PHOTOSYNTHETIC GAS EXCHANGE, ELECTRON TRANSPORT, FLUORESCENCE AND ELECTROLYTE LEAKAGE OF WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.) EXPOSED TO ENVIRONMENTAL STRESS

By

SENAYET ASSEFA

Bachelor of Science
Addis Abeba University
Alemaya, Ethiopia
1981

Master of Science
Addis Abeba University
School of Graduate Studies, Ethiopia
1984

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
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the Degree of
DOCTOR OF PHILOSOPHY
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Thesis Approved:

Thesis Advisor

Dean of the Graduate College

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CHAPTER I

PHOTOSYNTHETIC ELECTRON TRANSPORT, CHLOROPHYLL FLUORESCENCE, AND ELECTROLYTE LEAKAGE OF WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.). CHILLING LOW TEMPERATURE STRESS

SENAYET ASSEFA AND BJORN MARTIN

Abbreviations: BSA, bovine serum albumin; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DHQ, durohydroquinone (tetramethyl-*p*-hydroquinone); e^-, electron; Fo, initial fluorescence; Fm, maximal fluorescence; Fv, variable fluorescence (Fv = Fm - Fo); MV, methyl viologen; PAR, photosynthetically active radiation; PSI and PSII, photosystem I and PSII; SOD, superoxide dismutase; $t_{1/2}$, time required for fluorescence to rise from Fo to half its maximum value.

ABSTRACT

Three tests were used to determine photosynthetic competence and cellular membrane stability of the C_4 cereal tef (*Eragrostis tef Z.* cv. DZ-01-354) and the C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101). Plants were grown in growth chambers and exposed for periods of up to 168 h to chilling low temperature (2°C, 7°C, and 12°C) stress. The photochemical properties were evaluated at 25°C following chill exposure by measuring electron transport rates of isolated thylakoids and chlorophyll fluorescence of dark adapted leaves. Membrane integrity was quantified by measuring electrolyte leakage of leaf samples. Exposure of tef to chilling low temperatures reduced photosystem II (PSII) and whole chain (PSII + PSI) electron transport rates but not PSI electron transport rate. All components of photosynthetic electron transport were less affected by chilling in wheat. The greater susceptibility of electron transport of tef than wheat to chilling was consistent with susceptibility rankings based on reduced variable to maximum fluorescence (Fv/Fm). The decline in Fv/Fm was accompanied by increasing time required for Fv to rise from the minimal fluorescence (Fo) to half its maximum value ($t_{1/2}$). Fv/Fm correlated well with PSII and whole chain electron transport rates in both species. However, the slope of the dependence of Fv/Fm on electron transport was much greater in tef than in wheat. The high correlation suggests that Fv/Fm accurately estimated *in vivo* photosynthetic electron transport rates. Electrolyte leakage values confirmed the

greater susceptibility of tef than wheat to chilling low temperatures that had previously been found by measurements of electron transport and Fv/Fm. However, chlorophyll fluorescence and electron transport detected effects of chilling at mild stress levels whereas electrolyte leakage increased first at lower temperatures or longer exposure times.

INTRODUCTION

Chilling stress impairs numerous physiological functions of plants. Depending on the severity and duration of chilling, chill susceptible plants suffer from impaired respiration and membrane leakage (King and Ludford, 1983), reduced protoplasmic streaming and chloroplast activity (Dai et al., 1987), diminished PSII electron flow (Labate et al., 1990), and lowered photochemical quenching (Krall and Edwards, 1991; Labate et al., 1990). The primary cause of these changes has not been clearly established. Damage to the oxidizing side of PSII (Dai et al., 1987) and to whole chain electron transport (Bruggemann, 1992) in chilled cucumber and *Vigna* species, respectively, has been reported. The photochemical quenching coefficient (q_p) as well as CO_2 assimilation decreased in parallel with decreasing temperature (Krall and Edwards, 1991). Labate et al. (1990) suggested that thylakoid phase transitions interfere with electron flow. According to Bruggemann (1992) and Labate et al. (1990) neither the PSII reaction center nor the water splitting system limit electron flow.

Quantifying the effects of temperature stress is difficult. Chlorophyll fluorescence has been measured (van Kooten and Snel, 1990) in many detailed studies on photosynthesis (for reviews see Krause and Weis, 1991; Krall and Edwards, 1992). The fluorescence parameter most often employed in stress studies is Fv/Fm, a measure of the quantum efficiency of PSII photochemistry (Oquist and Wass, 1988; Krause and Weis, 1991). Considerable advances have been made in interpreting

fluorescence data as they relate to temperature effects on PSII electron transfer (Hetherington et al., 1983; Krause and Weis, 1984; Hetherington and Oquist, 1988; Neuner and Larcher, 1990). Critchley (1981) suggested that the effects of chilling on Fv/Fm followed from inhibition of PSII electron transport. Chilling stress also leads to increased plasma membrane permeability (Parkin and Kuo, 1989; Bergevin et al., 1993). However, information is lacking on whether the temperature that causes such changes is identical to the temperature causing loss of photosynthetic activity. Lyons and Raison (1970) proposed involvement of membrane lipids in chilling injury. Consequently, the relationship between chilling sensitivity and lipid composition (Bergevin et al., 1993) and eventually electrolyte leakage, has received attention. Electrolyte leakage has been used to quantify cell membrane injury in various plant parts such as leaves, pericarp and fruits (King and Ludford, 1983; MacRae et al., 1986; Bergevin et al., 1993).

In many studies leaf samples have been exposed to cold for short periods, usually a few minutes or hours. Fewer attempts have been made to examine effects of extended chilling exposure on photosynthetic activities and growth. The relationship, if any, between changes in (plasma) membrane stability and photochemical components is poorly understood.

The objective of this study was to compare photosynthetic properties and cellular membrane stability of two cereal crops subjected to different magnitudes and durations of chilling low temperature stress, and to asses the correlation between the

various measurements of stress injury. For this purpose three properties were measured: 1) *in vivo* chlorophyll fluorescence, 2) photosynthetic electron transport, and 3) electrolyte leakage of leaf tissue. We investigated the effects following various durations and levels of chilling low temperature.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Exposure

Seeds of the temperate C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101) and the subtropical C_4 cereal tef (*Eragrostis tef* Z. cv. DZ-01-354) were grown in controlled environment growth chambers (Conviron CMP 3244, Winnipeg, Manitoba, Canada) in pots containing a mixture of peat moss and top soil (1:1, v/v). Wheat and tef were maintained at 25°C/18°C and 30°C/23°C, respectively, day/night temperature and 14 h photoperiod. Thirty days after planting, plants were exposed to chilling low temperatures (2°C, 7°C, and 12°C) for up to 168 h. Control plants remained at the original temperature. Measurements were made at 24 h intervals.

Chlorophyll Fluorescence

Measurements were made with a portable fluorometer (Polar Tech, Umea, Sweden) (Oquist and Wass, 1988). Four fully expanded leaves were sampled from each pot (4 leaves/pot x 4 pots = 16 leaves) and placed in black plastic sleaves with holes to guide the measuring probe. The leaves were dark adapted for 10 min prior to a 5 s exposure to 100 μmol m^{-2} s^{-1} PAR. Fo, Fm, Fv, Fv/Fm and $t_{1/2}$ were measured.

Chloroplast Isolation

Chloroplasts were isolated from leaves of 30-day-old wheat and tef plants using the procedure of Kee et al. (1986). Twenty g leaves from a five-plant-pot were homogenized for 3-4 s in a Waring blender (Model 7011-31b192, Waring, Hartford,

CT) in 200 mL ice cold isolation medium containing 30 mM Tricine (pH 7.8), 300 mM NaCl, 3 mM $MgCl_2$ and 0.5 mM EDTA. The homogenate was filtered through 16 layers of cheesecloth (Veratec, Inc., Walpole, MA) and centrifuged at 1500 g for 2 min in a high speed refrigerated centrifuge (ICE Model B-201, Damon/ICE Division, Needham Heights, MA). Pellets were resuspended with a cotton tip in 60 mL resuspension medium containing 200 mM sorbitol, 2 mM $MgCl_2$, 5 mM HEPES-KOH (pH 7.5) and 0.05% (w/v) BSA. Course debris was removed by a short centrifugation. The supernatant was filtered through one layer of Kimwipe and recentrifuged at 1500 g for 2 min. The pellets were resuspended in a small volume of resuspension medium and stored on ice.

Chlorophyll Content

Chlorophyll content of chloroplast suspensions was determined spectrophotometrically (Spectronic 1201, Milton Roy, Rochester, NY) according to Arnon (1949) and converted from mass to molar values using a molecular weight of 900 for chlorophyll.

Photosynthetic Electron Transport

Electron transport rates of isolated thylakoids were measured by monitoring light saturated O_2 evolution or consumption with a temperature controlled Clark-type oxygen electrode assembly (Model LD-2, Hansatech Limited, King's Lynn, UK) at 25°C. Thylakoids were assayed for whole chain ($H_2O \rightarrow MV$), PSII ($H_2O \rightarrow DAD_{ox}$) and PSI ($DHQ \rightarrow MV$) electron transport rates. The procedures described by Kee et al. (1986) for tomato, Allen and Holmes (1986) and Sabat et al. (1991) for spinach, beet, and pea were employed with slight modifications. Briefly, whole chain electron transport rate was assayed in 1.5 mL reaction medium containing 50 mM HEPES-KOH

(pH 7.5), 100 mM sorbitol, 5 mM KCl, 2.5 mM $MgCl_2$, 5 mM Na_2HPO_4, 0.5 mM ADP, 100 μM MV, 100 mM NH_4Cl, 0.03 μM valinomycin, 3000 units (SOD), 5 mM sodium azide and chloroplasts containing 22.5 nmol chl. For assaying PSII the 1.5 mL reaction medium contained 50 mM HEPES-KOH (pH 7.5), 100 mM sorbitol, 5 mM KCl, 2.5 mM $MgCl_2$, 5 mM Na_2HPO_4, 0.5 mM ADP, 1.5 mM $K_3Fe(CN)_6$, 1 μM DBMIB, 0.5 mM DAD and 11.25 nmol chl. The PSI electron transport activity was assayed using an identical reaction medium to that for whole chain electron transport except that 0.5 μM nigericin was substituted for valinomycin and NH_4Cl and 0.5 mM DHQ replaced water as the electron donor. One-half μm DCMU was added to block electron flow from PSII. Unless otherwise specified electron transport rates were expressed in μmol e^- mg^{-1} chl h^{-1}. The electron transport rates were calibrated with a known amount of $K_3Fe(CN)_6$ just before the beginning of every measurement.

Electrolyte Leakage

For each temperature treatment and species, sixteen test tubes (4 samples/pot x 4 pots) containing 0.7 g leaf material and 20 mL deionized double distilled water (ddH_2O) were vacuum infiltrated for 15 min at 120 to 140 mm Hg. Prior to vacuum infiltration, the leaves were gently washed for approximately 90 min with 3 changes of ddH_2O and cut into 1 cm pieces (Premachandra and Shimada, 1987). Samples were agitated at room temperature for 1 h in a shaker (DUBNOFF metabolic shaking incubator, Precision Sci. Group, Model D/S 120-070, Chicago, IL). The initial electrical conductance (C_1) of the bathing solution was measured (Cole-Parmer conductivity meter Model 1481-60, Chicago, IL). Leaf samples were then autoclaved (NAPCO Model-9000-D, Portland, OR) for 20 min at 121°C, agitated for 1 h, and the electrical

conductance measured again (C_2). The ratio of C_1 to C_2 was used as a measure of stress injury.

All measurements were repeated three times with four replicate measurements per treatment. A completely randomized design was used.

Chemicals

Dihydrochloride salt of DAD was purified from a slurry of charcoal and cellite in ethanol:water (1:1, v/v) containing 0.4% (w/v) ascorbate following the procedures described by Kee (1984) for tomato and Sabat et al. (1991) for beet. The slurry was filtered through Whatman No. 42 filter paper. The stirring step with charcoal and cellite was repeated until a colorless filtrate was obtained. Very cold concentrated HCl was added dropwise to the clear filtrate until crystals appeared. The solution was chilled to -20°C and crystals allowed to grow. Excess concentrated HCl was added. Finally, crystals were collected on Whatman No. 542 filter paper. The collected crystals were rinsed with very cold 100% ethanol, dried, and stored at -20°C in a desiccator. Fresh solutions of recrystallized DAD.2HCl were prepared in 10 mM HCl before the beginning of each experiment. DBMIB was a gift from Dr. D.R. Ort, University of Illinois. All other chemicals were of analytical grade.

RESULTS

Photosynthetic Electron Transport and Chlorophyll Fluorescence

Exposure of tef plants to chilling low temperatures (2°C, 7°C, and 12°C) reduced the capacity of PSII (Fig. 1a) and whole chain (Fig. 1b) electron transport in isolated thylakoids. The reduction was greater in PSII than in whole chain electron transport (Fig. 1, a and b). Complete inhibition of PSII was observed after 96 h and 144 h of chilling when tef plants were exposed to 2°C and 7°C, respectively (Fig. 1a). These temperatures and exposure times led to losses of 63% and 53% of the whole chain activity (Fig. 1b). PSII and whole chain electron transport rates (Fig. 2, a and b) were less reduced by chill-exposure in wheat. PSI electron transport was largely unaffected by chill-exposure in both wheat and tef (data not shown).

Chilling temperatures that affected PSII and whole chain electron transport also altered chlorophyll fluorescence in tef. Even short duration chilling treatment substantially reduced Fv/Fm (Fig. 3a). PSII electron transport in thylakoids isolated from tef leaves and Fv/Fm of intact leaves were reduced by similar extents. For example, after 24 h at 2°C the reductions were 34% and 38%, respectively. Chlorophyll fluorescence (Fig. 3b) was much less reduced by chill exposure in wheat. In tef the decline in Fv/Fm was correlated with decreasing Fm (data not shown, $r = 0.95$, $P \leq 0.05$) and Fv (data not shown, $r = 0.95$, $P \leq 0.05$), a substantial increase in $t_{1/2}$ (Fig. 4, $r = -0.89$, $P \leq 0.05$) and a slight but significant change in Fo (data not

shown, $r = -0.65$, $P \leq 0.05$). Changes in Fv/Fm were closely correlated with PSII and whole chain electron transport in both species, and the slope of the relationship was greater in tef than in wheat (Fig. 5).

Electrolyte Leakage

The relationship between electrolyte leakage and chilling temperature in tef was best described by power response functions ($y = ax^b$) (Fig. 6a). The leakiness increased with decreasing temperature and increasing chilling time. Thus, enhanced membrane permeability accompanied the altered chlorophyll fluorescence properties and reduced electron transport rates described above. However, the latter two parameters detected temperature stress at shorter duration of exposure and less extreme temperatures than did electrolyte leakage. Like electron transport and chlorophyll fluorescence of chilled wheat, electrolyte leakage from chilled wheat leaves was not significantly different from the control (Fig. 6b).

Regression analysis was carried out to evaluate the association between Fv/Fm and electrolyte leakage. It showed that the correlation between Fv/Fm and electrolyte leakage of chilled tef was significant ($r = -0.93$ $P \leq 0.05$) and exponential as depicted in Fig. 7a. Also, the correlations between electrolyte leakage and PSII and whole chain electron transport activities were significant (Fig. 7, b and c; $r = -0.89$, $P \leq 0.05$).

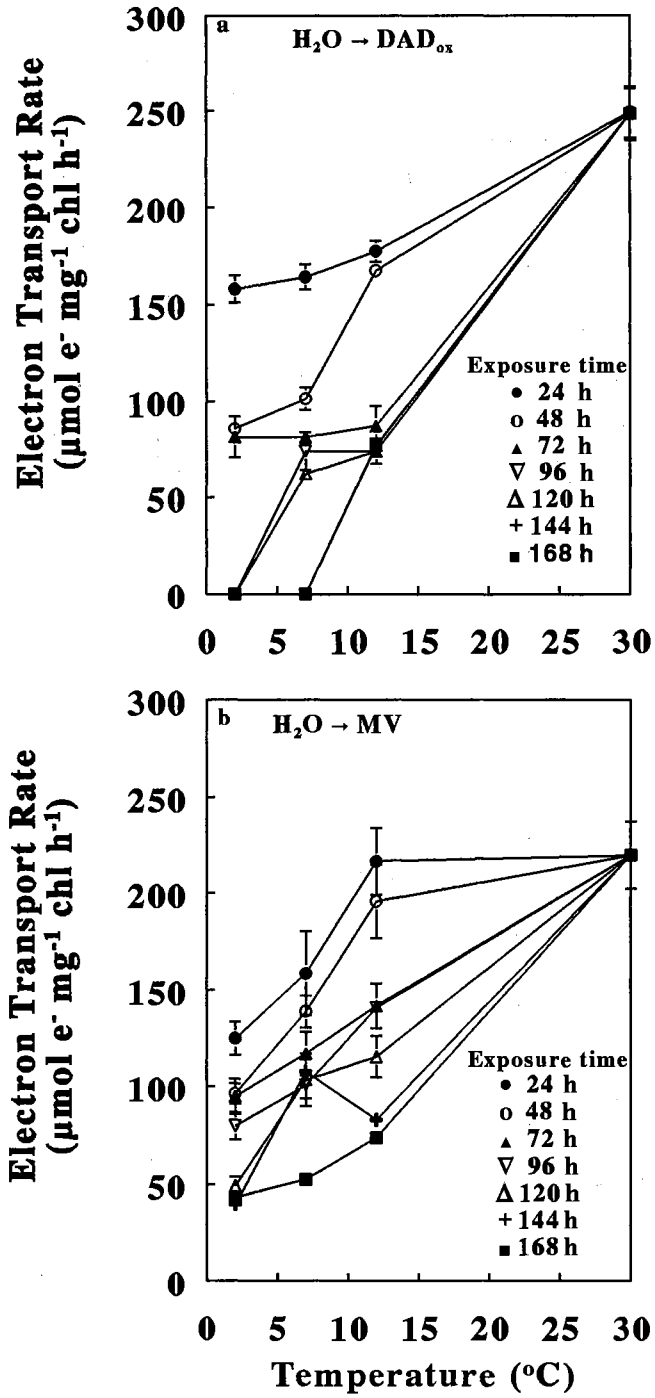

Figure 1. PSII (a) and whole chain (b) electron transport rates of tef as affected by temperature at different exposure times. Vertical bars indicate ± SE. The experiment was performed three times with four replications for each treatment.

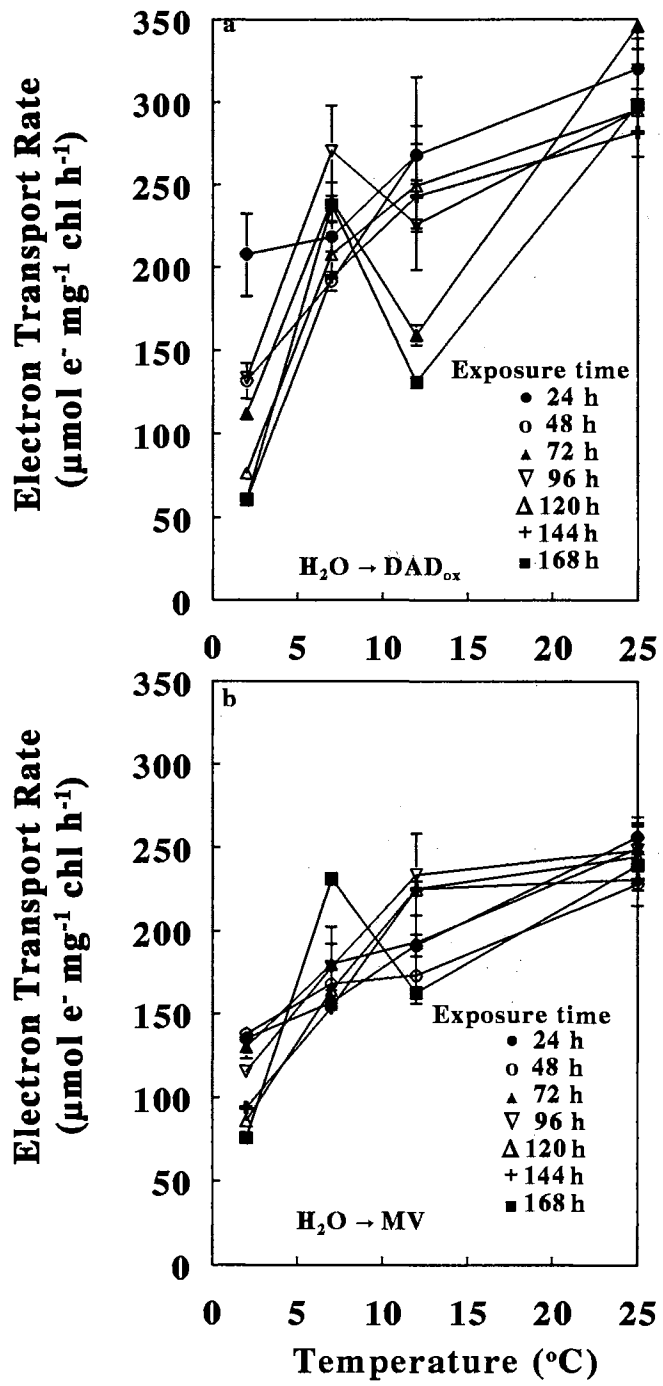

Figure 2. PSII (a) and whole chain (b) electron transport rates of wheat as affected by of temperature at different exposure times. Vertical bars indicate ± SE. The experiment was performed three times with four replications for each treatment.

