PHYSIOLOGICAL MECHANISMS OF HEAT STRESS TOLERANCE IN TURFGRASS

A THESIS SUBMITTED IN PARTIAL FULFILMENT
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

WAGDI SABER AHMED SOLIMAN

THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES,
IWATE UNIVERSITY
SCIENCE OF CRYOBIOSYSTEMS
(HIROSAKI UNIVERSITY)

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Chapter 1
GENERAL INTRDUCTION

During their life cycle, most plants are frequently exposed to one or more types of biotic or abiotic stresses. Biotic stresses are caused by pathogenic organisms such as bacteria, fungi, insects, nematodes, and viruses (Dangl and Jones 2001), while abiotic stresses are results of exposure to unfavorable physical and chemical environmental conditions such as extremely high temperature, radiation, drought, salinity, heavy metals, and others (Nilsen and Orcutt 1996). These stressors are main environmental factors in determining the distribution and productivity of plants.

In this study, plant tolerance to high temperature stress was focused because the anticipated climate change is expected to cause serious damages to the growth and yield of C_3 crops (Lobell and Asner 2003; Tester and Bacic 2005; Lobell and Field 2007). Heat or high temperature stress is expected to be more likely occurring as a result of global climate change resulting from the substantial increases in the concentration of greenhouse gases caused by human activities. Solomon et al. (2007) reported that temperature from 1995 to 2006 excluding 1996 ranked the 11 warmest years among the past historical records since 1850. The report also predicted that global air temperature will rise by 0.2 °C per decade to reach 1.8 °C to 4.0 °C above the current level by 2100 depending on climate change scenario.

Development of new cultivars that are tolerant to high summer temperatures is a major challenge to breeders working on many crops, especially C_3 crops, under ongoing climate change (Zhang et al. 2006; Barnabás et al. 2008; Semenov and Halford 2009). High summer temperature can induce oxidative stress as well as thermal stress (Basra 2001; Wahid et al. 2007). Clear-sky insolation during midday can increase leaf temperature above 40°C (Singsaas and Sharkey 1998), which could cause thermal damage to leaf tissues resulting from protein denaturation and fluidity increase of membrane lipid (Wahid et al. 2007; Huang and Xu 2008). On the other hand, functional decrease in photosynthetic light reaction, which is likely to occur even under moderately high temperatures, can induce oxidative stress by producing reactive oxygen species (ROS) that is mainly caused by increased electron leakage from the thylakoid membrane (Peñuelas et al. 2005; Almeselmani et al. 2006; Camejo et al. 2006).

In this study, stress resulting from high temperature during summer is referred to as heat stress. The impact of heat stress includes; structural changes at the tissue, cellular and sub-cellular levels (Wahid et al. 2007), reduction of photosynthesis by affecting photochemical and biochemical reactions as well as photosynthetic membrane (Schrader et al. 2004; Wise et al. 2004; Haldimann and Feller 2005), integrity and fluidity of membrane as well as peroxidation of membrane lipids (Liu and Huang 2000; Larkendale and Huang 2004; Xu et al. 2006), degradation and denaturation of proteins (He and Huang 2007; Huang and Xu 2008; Xu and Huang 2008), and changes in the scavenging system either antioxidants or metabolites (Jiang and Huang 2001; Xu and Huang 2004; Almeselmani et al. 2006; Guo et al. 2006). Although these physiological mechanisms of heat stress tolerance of plant have been extensively reviewed (Wahid et al. 2007; Barnabás et al. 2008), the key traits that confer greater tolerance to heat stress have not been clearly identified because of complex effects of heat on plant physiological processes (Tester and Bacic 2005; Zhang et al. 2006; Wahid et al. 2007; Barnabás et al. 2008).

Photosynthesis, leaf properties and heat tolerance

Reduction of photosynthetic rate under heat stress was observed in many crops. This decrease in photosynthetic rate is the main cause for reduced growth and lower yield (Kurek et al. 2007), and thus increasing tolerance of photosynthesis to heat stress is an imperative challenge (Raines 2011). The increase in the carbon flow toward the photorespiratory pathway is an important cause for reduction of photosynthetic rate. One mechanism to maintain high photosynthetic capacity under heat stress in C_4 pathway is the concentration of CO_2 in the bundle sheath cells and resulting suppression of the photoinhibition (Taiz and Zeiger 2002). Inhibition of photosynthesis under heat stress in C_3 species is also caused by decreased activity of ribulose-1,5-*bis*phosphate carboxlase/oxygenase (Rubisco) due to inactivation of Rubisco activase (Law and Crafts-Brandner 1999; Crafts-Brandner and Salvucci 2000; Haldimann and Feller 2005; Salvucci and Crafts-Brandner 2004; Yin et al. 2010). On the other hand, structural leaf properties play important roles in responses of photosynthesis to environmental stress by maintaining high CO_2 concentration in the chloroplast stroma (Bussotti 2008; Atkinson et al. 2010) and/or by increasing Rubisco activity and mesophyll surface area

per unit leaf area (Evans and Poorter 2001; Terashima et al. 2006, 2011).

Effect of prolonged inhibition of Rubisco activity under elevated temperature is the generation of ROS resulting from disrupted balance between photochemical and biochemical reactions, which are major cause for higher mortality under abiotic stress conditions (Wahid et al. 2007). Additionally, photorespiration also produces hydrogen peroxide (H_2O_2) as an end product of metabolic process in a peroxisome.

Reactive oxygen species and antioxidants

One mechanism of injury during heat stress involves the generation and reactions of ROS in plant tissue (Liu and Huang 2000; Kocsy et al. 2004; Xu et al. 2006). ROS are reduced forms of atmospheric oxygen including singlet oxygen (O_2^1), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Mittler 2002). Plants produce ROS through specific metabolic pathways including photosynthesis, through electron transport chain, and photorespiration (Noctor et al. 2002; Queval et al. 2008). Although there are many sites for production of ROS such as chloroplast, mitochondria and peroxisomes (Mittler 2002), the reaction centers of photosystem I (PSI) and photosystem II (PSII) are main sites of ROS production which enhanced when photon intensity is in excess of that required for CO_2 assimilation (Asada 2006).

The ROS play a dual role in plants either as toxic by-products leading to oxidative damages to plant cell or as signaling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defense and system signaling (Mittler 2002; Foyer and Noctor 2005). The accumulation of ROS causes peroxidation of membrane lipids which is considered to be one of the most important damages to cell membrane (Xu et al. 2006).

Plants exposed to heat stress frequently suffer from oxidative stress (Klueva et al. 2001; Sharkey 2005; Velikova and Loreto 2005; Barnabás et al. 2008; Locato et al. 2008). Survival of plants under stress conditions is determined mainly by protection against oxidative stress (Wahid et al. 2007). Fortunately, plants have developed enzymatic and non-enzymatic antioxidant systems to protect plant cell by controlling the intracellular ROS content (Liu and Huang 2000; Fu and Huang 2001). There are antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (Mittler 2002; Almeselmani et al. 2006) and

metabolites like glutathione and ascorbic acid (Mittler 2002; Xu et al. 2006). Oxidative stress occurs when the rate of ROS production is greater than the capacity of scavenging defence system to detoxify them.

Chloroplast has a crucial system to control the level of ROS in PSI, namely water-water cycle (Mittlet 2002; Guo et al. 2006). The primary step of the water-water cycle in PSI of the chloroplast is the reduction of O_2 to O_2^- which is rapidly catalyzed to H_2O_2 with SOD. The H_2O_2 generated by SOD is reduced to water by ascorbate (AsA) catalyzed with APX (Asada 1999; Asada 2006). The ability to cope with heat stress is associated with higher activities of catalase and SOD, higher AsA content and less oxidative damage (Sairam and Tyagi 2004; Almeselmani et al. 2006).

Mechanism of heat tolerance

The heat tolerance of plant is a complex trait controlled by a complex array of metabolic process. Two mechanisms are involved into resistance to heat stress; avoidance and tolerance. Avoidance mechanism includes transpirational cooling, morphological changes, leaf orientation and differences in reflection of solar radiation (Nilsen and Orcutt 1996; Buchanan et al. 2000). Once the plant tissues are heated to supra-optimal temperature, heat tolerance mechanism becomes relevant. Tolerance mechanism implies various mechanisms, including maintenance of membrane stability, scavenging ROS, production of antioxidants and chaperone signaling and transcriptional activation (Wahid et al. 2007). Heat-tolerant genotypes respond to stress by increasing the expression of genes participating in photosynthesis, protein synthesis and preservation of cell status, and transcription factors (Barnabás et al. 2008). At high temperatures, plants induce production of new type of proteins called heat shock proteins which help to cope with the stress through acting as chaperone (Taiz and Zeiger 2002; Wang et al. 2004). The compositions and saturation level of membrane lipids also play important roles in plant tolerance to heat stress (Larkindale and Huang 2004). Heat stress reduces the proportion of unsaturated fatty acid lipids. Decrease in the proportion of double bonds in creeping bentgrass was associated with increases of the membrane leakage and membrane lipid peroxidation under the stress (Larkindale and Huang 2004).

Genetic basis for heat tolerance trait

The severity of heat stress on plants can be reduced by genetic improvement and agricultural practices. The genetic improvement means the development of new cultivars which are tolerance to heat stress. Breeding programs for such complex trait depend on understanding the physiological mechanism and genetic basis of stress tolerance not only at the whole plant level but also at the cellular and molecular levels (Wahid et al. 2007). The molecular and genetic approaches contribute substantially to understand the complexity of plant response to stress (Sreenivasulu et al. 2007). A great number of functionally characterized genes were introduced into crop plants to improve its tolerance to various abiotic stress conditions. Most of the studies recorded higher tolerance to stress of the transgenic lines in different crop plants compared to the controls in the laboratory (Sreenivasulu et al. 2007). Heat tolerance is controlled by sets of genes, not a single gene, in cereals (Maestri et al. 2002) and in the common wheat (Yang et al. 2002). The genetic approach to enhance crop tolerance to heat stress can lead to economically increasing crop yield. However, information about the genetic basis of heat tolerance in plants excluding the major crops is rare (Zhang et al. 2006; Wahid et al. 2007). There are several methods of genetic approaches in cereals reviewed recently (Barnabás et al. 2008), such as using of molecular markers, functional genomics, proteomics and genetic engineering.

Methods used to study heat tolerance

Various methods have been used to assess the physiological damage on plants exposed to heat stress. The maximal efficiency of PSII represented by chlorophyll fluorescence (Fv/Fm) is a widely used technique to measure the physiological damage under the stress. Fv/Fm gives information about the state of PSII reaction centers (Maxwell and Johnson 2000). Under stress conditions, Fv/Fm decreases as a result of damage to reaction centers of PSII and induction of photoinhibition (Long et al. 1994). Measurement of ion leakage (IL) also has been used widely as an index of cellular membrane thermostability to identify heat tolerant populations in many species (Liu and Huang 2000; Xu et al. 2006). In addition to Fv/Fm and IL, measurement of the end product of lipid peroxidation by malondialdehyde (MDA) is a well-known method to prove the occurrence of lipid peroxidation and thus has been used widely as an indicator

of oxidative stress (Larkindale and Huang 2004; Xu et al. 2006). On the other hand, measuring the photosynthesis and its parameters give information not only about the injury observed under stress conditions, but also about occurrence of imbalance between photochemical and biochemical reactions which is a source of ROS generation (Wahid et al. 2007). The electron transfer rate (ETR), which estimates the actual flux of photons driving PSII, and non-photochemical quenching (q_N), which represents excess energy dissipation through the xanthophyll cycle (Maxwell and Johnson 2000), are used to test the possibility of excess energy flow through thylakoid membrane into ROS production.

In contrast, H_2O_2 content, the most stable of the ROS (Ślesak et al. 2007), is used widely to examine the induction of oxidative stress. Scavenging system, a mechanism of tolerance to oxidative stress, is examined by measuring the activity of antioxidants such as APX which has affinity for H_2O_2 and is considered as the major H_2O_2-metabolizing enzymes (Asada 1999) as well as AsA which plays important roles in the biochemical functions not only as an antioxidant but also plays a central role in photosynthesis as an electron transport or donor, and involves in cell wall metabolism and cell expansion (Smirnoff 1996). Also, structural leaf traits are measured because of their important roles in acclimation of plants under the environmental conditions (Bussotti 2008; Atkinson et al. 2010; Terashima 2006, 2011).

The objectives of the study

The plant response to heat stress varies depending on the species, genotypes within species, the duration and severity of the stress, the age and development stage, organ, and type of cells and sub cellular (Basra 2001). Here an important question is whether the cause of damage and/or the tolerance mechanism are similar or different among species, populations or genotypes? Given the differences, how hierarchies of variability influence tolerance mechanism?

Perennial ryegrass (*Lolium perenne* L.) is major forage and turfgrass used in temperate climates and is frequently exposed to heat stress as temperature increase in global warming. *L. perenen* is known to be more sensitive to heat stress compare to other turf grasses (Xu et al. 2006). In this study, main attention will give to heat tolerance of *L. perenne* in the first four experiments.

This study consists of six experiments to enhance understanding on the physiological mechanism of heat stress tolerance. The differences in response to heat stress were examined at different level including; among cultivars (Chapter 2-3), among progenies (Chapter 4) and among species (chapter 5) as well as ploidy differences (Chapter 3) and difference between C_3 and C_4 species (Chapter 5.2). The main purposes of this study were to understand (1) the main cause of physiological damage under heat stress, (2) the intra- and the inter-specific variations in response to heat stress, (3) how the populations in different level (progenies, cultivars and species) response to and cope with the stress, and (4) the genetic basis of heat tolerance in *L. perenne*.

Chapter 2 demonstrates two experiments that attempted to examine the response of photosynthesis and its parameters to heat stress (36/30°C) for 10 days (Chapter 2.1) and to examine the physiological mechanism of functional damages under two types of heat stress; 36/30°C for two months and 40/36°C for two weeks (Chapter 2.2). Chapter 3 focuses on the tolerance mechanism of heat tolerance. In the previous three experiments, *L. perenne* cultivars which differ in their heat tolerance under field conditions were used. Chapter 4 describes the genetic basis of heat tolerance in *L. perenne* progenies derived from a cross between tolerant and sensitive genotypes. Chapter 5 states two experiments and pays special concern about the relationship between heat tolerance and oxidative tolerance at interspecific level both among C_3 species (Chapter 5.1) and between C_3 and C_4 species (Chapter 5.2).

