of proline content after both high and low temperature treatments, whereas some had increased proline content for either low or high temperature stress. Proline content can be used as a screening tool for tolerance, but it must be used in conjunction with other screening methods like TTC.

Opsomming

Uiterste temperature is nadelig vir plant groei en ontwikkeling, en affekteer dus produktiwiteit van verskeie gewasse in die wêreld. In Suid Afrika is amper 544 000ha koring teen koue skade verseker in die laaste 15 jaar. Koring in die meeste westelike dele van die Vrystaat ondervind hoë temperatuur stremming in later groei stadia. In hierdie studie is 15 cultivars geëvalueer om hulle reaksie op hoë en lae temperatuur stremming te bepaal.

Tien van die cultivars is getoets om die effek van hoë en lae temperatuur stremming op opbrengs en opbrengs komponente te bepaal. Daar was 'n betekenisvolle positiewe korrelasie tussen opbrengs en opbrengs komponente gewees. Sommige cultivars het goeie opbrengs onder beide hoë- en lae temperatuur stremming gehad, wat stremming toleransie aandui. Ander cultivars was sensitief en het swak opbrengste gelewer. Lae temperatuur stremming het die grootste effek gehad deurdat sommige cultivars geen saad gevorm het nie, terwyl van die ander sade klein en verkrimp was.

Die akkumulاسie van metaboliete tydens stremming is met die vlak van stremming toleransie gekorreleer. 'n Studie is gedoen om die effek van hoë- en lae temperatuur stremming op proteïene en koolhidrate (glukose en sukrose) in saailinge te bepaal. Daar was betekenisvolle verskille in die toename van proteïene tussen hoë- en lae temperatuur stremming. Proteïen inhoud het betekenisvol meer toegeneem onder die lae temperatuur as hoë temperatuur stremming. Alle cultivars behalwe PAN 3349 het meer proteïen onder die lae temperatuur behandeling gehad, maar 'n paar cultivars het hoë temperatuur sensitiwiteit getoon deur nie hulle proteïen inhoud te verhoog nie. Verandering is ook in die vlak van koolhidate gesien. Daar was 'n betekenisvolle toename in glukose en sukrose inhoud in sekere cultivars. Die sukrose inhoud het ook betekenisvol meer as die glukose inhoud gestyg. Nie alle cultivars wat verhoogde sukrose inhoud getoon het, het ook verhoogde glukose inhoud getoon nie.

Verskillende evaluasie metodes is vergelyk vir hulle vermoë om stremming toleransie te meet. Resultate van die selmembraan stabiliteits toets in hierdie studie was onbevredigend en het vrae laat ontstaan oor die betroubaarheid en effektiwiteit

van hierdie toets as 'n meting van hitte stremming. Nog 'n toets wat gebruik is, is die TTC metode wat goeie en betroubare resultate gelewer het. Die toets het gewys dat daar verskillende vlakke van hitte toleransie in Suid Afrikaanse cultivars bestaan. Sommige cultivars was hitte tolerant omdat hulle hoë TTC reduksie het onder stremming gehad, terwyl ander cultivars sensitief was met lae TTC reduksie. Ander cultivars het stremming vermy deur toleransie vir kort tye te toon.

'n Kroon oorlewings studie is ook gedoen en blaar lengte en persentasie oorlewing is gebruik om koue toleransie te meet. Daar was 'n hoë persentasie koue toleransie in die cultivars, aangesien hulle oorleef het en nuwe blare na koue behandeling gevorm het. Die cultivars in die studie kon as tolerant, intermediêr en sensitief groepeer word. In hierdie studie was hierdie metodes betroubaar en kan gebruik word om koue toleransie te evalueer.

Die gebruik van prolien inhoud as 'n indikasie van stremmings toleransie is lank reeds gevestig en steeds in gebruik. Die effek van hoë- en lae temperatuur stremming is geëvalueer om te bepaal of prolien as 'n metode om toleransie te toets gebruik kan word. Resultate het 'n toename in prolien inhoud gewys na temperatuur stremming in vergelyking met 'n kontrole behandeling. Sommige cultivars het 'n toename in prolien inhoud na hoë- en lae temperatuur stremming getoon, terwyl ander verhoogde prolien inhoud vir of hoë- of lae temperatuur stremming gehad het. Prolien inhoud kan as 'n evaluasie metode vir toleransie gebruik word, maar dit moet liewer saam met ander metodes soos die TTC gebruik word.

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