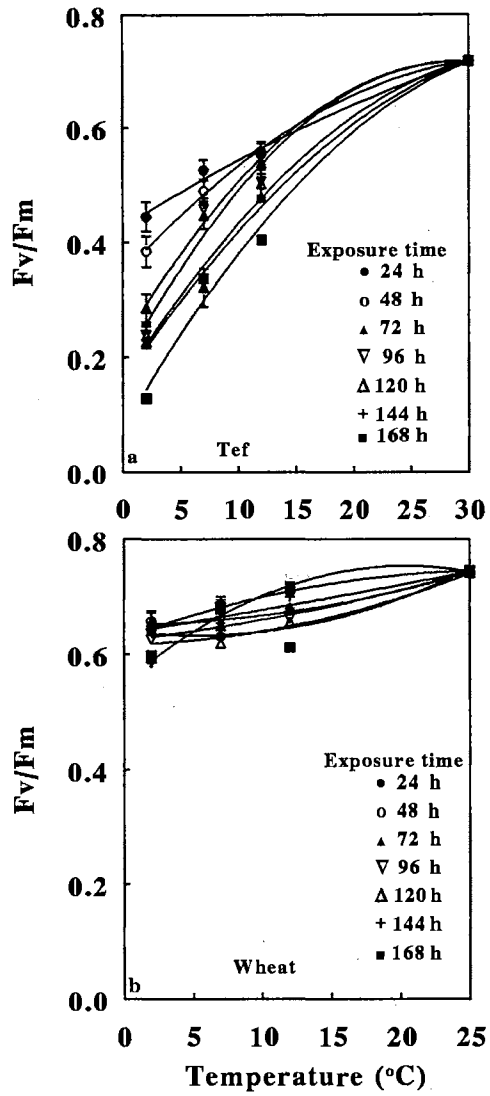

Figure 3. Ratio of variable to maximum fluorescence (Fv/Fm) of tef (a) and wheat (b) as functions of temperature at different exposure times. Intact leaves were dark adapted for 10 min prior to exposure to an excitation light intensity of 100 μmol m^{-2} s^{-1} PAR. Vertical bars indicate ± SE. The experiment was performed three times with four replications for each treatment. The best least square fits of the data were quadratic response functions of the general form $y = a + bx + cx^2$. The equations are for tef **24 h:** $y = 0.4267 + 0.0125x - 0.00009x^2$, $r = 0.99$; **48 h:** $y = 0.3447 + 0.0219x - 0.00031x^2$, $r = 0.99$; **72 h:** $y = 0.2261 + 0.0336x - 0.00057x^2$, $r=0.99$; **96 h:** $y = 0.1861 + 0.0367x - 0.00063x^2$, $r = 0.98$; **120 h:** $y = 0.1594 + 0.0316x - 0.00043x^2$, $r=0.99$; **144 h:** $y = 0.1569 + 0.0298x - 0.00036x^2$, $r=0.99$; **168 h:** $y = 0.0778 + 0.0346x - 0.00044x^2$, $r = 0.99$; and wheat, **24 h:** $y = 0.6280 + 0.0087x - 0.00016x^2$, $r=0.99$; **48 h:** $y = 0.6370 + 0.0038x + 0.00002x^2$, $r=0.86$; **72 h:** $y = 0.6509 + 0.0003x + 0.00013x^2$, $r=0.98$; **96 h:** $y = 0.6240 + 0.0028x + 0.00008x^2$, $r=0.99$; **120 h:** $y = 0.6415 - 0.0032x - 0.00030x^2$, $r=0.98$; **144 h:** $y= 0.5510 + 0.0190x - 0.00049x^2$, $r=0.99$; **168 h:** $y = 0.6170 + 0.0005x + 0.00019x^2$, $r=0.98$.

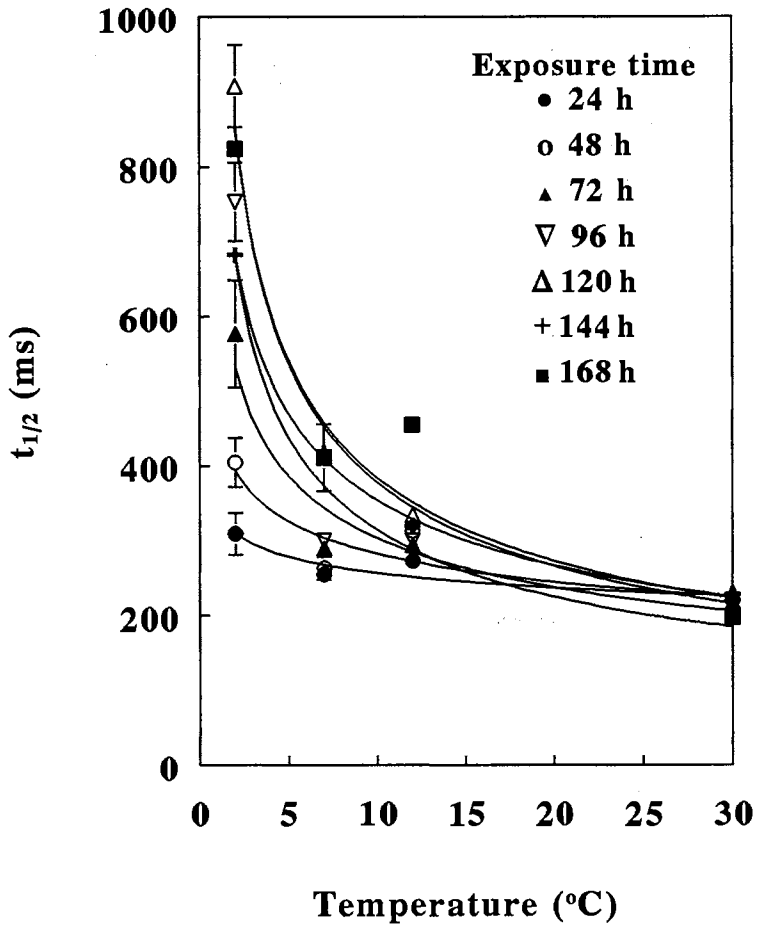

Figure 4. Fluorescence rise time ($t_{1/2}$) of tef as functions of temperature at different exposure times. For experimental details, see Fig. 3. The best least square fits of the data were power response functions of the general form $y = ax^b$; **24 h:** $y = 334.790x^{-0.115}$, r = -0.91; **48 h:** $y = 452.210x^{-0.204}$, r = -0.89; **72 h:** $y = 677.820x^{-0.349}$, r = -0.96; **96 h:** $y = 947.776x^{-0.479}$, r = -0.96; **120 h:** $y = 1212.944\ x^{-0.506}$, r = -0.99:, **144 h:** $y = 907.558x^{-0.409}$, r = -0.99, **168 h:** $y = 1197x^{-0.490}$, r=-0.95.

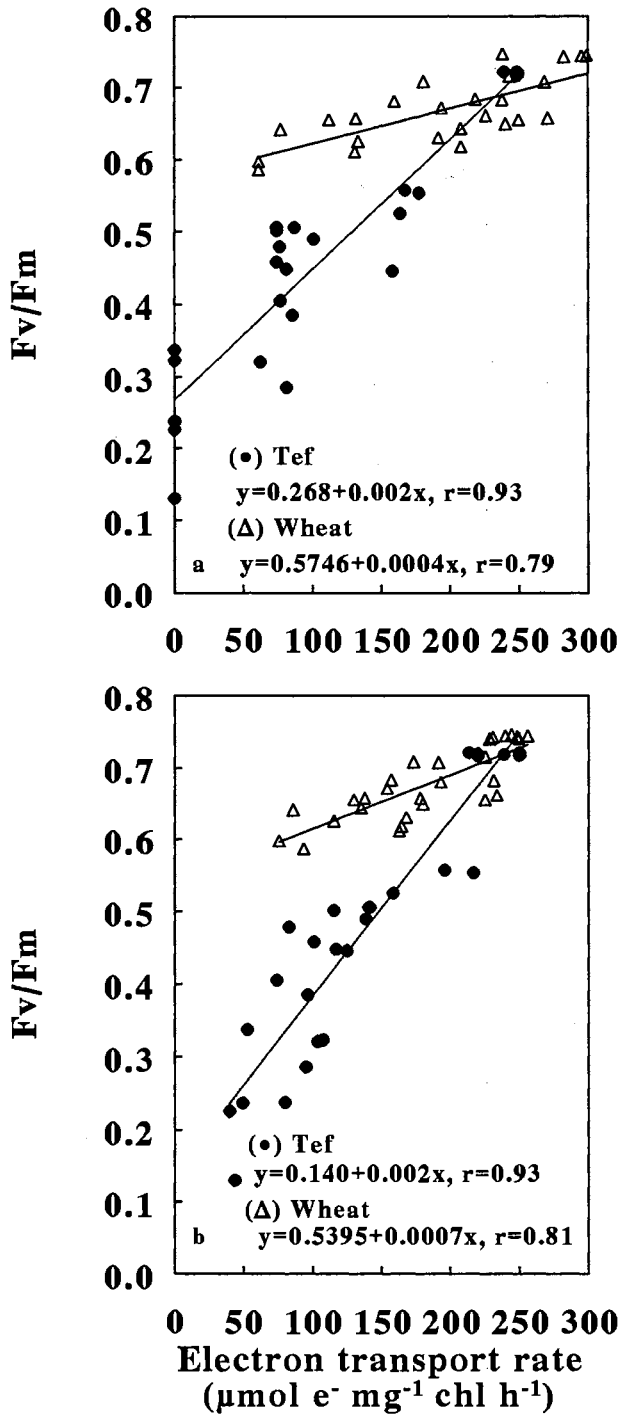

Figure 5. Relationship between Fv/Fm and PSII (a) and whole chain (b) electron transport rates in tef and wheat. Symbols represent mean values for each day and temperature treatment of three repeated experiments.

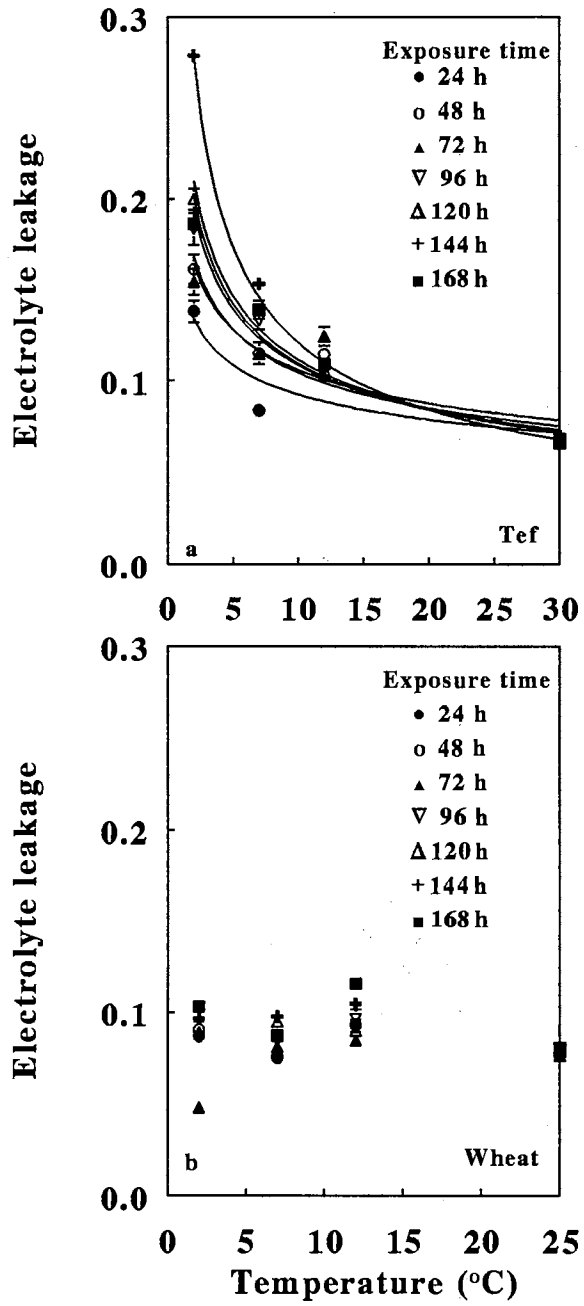

Figure 6. Electrolyte leakage as functions of temperature in tef (a) and wheat (b) at different exposure times. The experiment was performed three times with each treatment replicated four times. Vertical bars indicate ± SE. The best least square fits of tef data were power response functions of the general form $y = ax^b$; **24 h:** $y = 0.157x^{-0.229}$, r=-0.85; **48 h:** $y = 0.206x^{-0.296}$, r=-0.96; **72 h:** $y = 0.198x^{-0.271}$, r=-0.90; **96 h:** $y = 0.248x^{-0.359}$, r=-0.98; **120 h:** $y = 0.276x^{-0.393}$, r=-0.99; **144 h:** $y = 0.401x^{-0.521}$, r=-0.99; **168 h:** $y = 0.261x^{-0.378}$, r=-0.97.

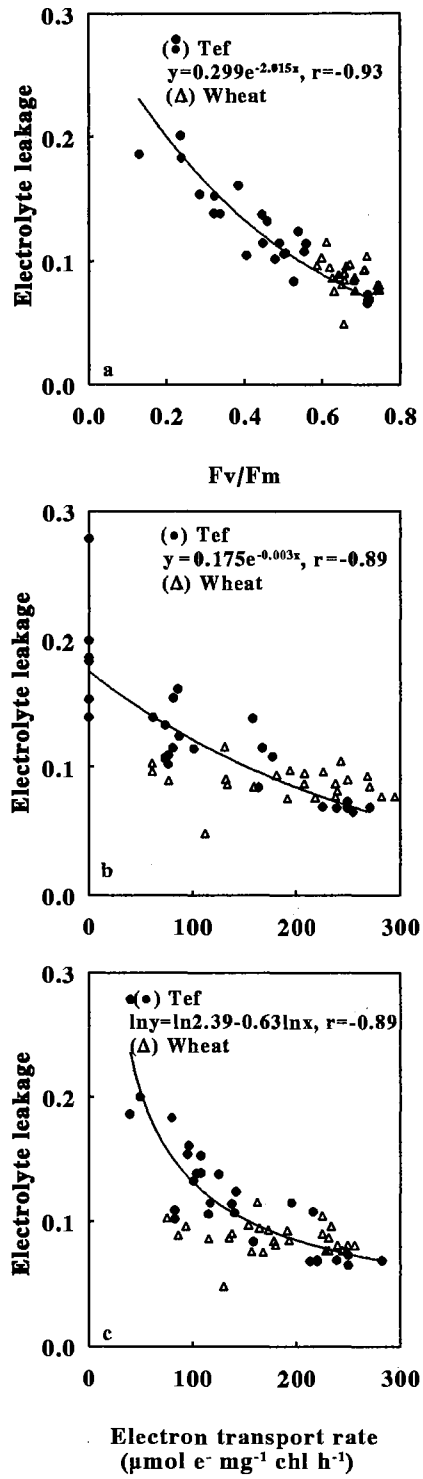

Figure 7. Relationship between electrolyte leakage and Fv/Fm (a), PSII (b) and whole chain electron transport rates (c) in tef and wheat exposed to chilling low temperature. Symbols represent mean values for each day and temperature treatment obtained from experiments performed three times. Regressions are for tef data only.

DISCUSSION

Tef (*Eragrostis tef* Z.), a cereal member of the NAD-malic enzyme subgroup of C_4 plants (Gutierrez et al., 1974; Edwards and Walker, 1983), has considerable PSII activity also in the bundle sheath cells (Edwards and Walker, 1983). There has been limited efforts to quantify PSII electron transport in isolated chloroplasts and fluorescence of intact leaves among C_4 plants. The presence of PSII in two biochemically and photochemically distinct types of photosynthetic cells (mesophyll and bundle sheath) in tef makes the determination of electron transport activities of isolated chloroplasts and chlorophyll fluorescence of intact leaves particularly interesting. We made no effort to isolate pure mesophyll chloroplasts or pure bundle sheath chloroplasts, so the electron transport data we present represent a mixed chloroplast population, albeit not necessarily at a ratio typical of the leaf. A mixed chloroplast population is desirable because chlorophyll fluorescence emitted by intact leaves does likely originates among both types of chloroplasts. The strong correlation between fluorescence and electron transport (Fig. 5) confirms a link between the two characteristics.

To study effects of chilling on wheat and tef, measurements of chlorophyll fluorescence, photosynthetic electron transport and electrolyte leakage were performed. These measurements showed susceptibility of tef to chilling low temperature stress. The activity of photosynthesis revealed by PSII (Fig. 1a) and

whole chain (Fig. 1b) electron transport, and Fv/Fm (Fig. 3a) were lowered by decreasing temperature (12°C-2°C) and increasing the time of chill exposure (24 h to 168 h). Thus, the classification of this species as a thermophilic plant (Edwards and Walker, 1983) was consistent with its temperature dependence of photosynthesis and not unexpected of a subtropical C_4 grass (Miedema, 1982). The greater chill inhibition of whole chain than PSI electron transport was previously demonstrated by Bruggemann (1992) with *Vigna*. However, PSII is the most chill-sensitive component of electron transport (Fig. 1a). A common observation of chilled plants is inefficient use of reducing equivalents (Bruggemann, 1992). It is thought that thylakoid phase transitions occur at low temperature in chilling sensitive species (Lyons and Raison, 1970; Labate et al., 1990). If so, transitions could have diminished the reduction of Q_A by inactivating electron transport. Fluorescence should then be depressed by photochemical quenching associated with oxidized Q_A. The greater reduction of Fv/Fm and PSII electron transport of tef than wheat likely resulted from chill-induced inhibition of PSII (Csapo et el., 1991) that led to inefficient reduction of Q_A (Bruggemann, 1992). As previously reported by Hetherington et al. (1983) for maize reduced Fv/Fm was accompanied by increased $t_{1/2}$ (Fig. 4), and decreased Fm and Fv (Csapo et al., 1991). The increase in $t_{1/2}$ reveals that reducing equivalents accumulate slower at Q_A. Either electrons arrive at Q_A at a reduced rate in chilled tef plants or, less likely, they leave Q_A more rapidly. In any event, fewer electrons reside in the primary acceptor of PSII, Q_A, and as a result the level of Fm declines. The lack of effect on Fo indicates that the reaction centers of PSII remained open even after severe chilling stress. Bruggemann (1992) suggested that the decline in electron transport by low temperature is a cooperative effect of multiple limiting processes. A

similar conclusion was drawn from photoinhibition studies on maize grown in bright light at sub-optimal temperature (Greer and Hardacre, 1989). This is consistent with the notion that plants are especially susceptible to photoinhibition when they are simultaneously exposed to other stresses. There are published reports of photoinhibition developing in the presence of bright light and chilling low temperature (Long et al., 1994). In our study plant performance might have been limited by concurrent low temperature and photoinhibitory damage.

Our findings on electron transport and fluorescence imply that PSI remained largely unaffected by exposure to chilling low temperature, but that PSII-dependent electron transport was impaired. In wheat both photosystems were less affected by chill exposure than in tef. It remains to be determined whether reduced PSII electron transport rate constitutes a greater limitation to net photosynthesis (CO_2 fixation) in chilled than in unchilled tef. That was not found to be the case in chilled tomato, although both CO_2 fixation and electron transport were reduced by low temperature treatment (Martin et al., 1981; Ort and Martin, 1983).

Electrolyte leakage of leaf tissue of tef increased with the extent of chilling exposure (Fig. 6a) whereas wheat, which better withstands low temperatures in the field, was unaffected (Fig. 6b). The increase in electrolyte leakage in tef indicates a deleterious change in membrane integrity caused by low temperature. Enhanced leakiness at low temperatures was previously reported in several chilling sensitive species by MacRae et al. (1986) and Bergevin et al. (1993). The likely cause of the increase in leakiness in chilling sensitive species is temperature induced phase transitions (Raison and Orr 1986; Raison and Orr, 1990) of possibly minor membrane domains. A change in plasma membrane lipid composition during chill exposure

coincided with the loss of cell membrane integrity (Parkin and Kuo, 1989; Nguyen and Mazliak, 1990) in susceptible plants. Murata et al. (1992) used tobacco transformed with the gene for glycerol-3-phosphate acyltransferase from *Arabidopsis thaliana*. They correlated chilling sensitivity of plants with degree of unsaturation of fatty acids in the phosphatidylglycerol of chloroplast membranes. Perhaps a small proportion of unsaturated fatty acids were present in the lipids of tef chloroplast membranes than in wheat. In chilling resistant plants lipid changes also take place, such as increased unsaturation of fatty acids and altered relative amounts of various lipid classes and species (Murata et al., 1992). These lipid changes may be related to acclimation to low temperature (Kasamo et al., 1992).