Chapter 2

PATTERN OF PHYSIOLOGICAL RESPONSES TO HEAT STRESS IN *Lolium perenne*

2.1 PHOTOSYNTHESIS RESPONSES TO HEAT STRESS

INTRODUCTION

Photosynthesis is one of the most sensitive physiological processes to climate warming, as shown by a large reduction of photosynthetic rate under heat stress reported in many species (Salvucci and Crafts-Brandner 2004; Sage and Kubien 2007; Nagai and Makino 2009). The expected increase in temperature at the end of this century is likely to have adverse effects on photosynthetic rate of C_3 crops. Although the regulation mechanism of photosynthesis at the optimum temperature is well understood, the mechanism of photosynthesis reduction under heats has not been clearly identified so far (Sage and Kubien 2007). Photosynthesis rate is largely determined by electron transport rate (light response) and ribulose-1,5-*bis*phosphate carboxylase/oxygenase activity (Rubisco, dark response). The response of gas exchange rate to a series of differing concentration of internal CO_2 provides the two key parameters limiting these two processes (Long and Bernacchi 2003): $V_{c,max}$, which represents the maximum rate of Rubisco, and J_{max}, which represents the maximum rate of regeneration of ribulose-1,5-*bis*phosphate (RuBP). However, it is not well clarified how these two parameters are influenced by heat stress. At present, two hypotheses have been proposed to explain heat-induced decline in photosynthesis: (1) limitation of capacity of Rubisco through inactivation of Rubisco activase and (2) increased electron leakage from electron transport chain driving regeneration of RuBP (Law and Crafts-Brandner 1999; Long and Bernacchi 2003; Sage and Kubien 2007; Sage et al. 2008).

Perennial ryegrass (*Lolium perenne* L.), widely used as pasture grass as well as turf grass in temperate climates, is sensitive to heat stress (Xu et al. 2006). In this chapter, photosynthetic rate and its two parameters were compared between two cultivars of *L. perenne*, one known to be tolerant to summer stress in the field and the other known to be sensitive to it. Also, I measured chlorophyll fluorescence (Fv/Fm) and membrane lipid peroxidation, as indicators of physiological damage, as well as hydrogen peroxide

(H_2O_2) content, a reactive oxygen species, to examine contribution of oxidative stress.

MATERIALS AND METHODS

Field evaluation

Field tolerance of 100 different cultivars of perennial ryegrass, which were developed in different countries, was evaluated at the Yamanashi Dairy Agricultural Station, Japan, where summer temperatures are higher than what is optimal for the species. Ten seedlings of each cultivar were planted in a row on 4 October 2000. The row spacing was 0.8 m and the inter-plant distance within each row was 0.5 m. A randomized block layout was used with two replications (rows). The plants were raised until the following summer, and each plant was visually assessed for shoot growth on a scale ranging from 0 (poorest) to 9 (best) on 13 September 2001. The mean maximum temperature in July and August 2001 was 30.4 °C, which was 2.6 °C higher than that in an average year; the mean monthly precipitation during the same period was 106 mm, which was 57 mm lower than the average.

Plant materials

From those cultivars, the most and the least tolerant populations were chosen for this study. Yatugadake-24 (Ya-24) turned out to be the most tolerant cultivar to heat stress and Norlea turned out to be the least tolerant cultivar. Ya-24 is a tetraploid cultivar recently developed in Japan, whereas Norlea is a diploid cultivar originally from Canada.

Growth conditions

Seeds of the two cultivars were germinated on wet filter paper in Petri dishes. When the coleoptile was at least 2 cm long, the seedlings were transplanted into pots. Each pot was 7.5 cm in diameter and 8 cm deep, filled with sandy loam containing 0.35 g each of N, P_2O_5 and K_2O for every kilogram of soil. The plants were grown in a controlled growth chamber with day/night temperatures of 23/16°C, a 16-h (4:00 to 20:00 h) photoperiod with photon flux of 250 μmol m^{-2} s^{-1} and relative humidity of 70% during the day and at night. Forty days after transplanting, all the plants were exposed to 30°C

for 3 days for acclimation, after which plants were exposed to high temperature stress (36/30°C) for 10 days. Water was supplied daily to avoid water stress. The experiment was set up in a randomized block layout incorporating six replications.

Gas exchange measurements

Gas exchange rates were measured using a portable photosynthesis measuring system (LI-6400, Li-cor, Lincoln, Nebraska, USA). Leaf CO_2 assimilation rate (A) versus intercellular CO_2 concentration (C_i) curves (A/C_i) were obtained using the protocol described by Long and Bernacchi (2003). Firstly, CO_2 assimilation rate (A) was measured at air CO_2 concentration (C_a) of 400 μmol mol^{-1} CO_2 and photon flux (1200 μmol m^{-2} s^{-1}) until A is steady-state. Values of A and C_i were recorded and then C_a was decreased to 200, 100, 50 μmol mol^{-1}. Values of A and C_i were recorded as soon as C_a is constant. C_a was returned to 400 μmol mol^{-1} to restore the original A and then C_a was increased stepwise to 600, 800, 1000 and 1200 μmol mol^{-1} recording A and C_i at steady-state of each step. A/C_i responses were measured before exposure to stress and twice after exposure to stress, at 5 day and 10 day.

The capacity of Rubisco activity was obtained from the initial slope of A/C_i curve. The equations of Long and Bernacchi (2003) were used to estimate $V_{c,mac}$ and J_{max} values and expressed as;

$$A = f'V_{c,\max} - R_d \quad (1)$$

$$A = g'J_{\max} - R_d \quad (2)$$

where f' and g' were calculated from fitting A/C_i curve with the following equations;

$$f' = \frac{C_i - \Gamma^*}{C_i + K_c(1 + O/K_o)} \quad (3)$$

$$g' = \frac{C_i - \Gamma^*}{4.5C_i + 10.5\Gamma^*} \quad (4)$$

where Γ^* is the photosynthetic compensation point when photorespiratory efflux of CO_2 equals to photosynthetic CO_2 uptake rate, K_c and K_o are the Michaelis constant of Rubisco for carboxylation and oxygenation, and O is the practical pressure of oxygen at Rubisco.

Physiological measurements

Chlorophyll fluorescence (Fv/Fm), peroxidation of membrane lipids and hydrogen peroxide content (H_2O_2) were measured before exposure to stress and at 5 day and 10 day of exposure to 36/30°C treatment. Six individual plants of each cultivar were maintained in the dark for 20 min for dark adaptation and then the minimum (F_0) and maximal (F_m) levels of fluorescence were measured with a portable photosynthesis measuring system (LI-6400, Li-cor, Lincoln, Nebraska, USA). The maximal photochemical efficiency of photosystem II (PSII), the most heat-sensitive component in photosynthesis, was calculated as $F_v/F_m = (F_m - F_0)/F_m$.

Membrane lipid peroxidation was estimated by determining the level of malondialdehyde (MDA) using the method described by Liu and Huang (2000) with a slight modification. Fresh leaves (50 mg samples) were ground in 1.5 mL of 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10,000 rpm (8,385 *g*) at 3°C for 5 min and 1 mL of the supernatant was mixed with 2 mL of 0.5% 2-thiobarbituric acid (TBA) in 20% TCA. The mixture was heated in a water bath for 20 min, quickly cooled in an ice bath and the supernatant was used for spectrophotometric determination of MDA. Absorbance at 532 nm was recorded and corrected for nonspecific absorbance at 600 nm. MDA concentrations using an extinction coefficient of 155 $mmol^{-1}$ cm^{-1} were calculated by the following formula on a fresh weight (FW) basis (Xu et al. 2006):

$$\text{MDA } (\mu\text{mol g FW}^{-1}) = [(A_{532} - A_{600})/155] \times 10^3$$

Hydrogen peroxide (H_2O_2) content of leaves was measured using a modified version of the ferrous ammonium sulphate/xylenol orange (eFOX) method described by Cheeseman (2006) and Queval et al. (2008). Leaf extracts were prepared by grinding 50 mg leaf samples first in liquid nitrogen and then in 500 µL of 0.1 M potassium phosphate buffer (pH 6.5) containing 5 mM NaN_3. The extracts were centrifuged at 10,000 rpm (8,385 *g*) at 5°C for 5 min. For every 200 µL of the extract was added 5 mL of the solution containing 250 µM ferrous ammonium sulphate, 100 µM sorbitol, 100 µM xylenol orange, 1% ethanol and 25 mM H_2SO_4. The assay consisted of measuring the difference in absorbance between 550 nm and 800 nm after 15 min with a spectrophotometer. H_2O_2 content was calculated by a standard curve using a series of diluted solutions of commercial, high-grade 30% H_2O_2.

Statistical analysis

The differences between the cultivars and between the days were tested by analysis of variance (ANOVA). Statistical analysis was carried out using JMP (ver 4. SAS Institute, Cary, NC, USA).

RESULTS

Figure 2.1.1 showed the A/C_i response of the two cultivars before and after the exposure of stress. Ya-24 had higher assimilation rate of CO_2 (A) than Norlea in most internal CO_2 (C_i) concentration before and after the stress. The photosynthetic rate at 400 μmol mol^{-1} and Rubisco activity was much higher in Ya-24 than Norlea in all measurement times (Figures 2.1.1a and 2.1.2b). Photosynthesis rate showed slight decreases, but not be significant, under stress relative to the control for both cultivars (Figure 2.1.2a). In contrast, Rubisco activity showed slight increases, but not significant, after exposure to stress relative to the control for both cultivars (Figure 2.1.2b). $V_{c,max}$ and J_{max} values estimated from the equation (1 and 2, respectively) showed significant increases under stress conditions for the two cultivars, while the ratio of J/V_{max} decreased under stress conditions for the two cultivars except for at 10 day for Norlea (Figure 2.1.3). $V_{c,max}$ showed no significant differences between the two cultivars before stress and at 10 day of the stress, but Ya-24 had significantly higher $V_{c,max}$ than Norlea at 5 day of the stress. On the other hand, Ya-24 had higher J_{max} value and J/V_{max} ratio than Norlea before the stress and at 5 day of the exposure to stress, while Norlea had significantly higher values of both traits than Ya-24 at 10 day of the stress.

Analysis of variance (ANOVA) in Table 2.1.1 showed that there were highly significant differences both between cultivars and between number of days after the exposure to stress for chlorophyll fluorescence (Fv/Fm) and hydrogen peroxide (H_2O_2), but not for malondialdehyde (MDA). The two cultivars showed significant decreases in Fv/Fm and significant increases in H_2O_2 beginning at 5 day, but no significant changes were shown in MDA content after the exposure to stress (Figure 2.1.4). Also, significant differences between Norlea and Ya-24 appeared at 10 day of the stress for Fv/Fm and H_2O_2 but not for MDA (Figure 2.1.4). Norlea had significantly lower Fv/Fm value and significantly higher content of H_2O_2 at 10 day of the stress than Ya-24 (Figure 2.1.4a and 2.1.4c, respectively).

DISCUSSION

Although Ya-24 had higher photosynthesis rate and Rubisco activity than Norlea both before and after the exposure to stress (Figure 2.1.2), both cultivars did not show declines in both traits under heat stress. Maintenance of high photosynthesis rate under stress conditions was results of maintaining high Rubisco activity and increasing $V_{c,max}$ and J_{max} (Figure 2.1.2b, 2.1.3a and 2.1.3b, respectively). In contrast, Ya-24 showed a significant decrease in J/V_{max} ratio under stress, while Norlea showed decrease in J/V_{max} ratio at 5 day followed by significant increase at 10 day of the stress. These results suggest that 10 days of exposure to heat stress (36/30°C) does not limit photosynthesis and Rubisco activity. On the other hand, because of technical problem and stomata closure, it was difficult to measure A/C_i response after 10 days of the exposure to stress.

Although the two cultivars maintained high level of photosynthesis rate and Rubisco activity under stress, they showed physiological damages under the stress as explained by significantly decreases in Fv/Fm as well as significantly increase in H_2O_2 content (Figure 2.1.4a and 2.1.4c). Significant differences were found between the two cultivars in Fv/Fm and H_2O_2 content. Norlea which is sensitive to summer stress under field showed lower value of Fv/Fm and higher content of H_2O_2 than the tolerant cultivar, Ya-24, at 10 day of the stress. No significant change and difference were observed for MDA content between the two cultivars. The declines in Fv/Fm under stress condition seemed to be associated with increases in H_2O_2 content. These results suggest that oxidative stress is related to physiological damage under heat stress. Furthermore, even sensitive cultivar had the ability to protect membrane against lipid peroxidation until 10 days of exposure to (36/30°C) stress which might prevent decrease in photosynthesis.

To summarize the results, higher tolerance of Ya-24 than Norlea is consistent with the result of field conditions in Yamanashi Experimental Station. The difference in physiological damage under stress seems to be associated with the excessive generation of reactive oxygen species (ROS).

Table 2.1.1 Analysis of Variance (ANOVA) of chlorophyll fluorescence (Fv/Fm), malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content for two *Lolium perenne* cultivars.

Source	**df**	Fv/Fm			MDA			H_2O_2		
		SS	***F* value**	***Prop***	**SS**	***F* value**	***Prop***	**SS**	***F* value**	***Prop***
cultivars	1	0.0017	10.10	0.0033	0.7482	0.05	0.83	0.1024	11.60	0.0018
days	2	0.0193	56.42	< 0.0001	1.3939	0.04	0.96	0.5131	29.05	< 0.0001

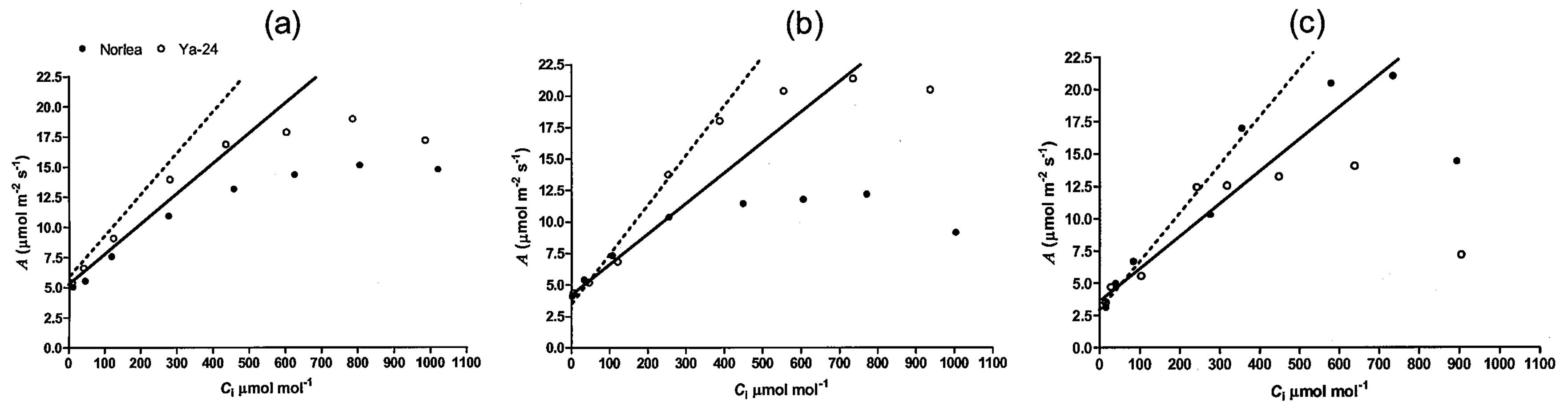


Figure 2.1.1 The response of CO_2 assimilation rate (A) to intercellular CO_2 concentration (C_i) before exposure to heat stress (a), at 5 day of the stress (b) and at 10 day of the stress (c) in Norlea (●) and Yatugadake-24 (Ya-24, ○) under heat stress (36/30°C). The initial slope was represented with the straight line for Norlea and the dotted line for Ya-24.

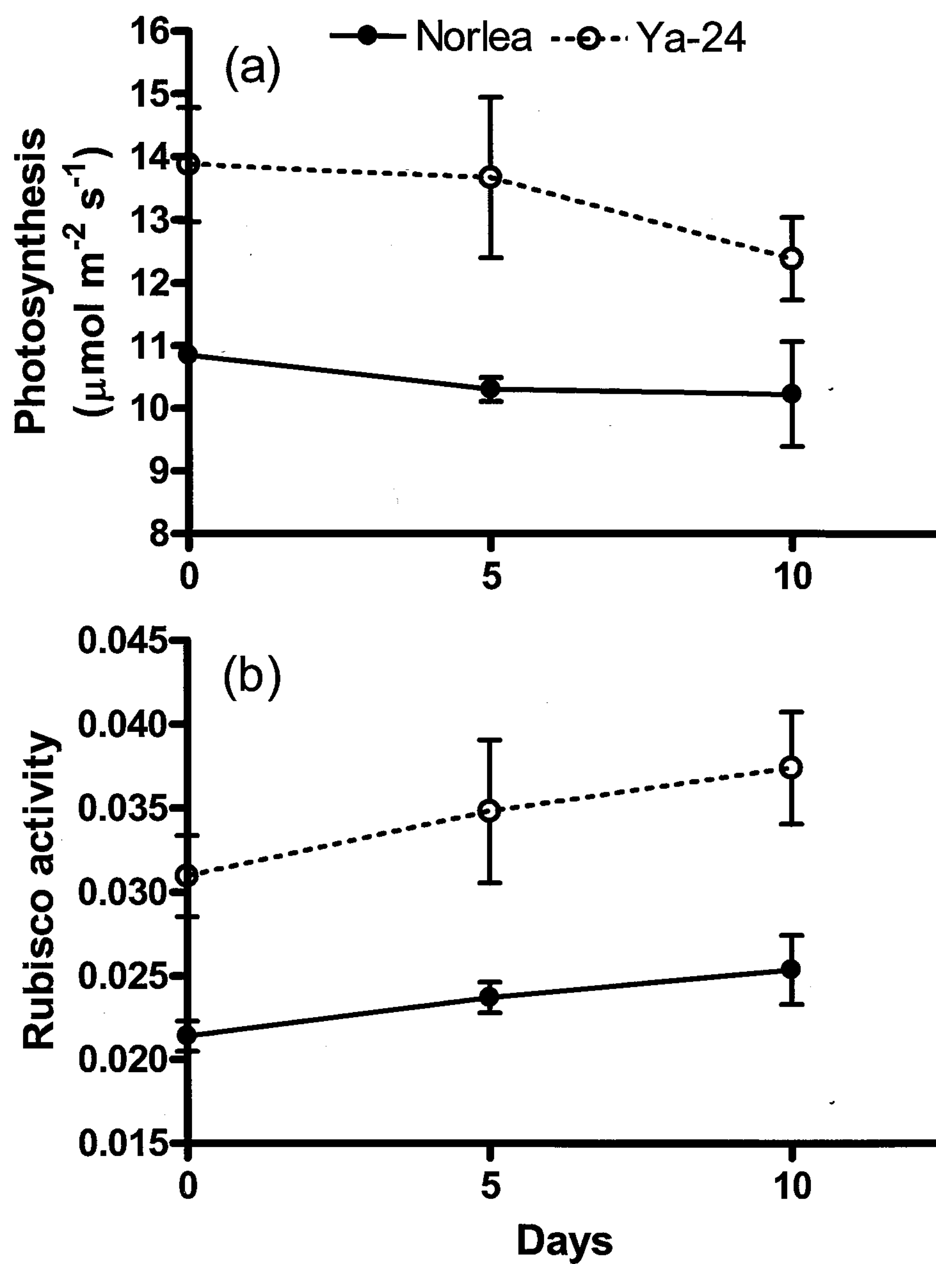


Figure 2.1.2 Temporal changes in photosynthesis (a) and Rubisco activity obtained from the initial slope of A/C_i curve (b) in Norlea (●) and Yatugadake-24 (Ya-24, ○) under heat stress (36/30°C). Photosynthesis rate was measured at $C_a = 400$ μmol mol^{-1}.

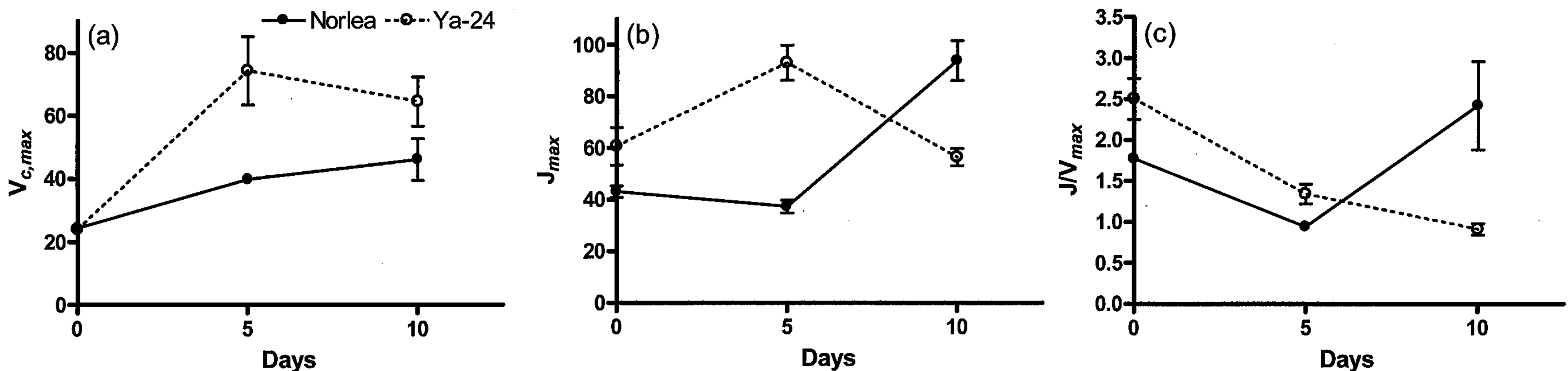


Figure 2.1.3 Temporal changes in gas exchange parameters; the maximum capacity of Rubisco ($V_{c,max}$, a), the maximum capacity of electron transport driving regeneration of RuBP (J_{max}, b), and J/V_{max} ratio (d) in Norlea (●) and Yatugadake-24 (Ya-24, ○) under heat stress (36/30°C).

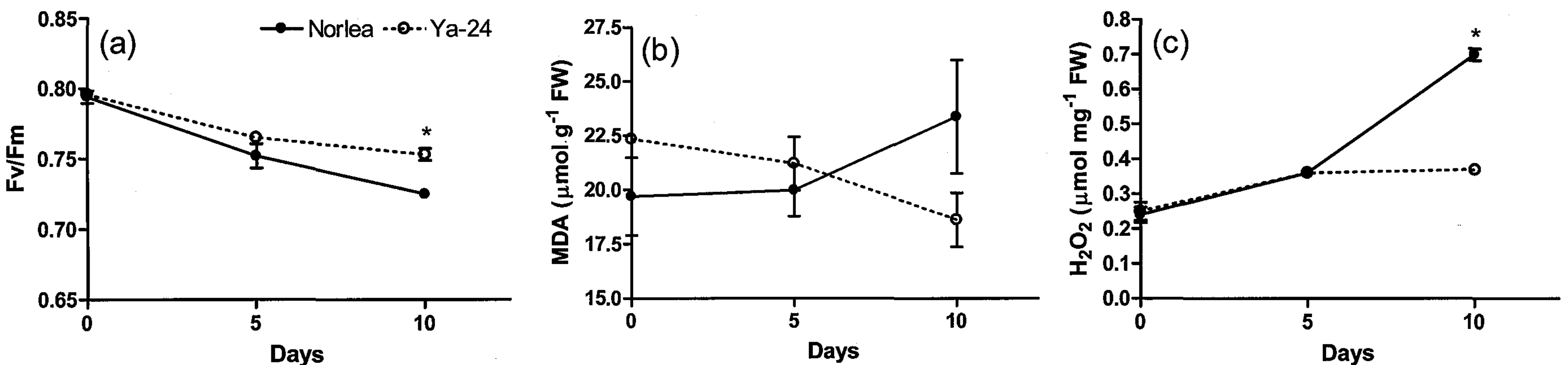


Figure 2.1.4 Temporal changes in chlorophyll florescence (Fv/Fm, a), membrane lipid peroxidation (MDA, b) and H_2O_2 content in leaves (c) in Norlea (●) and Yatugadake-24 (○) under heat stress (36/30°C). * indicates a significant difference between the three populations at $P < 0.05$.

2.2 HEAT STRESS AND OXIDATIVE DAMAGE IN *Lolium perenne* CULTIVARS

INTRODUCTION

Many studies have explored the molecular and physiological mechanisms that govern heat tolerance in response to short–term exposure to heat stress (Scharader et al. 2004; Haldimann and Feller 2005; Xu et al. 2006; Xu and Huang 2010). However, such responses to short-term stress may not represent the physiological mechanisms of heat tolerance under field conditions because in summers, field-grown plants are exposed to temperature stresses that take the form of long-term exposure to moderately high air temperature and intense midday solar radiation. To understand physiological mechanism of summer tolerance in fields, it is crucial to examine plant responses to prolonged exposure to heat stress.

In this chapter, I used the same cultivars of *Lolium perenne* grass as Chapter 2.1 to monitor the physiological damage and the content of hydrogen peroxide (H_2O_2) in leaves of plants exposed to two types of high-temperature stress, namely moderately high temperature (36/30°C; day/night temperatures) for 60 days and extremely high temperature (40/36°C) for 14 days. The purposes of this study were to examine (1) how H_2O_2 accumulation and physiological damage change under prolonged stress, (2) how the relationship between H_2O_2 accumulation and physiological damage differs between the tolerant and the sensitive populations, and (3) how the different temperature treatments influence H_2O_2 accumulation and physiological damage.

MATERIALS AND METHODS

Plant materials, growth conditions and heat stress treatments

The same cultivars used in the previous part (Chapter 2.1), Norlea and Ya-24, were germinated and grown in the same conditions as mentioned previously. After acclimation of the plants at 30°C for 3 days, the two treatments were introduced, namely exposure to 36/30°C for 60 days or to 40/36°C for 14 days (referred to respectively as 36°C and 40°C treatments hereafter). The two treatments did not run concurrently but had to be conducted at different times because of limited space. Water was supplied daily to avoid water stress. The experiment was set up in a randomized block layout

incorporating three replications.

Physiological measurements

Physiological damage was assessed in terms of chlorophyll fluorescence (Fv/Fm), peroxidation of membrane lipids (malondialdehyde, MDA) and cell membrane stability. These parameters as well as hydrogen peroxide content (H_2O_2) were recorded at 5-day intervals for the 36°C treatment and at 2-day intervals for the 40°C treatment. Fv/Fm, MDA and H_2O_2 content were measured using the same methods described in Chapter 2.1.

Cell membrane stability was measured by ion leakage (IL) from leaf tissues using the method described by Jiang and Huang (2002). The sampled leaves were cut into discs 2 mm in diameter. The discs were rinsed 3 times with distilled water and 10-15 discs were put in a test tube containing 6 mL distilled water. The test tubes were agitated on a shaker for about 1 h and conductivity (C_1) of the solution was measured with a conductivity meter (Cyberscan100, Iuchi, Tokyo, Japan). Leaf discs then were heated in an oven at 70–80°C for 1 h, and the conductivity of the solution containing the dead tissue (C_2) was measured after the tubes had cooled down to room temperature and had been agitated on a shaker for 1 h. The relative ion leakage was calculated as $(C_1/C_2) \times 100$.

Statistical analysis

Because the same individual plants were used repeatedly for each measurement - which meant that the data between different measurement times were not independent of each other - I used repeated measures of multiple analysis of variance (MANOVA) to test whether the two populations were significantly different (Weinfurt 2004). The statistical difference between the two populations for each measurement was tested by the *t*-test. The analysis was carried out using JMP (ver 4. SAS Institute, Cary, NC, USA).

RESULTS

The two populations revealed highly significant differences in the chlorophyll fluorescence (Fv/Fm), cell membrane stability (ion leakage %), lipid peroxidation

(MDA) and H_2O_2 content under the 36°C treatment, but none except Fv/Fm under the 40°C treatment (Table 2.2.1). Although the differences in Fv/Fm between the two populations were not evident at the early stage of the treatments, significant differences between the two populations appeared at 10 days for the 40°C treatment and at 45 days for the 36°C treatment (Figures 2.2.1a and 2.2.2a). For ion leakage and MDA, significant differences appeared at 40 days and 35 days respectively (Figures 2.2.1b, 2.2.1c) and for H_2O_2 content at 15 days for the 36°C treatment (Figure 2.2.1d). On the other hand, as mentioned earlier, no significant differences were found for the 40°C treatment (Figures 2.2.2b, 2.2.2c and 2.2.2d). H_2O_2 content was much higher in the 36°C treatment than in the 40°C treatment. Final H_2O_2 content in Norlea was 4 μmol mg^{-1} FW of leaf tissue for the 36°C treatment and 1.0 μmol mg^{-1} FW for the 40°C treatment.