There was a close relationship between electrolyte leakage and photosynthesis as revealed by electron transport (Fig. 7, b and c) and Fv/Fm (Fig. 7a). Excessive membrane leakage during chilling exposure correlated with substantially decreased rates of electron transport and lowered Fv/Fm. The parallel effects on tissue leakiness, electron transport and fluorescence suggest that cellular membranes, in particular the plasma membrane and the thylakoid membrane, are key sites of injury during chill-exposure of thermophilic plants. Alternatively, damage to the plasma membrane may alter the composition of the cytosol and indirectly interfere with chloroplast function. Yet another alternative is that dysfunction of the chloroplast, which is the main site of fatty acid biosynthesis, interferes with membrane lipid biosynthesis and therefore, also indirectly with the function of nonchloroplastic membranes (Whitaker, 1992). Clearly, disturbance of the membrane structure in leaves could lead to many secondary injuries because numerous crucial physiological and biochemical processes depend on membrane integrity (Bewley, 1979).

In summary, we show agreement between the assessments of chilling injury by measurements of chlorophyll fluorescence, electron transport and electrolyte leakage. The sensitivity of tef to chilling low temperature is characterized by limited photosynthetic capacity as revealed by lowered electron transport rate and Fv/Fm, and elevated membrane leakage. Because of the theoretical link between Fv/Fm and electron transport it is not unexpected that Fv/Fm predicts PSII activity well. Fv/Fm has the advantage of detecting chilling injury more rapidly and sensitively than electrolyte leakage. We propose that Fv/Fm is a good indicator of chilling stress also in C_4 plants, and it has the advantage of being nondestructive, sensitive, and rapid. Also electron transport measurements are a sensitive but indicator of chilling injury, but they are more time consuming to make.

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CHAPTER II

PHOTOSYNTHETIC ELECTRON TRANSPORT, CHLOROPHYLL FLUORESCENCE, AND ELECTROLYTE LEAKAGE OF WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.). HEAT STRESS

SENAYET ASSEFA AND BJORN MARTIN

Abbreviations: BSA, bovine serum albumin; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DHQ, durohydroquinone (tetramethyl-*p*-hydroqunione); e^-, electron; Fo, initial fluorescence; Fm, maximal fluorescence; Fv, variable fluorescence (Fv = Fm - Fo); MV, methyl viologen; PAR, photosynthetically active radiation; PSI and PSII, photosystem I and II; SOD, superoxide dismutase; $t_{1/2}$, time required for fluorescence to rise from Fo to half its maximum value.

ABSTRACT

Effects of heat stress on photosynthetic properties and cellular membrane stability of the temperate C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101) and the subtropical C_4 cereal tef (*Eragrostis tef* Z. cv. DZ-01-354) were studied. Both O_2 evolution of isolated thylakoids and chlorophyll fluorescence of leaves were measured to evaluate photochemical properties, whereas membrane integrity was determined by electrolyte leakage of leaf samples. Heat stress reduced photosystem II (PSII) dependent electron transport of wheat and tef more than it reduced whole chain (PSII + PSI) electron transport. PSI electron transport was largely unaffected. Electron transfer rates of wheat were reduced at lower temperature and after shorter exposure time than was observed for tef. The decrease in variable to maximum fluorescence ratio (Fv/Fm) indicated damage to the function of PSII photochemistry. In heat stressed wheat leaves the decline in Fv/Fm was accompanied by increasing minimal fluorescence (Fo) and time required for Fv to rise to half its maximum value ($t_{1/2}$). In tef Fo was unaltered. Fv/Fm was closely correlated with PSII and whole chain electron transport rates in both species. Electrolyte leakage of wheat leaf tissue exposed for various durations to high temperature fit an exponential sigmoidal response curve, whereas electrolyte leakage increased more monotonously with temperature in tef. These measurements corroborated the greater sensitivity of wheat than tef to high temperatures that had been previously observed by measurement of

photochemical properties. The decrease in electron transport activity and Fv/Fm was correlated with increased electrolyte leakage of the plasma membrane.

INTRODUCTION

Photosynthesis, both at the leaf and organelle levels, is sensitive to high temperature (Berry and Bjorkman, 1980). High temperature alters the efficiency of photosynthesis and the relative proportion of light energy reemitted as chlorophyll fluorescence (Weis, 1982). Earlier studies have found heat sensitivity of thylakoid membrane activities (Santarius, 1975). The exceptional susceptibility of PSII (Berry and Bjorkman, 1980; Inoue et al., 1987; Sabat et al., 1991; Havaux, 1992), particularly the oxygen-evolving complex (Critchley and Chopra, 1988; Nash et al., 1985, Thompson et al., 1989), is today accepted. PSI activity (Thomas et al., 1986; Havaux, 1992; Sabat et al., 1991) is less affected by high temperature. Two of the four Mn^{2+} of the water-splitting complex (Nash et al., 1985; Thompson et al., 1989) and Cl^- are thought to be released at high temperature (Critchley and Chopra, 1988), and denaturation of membrane proteins of the oxygen-evolving complex has been suggested (Thompson et al., 1989). Thus, one site of heat injury is located in the thylakoid membrane (Santarius, 1975). Early analysis of chlorophyll a fluorescence kinetics suggested that high temperature stress leads to blockage of the PSII reaction center and dissociation of the antennae pigment-protein complex from the central core of PSII (Armond et al., 1978). This view was further strengthened by freeze fracture studies (Gounaris et al., 1983, 1984) demonstrating the loss of grana stacking and redistribution of intramembrane particles.

Several techniques have been used to analyze stress effects on plants. Chlorophyll fluorescence techniques have been recently used (van Kooten and Snel, 1990) and revealed details of various aspects of photosynthesis (for a review see Krause and Weis, 1991; Schreiber and Bilger, 1993). Among the many parameters derived from the fluorescence induction curve, the parameter most often used is Fv/Fm, a quantitative measure of the quantum efficiency of PSII photochemistry and photon yield of oxygen evolution (Oquist and Wass, 1988; Adams et al., 1990; Krause and Weis, 1991). It is now possible to interpret chlorophyll fluorescence changes caused by high temperature in terms of altered PSII electron transfer (Krause and Weis 1984, Adams et al., 1990; Cao and Govindjee, 1990; Havaux, 1992).

A simple and rapid measurement that has received some attention is the cellular membrane thermostability as revealed by electrolyte leakage (Shanahan et al., 1990). Electrolyte leakage measurements have been used to quantify cell membrane injury (Ingram, 1985; Shanahan et al., 1990) in leaf, fruit and root tissue. The technique has also been used to find the critical temperature causing direct heat injury (Levitt 1980; Ingram, 1985). However, there is no clear evidence in the literature whether the temperature that causes such a change is appreciably different from that associated with loss of the photosynthetic activity.

Often leaf disks have been exposed to heat shock treatment for a few minutes or hours. Fewer attempts have been made to quantify the effects of extended heat stress. Also, the relationship between changes in photochemical properties of isolated chloroplasts and leaves, and membrane stability as revealed by electrolyte leakage, is poorly understood.

The objective of this study was to evaluate photosynthetic properties and cellular membrane stability of two plant species of contrasting thermal tolerance, and to identify the most sensitive and rapid technique(s) to assess heat stress injury. For this purpose we measured three properties: 1) *in vivo* chlorophyll fluorescence, 2) photosynthetic electron transport, and 3) electrolyte leakage of leaf tissue.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Exposure

Seeds of the temperate C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101) and the subtropical C_4 cereal tef (*Eragrostis tef* Z. cv. DZ-01-354) belonging to the NAD-malic enzyme subgroup were grown in controlled environment growth chambers (Conviron, CMP 3244, Winnipeg, Ltd., Manitoba, Canada) in pots containing a mixture of peatmoss and top soil (1:1, v/v). Wheat and tef were maintained at 25°C/18°C and 30°C/23°C day/night temperatures, respectively and 14 h photoperiod. Thirty days after planting, exposure to high temperatures (35°C, 40°C, and 45°C) lasting up to 168 h commenced. Control plants remained at the original temperature. Measurements were made at 24 h intervals.

Chlorophyll Fluorescence

Measurements were made with a portable fluorometer (Polar Tech Umea, Sweden) (Oquist and Wass, 1988). Four fully expanded leaves were sampled from each pot (4 leaves/pot x 4 pots) and placed to dark adapt for 10 min between black plastic sheets. The top sheet contained holes to guide the measuring probe. Dark-adapted leaves were exposed for 5 sec to an excitation light intensity of 100 μmol m^{-2} s^{-1} PAR. Fo, Fm, Fv, Fv/Fm and $t_{1/2}$ were measured.

Chloroplast Isolation

Chloroplasts were isolated from leaves of 30-day-old wheat and tef plants using

the procedures described by Kee et al. (1986). Twenty g freshly cut fully expanded leaves of five plants were homogenized in a Waring blender (Model 7011-31b192, Waring, Hartford, CT) for 3 to 4 s at high speed in 200 mL ice cold isolation medium that contained 30 mM Tricine (pH 7.5), 300 mM NaCl, 3 mM $MgCl_2$ and 0.5 mM EDTA. The resulting homogenate was filtered through 16 layers of cheesecloth (Veratec, Inc., Walpole, MA) and centrifuged (ICE Model B-20A centrifuge, Damon/ICE Division, Needham Heights, MA) at 1500 g for 2 min at 2°C. Pellets were resuspended in 60 ml resuspension medium containing 5 mM HEPES-KOH (pH 7.5), 200 mM sorbitol, 2 mM $MgCl_2$, and 0.05% (w/v) BSA. Course debris was removed by a short centrifugation step. The supernatant was filtered through a layer of Kimwipe tissue and recentrifuged at 1500 g for 2 min. The pellets were gently resuspended with a cotton tip in a small volume of resuspension medium and stored on ice.

Chlorophyll Content

Total chlorophyll content of chloroplast suspensions was determined spectrophotometrically (Spectronic 1201, Milton Roy, Rochester, NY) according to Arnon (1949). Units of mass were converted to molar units using a molecular weight of 900 for chlorophyll.

Photosynthetic Electron Transport

Electron transport rates were measured by monitoring oxygen evolution or consumption with a temperature controlled Clark-type oxygen electrode assembly (Model LD-2, Hansatech, Ltd., King's Lynn, Norfolk, England) at 25°C and saturating light from a 100 W projector lamp (Quartizine 12 V, West Germany). Thylakoids were assayed for whole chain ($H_2O \rightarrow MV$), PSII ($H_2O \rightarrow DAD_{ox}$), and PSI ($DHQ \rightarrow MV$) electron transport rates. The procedures described by Kee et al. (1986) for tomato,

and Allen and Holmes (1986) and Sabat et al. (1991) for spinach, beet and pea were employed with slight modifications. For details of the reaction media see Chapter I. The rates of all three activities were expressed in μmol e^- mg^{-1} chl h^{-1}. The electron transport rates were calibrated with a known amount of $K_3Fe(CN)_6$ just before the beginning of every measurement (Allen and Holmes, 1986).

Electrolyte Leakage

For each temperature treatment sixteen test tubes containing 0.7 g leaf sample and 20 mL deionized double distilled water (ddH_2O) were vacuum infiltrated at 120 - 140 mm Hg for 15 min. Prior to vacuum infiltration, sampled leaves were gently washed for 90 min with 3 changes of ddH_2O and cut into 1 cm pieces (Premachandra and Shimada, 1987). Samples were agitated at room temperature for 1 h in a shaker (DUBNOFF shaking incubator, Precision Sci. Model D/S 120-070, Chicago, IL). The initial electrical conductance (C_1) of the bathing solution was measured with a conductivity meter (Cole-Parmer Instrument Co. Model 1481-60, Chicago, IL). Leaf samples were then autoclaved (NAPCO Model-9000-D, Portland, OR) for 20 min at 121°C, agitated for 1 h, and a second electrical conductance (C_2) measured. The ratio of C_1 to C_2, was used as a measure of stress injury.

All measurements were repeated three times with four replicate measurements for each treatment. A completely randomized design was used.

Chemicals

Dihydrochloride salt of DAD was recrystallized from ethanol-water (1:1, v/v) containing 0.4% (w/v) ascorbate. See Chapter I for details. Fresh solutions of DAD.2HCl were prepared in 10 mM HCl immediately before the beginning of each

experiment. DBMIB was a gift by Dr. D.R. Ort, University of Illinois. All other chemicals were of analytical grade.

RESULTS

Effects of Heat Stress on Photosynthetic Electron Transport

The activity of thylakoid membranes isolated from wheat and tef leaves exposed to high temperature was evaluated by measuring PSII ($H_2O \rightarrow DAD_{ox}$), PSI (DHQ → MV) and whole chain (H_2O → MV) electron transport rates. Heat stress significantly reduced PSII and whole chain electron transport of wheat and tef (Fig. 1, a and b; 2, a and b). The extent of the reduction was greater in PSII than in whole chain, and greater in wheat than in tef (Fig. 1, a and b; 2, a and b). In agreement with earlier studies (Thomas et al. 1986; Sabat et al., 1991) the 35°C and 40°C treatments were very damaging to PSII and whole chain electron transport of wheat. For example, in wheat 48 h at 40°C caused complete loss of PSII activity, whereas 96 h was needed to eliminate whole chain activity (Fig. 1, a and b). Tef required 144 h at 40 °C for complete loss of PSII activity and 168 h for elimination of whole chain activity (Fig. 2, A and B). At 45°C, which was very harmful to both species, wheat lost its PSII and whole chain activities within 24 h and 48 h, respectively. In tef elimination of these activities occurred within 48 h and 96 h. PSI-dependent electron transport remained largely unaffected, or it was even stimulated in tef (Fig. 3, a and b) at temperature at which PSII and whole chain electron transport was partially or completely inhibited. Thus, the decline in whole chain electron transport of heat stressed leaves was probably caused by injury to PSII.

Effects of Heat Stress on *in vivo* Chlorophyll Fluorescence

Fv/Fm was significantly reduced by high temperature (Fig. 4, a and b) indicating damage to the function of PSII photochemistry. In heat stressed wheat leaves the decline in Fv/Fm was accompanied by an increase in Fo, a decrease in Fv and an increase in $t_{1/2}$ (Table I). The same was generally true for tef, but the temperature effect on the fluorescence components was smaller than in wheat (Table II). Also Fo was largely unaltered in tef (Table II). Thus, both reduced Fv and elevated Fo contributed to the reduction in Fv/Fm of wheat.

Effects of Heat Stress on Electrolyte Leakage

Electrolyte leakage of wheat leaf tissue exposed for various durations to high temperatures showed an exponential sigmoid time-response curve (Fig. 5a) represented by the equation $y = [(y_{max} + y_{min}) + (y_{max} - y_{min})(1 - e^{(-k|(x-x50)|)(x-x50)}]0.5$, where y is electrolyte leakage, y_{max} and y_{min} are the maximum and minimum levels of electrolyte leakage, K is the slope at the inflection point, and x and x_{50} are the treatment temperature and temperature corresponding to the mid point (i.e. the inflection point). In tef electrolyte leakage increased much more monotonously with increasing temperature (Fig. 5b). A least square approach was used to find best fit functions in Figures 5a and 5b. Both species showed greatly enhanced electrolyte leakage with increasing temperature and heating time. However, the extent of membrane leakiness in wheat leaf tissue was much greater than in tef at high temperatures. The maximum leakage value under our experimental conditions was 0.81 in wheat and 0.56 in tef (Fig. 5, a and b). The temperature causing 50% leakage in wheat ranged between 35°C and 40°C dependent on duration (Fig. 5a). These differences may reflect that tef is adapted to higher growth temperature than wheat.

At these temperatures electron transport and Fv/Fm of wheat are partially or completely inhibited dependent on duration (Fig. 1, a and b; Fig. 4a).

In both wheat and tef the decline in Fv/Fm was closely positively correlated with PSII ($r = 0.99, 0.96, P \leq 0.05$) and whole chain ($r=0.97, 0.85, P \leq 0.05$) electron transport (Fig. 6, a-d). Increased membrane leakage of wheat and tef was also correlated with PSII ($r= -0.72, -0.96, P\leq 0.05$) and whole chain ($r = -0.93, -0.85, P\leq 0.05$) electron transport (Fig. 6, a-d) and Fv/Fm (Fig. 7, a and b).

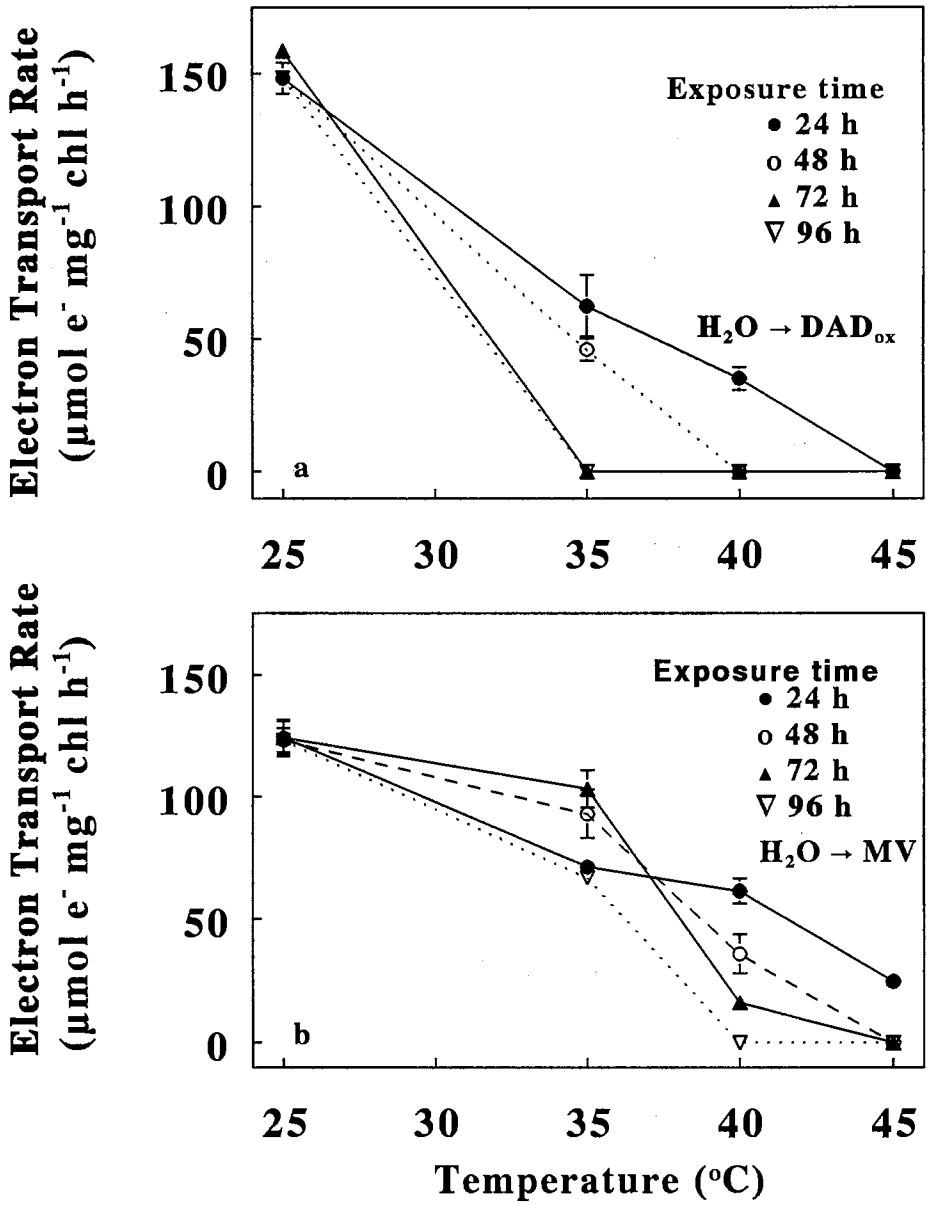

Figure 1. PSII (a) and whole chain (b) electron transport rate of wheat as affected by temperature at different exposure times. Bars indicate ± SE. The experiment was performed 3 times with 4 replications for each treatment.

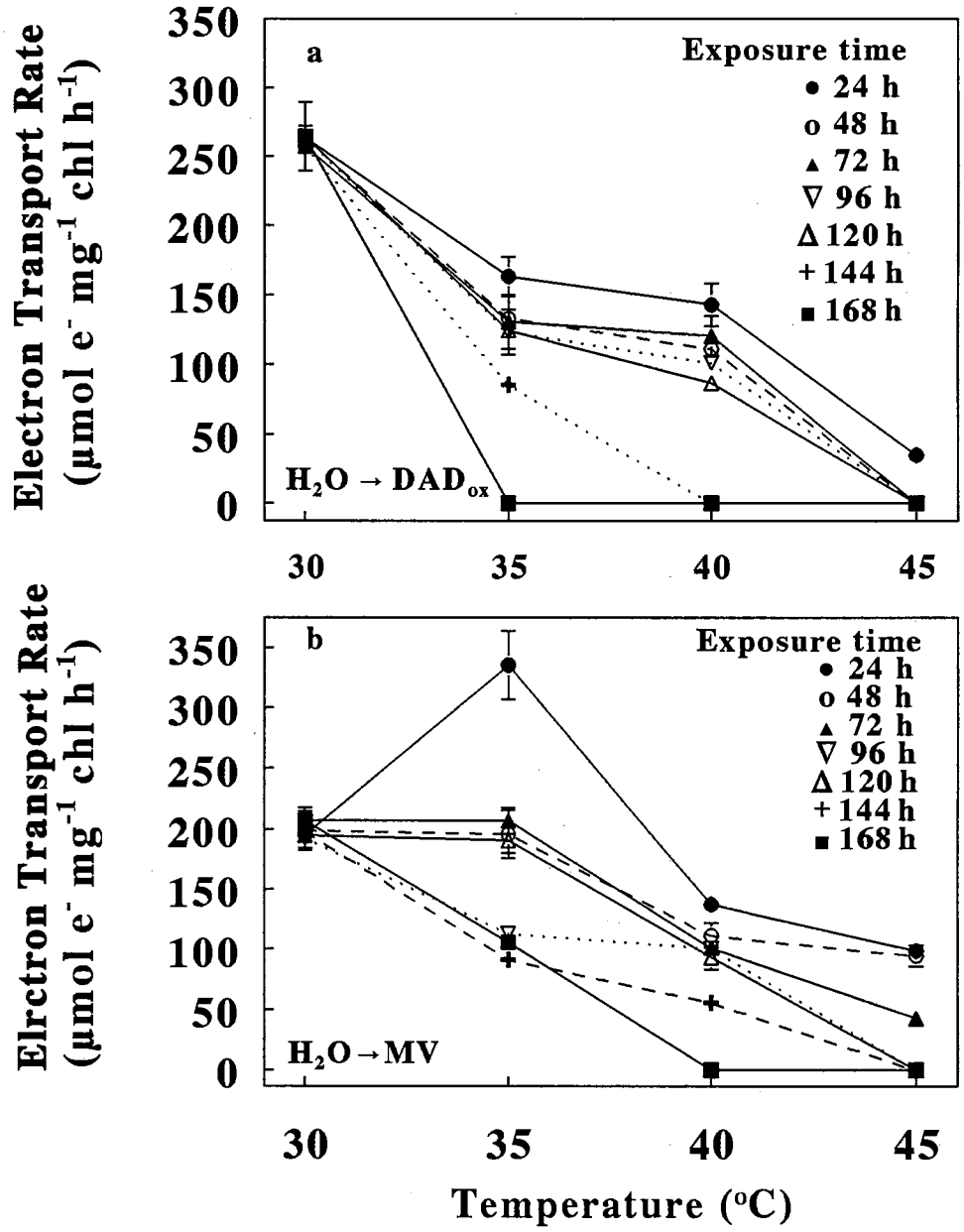

Figure 2. PSII (a) and whole chain (b) electron transport of tef as affected by temperature at different exposure times. Details are as for Figure 1a and 1b.

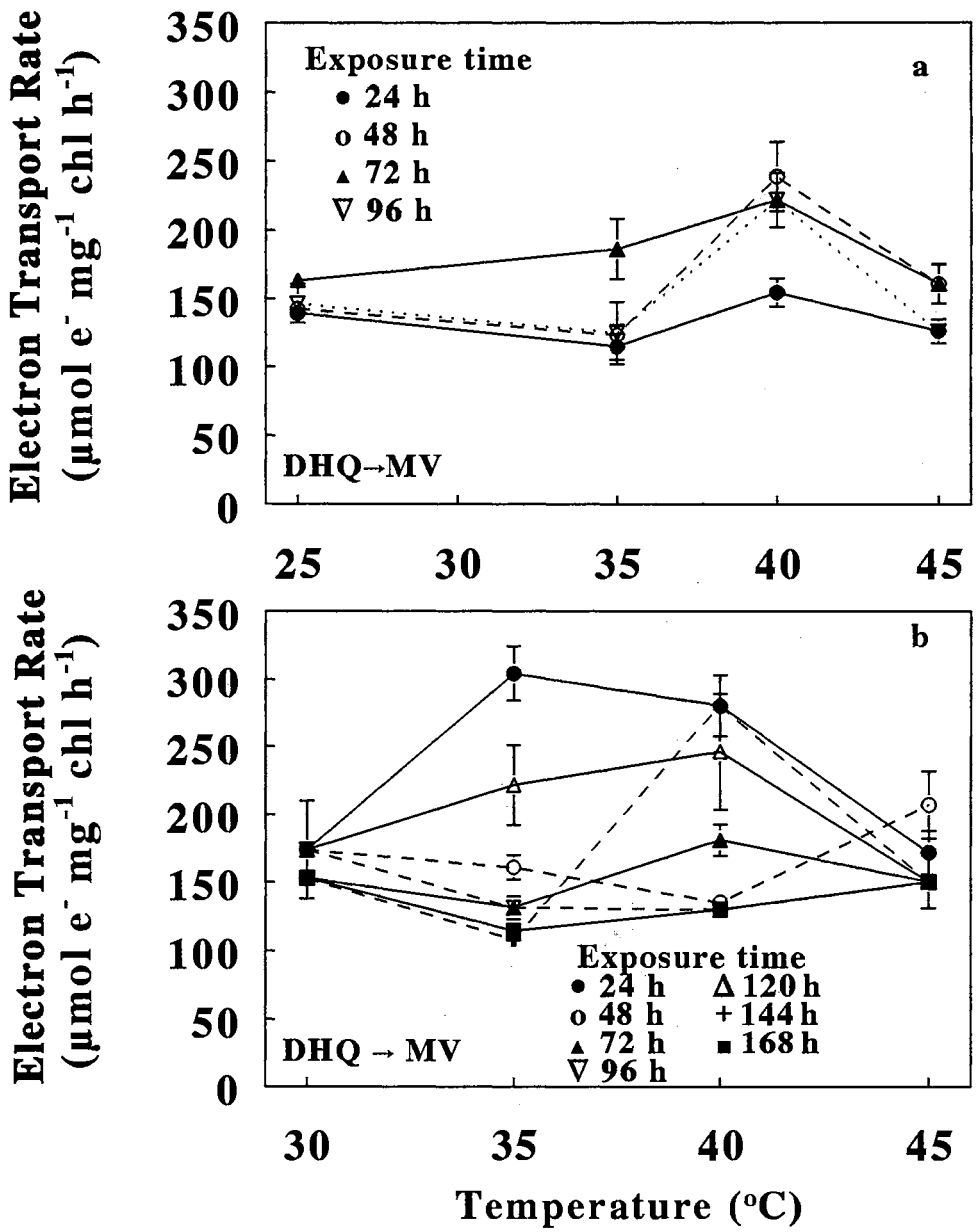

Figure 3. PSI electron transport rate of wheat (a) and tef (b) as affected by temperature at different exposure times. Bars indicate ± SE. The experiment was performed three times with four replication for each treatment.

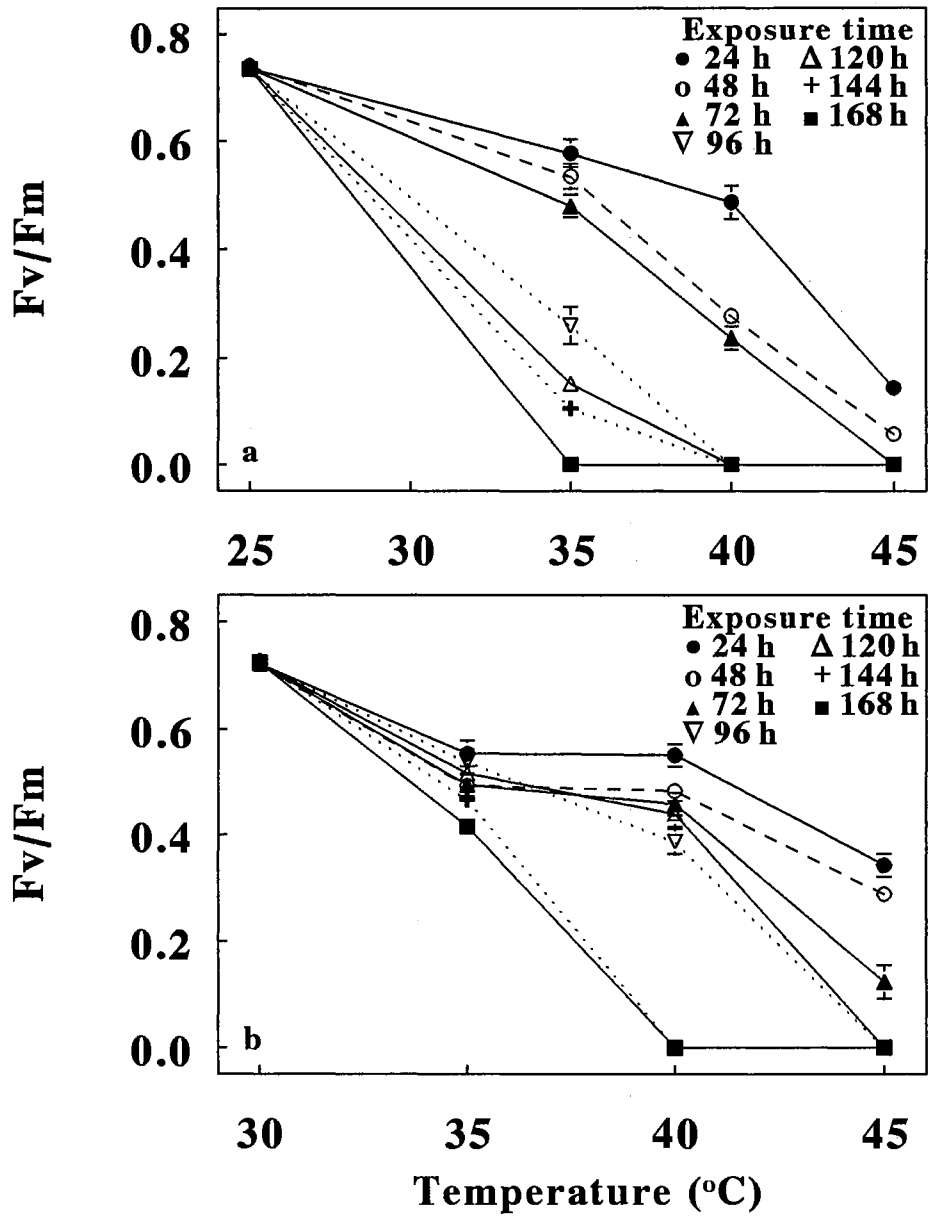

Figure 4. Ratio of variable to maximum fluorescence (Fv/Fm) as affected temperature treatment in wheat (a) and tef (b). Intact leaves were dark adapted for 10 min prior to exposure to excitation light with an intensity of 100 µmol m^{-2} s^{-1} PAR. Bars indicate ± SE. The experiment was performed three times with four replications for each treatment.

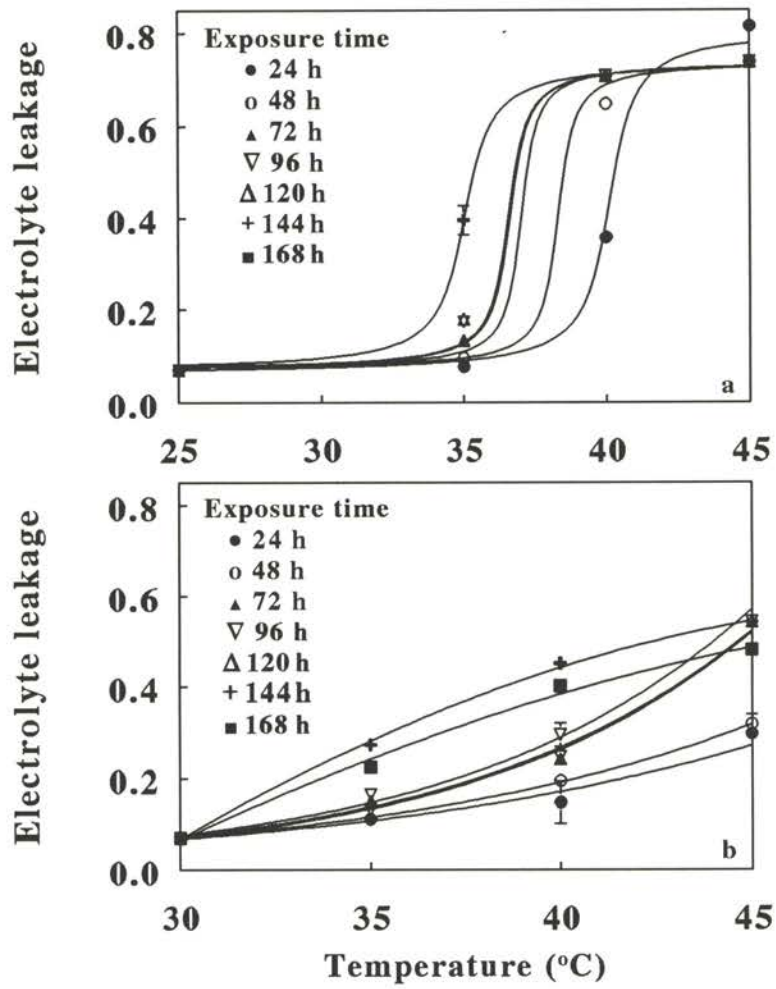

Figure 5. Electrolyte leakage of wheat (a) and tef (b) as functions of temperature at different exposure times. The experiment was performed three times with four replications for each treatment. Vertical bars indicate ±SE. A least square approach was used to find the best fitting regression functions. Below are best fitting equations for exposure times from 24 to 144 h for wheat,

(24 h) $y = [(0.877+0.749(1-e^{(-0.490|(x-40.111)|)})^{(x-40.111)}]0.5$, $r=0.99$;
(48 h) $y = [(0.809+0.669(1-e^{(-0.774|(x-38.317)|)})^{(x-38.317)}]0.5$, $r=1.00$;
(72 h) $y = [(0.808+0.668(1-e^{(-0.814|(x-37.035)|)})^{(x-37.035)}]0.5$, $r=1.00$;
(96 h) $y = [(0.808+0.668(1-e^{(-0.715|(x-36.642)|)})^{(x-36.642)}]0.5$, $r=1.00$;
(120 h) $y = [(0.808+0.668(1-e^{(-0.703|(x-36.586)|)})^{(x-36.586)}]0.5$, $r=1.00$;
(144 h) $y = [(0.807+0.673(1-e^{(-0.472|(x-35.049)|)})^{(x-35.049)}]0.5$, $r=1.00$; and for tef
(24 h) $y = 0.00399e^{0.0938x}$, $r=0.99$; **(48 h)** $y = 0.00320e^{0.1021x}$, $r=0.99$;
(72 h) $y = 0.00125e^{0.1340x}$, $r=0.99$; **(96 h)** $y = 0.00110e^{0.1365x}$, $r=0.99$;
(120 h) $y = 0.00126e^{0.1359x}$, $r=0.99$; **(144 h)** $y = -2.242 + 0.1168x - 0.0011x^2$, $r=0.99$;
(168 h) $y = -1.828 + 0.0862x - 0.0007x^2$, $r=0.99$.

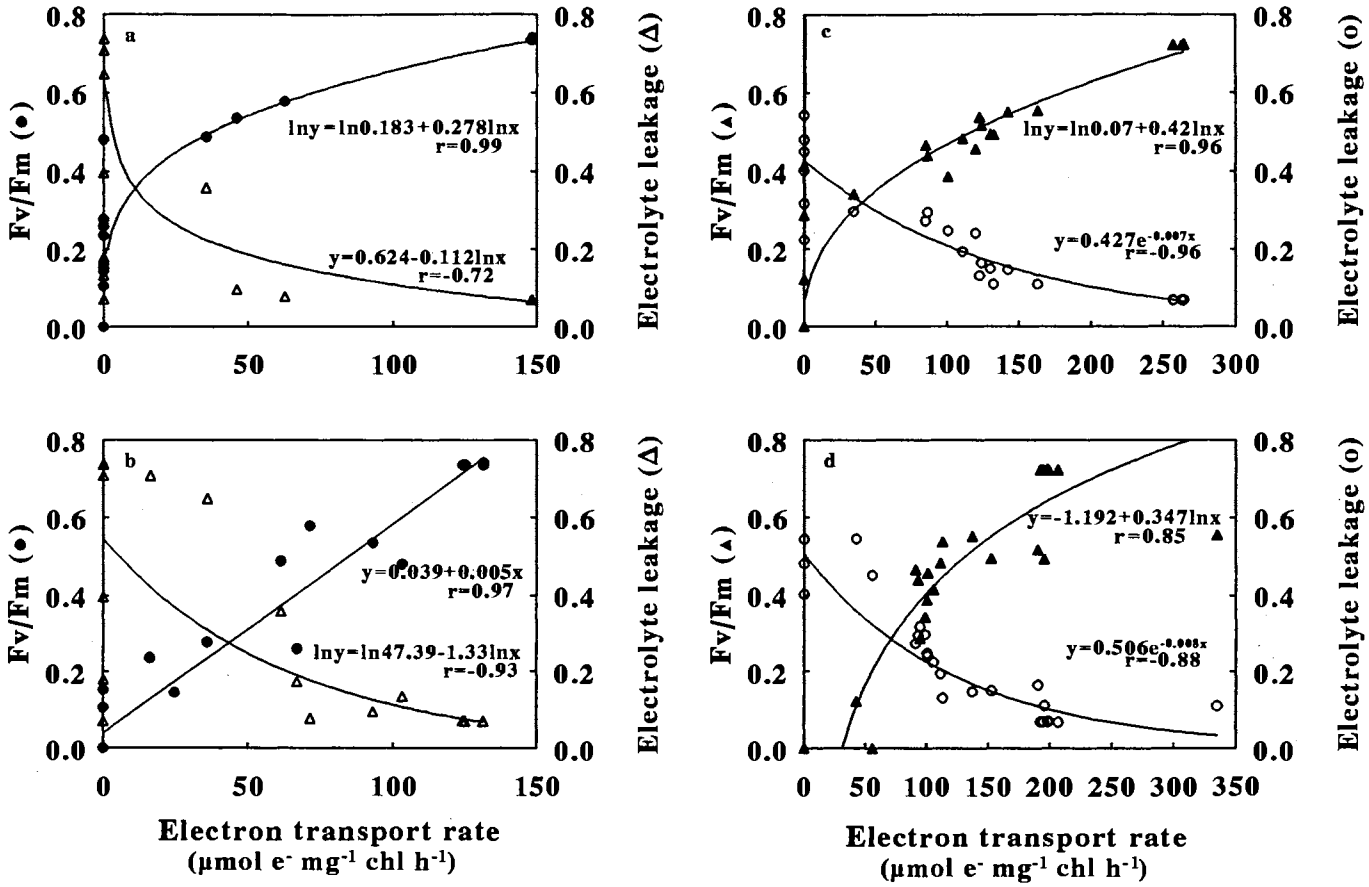

Figure 6. Relationship between Fv/Fm, electrolyte leakage and electron transport in heat stressed wheat of PSII (a), wheat of whole chain (b), tef of PSII (c), and tef of whole chain (d).