The magnitude of plant functional damage by H_2O_2 is influenced by the total amount of exposure over a given time of period rather than by a level at a certain time. I examined the relationships of Fv/Fm, ion leakage and MDA with H_2O_2 content at each measurement time as well as with accumulated H_2O_2 content (aH_2O_2), which was calculated by adding up the H_2O_2 contents determined on all the earlier occasions (Figure 2.2.3). Fv/Fm, ion leakage and MDA did not show clear relationships with H_2O_2 content at each measurement time for a tolerant cultivar, Ya-24. Although no consistent relationships were found until 20 days, linear relationships of all the three parameters with accumulated H_2O_2 content were evident thereafter for the two cultivars. ANCOVA showed that if the effects of accumulated H_2O_2 contents were removed, the two populations showed no significant differences after 20 days with respect to any of the three parameters (Table 2.2.2). These results indicate that the differences in Fv/Fm, ion leakage and MDA between the two populations were due to the difference in accumulated H_2O_2 content rather than to the difference in tolerance to H_2O_2.

DISCUSSION

In this chapter, the plants were exposed to two types of high-temperature stress: 36/30°C for 60 days and 40/36°C for 14 days. The significant difference between the two populations was found only under the 36/30°C treatment except Fv/Fm under the 40°C treatment. Plants exposed to the 40/36°C treatment showed physiological damage on the

seventh day of exposure to the stress (Figure 2.2.2a). However, leaf temperatures under field conditions rarely remain at 40°C for long even on sunny days because of the combined effects of transpirational cooling and wind (Wise et al. 2004). These results demonstrate that the prolonged exposure to moderately high temperature in summer can simulate summer damage under field conditions more efficiently than short-term extremely high temperature does.

Although ROS in leaves are generated mainly in organelles such as chloroplasts, peroxisomes and mitochondria, the reaction centers of photosystem I (PSI) and photosystem II (PSII) in chloroplasts are the major sites of ROS generation (Asada 2006). Under stress conditions where photon intensity absorbed by PS1 and PSII is in excess of that required for CO_2 assimilation, surplus electrons serve as the source of toxic oxygen species. The linear relationship between maximal efficiency of PSII (Fv/Fm) and the accumulated H_2O_2 content (Figure 2.2.3a) suggests that prolonged generation of ROS under long-term exposure to moderately high temperatures caused physiological damage. On the other hand, plants that had been exposed to higher temperatures for a shorter duration contained less H_2O_2 in their leaves, probably because thermal damage to photosystems under such high temperatures resulted in less photon being absorbed and, consequently, in lower rate of H_2O_2 generation. The degree of lipid peroxidation and ion leakage showed temporal fluctuations until 20 days but increased thereafter and was significantly different in the two populations with markedly greater levels of H_2O_2 in the sensitive one. All these results demonstrate that oxidative stress is the main cause of physiological damage seen under long-term exposure to moderately high temperature.

Plants develop several defense mechanisms against toxic reactive oxygen molecules. These mechanisms include suppressing ROS production, scavenging the produced ROS and repairing the damage caused by ROS (Asada 1999). The results of ANCOVA, including the accumulated H_2O_2 content as a covariate in leaves (Table 2.2.2), suggest that the difference in the extent of physiological damage between the two populations was mainly due to the ability to suppress H_2O_2 accumulation in leaves rather than to the difference in tolerance to H_2O_2 accumulation. The difference in H_2O_2 content in leaves suggests the involvement of the first two mechanisms, namely suppression and scavenging of ROS.

Understanding the physiological mechanisms involved in the tolerance of C_3 crops to heat stress is crucial to the improvement of these crops. This study suggests that oxidative stress resulting from generation of ROS is a major cause of damage to *L. perenne* populations in summer.

Table 2.2.1 Effect (*F*-value) of population (Norlea and Yatugadake-24) on chlorophyll fluorescence (Fv/Fm), ion leakage, MDA and H_2O_2 content in 36°C and 40°C treatments.

Variable	Treatment	
	36°C	40°C
Fv/Fm	114.5***	43.7**
Ion leakage	64.0***	0.3
MDA	27.9***	4.9
H_2O_2 content	66.3***	0.9

$**P < 0.01$, $***P < 0.001$.

Table 2.2.2 Effect (*F*-value) of population (Norlea and Yatugadake-24) and accumulated H_2O_2 content on chlorophyll fluorescence (Fv/Fm), ion leakage (IL%) and MDA, with overall coefficient of determination (R^2) (data after 20 days in 36°C treatment).

Item	df	Fv/Fm	IL%	MDA
F-value				
Population	1	0.6	1.4	0.3
aH_2O_2	1	239.5***	97.4***	35.1***
R^2 (%)		94.0	82.6	62.9

***$P < 0.001$.

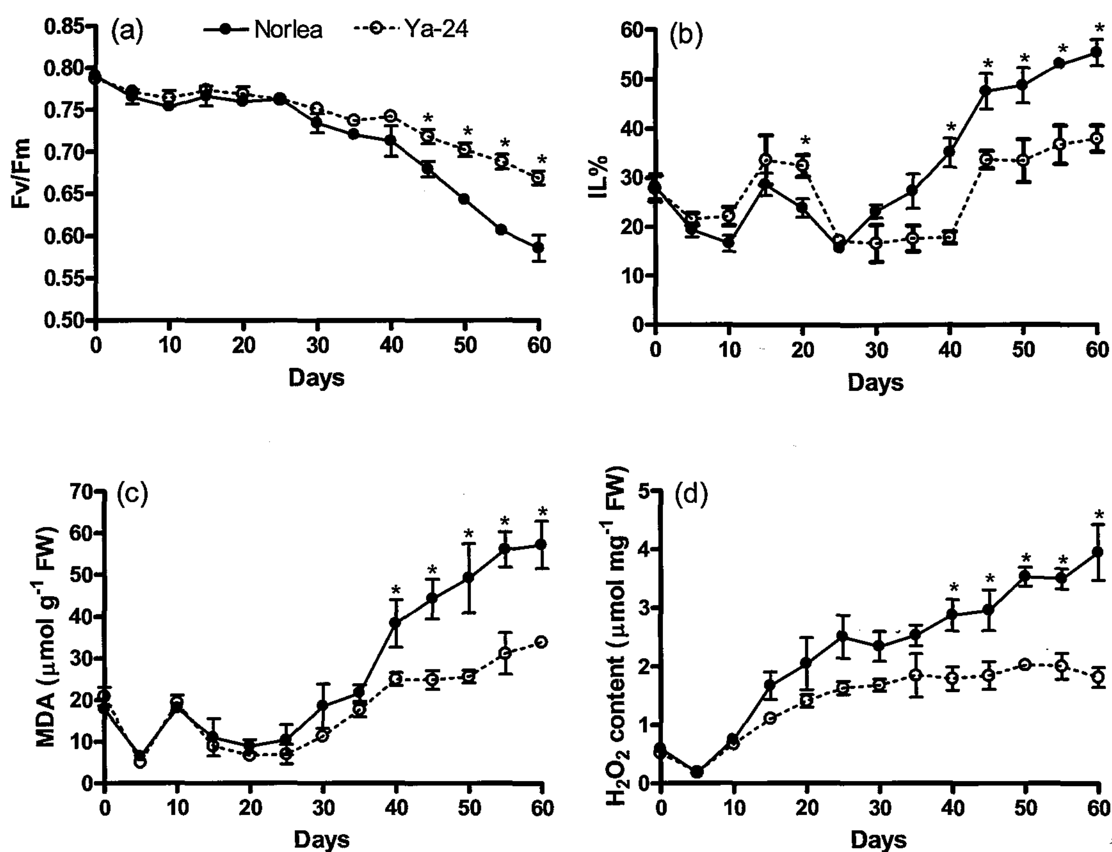


Figure 2.2.1 Temporal changes in chlorophyll florescence (Fv/Fm, a), ion leakage (IL%, b), lipid peroxidation (MDA, c) and H_2O_2 content in leaves (d) in Norlea (●) and Yatugadake-24 (○) under moderately high temperature (36°C). * indicates a significant difference between the two populations at $P < 0.05$.

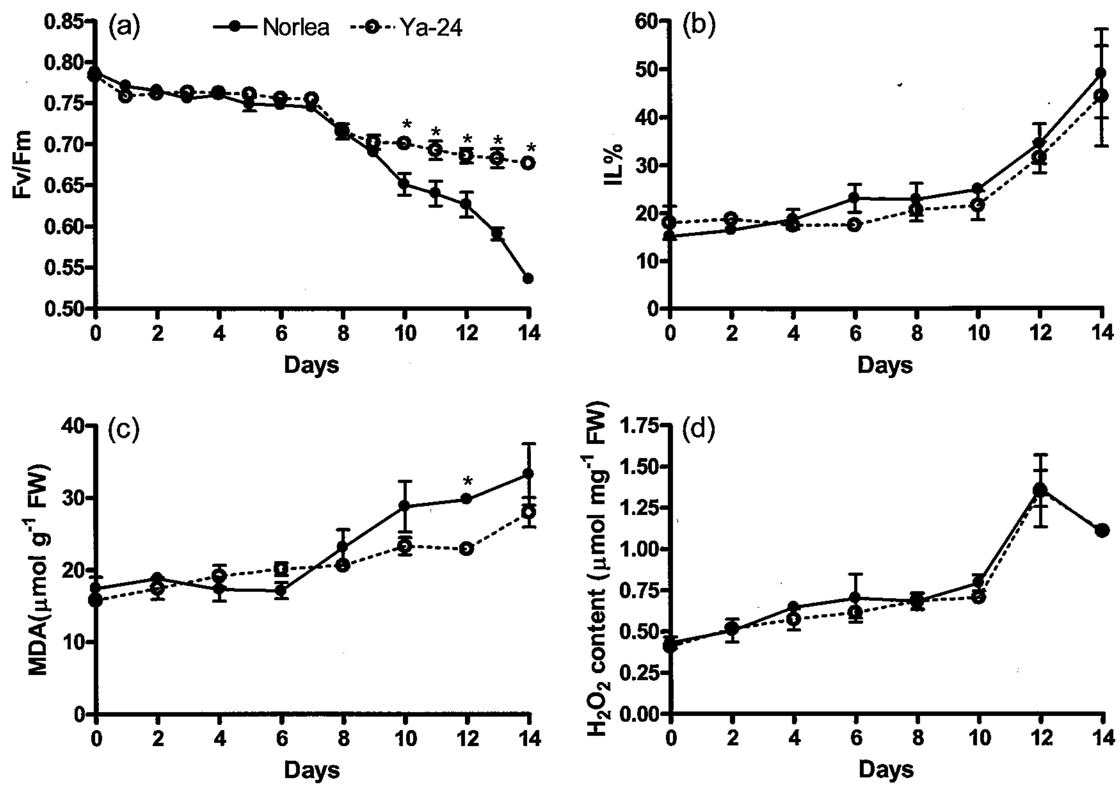


Figure 2.2.2 Temporal changes in chlorophyll florescence (Fv/Fm, a), ion leakage (IL%, b), lipid peroxidation (MDA, c) and H_2O_2 content in leaves (d) in Norlea (•) and Yatugadake-24 (○) under extremely high temperature (40°C). * indicates a significant difference between the two populations at $P < 0.05$.

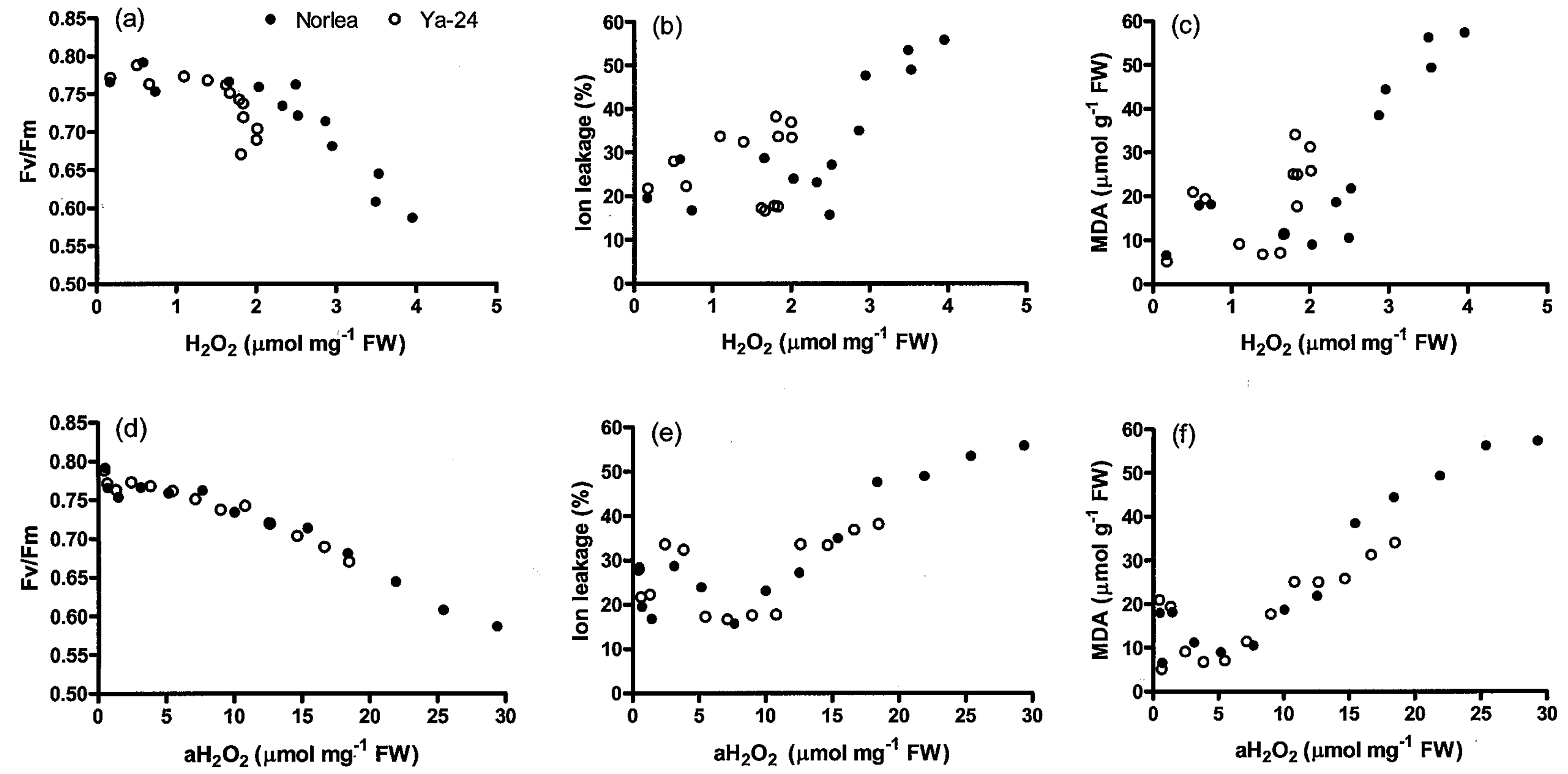


Figure 2.2.3 The relationships of chlorophyll fluorescence (a, d), ion leakage (b, e) and lipid peroxidation (c, f) against H_2O_2 content (a–c) and accumulated H_2O_2 content (d–f) in leaves in Norlea (●) and Yatugadake-24 (○) under moderately high temperature (36°C).