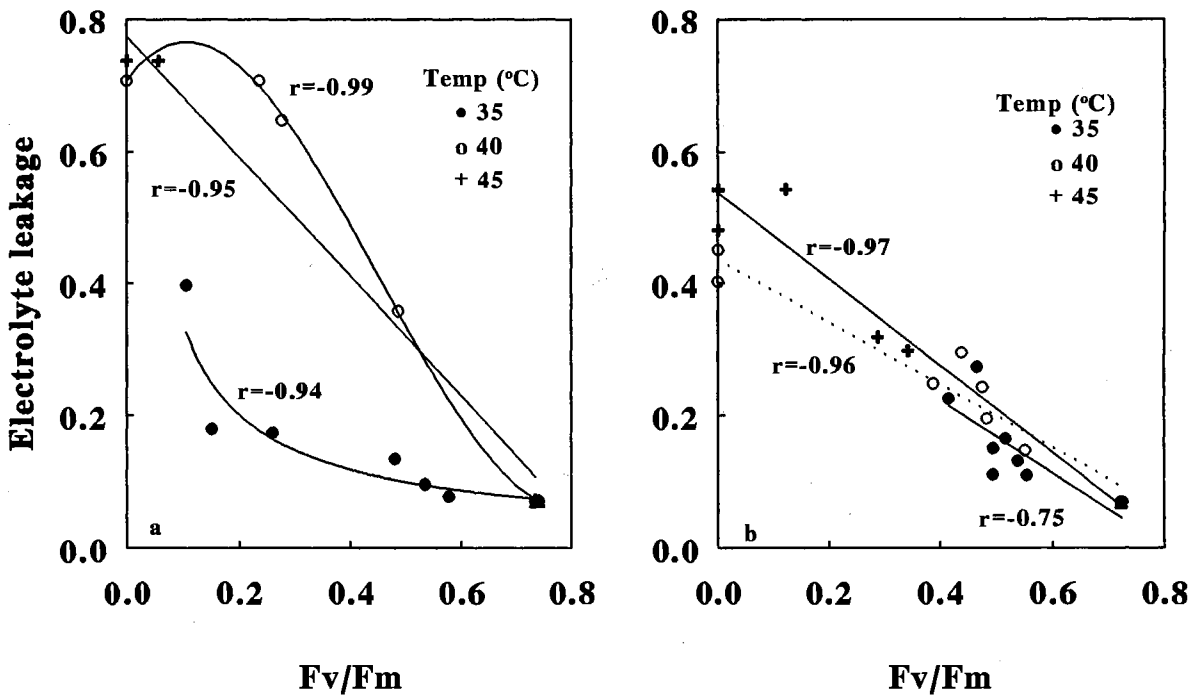

Figure 7. Relationship between Fv/Fm and electrolyte leakage in heat stressed wheat (a) **35°C**, lny=ln0.058 - 0.763lnx, r=-0.94; **40°C**, y=0.708 + 1.120x-5.942x² - 4.409x³, r=-0.98; and **45°C**, y=0.775 - 0.908x, r=-0.95) and tef (b) **35°C**, 0.441 - 0.545x, r=-0.75: **40°C**, y=0.437 - 0.474x, r=-0.96; **45°C**, y=0.538 - 0.657x, r=-0.97. Symbols represent mean values for each day and temperature treatment obtained from experiments repeated three times.

Table I. Fo, Fm, Fv and $t_{1/2}$ of wheat exposed to high temperature. Blanks indicate that F_p (peak fluorescence) was not detected during the 5 s duration of the measurement. Intact leaves were dark adapted for 10 min prior to exposure to excitation light with an intensity of 100 µmol m^{-2} s^{-1} PAR. The experiment was performed three times with four replications for each treatment.

Temp (°C)	Time (h)	Fo	Fm (relative units)	Fv	$t_{1/2}$ (ms)
25	0	0.106 ± 0.000	0.404 ± 0.011	0.306 ± 0.013	210 ± 6
35	24	0.117 ± 0.013	0.282 ± 0.007	0.175 ± 0.012	211 ± 12
	48	0.126 ± 0.007	0.265 ± 0.010	0.143 ± 0.009	247 ± 79
	72	0.223 ± 0.015	0.319 ± 0.010	0.096 ± 0.009	295 ± 58
	96	0.346 ± 0.007	0.384 ± 0.011	0.057 ± 0.019	706 ± 211
	120	0.412 ± 0.000	0.484 ± 0.010	0.069 ± 0.010	555 ± 16
	144	-	-	-	-
40	24	0.135 ± 0.002	0.316 ± 0.017	0.184 ± 0.013	234 ± 13
	48	0.202 ± 0.001	0.327 ± 0.010	0.124 ± 0.019	470 ± 64
	72	0.350 ± 0.000	0.373 ± 0.010	0.025 ± 0.009	495 ± 48
	96	-	-	-	-
45	24	0.137 ± 0.002	0.164 ± 0.011	0.029 ± 0.010	1001 ± 304
	48	-	-	-	-

Table II. Fo, Fm, Fv and $t_{1/2}$ of tef exposed to high temperature. Blanks indicate the equipment did not detect F_p (peak fluorescence) during the 5 s duration of the measurement. For experimental detail, see Table I.

Temp (°C)	Time (h)	Fo	Fm	Fv	$t_{1/2}$ (ms)
		(relative units)			
30	0	0.084 ± 0.001	0.313 ± 0.005	0.228 ± 0.005	222 ± 5
35	24	0.087 ± 0.001	0.210 ± 0.011	0.123 ± 0.009	265 ± 29
	48	0.113 ± 0.001	0.234 ± 0.012	0.117 ± 0.013	282 ± 12
	72	0.096 ± 0.008	0.201 ± 0.008	0.105 ± 0.008	268 ± 14
	96	0.110 ± 0.000	0.220 ± 0.015	0.110 ± 0.009	256 ± 14
	120	0.088 ± 0.001	0.199 ± 0.008	0.110 ± 0.010	269 ± 14
	144	0.087 ± 0.000	0.170 ± 0.005	0.083 ± 0.007	262 ± 11
	168	0.093 ± 0.001	0.170 ± 0.006	0.082 ± 0.004	301 ± 10
40	24	0.095 ± 0.001	0.222 ± 0.016	0.128 ± 0.016	243 ± 11
	48	0.099 ± 0.001	0.198 ± 0.004	0.098 ± 0.004	289 ± 13
	72	0.090 ± 0.000	0.174 ± 0.010	0.083 ± 0.011	257 ± 10
	96	0.103 ± 0.001	0.175 ± 0.009	0.074 ± 0.011	368 ± 44
	120	0.107 ± 0.001	0.187 ± 0.011	0.080 ± 0.010	327 ± 33
	144	-	-	-	-
45	24	0.104 ± 0.001	0.164 ± 0.005	0.060 ± 0.005	402 ± 47
	48	0.107 ± 0.001	0.151 ± 0.004	0.046 ± 0.005	682 ± 129
	72	0.123 ± 0.000	0.150 ± 0.011	0.030 ± 0.011	637 ± 89
	96	-	-	-	-

DISCUSSION

Berry and Bjorkman (1980) and others found that the water splitting apparatus of PSII is susceptible to heat damage. Our chlorophyll fluorescence results on wheat are consistent with heat stress damage to the oxidizing side of PSII. The reduction in Fv/Fm (Fig. 4a) brought about by the combination of a decrease in Fv and an increase in Fo (Table I) agrees with this interpretation. The increase in Fo is indicative of damage to the reaction center of PSII while decreased Fv suggests an increase in non-radiative energy dissipation (Bolhar-Nordenkampf et al., 1989). These findings suggest that high temperature stress diverts excitation energy from photosynthesis to dissipation of heat. In contrast, in tef where there was a more gradual reduction in Fv/Fm, the reduction was predominantly due to a decrease in Fm without a significant effect on Fo. Thus, in tef the inactivation of PSII may not have resulted from loss of active reaction centers and emergence of a population of closed centers in dark-adapted leaves. Perhaps heat stress in tef causes inefficient energy transfer within the pigment bed. The heat stress effects on the photosynthetic apparatus of tef may be less specifically localized on the oxidizing side of PSII than in wheat. The effect of heat stress on Fm of wheat varied with temperature (Table I). From 25°C to 35°C Fm decreased sharply followed by an increase and, finally, again a sharp decrease at higher temperatures (45°C). The water oxidizing side of PSII is susceptible to high temperature so presumably electron supply was slowed from water to the

plastoquinone pool and this resulted in low Fm. However, the reason behind the transitory increase in Fm is not known. In tef Fm declined greatly (Table II). There has been speculations that lowered Fm could be caused by structural alterations taking place in the PSII complex, which lead to an overall decrease in PSII photochemistry (Mishra and Singhal, 1992).

Thylakoid membranes isolated from heat stressed wheat and tef leaves showed drastic inhibition of the activities of PSII and whole chain electron transport (Figs. 1, a and b; Fig. 2a; Fig.3b). In contrast PSI remained largely unaffected. These observations are consistent with the conclusion by Critchley and Chopra (1988) and Mishra and Singhal (1992) that the oxygen-evolving complex of higher plants is one of the target sites for high temperature damage. Nash et al. (1985), Sundby et al. (1986) and Thompson et al. (1989) reported that heat inactivation of oxygen evolution occurred within the 30°C-40°C range. Several of the studies used short heating times, usually 5 to 30 min. However, also in our experiments with a minimum duration of heat exposure of 24 h, both PSII and whole chain electron transport of wheat was greatly inhibited at temperatures between 30°C and 40°C. Heat inactivation of oxygen evolution has been attributed to loss of Cl^- from the oxygen evolving complex (Critchley and Chopra, 1988; Coleman et al., 1988; Nash et al., 1985), release of manganese, and loss of 33, 24, and 18 KD extrinsic polypeptides (Nash et al., 1985; Thompson et al., 1989). Also thermal denaturation of proteins essential for water oxidation and electron transport has been proposed (Thompson et al. 1989). The heat-induced inhibition of PSII electron transport can be partially restored by use of artificial electron donors in place of water (Mishra and Singhal, 1992). However, even in the presence of an artificial donor, PSII activity was lost by heating above 44°C

(Gounaris et al., 1983). Complete destacking of the grana at temperatures between 35°C and 45°C (Gounaris et al., 1983) and thermal dissociation of light-harvesting complexes from the central core of PSII revealed by transition from $PSII_{\alpha}$ to $PSII_{\beta}$ (Sundby et al., 1986) may also account for inhibition of PSII electron transport.

Increasing the temperature above 35°C for a short period (5 min) has been documented to stimulate PSI dependent electron transport rate (Thomas et al., 1986; and Sabat et al., 1991). This stimulation was explained in terms of rearrangement of the thylakoid membrane resulting in the exposure of new donor sites within the $cytb_6/f$ complex (Thomas et al., 1986) or enhancement of spill over (Velitchkova et al., 1988). In our experiments only a small increase in PSI electron transport rate was observed at exposure temperatures between 35°C and 40°C (Fig. 3, a and b). The lack of greater and more consistent heat stimulation of PSI could be due to use of a different electron donor, or alternatively the effect was offset by the extended periods of heat stress used in our study. Velitchkova et al. (1988) also failed to find heat stimulation of PSI in stromal thylakoids, where the major proportion of PSI complexes are located.

Electrolyte leakage of wheat and tef leaf tissue increased with increasing temperature and heating time (Fig. 5, a and b). Membrane leakiness in wheat leaf tissue, was much greater than in tef, however, suggesting greater loss of membrane integrity in wheat compare to tef. As the heating time and temperature increased, the difference in electrolyte leakage between the two species narrowed. Loss of membrane integrity at high temperature correlated with reduced photosynthetic activity. There were close negative correlations between photosynthetic activity, as revealed by electron transport (Fig. 6, a-d) and Fv/Fm (Fig. 7, a-b), and membrane

leakage. The exact implication of plasma membrane dysfunction for the activities of chloroplast membranes are not understood and there may not be a direct functional relationship at all. There could be a separate but analogous effect of high temperature directly on the chloroplast membranes that is responsible for changes in electron transport and fluorescence. Alternatively, plasma membrane leakiness might cause changes in solute composition inside the cell, which indirectly could cause negative effects on thylakoid function. Electron transport and Fv/Fm of wheat was almost fully inhibited at temperatures causing 50% leakage suggesting that either thylakoid function is very sensitive to plasma membrane leakiness, or thylakoid injury precedes plasma membrane injury. The physiological relationship between plasma membrane permeability and thylakoid membrane function needs further study.

In summary, this study shows that wheat is more susceptible to heat injury than tef and that there is a general agreement between determinations of heat stress injury assessed by chlorophyll fluorescence, electron transport, and electrolyte leakage. Measurements of fluorescence and electrolyte leakage have the advantage of being especially simple to make and rapid. Electrolyte leakage does not detect heat injury as early as fluorescence and electron transport.

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CHAPTER III

PHOTOSYNTHETIC ELECTRON TRANSPORT, CHLOROPHYLL FLUORESCENCE, CHLOROPHYLL CONTENT, AND ELECTROLYTE LEAKAGE OF OF WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.): WATER DEFICIT STRESS

SENAYET ASSEFA AND BJORN MARTIN

Abbreviations: BSA, bovine serum albumin; chl, chlorophyll; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (dibromothymoquinone); DCMU 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DHQ, durohydroquinone (tetramethyl-*p*-hydroquinone); e^{-1}, electron; Fo, initial fluorescence; Fm, maximum fluorescence; Fv, variable fluorescence (Fv = Fm - Fo); Ψ, leaf water potential; MV, methyl viologen; PAR, photosynthetically active radiation, PSI and PSII, photosystem I and II; $t_{1/2}$, time required for fluorescence to rise from Fo to half its maximum value.

ABSTRACT

Experiments were conducted to study photochemical properties, pigment content, and cellular membrane stability of the C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101) and the C_4 cereal tef (*Eragrostis tef* Z. cv. DZ-01-354) subjected to water deficit stress. Evaluation of photochemical properties were made by measurement of the rate of photosynthetic electron transport of isolated thylakoids, *in vivo* chlorophyll fluorescence of dark adapted leaves, and chlorophyll content of leaf tissue. Water stress reduced photosystem II (PSII) and whole chain (PSII+ PSI) electron transport rates of wheat and tef, whereas the rate of PSI electron transport was unaffected. Water stress reduced electron transport of wheat at lower stress level (higher leaf water potential, Ψ) compared to tef. The relative sensitivities of electron transport to water stress in the two species were corroborated by fluorescence measurements. The changes in Fv/Fm, Fv, and Fm of wheat indicated that water stress eventually caused inactivation of the primary photochemistry of PSII. Fv/Fm was closely correlated with both PSII and whole chain electron transport rates. Chlorophyll content remained unchanged by water stress. Electrolyte leakage from water stressed wheat and tef leaves was generally low above Ψ = -1.0 MPa. However, a slow and gradual increase was observed as Ψ fell from -1.0 MPa to -2.4 MPa. These measurements confirmed the greater sensitivity to water stress of wheat than of tef. Electron transport measurements detected water deficit stress with as great sensitivity

as did Fv/Fm. Electrolyte leakage and chlorophyll contents only responded to water stress outside the physiologically meaningful levels.

INTRODUCTION

Water deficit stress is one of the main environmental stresses that limit photosynthesis. Many effects of water deficit stress are well documented (for reviews see Hsiao, 1973; Kaiser, 1987). Until recently inhibition of photosynthesis was largely ascribed to stomatal closure and its effect on the supply of CO_2 to the leaf interior. However, there is now strong evidence that components of photosynthesis unrelated to stomata are also affected by reduced Ψ. Water stress may inhibit photosynthetic electron transport (Boyer and Bowen, 1970; Keck and Boyer, 1974; Mayoral et al., 1981; Boyer and Younis, 1984; Bjorkman and Powles, 1984), photophosphorylation (Mayoral et al., 1981), and assimilatory enzymes of the Calvin cycle (Sharkey and Seemann, 1989; Kicheva et al., 1994).

According to Boyer and Younis (1984), neither chloroplast degradation, nor structural disintegration are factors that limit chloroplast activity under water stress. Boyer and Younis (1984) and Fellow and Boyer (1976) suggested the involvement of a conformational change in thylakoid membranes.

The intensity and kinetics of fluorescence emitted by chlorophyll a provides an indication of the primary photochemistry of photosynthesis. Advances have been made in interpreting fluorescence data as they relate to effects of water stress on PSII electron transfer (Govindjee et al., 1981; Bjorkman and Powles, 1984; Bukhov et al., 1989; Ogren, 1990). Some studies have questioned the effects of water stress on the

function of PSII (Genty et al., 1987; Havaux, 1992; Jefferies, 1994; Kicheva et al., 1994). Ogren and Oquist (1985), Bukhov et al. (1989), and Ogren (1990) proposed that the slow kinetics of fluorescence induction was relatively more informative than the fast kinetics. Govindjee et al. (1981) have demonstrated that the amplitude of Fv was most affected by water stress. It was suggested that the photochemistry of PSII was inhibited early, but that ultimately also CO_2 fixation was impaired (Bukhov et al., 1989).

Light harvesting and photochemistry depend on chlorophyll assemblage into pigment protein complexes. Mayoral et al. (1981) and Baisak et al. (1994) reported rapid decline of chlorophyll content of water stressed wheat leaves. Others (Bjorkman and Powles, 1984; Stuhlfauth et al., 1990) have suggested that chlorophyll content is unaffected by water stress in the physiological range.

In addition to the effects on photosynthesis, water stress may cause electrolyte leakage from leaf tissue (Sullivan and Ross, 1979; Leopold et al., 1981; Martin et al. 1987; LeBlanc and Dhindsa, 1993). Electrolyte leakage from leaf disks increased as Ψ of excised leaves declined (Martin et al., 1987). Several studies have suggested that cell membranes are the initial site of injury. The review by Bewley (1979) and other reviews pointed out the central role of cell membrane stability in drought tolerance and drought adaptation (Sullivan and Ross, 1979; Blum and Ebercon, 1981; Premachandra and Shimada, 1987). Sullivan and Ross (1979), Blum and Ebercon (1981), and Premachandra and Shimada (1987) applied electrolyte leakage as a measure of drought tolerance in sorghum, wheat and orchard grasses. The method was based on dehydration of leaf disks by incubation in a solution of polyethylene glycol and subsequent measurement of electrolyte leakage into an aqueous medium.

Studies differ regarding the tolerance level of wheat compared to corn, sorghum, and millet (Sullivan and Ross, 1979; Blum and Ebercon, 1981).

Many studies on chlorophyll fluorescence (Kicheva et al., 1994), electrolyte leakage (Premachandra and Shimada, 1987) and chlorophyll content (Baisak et al., 1994) have used leaf disks that were water stressed by bathing in polyethylene glycol solutions. Fewer studies have compared effects of gradual water loss on photochemical activities of isolated thylakoids and *in vivo* chlorophyll fluorescence of intact leaves at the same Ψ. Also the relationship between changes in photochemical function and electrolyte leakage is poorly understood. The study by Gupta (1977) on bryophytes is to our knowledge, the only one that compares solute leakage and inhibition of photosynthesis.

The objectives of this study were to determine effects of stress on photosynthetic properties, cellular membrane stability and pigment content of two plant species that vary in their levels of drought resistance. The study was designed to test the association between the parameters in order to identify the most sensitive and rapid technique(s) to asses water stress injury. The following properties were tested in the laboratory: 1) *in vivo* chlorophyll fluorescence, 2) photosynthetic electron transport, 3) electrolyte leakage, and 4) chlorophyll content.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Exposure

Seeds of the C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101) and the C_4 cereal tef (*Eragrostis tef* Z. cv. DZ-01-354) were grown in controlled environment growth chambers (Conviron CMP, Winnipeg Ltd., Manitoba, Canada) in pots containing a mixture of peat moss and top soil (1:1, v/v). Wheat and tef were maintained at 25°C/18°C and 30°C/23°C, respectively, day/night temperature and 14 h photoperiod. The irradiance at plant height was 460 to 500 μmol m^{-2} s^{-1} PAR. The chambers were humidified to maintain at least 50% RH. A week after emergence the plants were thinned to 5 plants per pot. The plants were watered daily and received Peters 20-20-20 fertilizer (W.R. Grace and Company, Allentown, PA) once a week. Thirty days after planting, plants were grouped into control (which was continually watered) and stressed groups. Water deficit stress was induced gradually by withholding watering following the initial measurement. Measurements were made every day.

Water Potential

Leaf water potential (Ψ) was measured on fully expanded leaves (4 leaves/pot) using leaf cutter thermocouple psychrometers (J.R.D. Morrill Specialty Equipment, Logan, UT) connected to a Wescor HP-115 (Wescor Inc., Logan, UT) automatic water potential measurement system. The Ψ values (MPa) reported here are averages of

fifteen 0.31 cm^2 leaf disks measured after two hours of equilibration of the thermocouple psychrometers in a water bath at 30°C.

Chlorophyll Fluorescence

Immediately after collecting tissue samples for determination of Ψ, measurement of chlorophyll fluorescence was made with a portable fluorometer (Polar Tech, Umea, Sweden) (Oquist and Wass, 1988). Four fully expanded leaves were sampled from each pot (4 leaves/pot x 4 pots = 16 leaves) and immediately placed in sleaves of black plastic with holes to guide the measuring probe. The leaves were dark adapted for 10 min prior to exposure for 5 s to excitation light (100 $\mu mol\ m^{-2}\ s^{-1}$ PAR). Fo, Fm, Fv, Fv/Fm, and $t_{1/2}$ were measured.

Chloroplast Isolation

Chloroplasts were isolated from leaves of 30-day-old wheat and tef plants using the procedure of Kee et al. (1986). Twenty g freshly cut fully expanded leaves were homogenized in a Waring blender (Model 7011-31b192, Waring, Hartford, CT) for 3 to 4 s at high speed in 200 mL ice cold isolation medium contained 30 mM Tricine (pH 7.5), 300 mM NaCl, 3 mM $MgCl_2$ and 0.5 mM EDTA. The resulting homogenate was filtered through 16 layers of cheesecloth (Veratec, Inc, Walpole, MA) and centrifuged (ICE Model B-201, Damon/ICE Division Needham Heights, MA) at 1500 g for 2 min. Pellets were resuspended with a cotton tip in 60 ml resuspension medium containing 5 mM HEPES-KOH (pH 7.5), 200 mM sorbitol, 2 mM $MgCl_2$, and 0.05% (w/v) BSA. The coarse debris was removed by a short centrifugation. The supernatant was filtered through one layer of Kimwipe tissue and recentriuged at 1500 g for 2 min. The pellets were resuspended in a small volume of resuspension medium and stored on ice. All the isolation steps were carried out on ice.

Chlorophyll Content

Chlorophyll content of the chloroplast suspensions was determined spectrophotometrically (Spectronic 1201, Millton Roy, Rochester, NY) according to Arnon (1949). To determine the chlorophyll content of leaves, sixteen 0.39 cm^2 leaf disks from sixteen different leaves (4 leaves/pot x 4 pots = 16 leaves) were punched out from the middle portion of the leaves and ground in a Pyrex tissue grinder (Corning 7727-7, UK) containing 2 mL 80% acetone (v/v). The extracts were centrifuged at full speed in a table-top centrifuge (Clay Adams, No. 0005, Parsippany, NJ) for 2 to 3 min. Absorbances of the extract at 663 nm (A_{663}), 645 nm (A_{645}) and 750 nm (A_{750}) were measured with a Spectronic 1201 spectrophotometer (Millton Roy, Rochester, NY). The contents of chl a, chl b, and chl (a+b) (mg L^{-1}) were calculated using the extinction coefficients and equations below from Arnon (1949) modified by using A_{750} as a zero absorbance baseline:

$$\text{chl a} = ((A_{663} - A_{750}) \times 12.7) - (A_{645} - A_{750}) \times 2.69))$$

$$\text{chl b} = ((A_{645} - A_{750}) \times 22.9) - (A_{663} - A_{750}) \times 4.68))$$

$$\text{Chl (a+b)} = ((A_{663} - A_{750}) \times 8.02) + (A_{645} - A_{750}) \times 20.2))$$

Chlorophyll contents in units of mass were converted to molar units using molecular weights of 893.5, 907.5 and 900 for chl a, chl b and chl (a+b), respectively (Nobel, 1991).

Photosynthetic Electron Transport

Electron transport activities of the isolated thylakoids were measured by monitoring O_2 evolution or consumption with a temperature controlled Clark-type oxygen electrode assembly (Model LD-2, Hansatech, Ltd., King's Lynn, Norfolk, UK) at 25°C and saturating light from a 100 W projector lamp (Quartizine 12 V, West

Germany). Thylakoids were assayed for whole chain ($H_2O \rightarrow MV$), PSII ($H_2O \rightarrow DAD_{ox}$) and PSI (DHQ→MV) electron transport rates. The procedures described by Kee et al. (1986) for tomato, and Allen and Holmes (1986) and Sabat et al. (1991) for spinach, beet, and pea were used with slight modifications. Details of the reaction media were presented in Chapter I. Electron transport rates are expressed in $\mu mol\ e^- mg^{-1}$ chl h^{-1}. The electron transport rates were calibrated with a known amount of $K_3Fe(CN)_6$ just before the beginning of every measurement (Allen and Holmes, 1986).

Electrolyte Leakage

On each day of the water stress treatment, sixteen test tubes containing 0.7 g leaf material and 20 mL deionized double distilled water (ddH_2O) were vacuum infiltrated at 120 to 140 mm Hg for 15 min. Prior to vacuum infiltration, sample leaves were first gently washed for approximately 90 min with 3 changes of ddH_2O and thereafter cut into 1 cm pieces (Permachandra and Shimada, 1987). The tubes were agitated at room temperature for 1 h in a shaker (DUBNOFF metabolic shaking incubator, Precision Sci. Group, Model D/S 120-070, Chicago, IL). The initial electrical conductance (C_1) of the bathing solution was measured (Cole-Parmer conductivity meter, Model 1481-60, Chicago, IL). The test tubes containing leaf samples were then autoclaved (NAPCO model-9000-D, Portland, OR) for 20 min at 121°C, agitated for 1 h, and a second electrical conductance measured (C_2). Electrolyte leakage was expressed as the C_1/C_2 ratio.

All measurements were repeated 3 times, and for each treatment 4 replicate measurements were made. A completely randomized design was used.

Chemicals

Dihydrochloride salt of DAD was purified from a slurry of charcoal and cellite in an ethanol-water (1:1, v/v) mixture containing 0.4% (w/v) ascorbate. For details see Chapter I. Fresh solutions of recrystallized DAD.2HCl were prepared in 10 mM HCl at the beginning of each experiment. DBMIB was a gift from Dr. D.R. Ort, University of Illinois. All other chemicals were of analytical grade.

RESULTS

Effects on Photosynthetic Electron Transport Rates of Isolated Thylakoids

The effect of water stress on leaves of wheat and tef was determined by measuring PSII ($H_2O \rightarrow DAD_{ox}$), PSI (DHQ→MV) and whole chain (H_2O→MV) electron transport rates of isolated thylakoids. Water deficit stress induced by withholding water resulted in significant reductions of PSII and whole chain electron transport of wheat and tef (Fig. 1, a and b). A decrease in Ψ from -0.3 MPa to -1.5 MPa resulted in 68% and 65% lower rates of PSII and whole chain electron transport of wheat (Fig. 1, a and b). Severe water stress, close to a Ψ of -2.5 MPa, resulted in complete loss of PSII activity and loss of 87% of whole chain electron transport activity (Fig. 1, a and b). PSII and whole chain electron transport rates of tef were also reduced by declining Ψ although not to the same extent as in wheat (Fig. 1, a and b). As Ψ decreased from -0.3 MPa to -2.5 MPa in this species PSII and whole chain activities declined by about 50% (Fig. 1, a and b). The difference in the relative sensitivity of electron transport of these two species is consistent with tef being adapted to cultivation in drier climates than wheat.

PSI electron transport was much less sensitive to water stress than whole chain or PSII electron transport in tef and it was unaffected by water stress in wheat (Table I).

Effects on *in vivo* Chlorophyll Fluorescence

Fm, Fv and Fm/Fo were relatively more responsive to water stress in wheat than in tef (Table II). PSII and whole chain electron transport was closely linked to changes in the chlorophyll fluorescence parameters. Fv/Fm (and also Fm/Fo) of wheat and tef was significantly affected by decreasing Ψ (Fig. 2 and Table II). Reducing Ψ to -2.5 MPa resulted in lowering Fv/Fm by 32% and 24% in wheat and tef, respectively. No consistent pattern was seen in Fo as Ψ gradually decreased (Table II). Also $t_{1/2}$ was largely unaffected by water stress (Table II). In both wheat and tef the decline in Fv/Fm was exponentially correlated with PSII and whole chain electron transport rates (Fig. 3, a and b).

Effect on Chlorophyll Content

The effect of water deficit stress on chlorophyll content of wheat and tef leaves is shown in Table III. On a leaf area basis chl a, chl b, and chl (a+b) contents and the chl a/b ratio of wheat remained largely unaffected by water stress until Ψ fell below -3.0 MPa. In tef a small chlorophyll peak was seen between -1.4 MPa to -2.1 MPa.

Effects on Electrolyte Leakage

Membrane damage was assessed by measuring electrolyte leakage of leaf samples. Electrolyte leakage of wheat and tef subjected to water deficit stress is shown in Figure 4. Membrane leakiness increased gradually as Ψ decreased to about -2.4 MPa in wheat. At even lower leaf Ψ electrolyte leakage increased greatly to a highest value of 0.47 (Fig. 4). In tef leakage increased rapidly as Ψ dropped to -2.4 MPa after which it remained constant (Fig. 4). The observed highest leakage in tef was 0.35 (Fig. 4).

The correlation between electrolyte leakage and photochemical properties was determined by regression analysis. The correlation between Fv/Fm and electrolyte leakage of wheat and tef was high ($r=-0.97$, -0.89 $P \leq 0.05$) as shown in Figure 5a). Both PSII and whole chain electron transport activities were significantly correlated with electrolyte leakage (Fig. 5, b and c). However, electron transport responded to water deficit stress already at greater Ψ than membrane leakage. Thus, it appears that substantially enhanced membrane leakage occurs first at sever water stress (below -1.0 to -2.0 MPa) whereas photochemical inhibition develops earlier.

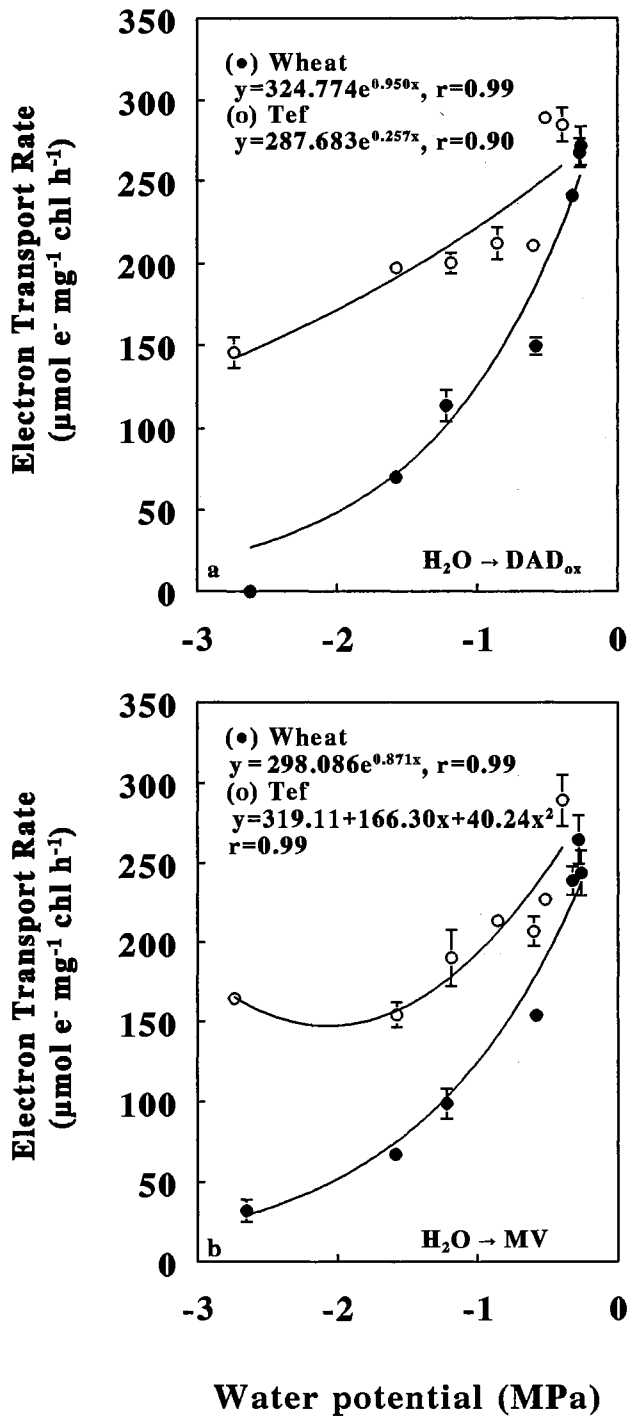

Figure 1. Response to Ψ of PSII (a) and whole chain (b) electron transport rates in wheat and tef. Bars indicate ± SE. The experiment was performed three times with four replications for each treatment.

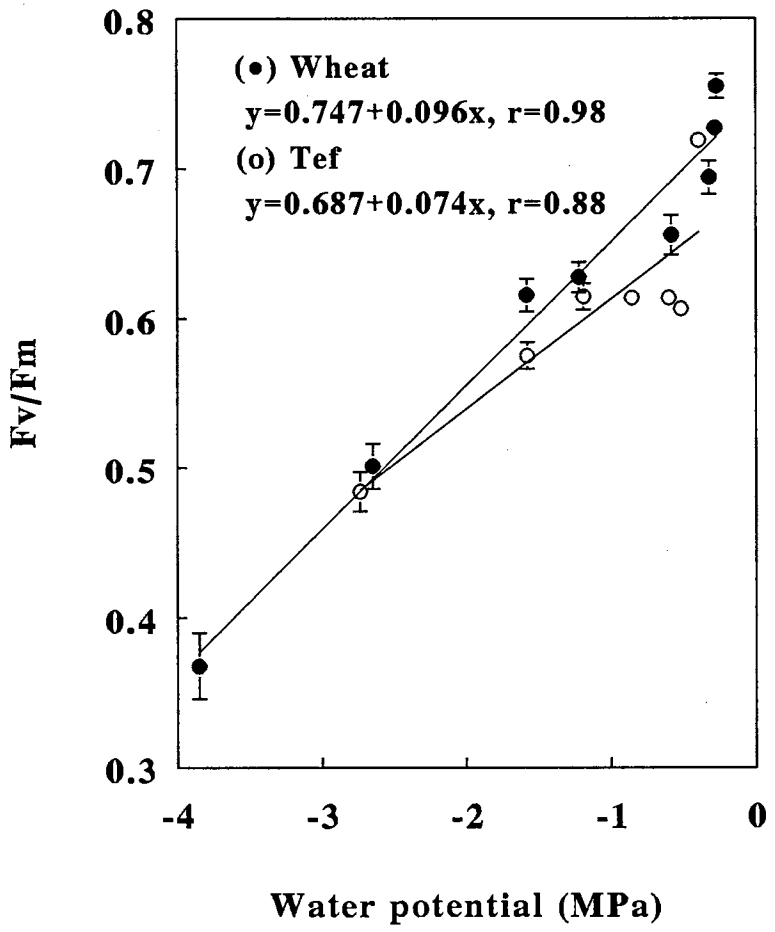

Figure 2. Response to Ψ of the ratio of variable to maximum fluorescence (Fv/Fm) of wheat and tef. Intact leaves were dark adapted for 10 minutes prior to exposure to excitation light with an intensity of 100 μmol m^{-2} s^{-1} PAR. Vertical bars indicate ± SE. The experiment was performed three times with four replications for each treatment.

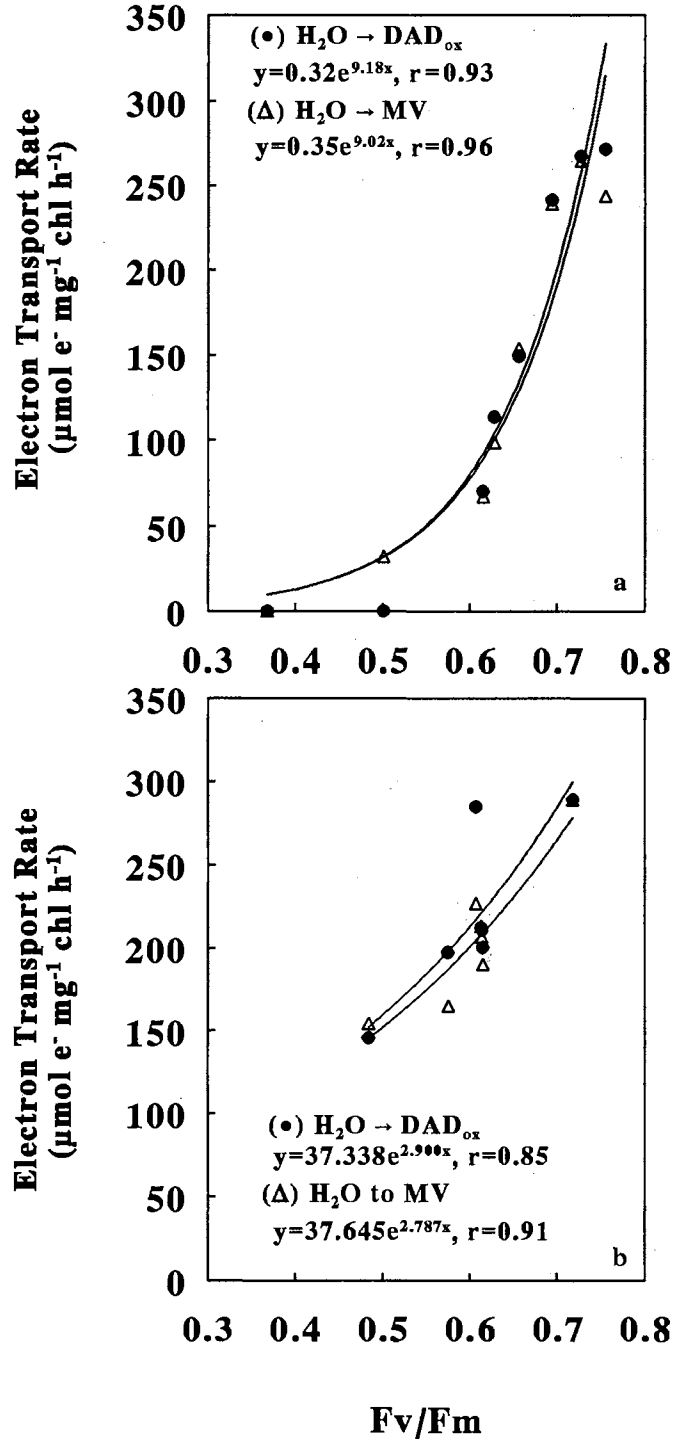

Figure 3. Relationship between electron transport and Fv/Fm in water stressed wheat (a) and tef (b) leaves. Symbols represent mean values for each day of three repeated experiments.

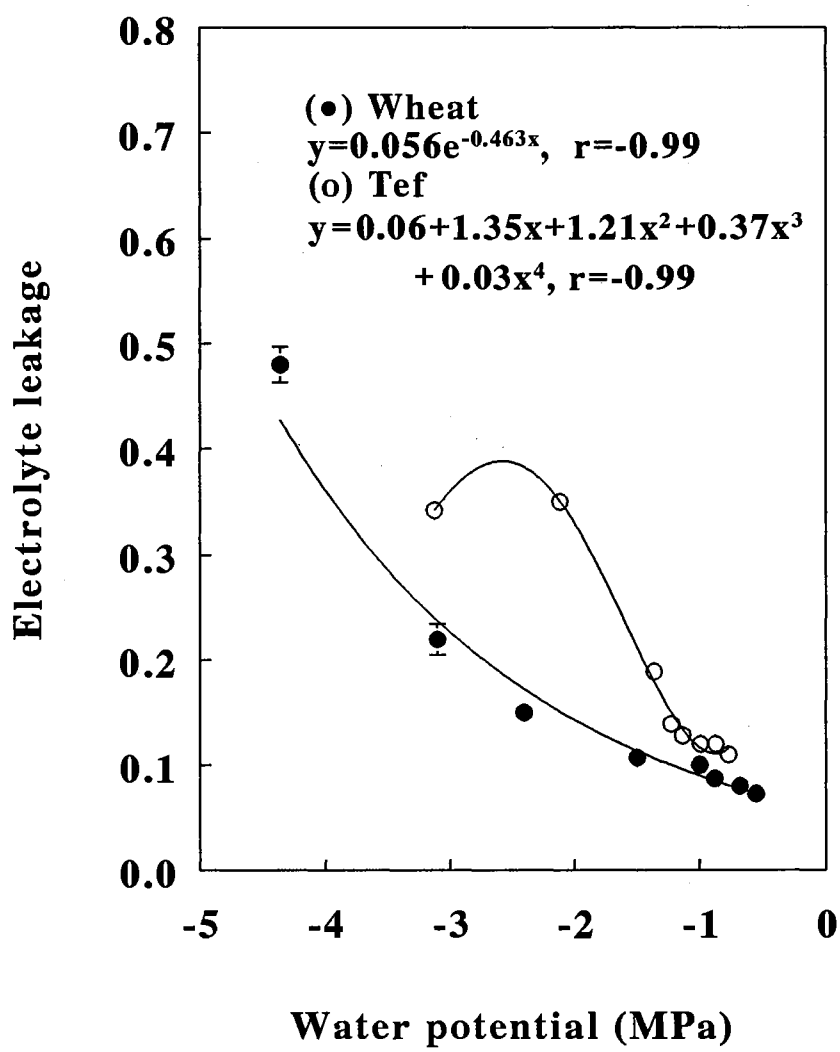

Figure 4. Electrolyte leakage of wheat and tef as functions of Ψ. Bars indicate ± SE. The experiment was performed three times with four replications for each treatment.