Chapter 3
TOLERANCE MECHANISMS TO HEAT STRESS IN C_3 GRASS, *Lolium perenne*

INTRODUCTION

Plants are more likely to suffer from oxidative stress than animals because reactive oxygen species (ROS) are produced not only by aerobic respiration in mitochondria but also by photosynthetic light reaction in chloroplasts and photorespiration in peroxisomes (Mittler et al. 2004; Miller et al. 2010). Content of ROS in leaves is kept in balance by production and scavenging of ROS. However, the oxidative balance in leaves is regulated by a redundant and complex biochemical network, not by a system composed of a limited number of factors working independently of one another. Rizhsky et al. (2002) and Miller et al. (2007) have shown that a breakdown of gene expression in two major scavenging enzymes, namely ascorbate peroxidase (APX) and catalase, does not bring substantial changes in oxidative balance. Furthermore, ROS can play a dual role, as damaging toxic compounds and as beneficial signal molecules that activate the defensive response to oxidative stress (Mittler 2002; Foyer and Noctor 2005; Suzuki and Mittler 2006; Jaspers and Kangasjarvi 2010). To understand a complex system such as oxidative balance, any given measure in a system has to be analyzed in relation to changes in rest of the components of the system (Hõrak and Cohen 2010). Building a structural equation model (SEM), which is a statistical method to construct causal relationships among the components of a system (Shipley 2000), is particularly suitable for analyzing the regulation of oxidative balance in plants.

Plants exposed to heat stress frequently suffer from oxidative stress. After a hot summer in 2002, a large difference was observed among cultivars of *Lolium perenne* L. in the extent to which the growth in field had been affected, and this chapter seeks to clarify the physiological mechanisms of the damage caused by heat stress in relation to oxidative stress in the species. I examined the variation in functional damage, photosynthetic properties, antioxidants, and H_2O_2 contents in 25 cultivars that differed in their degree of tolerance to prolonged exposure to moderately high temperatures in the laboratory and then, by constructing SEMs, I analyzed the causal relationships among the factors that affect H_2O_2 accumulation in leaves and its influence on

functional damage in the laboratory as well as in the field.

MATERIALS AND METHODS

Plant materials

Twenty-five cultivars (fourteen diploid and eleven tetraploid) of *L. perenne* were used in this study (Table 3.1). Those cultivars were selected from the cultivars which were evaluated for field tolerance at the Yamanashi Dairy Agricultural Station as described in Chapter 2.1.

Growth conditions and heat stress

Growth conditions were the same as described in Chapter 2.1. The plants were acclimated at 30°C for 3 days and then were exposed to heat stress (36/30°C, day/night) for 40 days, when the differences in heat tolerance among the cultivars became apparent (Chapter 2.2). The experiment was set up in a randomized block layout incorporating three replications.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence (Fv/Fm) were measured in three individual plants of each cultivar with a portable photosynthesis measuring system (LI-6400, Li-cor, Lincoln, Nebraska, USA) as described in Chapter 2.1. Measurements were made before the acclimation (control) and at 10-day intervals during the period of exposure to heat stress.

The minimal (F_0'), steady-state (F_s), and maximum (F_m') levels fluorescence in the light-adapted leaves were measured under actinic light of 200 μmol m^{-2} s^{-1} before the acclimation and at 40 days of stress treatment. The electron transfer rate (ETR) driving PSII was determined by the following formula:

$$ETR = \left(\frac{F_m' - F_s}{F_m'}\right) f I \alpha_{leaf}$$

where f is the fraction of absorbed quanta by PSII (0.5), I is incident photon flux (1200 μmol m^{-2} s^{-1}), and α_{leaf} is leaf absorptance (0.85). ETR was calculated on fresh weight (FW) basis by multiplying the specific leaf area and leaf water content.

Non-photochemical quenching (q_N), which represents excess energy dissipation through the xanthophyll cycle, is calculated by the following formula:

$$q_N = \frac{F_m - F_m'}{F_m - F_o'}$$

Physiological measurements

Membrane lipid peroxidation (MDA), H_2O_2 content, ascorbic acid (AsA) content, and APX enzyme activity were recorded twice, before the acclimation and at 40 days of stress exposure. Membrane lipid peroxidation was determined by malondialdehyde (MDA) content using the thiobarbituric acid (TBA) method and a modified version of the ferrous ammonium sulphate / xylenol orange (eFOX) method was used to measure H_2O_2 content of leaves as described in details in Chapter 2.1.

The content of AsA was assayed as described by Kampfenkel et al. (1995). Frozen leaf material (50 mg) was ground in 0.8 mL of cooled 6% TCA solution. The homogenate was made up to 2 mL using 6% TCA and centrifuged at 15,000 rpm (18,866 *g*) for 5 min at 4 °C. The homogenate (0.2 mL) in a glass tube was mixed with 0.6 mL 0.2 M phosphate buffer (pH 7.4), 0.2 mL double-distilled water, 1 mL 10% TCA, 0.8 mL 42% H_3PO_4, 0.8 mL 4% 2,2-dipyridyl, and 0.4 mL 3% $FeCl_3$. The solution was incubated at 42°C in a water bath for 40 min and the absorbance was read at 525 nm using a spectrophotometer.

Total APX activity was measured by the method described by Amako et al. (1994). Frozen leaf tissue (50 mg samples) was homogenized with 200 µL of the homogenizing solution containing 1 mM AsA, 1 mM EDTA, and 50 mM potassium phosphate (pH 7.0). The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM ascorbic acid, and an aliquot of the extract. The reaction was started by adding 0.5 mM hydrogen peroxide. Decrease in absorbance for a period of 30 s was measured at 300 nm using a spectrophotometer, with an absorption coefficient of 0.49 mM^{-1} cm^{-1}. The relative increase in activity following the stress treatment (APX %) was calculated as follows:

$$APX\ \% = (APX_{40} - APX_{control}) / APX_{control} \times 100.$$

moderate heat stress and (2) how H_2O_2 content and scavenging systems lead to physiological damage (summer damage seen in the field and Fv/Fm) as a result of heat stress. The models were refined by minimizing the root-mean-square error of approximation (RMSEA) and AIC. Maximum likelihood chi-square (χ^2) was used to test the goodness of fit of the models (probability (p) $\geq$ 0.05 is considered acceptable). SEM analysis was performed by using Amos (ver. 18, SPSS Institute, IBM, New York, USA).

RESULTS

Field tolerance, which ranged from 1.5 (Norlea) to 7.0 (Yatugadake-24), was significantly different among the 25 cultivars (P <0.001, Table 3.1). There was also a highly significant difference between the diploid and the tetraploid cultivars (P <0.001): the mean value of field tolerance of the tetraploid cultivars was 5.0 compared to that of 2.8 of the diploid cultivars.

Figure 3.1 shows temporal changes in the maximal PSII activity (Fv/Fm) during stress treatment in the laboratory. There were significant differences in Fv/Fm among the cultivars every time it was measured (Table 3.2). The value of Fv/Fm decreased gradually with increasing duration of the stress whereas differences among the cultivars widened at the same time, peaking at 40 days, and ranged from 0.58 to 0.75. The value of Fv/Fm at 40 days of stress was not significantly correlated with that before the treatment (r = 0.26). Although the difference between the diploid and tetraploid cultivars was not significant before the treatment, the tetraploid cultivars recorded significantly higher values of Fv/Fm when subjected to heat stress (Table 3.2). Although the correlation between field tolerance and Fv/Fm was not statistically significant before the treatment (r = 0.08), the correlation between them became significant 10 days (r = 0.53**) after the treatment began and then increased gradually from r = 0.47 (P <0.05) at 20 days to r = 0.70*** (P <0.001) at 40 days (Table 3.2). These patterns of correlation demonstrate that the value of Fv/Fm under prolonged exposure to moderate heat stress in the laboratory is a good indicator of field tolerance.

Although H_2O_2 content and the magnitude of lipid peroxidation (MDA) did not show any significant differences among the 25 cultivars before the treatment, the differences were significant at 40 days of stress exposure (Table 3.3), with the tetraploid cultivars

recording significantly lower H_2O_2 content and MDA than the diploid cultivars (Figure 3.2).

H_2O_2 content showed a significant correlation with field tolerance (r = –0.66**) and with Fv/Fm value at 40 days (r = –0.78**), as shown in Figure 3.2; MDA also showed a significant correlation with field tolerance (r = –0.65**) and with Fv/Fm value (r = –0.62**). These results demonstrate that ROS generated under prolonged exposure to moderate heat stress are a major cause of the differences in heat tolerance among the cultivars.

Electron transport rate (ETR), SLA, and its components also showed significant differences among the 25 cultivars, but no significant difference was found for non-photochemical quenching (q_N), as shown in Table 3.3. Only leaf thickness (LT) showed a significant difference between the diploids and the tetraploids. H_2O_2 content showed a significantly positive correlation with ETR (r = 0.56**) but not with q_N (r = –0.17), as shown in Figure 3.3 and Table 3.4. On the other hand, H_2O_2 content showed a negative correlation with LT (r = –0.63**), a positive correlation with leaf dry matter concentration (LD, r = 0.41*), and a negative correlation with leaf water content (LWC, r = –0.48*) but not with SLA (r = 0.19). The content of ascorbic acid (ASA), an antioxidant, showed significant differences among the cultivars before the treatment as well as at 40 days of stress exposure. However, AsA did not show any significant correlation with H_2O_2 content (r = –0.11). Stress-induced change in the activity of ascorbate peroxidase (APX%) also did not show a significant correlation with H_2O_2 (r = –0.30).

A stepwise multiple regression of H_2O_2 content as a dependent variable selected LT and ETR and explained 50.0% of the variation in H_2O_2 content (Table 3.4). A multiple regression of Fv/Fm as a dependent variable selected only two variables, H_2O_2 content and AsA, whereas field tolerance selected seven variables (H_2O_2, MDA, q_N, AsA, APX%, SLA, and LWC).

I constructed SEMs explaining the variation in Fv/Fm and field tolerance among the 25 cultivars (Figure 3.4a,b). The models selected six independent variables: H_2O_2 content, MDA, AsA, APX%, ETR, and LT. The model (a) explained 68% of the variation in Fv/Fm and showed that the maximum likelihood test was not significant (χ^2 = 8.578, 13 df, P = 0.804), which shows the consistency of the model with the data.

Main sites for ROS production in leaves are PSI and PSII in chloroplasts (Asada 1999). The redox state of the electron transport chain in thylakoids is determined by the total amount of photons trapped by the photosystem and by the system's capacity to dissipate excess energy through the xanthophyll cycle. Electron transport rate (ETR), which estimates the actual flux of photons driving PSII, showed a significant correlation with H_2O_2 content, whereas non-photochemical quenching (q_N), which represents the capacity of the xanthophyll cycle, did not show a clear correlation with H_2O_2 content, suggesting the involvement of excess flow of light energy through the thylakoid membrane into ROS production. On the other hand, leaf thickness contributed the most to H_2O_2 content. Leaf thickness characterizes the differences between sun and shade leaves in terms of their anatomy as well as their photosynthetic responses to light intensity (Terashima et al. 2006). Under high irradiance, the rate of photosynthesis in leaves under shade, which are thin, is restricted by low Rubisco content per unit leaf area (Evans and Poorter 2001). Lower Rubisco content of thin leaves under heat stress seems to lead to imbalance between biochemical activity and photochemical activity and, in turn, to accumulation of ROS. Furthermore, in thinner leaves, the thinner mesophyll and the resulting smaller surface area of chloroplasts leads to low CO_2 diffusion to Rubisco (Terashima et al. 2006). Such decreased CO_2 diffusion to Rubisco is also likely to increase the rate of photorespiration, which results in greater H_2O_2 generation in peroxisomes (Sharkey 2005). Although no significant reduction in Rubisco activity was observed until 10 days of the stress (Chapter 2.1), I suggest occurrence of imbalance between Rubisco activity and electron transport capacity, which is largely determined by leaf thickness, after prolonged exposure to the stress. Leaf thickness played a pivotal role in determining the variation in H_2O_2 content in leaves under prolonged exposure to moderately high temperatures.

Antioxidant activity (AsA content and APX%) did not show any significant correlation with H_2O_2 content. However, these low correlations do not imply that the effects of these antioxidants on heat tolerance are negligible because antioxidant activity was significantly correlated with field tolerance. The inconsistent contribution of the two antioxidants with Fv/Fm in the laboratory and with field tolerance suggests complex networks of the antioxidant system in plants.

The comparison of ploidy difference in heat tolerance showed that the tetraploid

cultivars were significantly more tolerant in the field than diploid cultivars. The lower H_2O_2 content and lower lipid peroxidations in the tetraploids suggest that their greater ability to cope with heat lies in their higher capacity to suppress H_2O_2 accumulation. There were no significant differences in ETR, q_N, AsA, and APX% between the diploid and tetraploid cultivars (Table 3.3); the two differed in LT, however, and even among the tetraploids, cultivars with thinner leaves had higher H_2O_2 content and lower heat tolerance (Figure 3.4). These results demonstrate that the higher heat tolerance of tetraploid is due to their thicker leaves rather than to genetic effects of chromosome doubling.

Waines (1994) reported that polyploid species in wild wheat and spring wheat tend to have higher heat tolerance than diploid species do. The higher tolerance of tetraploid cytotype to drought stress has also been reported in *Arabidopsis thaliana* (Bouharmont and Mace 1972), *Phlox drummondii* (Garbutt and Bazzaz 1983), and *Chamerion angustifolium* (Maherali et al. 2009). Polyploid plants have a wider geographical range than their diploid ancestors (Stebbins 1971; Levin 2002), and the increased physiological tolerance of polyploids to abiotic sources of stress, which is probably due to their greater ability to suppress ROS production, is likely to play a role in their greater tolerance to ecological amplitude to cope with stress in natural habitats.

Since field-grown plants during summer are subject to complex sources of abiotic stress including high irradiance, prolonged heat stress, and unpredictable water deficit, plants require a range of mechanisms to protect them from heat-induced oxidative stress. This study demonstrates that low H_2O_2 production and high antioxidant activity (AsA and APX %) contribute to greater heat tolerance. Furthermore, heat-shock proteins have been shown to play a major role in heat tolerance in C_3 grasses under field conditions (Park et al. 1996; Queitsch et al. 2000). Among these potential mechanisms influencing heat tolerance, the role of structural properties of leaves such as leaf thickness and leaf dry matter concentration has been neglected. Leaf structure, particularly specific leaf area (SLA), is a key trait mediating the trade-off between the rate of photosynthesis per unit leaf mass and the life span of a leaf (Reich et al. 1997) which is the worldwide leaf economic spectrum (Wright et al. 2004). The significant correlation between such structural properties of a leaf as thickness and dry matter concentration and H_2O_2 content suggests that structural properties play an important role in tolerance to

oxidative stress by changing the balance between the electron transport capacity of thylakoids (photochemical response) and Rubisco activity in the stroma (biochemical response) under heat stress.

Table 3.1 Cultivars of *Lolium perenne* and the degree of their field tolerance to high summer temperatures evaluated at the Yamanashi Dairy Experimental Station. The high values of field data represent high tolerance.

Cultivar	Country of origin	Field tolerance
	Diploids (2n)	
Norlea	Canada	1.5
Barmilka	The Netherlands	2
Paddok	Belgium	2
Pagode	The Netherlands	2
Mongita	The Netherlands	2.5
Olaf	Czech Republic	2.5
Record	The Netherlands	2.5
Synerga	The Netherlands	2.5
Ilirka	Slovenije [Slovenia?]	3
Raidi	Estonia	3
Sponsor	The Netherlands	3
Tobago	The Netherlands	3
Weigra	Germany	3
Grasslands Samson	New Zealand	6.5
	Tetraploids (4n)	
Raigt	Norway	2
Phoenix	Netherlands	2.5
Roderick	Netherlands	2.5
Meretti	Belgium	3
Yatsuyutaka	Japan	5
Yatukaze	Japan	6
Merlov	Belgium	6.5
Pomerol	Belgium	6.5
Yatugadake-2	Japan	6.5
Merkem R.v.P	Belgium	7
Yatugadake-24	Japan	7
Statistical difference		
Population		***
Ploidy		***

Table 3.2 One-way ANOVA of chlorophyll fluorescence (Fv/Fm) among 25 cultivars of *Lolium perenne* and between their diploid and tetraploid populations as well as the changes in correlation coefficient between chlorophyll fluorescence and field tolerance at different durations of continuous exposure to heat stress.

Days of exposure	F value		Correlation coefficient
	Cultivars	Ploidy	
0	3.93***	0.92	0.08
10	2.31**	7.39**	0.53**
20	2.48**	2.78	0.47*
30	5.52***	6.31*	0.67***
40	14.28***	23.98***	0.70***

*, **, and *** represent significance at probability levels of 5%, 1%, and 0.1 %, respectively.

Table 3.3 Hydrogen peroxide content (H_2O_2), malondialdehyde (MDA), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA), ascorbate peroxidase activity (APX), leaf area (LA), specific leaf area (SLA), leaf water content (LWC), leaf thickness (LT), and leaf dry matter concentration (LD) of 25 cultivars of *Lolium perenne* and mean values for the diploid and tetraploid groups. ANOVA was conducted among the 25 cultivars and between the diploids and the tetraploids.

Variable	Population range	Mean values		F value	
		2n	4n	Cultivars	Ploidy
H_2O_2 (μmol mg^{-1} FW, 0 day)	0.34 – 0.57	0.43	0.42	0.91	0.61
H_2O_2 (μmol mg^{-1} FW, 40 day)	0.61 – 0.99	0.82	0.70	2.32**	15.86***
MDA (μmol g^{-1} FW, 0 day)	8.42 – 18.98	15.42	13.46	1.33	3.73
MDA (μmol g^{-1} FW, 40 day)	15.97 – 31.30	24.90	19.57	5.51***	22.11***
ETR (μmol g^{-1} FW s^{-1}, 0 day)	0.51 – 0.97	0.79	0.70	2.87***	7.13**
ETR (μmol g^{-1} FW s^{-1}, 40 day)	0.27 – 0.65	0.43	0.37	2.15*	3.74
q_N (0 day)	0.79 – 0.84	0.82	0.82	1.14	0.19
q_N (40 day)	0.85 – 0.93	0.89	0.88	0.82	2.88
AsA (μmol mg^{-1} FW, 0 day)	21.72 – 50.89	37.21	38.73	7.59***	0.50
AsA (μmol mg^{-1} FW, 40 day)	44.83 – 68.54	53.15	56.2	2.54**	2.61
APX (Unit mg^{-1} FW, 0 day)	22.35 – 41.64	29.97	27.45	1.16	2.22
APX (Unit mg^{-1} FW, 40 day)	30.33 – 55.03	42.89	42.02	1.08	0.15
Leaf trait (0 day)					
LA (cm^2)	5.88 – 15.42	8.79	10.97	6.00***	16.17***
SLA (mm^2 mg^{-1})	18.35 – 38.75	29.00	27.11	9.06***	1.66
LWC	0.69 – 0.83	0.77	0.79	10.92***	2.31
LT (μm)	153 – 217	175	193	2.75**	13.96***
LD (mg cm^{-3})	141 – 305	210	202	6.59***	0.42

Table 3.4 Correlation coefficients (r) of field tolerance and Fv/Fm with hydrogen peroxide content (H_2O_2), malondialdehyde (MDA), electron transport rate (ETR), non-photochemical quenching (q_N), ascorbic acid (AsA), ascorbate peroxidase change (APX%), leaf area (LA), specific leaf area (SLA), leaf water content (LWC), leaf thickness (LT), and leaf dry matter concentration (LD). Standardized regression coefficient (s) of the selected variables by stepwise multiple regression analysis and their proportion of variance explained (R^2) and Akaike's information criteria difference from the full model (ΔAIC) are shown. The correlations and the model explaining the variation in H_2O_2 content are also shown.

	Field tolerance		Fv/Fm		H_2O_2	
	r	s	r	s	r	s
H_2O_2	–0.66**	–0.46	–0.78**	–0.74	--	--
MDA	–0.65**	–0.31	–0.62**		--	--
ETR	–0.37		–0.34		0.56**	0.31
q_N	–0.42*	–0.34	–0.09		0.17	
AsA	–0.43*	0.29	0.39	0.31	–0.11	
APX%	0.38	0.24	0.05		–0.30	
LA	–0.07		0.02		–0.12	
SLA	0.40	0.76	0.20		–0.18	
LWC	0.53**	–0.67	0.39		–0.48*	
LT	0.43*		0.59**		–0.63**	–0.37
LD	–0.54**		–0.44*		0.4*	
R^2 (%)		76.0		67.1		56.0
ΔAIC		5.39		10.95		6.08

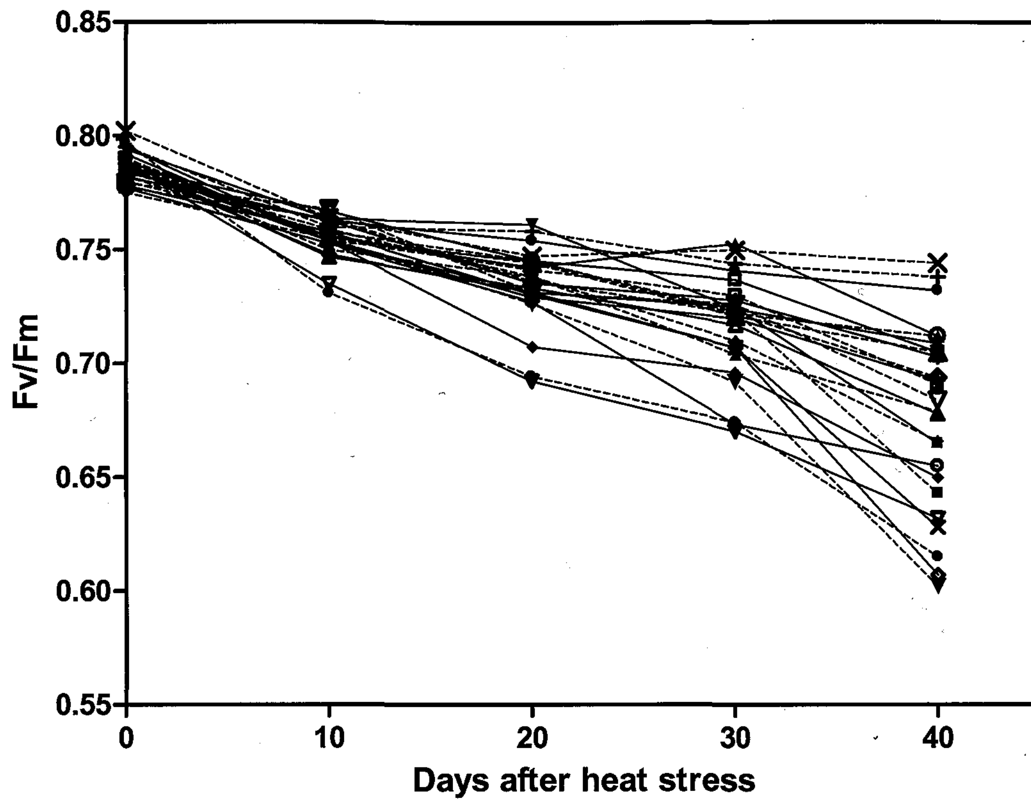


Figure 3.1 Response patterns of chlorophyll fluorescence in 25 cultivars of *Lolium perenne* at different durations (days) of continuous exposure to heat stress.

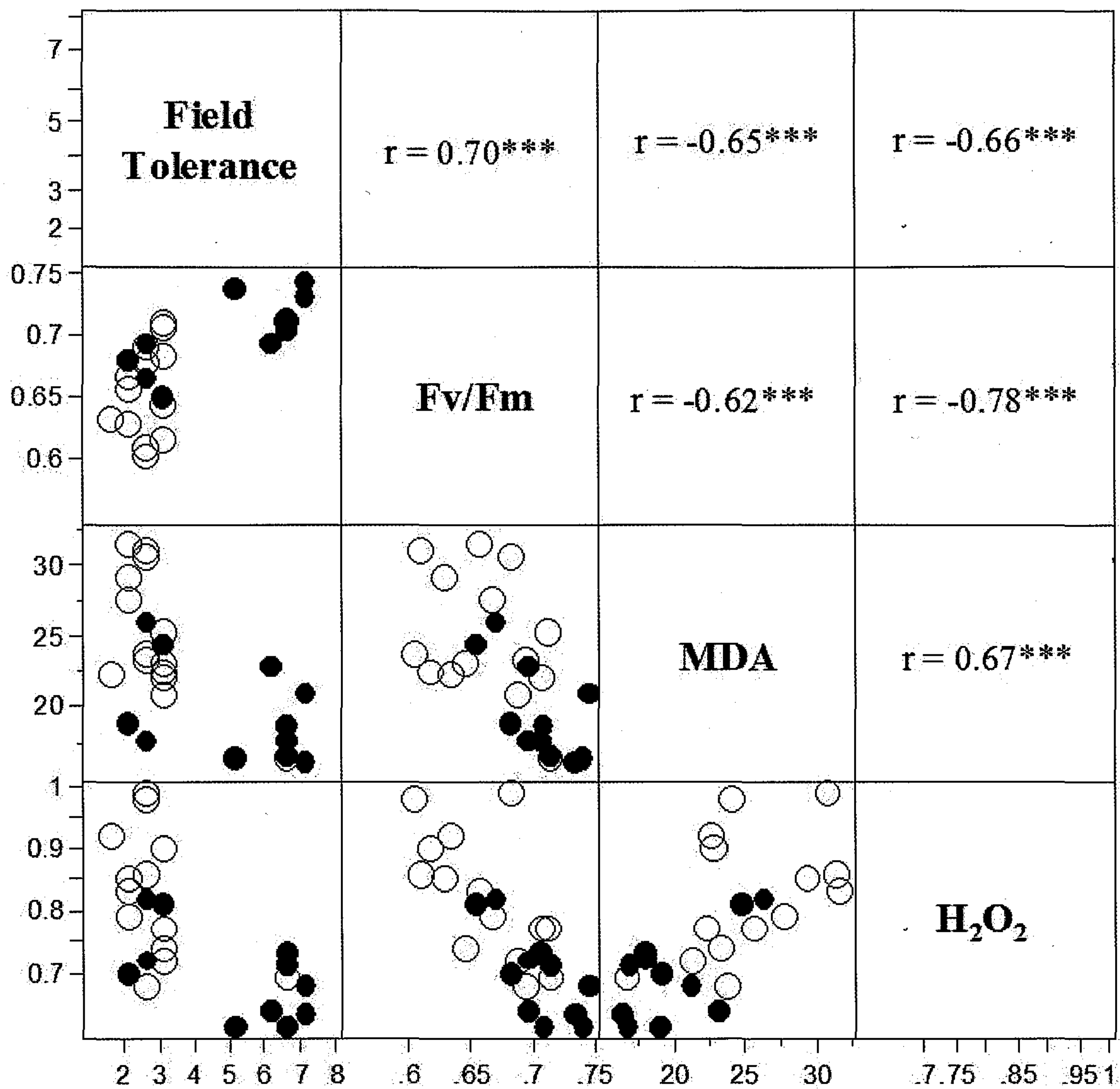


Figure 3.2 Scatterplot matrix of field tolerance, chlorophyll fluorescence (Fv/Fm), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2) of 25 cultivars of *Lolium perenne* (○ and ● are diploid cultivars and tetraploid cultivars respectively). Data at 40 days of heat stress were used for Fv/Fm, MDA and H_2O_2 (*** represents significance at p <0.001).