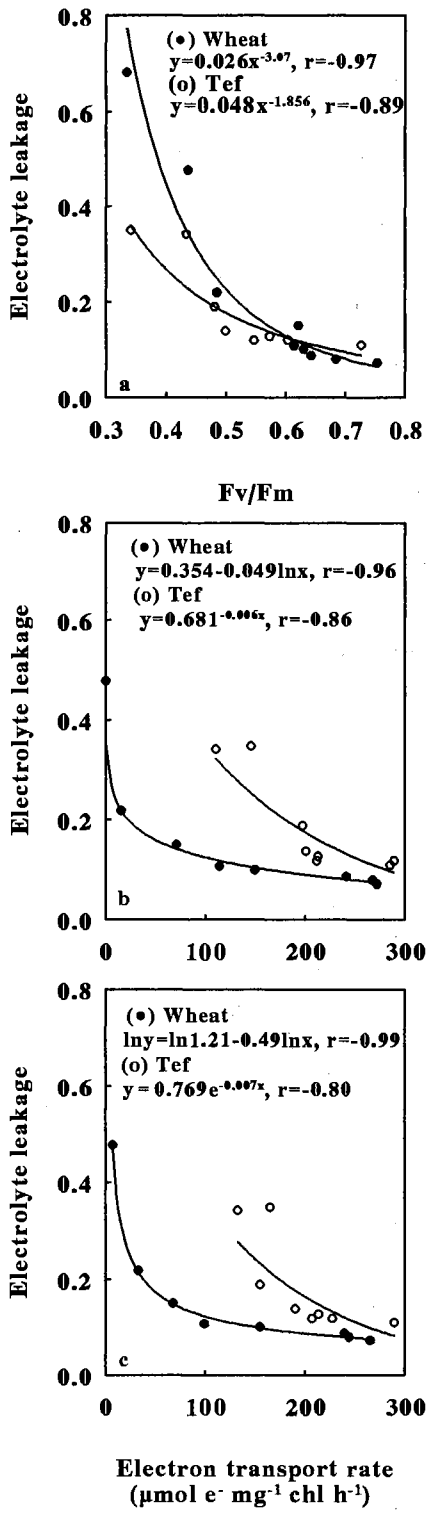

Figure 5. Relationship between electrolyte leakage and Fv/Fm (a), PSII (b) and whole chain (c) electron transport in water stressed wheat and tef leaves. Symbols represent mean values for each day of three repeated experiments.

Table I. Effects of water deficit stress on photosystem I (PSI) electron transport rate of thylakoids isolated from wheat and tef. The experiments were performed three times with four replications for each treatment. Data are means ± SE.

Species	Ψ (MPa)	PSI electron transport rate (μmol e^- mg^{-1} chl h^{-1})†
Wheat	-0.29±0.02	212±14
	-0.33±0.03	202±19
	-0.40±0.03	223±26
	-0.87±0.11	242±18
	-1.72±0.17	204±18
	-2.14±0.15	203± 5
	-3.73±0.20	231±23
Tef	-0.39±0.03	229± 7
	-0.51±0.03	202± 3
	-0.60±0.06	235± 7
	-0.85±0.05	183± 1
	-1.19±0.10	236± 5
	-1.58±0.06	182± 4
	-2.74±0.17	168± 2

† Electron transport rate from DHQ → MV was measured with Clark-type oxygen electrode.

Table II. Effects of water deficit stress on chlorophyll fluorescence (Fo, Fm, Fv, Fm/Fo and $t_{1/2}$) of dark adapted wheat and tef leaves. The experiment was performed three times with four replications for each treatment. Data are means ± SE. Intact leaves were dark adapted for 10 min prior to exposure to an excitation light intensity of 100 μmol m^{-2} s^{-1} PAR.

Species	Ψ (MPa)	Fo	Fm	Fv	Fm/Fo	$t_{1/2}$ (ms)
		(relative units)				
Wheat	-0.26±0.02	0.093±0.000	0.390±0.013	0.297±0.013	4.13±0.14	183± 5
	-0.28±0.02	0.122±0.000	0.430±0.009	0.311±0.009	3.51±0.08	188± 6
	-0.32±0.01	0.114±0.001	0.403±0.014	0.292±0.012	3.55±0.13	192± 3
	-0.58±0.02	0.122±0.002	0.368±0.013	0.246±0.012	3.02±0.11	189± 7
	-1.23±0.03	0.124±0.002	0.358±0.011	0.234±0.006	2.90±0.09	206± 7
	-1.59±0.07	0.106±0.001	0.309±0.011	0.198±0.012	2.92±0.11	163± 4
	-2.61±0.06	0.098±0.001	0.233±0.008	0.141±0.015	2.37±0.09	232±25
	-3.91±0.08	0.106±0.001	0.183±0.006	0.085±0.007	1.73±0.06	263±19
Tef	-0.39±0.03	0.085±0.001	0.296±0.006	0.215±0.005	3.49±0.08	220± 3
	-0.51±0.03	0.097±0.000	0.232±0.002	0.136±0.003	2.40±0.03	244± 4
	-0.60±0.06	0.093±0.000	0.229±0.003	0.139±0.004	2.45±0.04	250± 4
	-0.85±0.05	0.090±0.001	0.240±0.004	0.155±0.005	2.66±0.05	248± 5
	-1.19±0.10	0.077±0.000	0.207±0.003	0.130±0.004	2.69±0.05	234± 7
	-1.58±0.06	0.088±0.000	0.209±0.005	0.120±0.060	2.39±0.06	211± 9
	-2.74±0.17	0.093±0.000	0.189±0.004	0.099±0.003	2.02±0.05	261± 7

Table III. Effects of water deficit stress on the contents of chl a, chl b, and chl (a+b) and on the chl a/b ratio of wheat and tef. The experiment was performed 3 times with 4 replications for each treatment. Data are means ± SE.

Species	Ψ (MPa)	chl a	chl b	chl (a+b)	chl a/b
			(μmol m^{-2})		
Wheat	-0.56±0.04	406±15	120±4	526±19	3.36±0.03
	-0.69±0.03	366± 9	108±2	474±12	3.37±0.02
	-0.88±0.03	367±11	106±3	473±14	3.44±0.02
	-1.01±0.05	369±10	110±3	479±13	3.35±0.07
	-1.53±0.10	313±12	100±5	413±15	3.17±0.07
	-2.40±0.09	365±13	110±5	475±17	3.33±0.05
	-3.17±0.07	378±15	111±5	489±19	3.41±0.02
	-4.36±0.32	286±17	82±5	368±21	3.46±0.10
	-4.46±0.22	281±13	82±6	363±17	3.59±0.25
tef	-0.77±0.05	247± 6	75±6	322± 6	3.47±0.14
	-0.87±0.06	281±12	85±4	366±16	3.33±0.07
	-0.99±0.05	282± 5	77±2	359± 7	3.66±0.06
	-1.13±0.05	270± 5	84±5	354± 8	3.31±0.13
	-1.22±0.10	272± 8	75±2	357± 7	3.62±0.03
	-1.36±0.06	367± 9	102±2	469±16	3.59±0.03
	-2.11±0.07	337±10	99±3	436±12	3.39±0.08
	-3.12±0.28	248± 5	75±2	323± 7	3.29±0.04

DISCUSSION

Tef (*Eragrostis tef* Z.) is a cereal member of the NAD-malic enzyme subgroup of C_4 plants (Edwards and Walker, 1983). It belongs to the subfamily Eragrostideae (Gutierrez et al., 1974) and is adapted to warmer climates. Wheat (*Triticum aestivum* L.), being a temperate C_3 cereal, is more drought susceptible (Mayoral et al., 1981) and is grown in less dry conditions. To study the effects of water deficit stress on the two plant species, measurements of chlorophyll fluorescence and content, photosynthetic electron transport, and electrolyte leakage were made.

With the exception of chlorophyll content, all measurements indicated greater drought sensitivity of wheat than tef (Fig. 1, a and b; Fig. 2; Fig. 4). Water deficit stress caused a significant decline in PSII and whole chain electron transport (Figs. 1a, 1b) of isolated thylakoids indicating inhibition of the photosynthetic machinery at the level of thylakoid-mediated reactions. Boyer and Younis (1984) estimated that 45% water was lost from stressed sunflower leaves in the range of -0.2 MPa to -1.5 MPa. In our study 45% water was lost from -0.3 MPa to -1.6 MPa (data not shown) and this was sufficient to reduce PSII and whole chain electron transport of wheat by 71% and 68% and of tef by 30% and 43%, respectively (Fig. 1, a and b). These results are consistent with the notion that decreased photosynthesis under low Ψ is linked not only to stomatal closure but also to inhibition of chloroplast activity. Similar effects were reported under a variety of stress treatments and in a wide variety of plant

species (Boyer and Bowen, 1970; Keck and Boyer, 1974; Mayoral et al., 1981; Boyer and Younis, 1984; Bjorkman and Powles, 1984). However, the results of the present study contradict conclusions by Kaiser (1987) and Havaux (1992) that PSII is insensitive to water stress. The response of the photochemical activity both *in vivo* and in isolated thylakoids is species specific. Also, the relative magnitude of stress, light conditions, age of the plant and methodology might produce different results. PSI-driven electron transport was less sensitive to water stress (Table I), which is in agreement with earlier studies (Boyer and Younis, 1984; Bjorkman and Powles, 1984; Havaux, 1992) implying that the decline in whole chain activity of water stressed leaves was caused by inhibition of PSII.

The inhibition of chloroplast activity was previously demonstrated (Fellows and Boyer, 1976; Boyer and Younis, 1984). Changes in thylakoid membrane conformation and cellular constituents have been suggested to be the two major reasons. Under condition of low Ψ thylakoid lamellae become thinner (Fellows and Boyer, 1976). The Mg^{2+} concentration, which is typically in the 1-3 mM range in well watered plants, increases with decreasing Ψ to values as high as 9 mM at Ψ = -2.5 MPa (Boyer and Younis 1984). Such membrane changes could have diminished the rate of electron transport of isolated thylakoids and Fv/Fm of dark adapted leaves also in our study. Boyer and Bowen (1970) reported that inhibition of oxygen evolution by water stress was detectable within 5-60 min and occurred in the -0.8 MPa to -1.2 MPa range in sunflower and pea leaves. In our experiments with gradual loss of water both PSII and whole chain electron transport declined immediately as Ψ fell below that of the unstressed controls.

As previously reported by Bjorkman and Powles (1984) reduced PSII and whole chain electron transport of wheat was accompanied by changes in Fv/Fm (Fig. 2). Govindjee et al. (1981) and Bjorkman and Powles (1984) found that fluorescence emitted by chlorophyll a was lower in water stressed leaves of *Nerium oleander*, *Atriplex triangularis* and *Tolmiea menziesii* than in control leaves. Our results (Table II) support their conclusion that the lowering of Fm/Fo, Fv/Fm and Fm are an indication of inhibition of the primary photochemistry of PSII. Thus, the influence of water deficit stress on Fv/Fm of wheat is linked to the sensitivity of PSII electron transport to low Ψ. Govindjee et al. (1981) pointed out that when the inhibition is on the donor side of PSII, Fm/Fo is reduced. This suggests that electron transport in our study, especially in wheat, was blocked by water stress on the donor side of PSII (Govindjee et al., 1981; Kicheva et al., 1994). For photosynthesis as a whole, however, the range of sites sensitive to water stress may span from photochemistry to the dark reaction (Kaiser, 1984). There may be more than one simultaneous blockage (Kaiser, 1984).

The decline in Fv/Fm was accompanied by reduced Fm and Fv (Table II). In agreement with previous work by Govindjee et al. (1981), Bjorkman and Powles (1984) and Bjorkman (1989), no consistent pattern was seen in Fo as Ψ decreased (Table II). The absence of an effect on Fo, indicates that the reaction center of PSII remained open even at severe water stress. Reduced Fv and Fm reflect increased non-radiative energy dissipation and may result from degradation of the photosynthetic apparatus (Fock et al., 1992). Bjorkman and Powles (1984) made interesting observations on leaves subjected to water stress and bright light. They concluded that PSII is vulnerable to photoinibition during stress exposure and, thus, a combination of water deficit stress and bright light might inhibit the photosynthetic machinery at the

thylakoid level. Several investigators have now verified the occurance of photoinhibition in the presence of water stress (Powles, 1984; Long et al., 1994). It is possible the plants in our study suffered from secondary photoinhibitory damage.

Chlorophyll contents of wheat and tef remained unaffected by water deficit stress (Table III). The unchanged chlorophyll contents of wheat and tef are consistent with reports by Bjorkman and Powles (1984) and Stuhlfauth et al. (1990) but contradict work of Mayoral et al. (1981) and Baisak et al. (1994) on *Triticum aestivum*. Baisak et al. (1994) reported a rapid decline in chlorophyll content of water stressed wheat leaves. The stress was incurred by incubating excised leaf segments in polyethylene glycol (PEG 6000) solutions. Their findings were likely affected by the use of PEG to induce stress. Water stress might have accelerated senescence of the excised leaf samples and caused chlorophyll degradation. Attached leaves are likely less susceptible to chlorophyll breakdown. In our study chlorophyll bleaching was noted first below -3.0 MPa whereas CO_2 fixation was completely inhibited at substantially greater Ψ. Thus, we conclude that inhibition of photosynthesis by water stress is unrelated to chlorophyll destruction.

It has been reported previously in various species (Blum and Ebercon 1981, Martin et al., 1987; LeBlanc et al., 1993; Tan and Blake, 1993), that water deficit stress is related to increased electrolyte leakage of leaf tissue as we found to be the case in wheat and tef (Fig. 4). The extent of leakage was low at Ψ above -1.0 to -2.0 MPa although significant inhibition of photosynthetic activities occurred. According to Boyer and Younis (1984) reduction of Ψ to -2.0 MPa corresponded to a leaf tissue water loss of about 50%. The present study, therefore, agrees that leakage from dehydrated leaf tissues is low until relative cell volumes below 50% are reached

(Kaiser, 1987). Beyond -2.4 MPa the extent of membrane leakiness of wheat markedly increased with the decline in Ψ (Fig. 4). In tef that better tolerates warm and dry climates (Edwards and Walker, 1983), more extensive leakage below -2.4 MPa did not occur. Several reports indicate genotypic differences with regard to stress tolerance and electrolyte leakage (Blum and Ebercon, 1981; Martin et al., 1987; LeBlanc and Dhindsa, 1993; Tan and Blake, 1993). Tan et al. (1992) noted a negative correlation between membrane leakage and ability to accumulate sugars during water stress in the tolerant species black spruce. In tolerant species compatible solutes such as sugars are believed to play a role in osmoregulation and reduce membrane damage (Tan et al., 1992).

Increase in electrolyte leakage reflects disruption of membrane integrity by water stress (Gupta, 1977) presumably due to increased membrane contraction, potassium efflux (Shcherbakova and Kacperska 1983) or free radical formation (Dhindsa and Matowe 1981). A change in composition of plasmalemma and tonoplast was related to the loss of membrane integrity in sensitive plants (Bewley, 1979). Many studies have suggested membrane lipids as an initial site of injury (Chetal et al., 1983). Changes in sterol, phospholipid and glycolipid composition were correlated with electrolyte leakage in water stressed leaves of black spruce (Zwiazke and Blake, 1990).

The parallel effects of water stress on membrane leakiness and thylakoid activities (Fig. 5, a-c) suggest that cellular membranes are the target of water stress injury. Chetal et al. (1983) reported changes in phospholipid contents of chloroplasts in stressed leaves of wheat and barley at tillering. Among the changes of the different phospholipid components, there was a drastic decrease in phosphatidylglycerol (PG)

and a marked increase in phosphatidylcholine (PC, major constituents of non-photosynthetic membranes). The link between photosynthetic capacity and membrane leakage might be phosphatidylglycerol of the plasmalemma. Dysfunctioning of the chloroplast, which is the main site of fatty acid biosynthesis, may indirectly interfere with the function of nonchloroplastic membranes (Whitaker, 1992). Monogalactosylidiacylglycerol is the major component of the thylakoid membrane (Harwood, 1980). It is likely that this component is also affected by a general interference with fatty acid synthesis.

In conclusion, we here compare and contrast the responsiveness to water deficit stress of various photosynthetic and cellular properties of wheat and tef. There was good agreement between electron transport and chlorophyll fluorescence. Membrane leakage and chlorophyll content were affected only by severe stress. Thus, chlorophyll content and electrolyte leakage are not early sensors of water stress. They rather appear to reflect severe stress, perhaps even cell death. Of the four stress indicators we used, electron transport and chlorophyll fluorescence detected water stress injury at less sever stages of stress than did membrane leakage and chlorophyll content.

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CHAPTER IV

PHOTOSYNTHETIC GAS EXCHANGE OF LEAVES OF WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.) EXPOSED TO WATER DEFICIT STRESS

SENAYET ASSEFA AND BJORN MARTIN

Abbreviations: A, rate of net CO_2 fixation in ambient air; A_{max}, light and CO_2 saturated rate of A; c.e., initial slope of A/C_i curve (carboxylation efficiency); C_a, CO_2 concentration in the air; C_i, CO_2 concentration in the intercellular air spaces; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); DHQ, durohydroquinone (tetramethyl-*p*-hydroquinone); Fo, initial fluorescence; Fm, maximum fluorescence; Fv, variable fluorescence (Fv = Fm - Fo); g_s, stomatal conductance for water vapor; I_{ce}, percent reduction of A due to limiting c.e. (carboxylation efficiency limitation); I_{gs}, percent reduction of A due to limiting g_s (stomatal limitation); Ψ, leaf water potential; MV, methyl viologen; PSI and PSII, photosystem I and II.

ABSTRACT

Leaf gas exchange of the C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101), and the C_4 cereal Tef (*Eragrostis tef* Z. cv. DZ-01-354) subjected to water deficit stress was studied in the laboratory using an open gas exchange system. Net photosynthesis rate (A) and the photosynthetic components A_{max} (light and CO_2 saturated rate of photosynthesis), g_s (stomatal conductance) and c.e. (carboxylation efficiency) were determined. Reducing the leaf water potential (Ψ) from -0.5 MPa to -2.3 MPa lowered A, g_s, c.e., and A_{max} of wheat by 94%, 95%, 86%, and 78%, and tef by 85%, 60%, 84%, and 62%, respectively. Thus, in wheat there were proportional decreases in A and g_s, whereas in tef A declined significantly more than g_s. Stomatal limitation of photosynthesis (l_{gs}) increased more in water stressed wheat than in tef, indicating that A became relatively more limited by stomata at low Ψ in the former species. The dependence of the intercellular CO_2 concentration (C_i) on Ψ suggested that the non-stomatal limitation became relatively greater in tef over the entire range of declining Ψ. In contrast, in wheat the stomatal limitation increased in the range of moderate stress (down to about -1.8 MPa), whereas at severe stress the non-stomatal limitation grew relatively larger again. The greater inhibition of A, A_{max} and c.e. of wheat at low Ψ was consistent with observed reduced rates of PSII electron transport. A, A_{max} and electron transport were linearly correlated with Fv/Fm. Electron transport and Fv/Fm were as sensitive as gas exchange to water deficit stress. Membrane

leakage was greatly enhanced first at Ψ below -2.0 MPa, whereas inhibition of A appeared at much higher Ψ. These results suggest that water stress reduced A of tef primarily through biochemical inhibition, whereas both biochemical and stomatal inhibitions were prominent in wheat.

INTRODUCTION

Water stress inhibits A by inducing stomatal closure and by impairing chloroplast activity (Boyer and Bown, 1970; Matthews and Boyer, 1984; Kaiser, 1987; Johnson et al., 1987; Sharkey, 1990). Considerable decreases in A, c.e and g_s, and an increase in the CO_2 compensation point have been reported in water stressed wheat plants (Johnson et al., 1987; Steuer et al., 1988; Kicheva et al., 1994).

Stomatal closure starves the carboxylating machinery by elevating the diffusive resistance to CO_2 entering the leaf. Stomata may be the only major limitation at mild stress (down to -0.9 MPa, Cornic et al., 1992) caused by either withholding water or increasing the water vapor deficit (VPD) of the air (Steuer et al., 1988; Dai et al., 1992). Non-stomatal inhibition of A was suggested to occur at water deficits greater than 30% (Kaiser, 1987; Cornic et al., 1989). However, reduced A due to inhibition of chloroplast activity has also been reported at mild stress in sunflower (-0.5 MPa, Matthews and Boyer, 1984; Sharp and Boyer, 1986) and wheat (-0.8 MPa, Mayoral et al., 1981). As water stress develops, g_s declines causing reduced CO_2 diffusion rates into the leaf and reduced C_i (Johnson et al. 1987). Photosynthesis can be restored by elevating the partial pressure of CO_2 in the air surrounding the leaf (Sharkey, 1990; Graan and Boyer, 1990; Pospisilova et al., 1992). Impaired mesophyll capacity at low Ψ reduces the capacity to fix available CO_2. Therefore, inhibition of chloroplast activity can not be fully restored by increasing the partial pressure of CO_2.