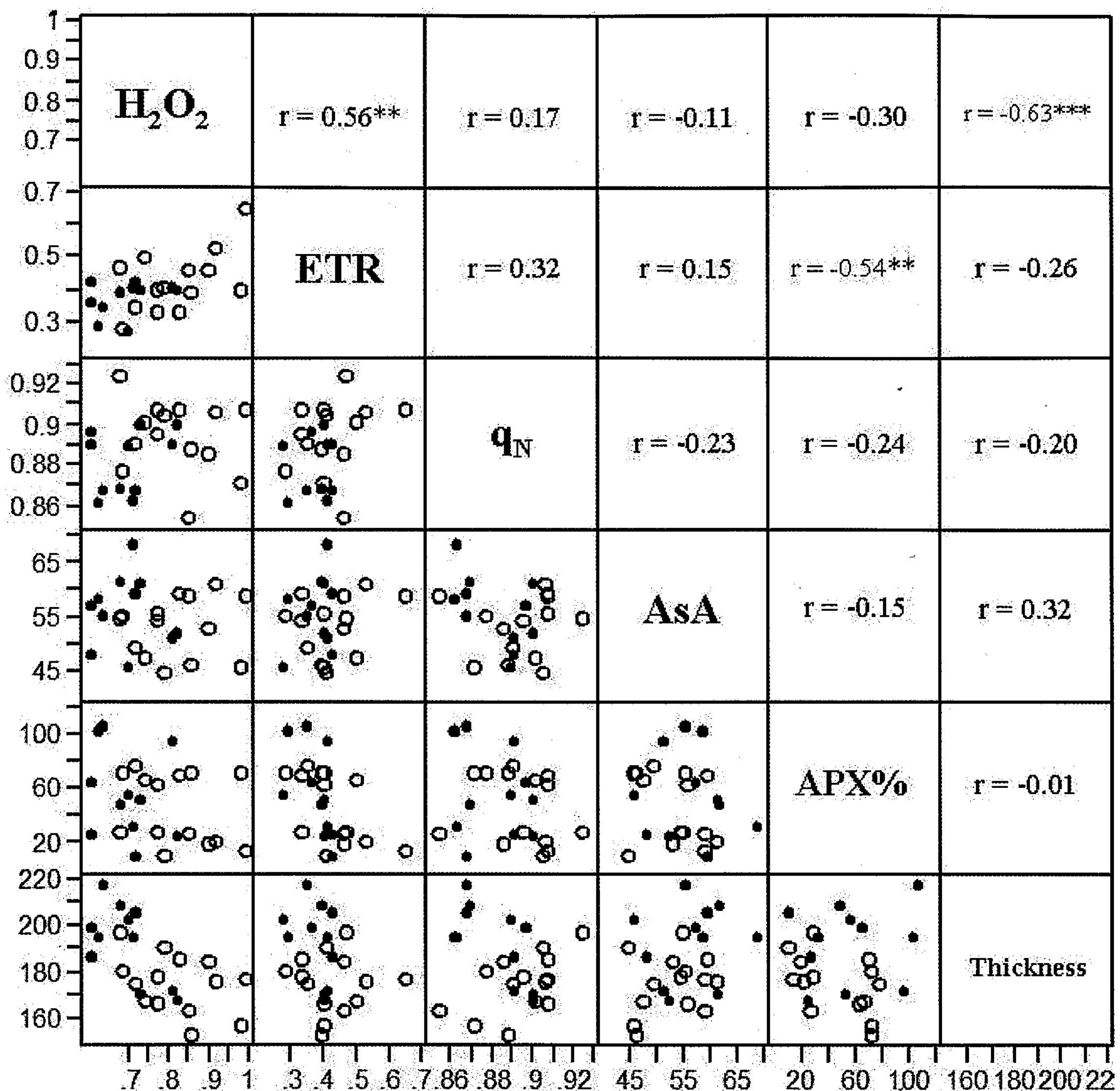


Figure 3.3 Scatterplot matrix of hydrogen peroxide (H_2O_2), electron transport rate (ETR), non-photochemical quenching (q_N), and ascorbic acid (AsA) at 40 days of heat stress as well as relative change in ascorbate peroxidase due to stress treatment (APX%) and leaf thickness before stress in 25 cultivars of *Lolium perenne* (○ and ● are diploid cultivars and tetraploid cultivars respectively). ** and *** represent significance at p <0.01 and 0.001 respectively).

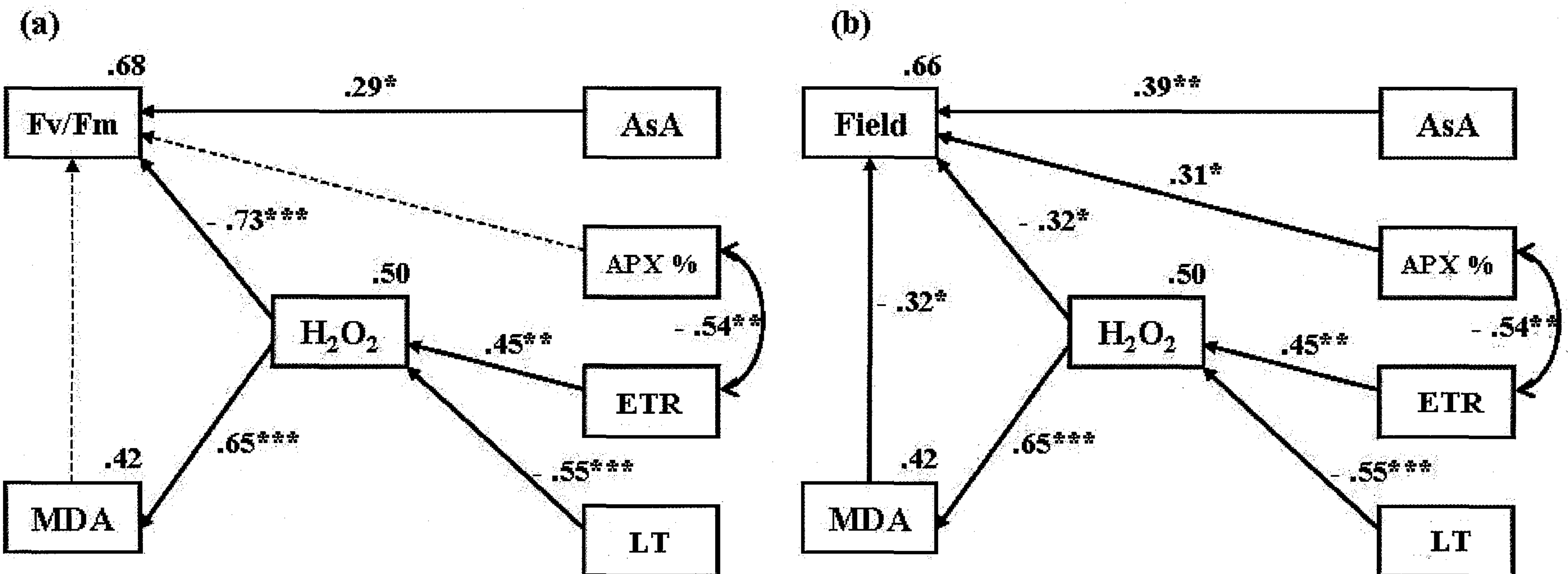


Figure 3.4 Causal modeling illustrating the effect of different traits on (a) Fv/Fm with [χ^2 = 8.578, 13 df, P = 0.804,TLI = 1.118, RMSEA = 0.000, AIC = 38.578] and (b) summer field tolerance with [χ^2 = 8.737, 13 df, P = 0.792, TLI = 1.118, RMSEA = 0.000, AIC = 38.737]. The data set contains malondialdehyde (MDA), hydrogen peroxide (H_2O_2), ascorbic acid (AsA), and electron transport rate (ETR) at 40 days of high-temperature treatment as well as relative increase in APX (APX%) and leaf thickness (LT, before exposure to stress).

Chapter 4

GENETIC BACKGROUND OF HEAT TOLERANCE IN PROGENIES FROM A CROSS BETWEEN TOLERANT AND SENSITIVE GENOTYPES IN *Lolium perenne*

INTRODUCTION

The potential for improving heat tolerance of plants through breeding is encouraging if the variation in heat tolerance is genetically controlled. Knowledge of the genetic basis of variation in heat tolerance would help to estimate the heritability of the trait and to select effectively tolerant plants. The extent of damage caused by exposure to heat stress differs between the genotypes (Basra 2000). In the previous study, *Lolium perenne* cultivars including diploids and tetraploids were compared (Chapter 3). In this study, the differences in response of genotypes derived from a cross between a heat-tolerant and a heat-sensitive cultivar were examined. The purpose of this study was to examine the genetic basis of variation in heat tolerance.

MATERIALS AND METHODS

A cross between a tolerant (Kangaroo) and a sensitive cultivar (Norlea) was conducted in Yamanashi Experimental Station and in this study 72 genotypes derived from a cross between them were used. Kangaroo was developed in Australia, while Norlea was developed in Canada and both of them are diploids. All plants were grown in the same conditions as mentioned in Chapter 2.1. After acclimation at 30°C for three days, the plants exposed to heat treatments (36/30°C, day/night temperatures) for 40 days.

Chlorophyll fluorescence (Fv/Fm) was measured before the acclimation and at 10-day intervals, while hydrogen peroxide (H_2O_2) content was measured twice, before and at 40 day of the stress. The leaf traits were measured before the stress. The methods of measurement were described in detail previously (Chapter 2.1 and Chapter 3).

Statistical analysis

Analysis of variance (ANOVA) was used to test the significance of differences either between the two parents or among the genotypes for each measurement. The statistical

analysis was carried out using JMP (ver 4. SAS Institute, Cary, NC, USA).

RESULTS

There were no significant differences between the two parent genotypes before the the exposure to stress in chlorophyll fluorescence (Fv/Fm) and hydrogen peroxide (H_2O_2). The two parents showed significant decreases in Fv/Fm and significant increases in H_2O_2 after the stress. Significant differences between the two parents appeared at 30 day of the stress for Fv/Fm and at 40 day of the stress for H_2O_2 (Figures 4.1 and 4.2). The 72 progenies showed highly significant differences in Fv/Fm at all measurement times (Table 4.1). The value of Fv/Fm decreased gradually with increasing duration of the exposure to stress and a broad genetic distribution was shown at 40 day of the stress (Figure 4.3a). Comparison between the 72 progenies and the parents showed that values of Fv/Fm of most progenies (72%) were intermediate between the two parents. On the other hand, the 72 progenies showed significant differences in H_2O_2 content both before and after the stress (Table 4.1). The content of H_2O_2 increased under the stress and a wide genetic distribution was shown among progenies at 40 day of the stress (Figure 4.3b). The genotypic distribution of H_2O_2 at 40 day of the stress showed that approximately two third of the progenies had H_2O_2 content in the range between the two parents. It is notable that Fv/Fm showed a highly significant correlation with H_2O_2 content at 40 day of the stress (Figure 4.4).

Structural leaf traits showed no significant differences between the two parents except for leaf water content and leaf thickness (Table 4.2). On the other hand, significantly differences were observed in all leaf traits among the 72 genotypes (Table 4.2). The genotypic distribution of leaf traits were shown in (Figure 4.5). Most progenies had values of leaf traits out of the range between the two parents except leaf water content (LWC), 65% of the progenies had LWC in the range between the parents (Figure 4.5c). About 60% of the progenies had leaf thickness out of the range between the parents (Figure 4.5d), while all the progenies excluding two had larger leaf area than the two parents (Figure 4.5a).

DISCUSSION

Maximal PSII efficiency (Fv/Fm) decreased with the duration of heat stress and highly

significant differences were observed either between the parents or among the progenies at 40 day of the stress (Figure 4.1 and Table 4.1, respectively). The decreases in Fv/Fm were associated with significant increases in H_2O_2 content at 40 day of the stress and a significant correlation was found between them (Figure 4.4). These results suggest that the difference in heat tolerance shown by progenies of *L. perenne* is closely associated with the ability to suppress oxidative stress. This is consistent with our findings among cultivars of *L. perenne* (Chapter 3). The vast majority of the progenies had Fv/Fm value and H_2O_2 content in the range between the two parents (Figure 4,3). This genetic distribution indicates the genetic effects and inheritance of the genes responsible for heat tolerance.

Leaf traits showed significantly differences among progenies but not between the parents except for leaf water content and leaf thickness. This indicates that the variation in leaf traits is less genetically controlled. No significant correlations of leaf traits with Fv/Fm and H_2O_2 content were shown at 40 day of the stress. This is not consistent with our finding in *L. perenne* cultivars (Chapter 3), which showed significant contribution of leaf traits, especially leaf thickness, to ROS generation and heat tolerance. This inconsistent pattern seems to result from the difference in genetic variability in leaf traits between the two parents and cultivars used in Chapter 3.

Table 4.1 Analysis of Variance for chlorophyll fluorescence (Fv/Fm) and hydrogen peroxide content (H_2O_2) among 72 genotypes at different durations of continuous exposure to heat stress.

Days of exposure	Range	*F* value
Chlorophyll fluorescence (Fv/Fm)		
0 day	0.762 ~ 0.807	2.71***
10 day	0.714 ~ 0.783	2.68***
20 day	0.603 ~ 0.778	3.01***
30 day	0.358 ~ 0.776	4.58***
40 day	0.483 ~ 0.767	25.43***
Hydrogen peroxide (H_2O_2)		
0 day	0.15 ~ 0.52	25.76***
40 day	0.32 ~ 1.74	27.95***

*** represents significance differences at 0.1%

Table 4.2 Analysis of Variance for leaf trait between the two parents and among the 72 genotypes derived from them before exposure to heat stress.

Leaf traits	The two parents			The 72 genotypes	
	Norlea	Kangaroo	*F* value	Range	*F* value
Leaf area (cm^2)	1.72	1.81	0.07^{ns}	0.87 ~ 7.44	7.48***
Specific leaf area (mm^2 mg^{-1})	26.96	24.43	1.01^{ns}	19.0 ~ 35.4	3.38***
Leaf water content (%)	81.3	77.5	8.96*	74.5 ~ 85.2	4.76***
Leaf thickness (μm)	169	198	7.75*	141 ~ 242	5.68***
Leaf density (mg cm^{-3})	221	211	0.27^{ns}	153 ~ 328	7.93***

*, *** represents the differences at 5 and 0.1%, respectively.

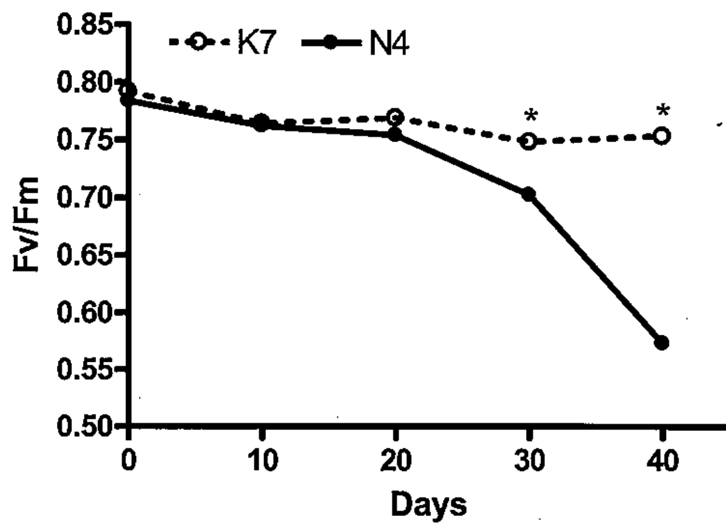


Figure 4.1 Temporal changes in chlorophyll florescence (Fv/Fm) for the two parents; Norlea (●) and Kangaroo (○) under hest stress. * indicates a significant difference between the three populations at $P < 0.05$.

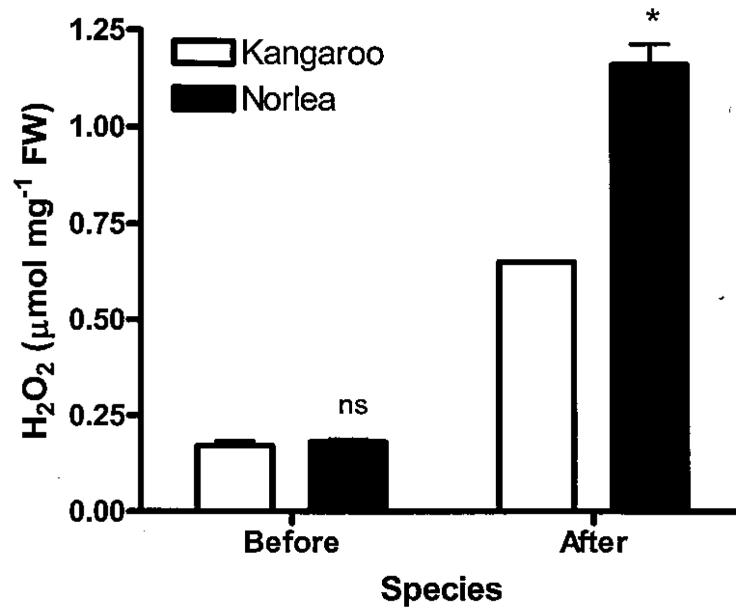
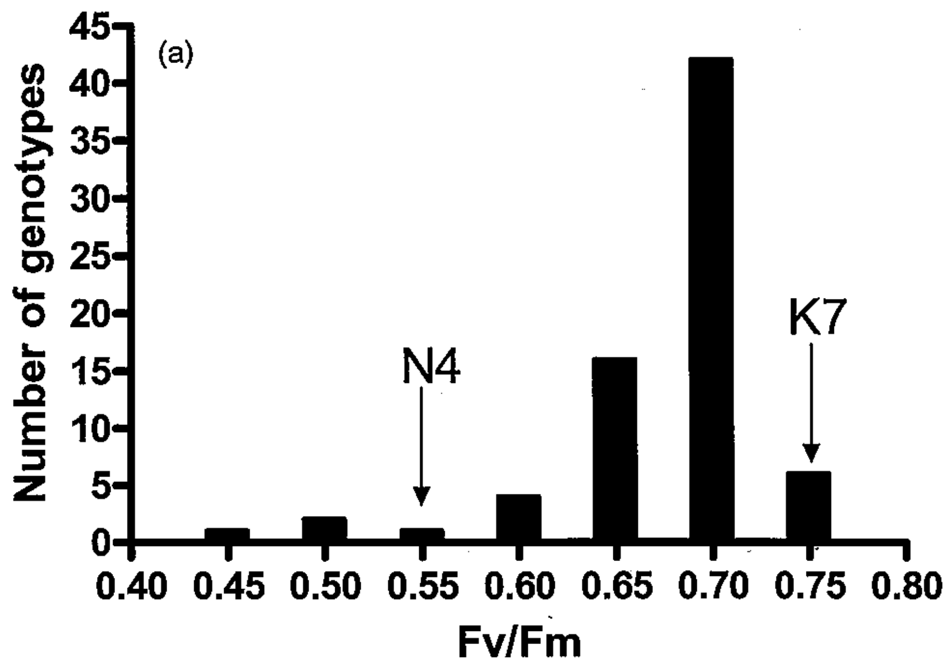


Figure 4.2 Hydrogen peroxide content (H_2O_2) before and after the exposure to heat stress for the two parents; Norlea (heat-sensitive) and Kangaroo (heat-tolerant). * represents the difference between parents at 0.1 %.



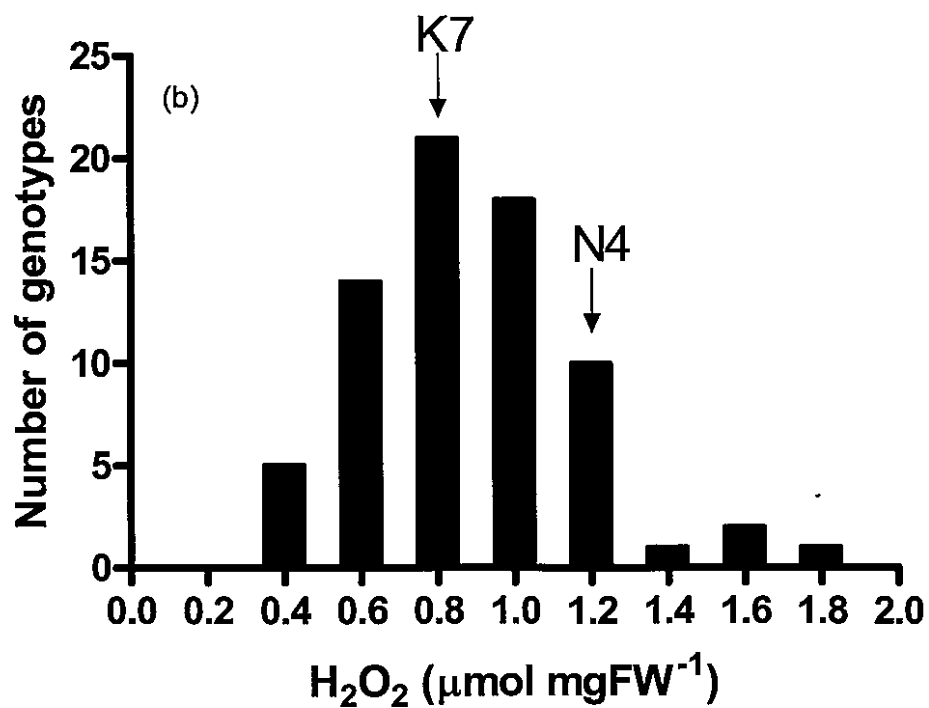


Figure 4.3 Frequency distribution of chlorophyll fluorescence (Fv/Fm, a) and hydrogen peroxide content (H_2O_2, b) at 40 days of exposure to stress in 72 progenies derived from a cross between Kangaroo and Norlea.

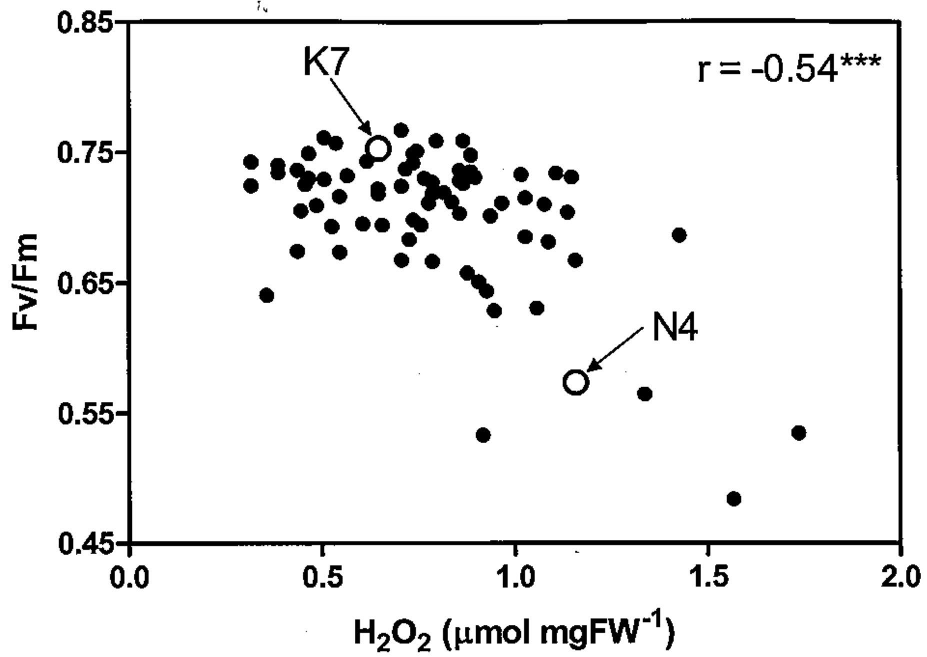


Figure 4.4 Correlation between chlorophyll fluorescence (Fv/Fm) and hydrogen peroxide (H_2O_2) at 40 days of exposure to stress among the 72 genotypes derived from the cross between Kangaroo (K7) and Norlea (N4).

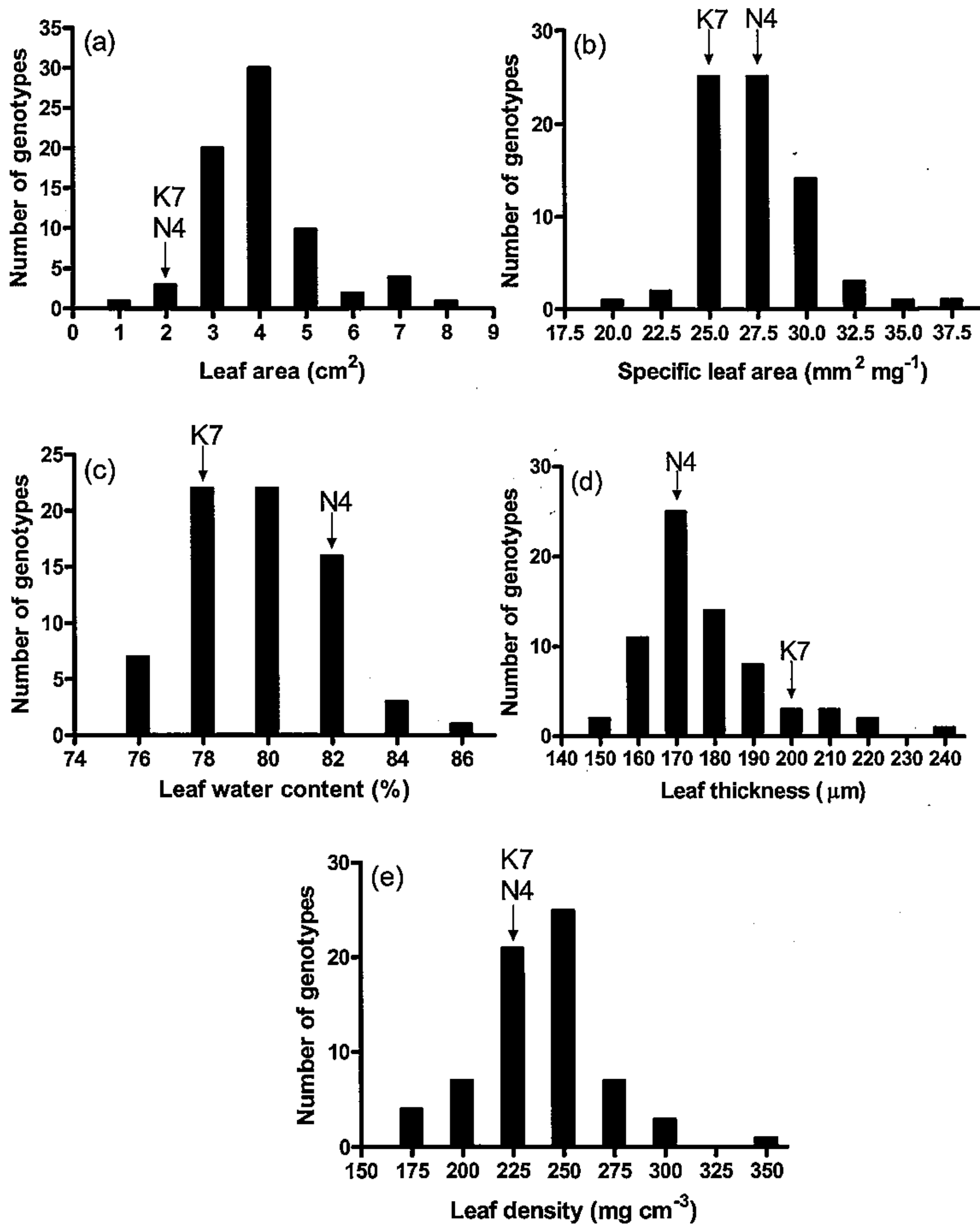


Figure 4.5 Frequency distributions of leaf area (a), specific leaf area (b), leaf water content (c), leaf thickness (d) and leaf density (e) before the exposure to stress in 72 progenies derived from a cross between Kangaroo (K7) and Norlea (N4).