Methods of distinguishing between stomatal and non-stomatal inhibition of A use the relationship between A and C_i (Johnson et al., 1987). There are reports that C_i decreases slightly when stomata close (Steuer et al., 1988; Cornic and Briantais, 1991; Cornic et al., 1992; Dai et al., 1992), whereas other report have found that C_i remains stable (Renou et al., 1990) or even increases (Ephrath et al., 1993). Non-stomatal factors are visualized by altered chloroplast ultrastructure, and inhibition of photosynthetic electron transport have been reported to contribute to the reduction in A (Matthews and Boyer, 1984). However, Downton et al. (1988), and Cornic et al. (1989) suggested that calculated C_i values are misleading if there are inhomogeneities in stomatal apertures across the leaf surface. Gunasekera and Berkowitz (1991) in wheat, Wise et al. (1992) in cotton, and Ni and Pallardy (1992) in woody angiosperms, used $^{14}CO_2$ pulses to evaluate the extent of non-homogenous stomatal closure in water stressed plants. Patchy deposition of radioactive photosynthate first occurred at Ψ as high as -1.3 MPa or as low as -2.6 MPa depending on species and preconditioning. Wise et al. (1992) reported that when patchiness occurred it was of stomatal nature because patchy deposition of radioactivity could be avoided by increasing the CO_2 concentration in the air.

Limitation of A at the level of the chloroplast has been proposed (Matthews and Boyer, 1984; Kaiser, 1987; Masojidek and Hall, 1992). In sunflower and sorghum Matthews and Boyer (1984) and Masojidek and Hall (1992), respectively, suggested that inhibition of PSII electron transport accounted for most of the inhibition of A at low Ψ. Inactivation of photophosphorylation (Sharkey and Badger, 1982) has also been reported. Boyer and Younis (1984) suggested that changes both in the concentration of cellular constituents (Mg^{+2}) and conformation of thylakoid

membranes were responsible for the decreased rate of electron transport of water stressed chloroplasts. Other studies using different species have not supported the contention of reduced electron transport at low Ψ (Stuhlfauth et al., 1988; Cornic et al., 1989; Castonguay and Markhart, 1991). The contrasting findings suggest variation among species, and the use of many different electron donors (DHQ, tetramethyl-p-phenylene diamine (TMPD)) and acceptors (ferricyanide ($Fe(CN)_6$, DAD, p-phenylene diamine (PD), MV) may also be partly responsible. Masojidek and Hall (1992) used three electron acceptors ($Fe(CN)_6$, PD, MV) in studying drought stressed sorghum plants. They noted the greatest damage in the $H_2O \rightarrow$ MV reaction. Some studies using chlorophyll fluorescence have suggested inactivation of the primary photochemistry by water stress (Bjorkman and Powles, 1984; Martiniello and Blum, 1989).

A decrease in the initial slope (c.e.) of the photosynthetic CO_2 response curve has also been reported to result from drought stress and suggests an effect on the Calvin cycle (Martin and Ruiz-Torres, 1992; Kicheva et al., 1994). It is not known whether this is a direct effect or downregulation in response to reduced g_s.

Increased membrane leakage have been proposed to result from water stress. A decrease in chloroplast phospholipid content (Chetal et al., 1983) could result in chloroplast membrane dysfunction. However, the understanding of the relationship between leakage and photosynthesis remains incomplete.

The objectives of the present study were: a) to investigate the response of photosynthesis and its components of a C_3 and a C_4 plant to water deficit stress and, b) to examine the relationship of photosynthesis to stomatal, biochemical and photochemical components, and electrolyte leakage. The gas exchange

measurements presented here were made on intact leaves. Chlorophyll fluorescence measurements were made on the same plants. Measurements of the electron transport rates and electrolyte leakage are from a parallel experiment that has been previously presented. Data points linking electron transport and electrolyte leakage to gas exchange were calculated at equal Ψ.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Exposure

Seeds of the temperate C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101), and the subtropical C_4 cereal tef (*Eragrostis Tef* Z. cv. DZ-01-354) belonging to the NAD-malic enzyme subgroup were grown in a controlled environment growth chamber (Conviron CMP 3244, Winnipeg Ltd., Manitoba, Canada) in pots containing a modified peat moss and top soil mix (1:1 v/v) (Metro Mix growing medium, Grace Sierra Horticultural Products Company, Milpitas, CA). Wheat and tef were maintained at 25°C/18°C and 30°C/23°C, respectively, day/night temperature and 14 h photoperiod. The irradiance at plant height was between 460 and 500 μmol m^{-2} s^{-1} PAR. The chambers were humidified to maintain at least 50% RH. To meet the continuous need of plants for experimentation, seeds of wheat and tef were planted every week. Therefore, when the growth chamber was full the RH was occasionally higher than 50%. A week after emergence the plants were thinned to 3 plants per pot. One plant was used for gas exchange measurement, and the remaining two plants for concurrent measurement of chlorophyll fluorescence. The plants were watered daily and fertilized with Peters 20-20-20 (W.R. Grace and Company, Allentown, PA) once a week.

Water Deficit Stress Exposure

Thirty days after seeding plants were split into a control group, which was

watered regularly, and a stressed group from which water was withheld to slowly induce water deficit stress. Every morning plants were brought from the growth chamber to the laboratory for gas exchange measurements. They were returned to the same chamber once measurements were completed. Measurements on the same plant continued until the end of the stress period.

Gas Exchange Measurements

Measurements were carried out on recently fully expanded attached leaves (2nd or 3rd from the top) held horizontally in the assmilation chamber of an open gas exchange system previously described by Johnson et al. (1987). The CO_2 concentration was measured using an infra-red gas analyzer (Horiba PIR 2000 R, Horiba Instrument Inc., Irvine, CA), and chamber humidity with a dew point hygrometer (System 1100DP, General Eastern Instruments Corporation, Watertown, MA). Dry, CO_2 free air from a compressed air cylinder mixed in various ratios with dry air from another cylinder containing 1700 $\mu L\ L^{-1}$ CO_2 was used. The measurements were made in air ranging from 0 to 1700 $\mu L\ L^{-1}$ CO_2. Air temperature was maintained at 25°C and 30°C for wheat and tef, respectively (identical to daytime growth temperatures). The irradiance incident on the leaf was 1800 $\mu mol\ m^{-2}\ s^{-1}$ PAR, measured with a quantum sensor (LI-19OSB, Li-Cor Instruments, Lincoln, NE), provided by a 1000 W multivapor lamp (R1000, General Electric, Cleveland, OH). Stress was reduced on the main part of the plant that remained outside the assimilation chamber by covering it with white cloth. The RH in the assimilation chamber was maintained at 50% by adjusting the leaf area and air flow rate. However, with the most stressed plants, having very small transpiration rates, RH occasionally fell below 50%. Ambient photosynthesis rate (A) was measured at 350±2

$\mu L\ L^{-1}\ CO_2$, and 350±1 $\mu L\ L^{-1}\ CO_2$ in the air (C_a) for wheat and tef, respectively. A_{max} was measured at 1600 to 1700 $\mu L\ L^{-1}\ CO_2$ in the air. Calculations of g_s and C_i followed the methods of von Caemmerer and Farquhar (1981). As soon as a gas exchange measurement was completed, the leaf was removed from the chamber and its area determined using a leaf area meter (LI-3000A Portable Area Meter combined with LI-3050A Transparent Belt Conveyer, Li-Cor Instruments, Lincoln, NE). The calculated C_i values were plotted against net rate of photosynthesis to produce A/C_i curves. The A/C_i curves were generated by measurements at about 15 ambient CO_2 concentrations ranging 0 to 1700 $\mu L\ L^{-1}$. The carboxylation efficiency (c.e.) was calculated from the initial slope of the A/C_i curve. Three to five data points within the linear part of the curve at C_i values below 200 $\mu L\ L^{-1}$ were used. The stomatal limitation of photosynthesis (l_{gs}) was calculated according to Farquhar and Sharkey (1982), whereas the limitation caused by carboxylation efficiency (l_{ce}) was calculated using the method of Martin and Ruiz-Torres (1992).

Water Potential Measurements

Leaf disks for measurement of Ψ were collected from the same leaves previously used for gas exchange measurements. Ψ was measured with leaf cutter thermocouple psychrometers (J.R.D. Morrill Specialty Equipment, Logan, UT) connected to a Wescor HP-115 (Wescor Inc., Logan, UT) automatic water potential measurement system. The Ψ values reported here are averages of 12 (3 disks per leaf x 4 replicate leaves) 0.31 cm^2 leaf disks measured after two hours of equilibration of the thermocouple psychrometers in a water bath at 30°C.

Chlorophyll Fluorescence

Measurements of chlorophyll fluorescence were made at room temperature with a portable fluorometer (Polar Tech, Umea, Sweden) (Oquist and Wass, 1988). Fully expanded leaves were dark adapted for 10 min prior to a 5 s exposure to excitation light with an intensity of 100 μmol m^{-2} s^{-1} PAR. Fo, Fm, Fv, Fv/Fm, and $t_{1/2}$ were measured.

Chloroplast Isolation

Chloroplasts were isolated from leaves of 30-day-old wheat and tef plants using the procedure of Kee et al. (1986). Details are in Chapters I, II and III.

Photosynthetic Electron Transport

Electron transport activities of the isolated thylakoids were measured by monitoring O_2 evolution or consumption with a temperature controlled Clark-type oxygen electrode assembly (Model LD-2, Hansatech, Ltd., King's Lynn, Norfolk, England) at 25°C and saturating light from a 100 W projector lamp. Thylakoids were assayed for whole chain ($H_2O \rightarrow MV$), PSII ($H_2O \rightarrow DAD_{ox}$) and PSI ($DHQ \rightarrow MV$) electron transport rates. The procedures described by Kee et al. (1986) for tomato, and Allen and Holmes (1986) for spinach and pea were used with slight modifications. See details in Chapter I.

Electrolyte Leakage

Test tubes containing 0.7 g leaf material and 20 mL deionized double distilled water (ddH_2O) were vacuum infiltrated at 120 to 140 mm Hg for 15 min. Prior to vacuum infiltration, sample leaves were gently washed for approximately 90 min with 3 changes of ddH_2O and cut into 1 cm pieces. The initial electrical conductance (C_1) of the bathing solution was measured (Cole-Parmer conductivity meter, Model 1481-60, Chicago, IL). Leaf samples were then autoclaved for 20 min at 121°C, and the

electrical conductance (C_2) measured again. Electrolyte leakage was expressed as the C_1/C_2 ratio.

All measurements were repeated at least twice, and for each treatment four replicate measurements were made. A completely randomized design was used.

RESULTS

Figure 1 shows the response to decreasing Ψ of ambient CO_2 fixation rate (A) and several related parameters. Exponential decreases in A with decreasing Ψ were observed in leaves of both wheat and tef (Fig. 1a). Although also A_{max} decreased substantially in both crops with declining Ψ (Fig. 1b) the reduction in A_{max} was proportionally smaller than the reduction in A (Fig. 1, a and b). The decrease in A ranged from about 21.0 μmol m^{-2} s^{-1} to 1.2 μmol m^{-2} s^{-1} (94% reduction) and from 26.7 μmol m^{-2} s^{-1} to 4.0 μmol m^{-2} s^{-1} (85% reduction) as Ψ decreased from -0.5 MPa to -2.3 MPa in wheat and tef, respectively (Fig. 1a). A_{max} decreased from 30.1 μmol m^{-2} s^{-1} to 6.9 μmol m^{-2} s^{-1} (78%) and 30.0 to 11.4 μmol m^{-2} s^{-1} (62%) in wheat and tef, respectively in the same range of Ψ (Fig. 1b). Regression analysis showed that the correlations between A and A_{max} in water stressed wheat ($A=-4.856+0.798A_{max}$, $r=0.99$, $P \leq 0.05$, data not shown), and tef ($A=-5.480+0.901A_{max}$, $r=0.96$, $P \leq 0.05$, data not shown) were linear. The negative y-intercepts of the regressions suggest that under stress a relatively smaller proportion of the photosynthetic potential was achieved in ambient air.

Like the response of A to water stress, g_s in wheat decreased significantly (Fig. 1c). When Ψ had declined to -2.3 MPa g_s was reduced by 95% compared to unstressed control leaves (Fig. 1c). The effect of reduced Ψ on g_s was much smaller in tef. At -2.3 MPa g_s was reduced by 60% compared to the control. Due to the

different sensitivities of g_s to Ψ in the two species, g_s of tef was higher than in wheat at low Ψ, whereas the opposite was true in control plants at high Ψ. The c.e. of both species declined significantly with decreasing Ψ (Fig. 1d), and tef had higher c.e. than wheat within the entire range of Ψ. Water stress that reduced Ψ from -0.5 MPa to -2.3 MPa in wheat and tef reduced c.e. from 0.105 mol m^{-2} s^{-1} to 0.015 mol m^{-2} s^{-1} (86%), and from 0.167 mol m^{-2} s^{-1} to -0.027 mol m^{-2} s^{-1} (84%), respectively (Fig. 1d).

C_i was higher in wheat than in tef control plants and at mild stress but quite similar in the two species at more sever stress (Fig. 1e). C_i of wheat declined with decreasing Ψ down to -1.8 MPa (Fig. 1e). When Ψ fell below this value, C_i increased again and in the most severely stressed plants approached the C_i of unstressed control plants. Tef leaves maintained more or less constant C_i values down to -1.4 MPa (Fig. 1e). Below -1.4 MPa, however, C_i exhibited a significant increase suggesting increasing mesophyll inhibition by decreasing Ψ. A of tef decreased as C_i increased whereas the opposite trend was noted in wheat (Fig. 2a).

The percent reduction of photosynthesis due to limiting g_s (l_{gs}) and c.e (l_{ce}) of wheat and tef are given in Table I. Both l_{gs} and l_{ce} were relatively low above -0.8 MPa and -1.4 MPa in wheat and tef, respectively (Table I). The maximum l_{ce} of wheat and tef was identical (76%) (Table I). Figure 2b and 2c shows the relationship between A and l_{ce} and between c.e. and l_{ce}. Both A and c.e. decreased as l_{ce} increased. The average l_{gs} in wheat and tef plants subjected to severe water stress (< -2.0 MPa) was 34% and 25% (Table I), respectively, and the increase with stress was relatively less

pronounced in tef than in wheat. The ratio of I_{gs} to I_{ce} increased with increasing stress in wheat whereas it decreased in tef (Table I). Figure 2d and 2e shows relationships between A and I_{gs}, and between g_s and I_{gs}, respectively. Any given I_{gs} was obtained at a smaller g_s in tef than in wheat (Fig. 2e).

A was linearly related to g_s as well as c.e. in both species (Fig. 3, a and b) and c.e. was linearly associated with g_s (Fig. 3c). The negative y-intercepts of the regression of A and c.e. on g_s (Fig. 3, a and c) show that stomata were not fully closed when A and the Calvin cycle activity were fully inhibited in severely stressed tef plants.

The decrease in photosynthesis in water stressed wheat and tef plants coincided with decreases in PSII ($H_2O \rightarrow DAD_{ox}$) (Fig. 4) and whole chain ($H_2O \rightarrow MV$) ($A=-2.081+0.086x$, $r=0.98$ in wheat and $A=-14.826+0.138x$, $r=0.87$ in tef, data not shown) electron transport rates of isolated thylakoids and Fv/Fm of intact leaves (Fig. 5). Considerable decreases in PSII and whole chain electron transport, but not in PSI (DHQ → MV) electron transport (data not shown) of wheat and tef were found in thylakoids isolated from water stressed leaves as compared to nonstressed leaves. In this paper only PSII electron transport is shown, but whole chain electron transport responded to water stress in an almost identical manner. PSII electron transport of wheat ($PSII=324.77e^{0.95\Psi}$, $r=0.99$) and tef ($PSII=287.683e^{0.257\Psi}$, $r=0.90$) decreased by about 85% and 49% of the control as Ψ dropped from -0.5 to -2.3 MPa, respectively. Inhibition of electron transport in isolated thylakoids depended in a similar manner on Ψ as did inhibition of A and A_{max} (Fig. 1, a and b). In wheat, PSII electron transport rates were completely abolished when Ψ dropped below about -2.5 MPa (see Chapter III) and A showed only a trace of residual activity (Fig. 1a). In tef, however, severe

water stress did not affect electron transport as much as it affected A and A_{max}. Thus higher residual activities of PSII still remained at this Ψ in tef. Regression analysis shows that the relationships between A, or A_{max} and PSII (Fig. 4, a and b) and whole chain (data not shown) electron transport rates were linear. Also c.e. decreased simultaneously with electron transport rates (Fig. 4c). When c.e. extrapolated to zero substantial electron transport remained in tef but not in wheat.

The correlations between Fv/Fm and A, A_{max} and c.e. were linear and high (Fig. 5, a-c). As is shown in Figure 5d, Fv/Fm of wheat decreased exponentially with decreasing g_s, whereas the relationship in tef was linear with a steep slope.

Photosynthesis of wheat and tef was negatively correlated with electrolyte leakage (Fig. 6). In tef the increase in leakage was rather monotonous with decreasing A. In wheat the increase was modest down to A around 5 μmol m^{-2} s^{-1}, but when A fell further leakage increased abruptly.

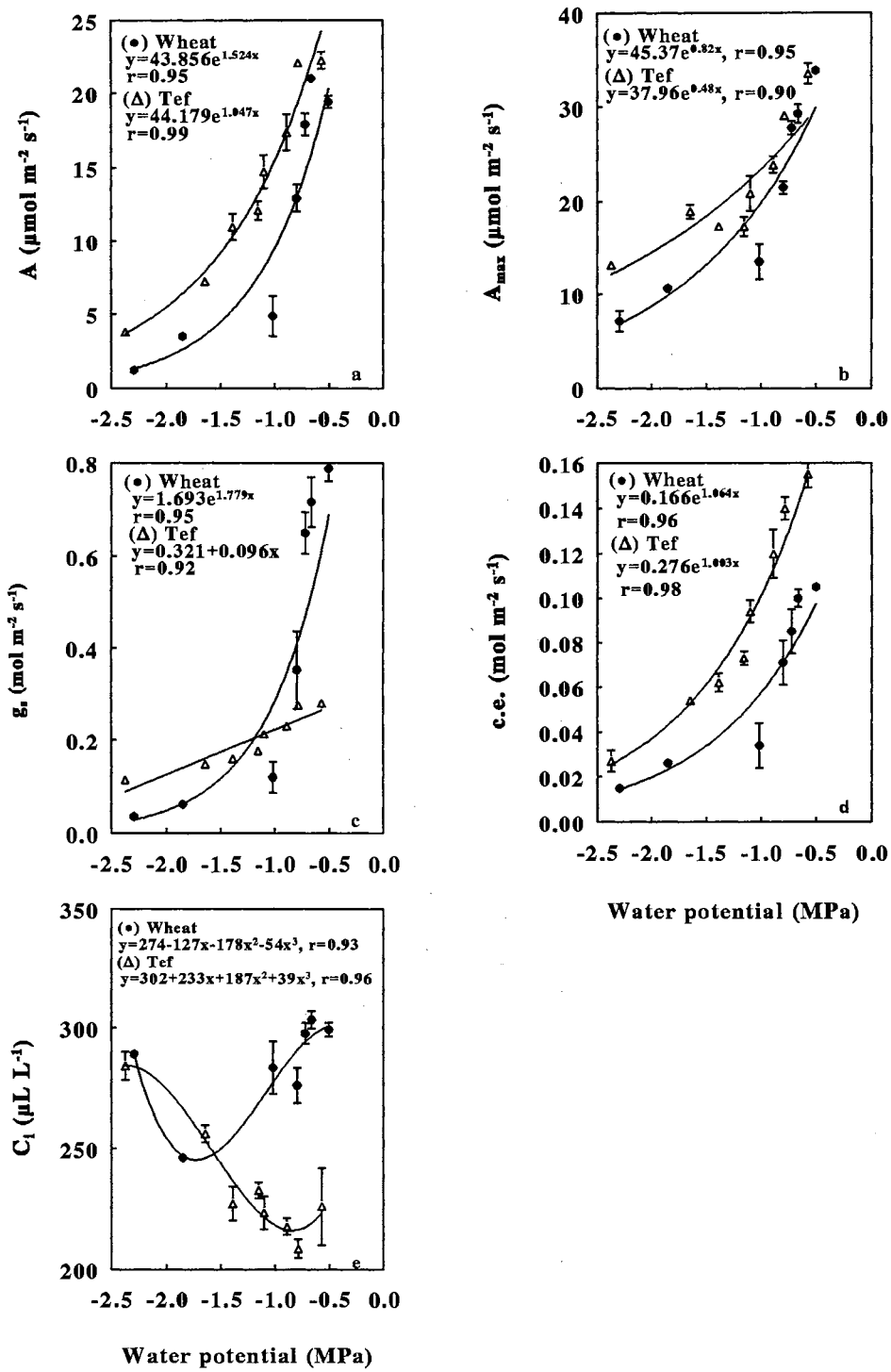

Figure 1. Response to Ψ of A (a), A_{max} (b), g_s (c), c.e. (d), and C_i (e) in wheat and tef. Each measurement was performed on a single leaf of wheat and on 2-3 leaves of tef. Leaves were measured at an irradiance of 1800 µmol m^{-2} s^{-1} PAR, 50% RH and air temperatures of 25°C for wheat and 30°C for tef. Bars indicate ± SE. The experiments were repeated 2 to 3 times with four replications for each Ψ.

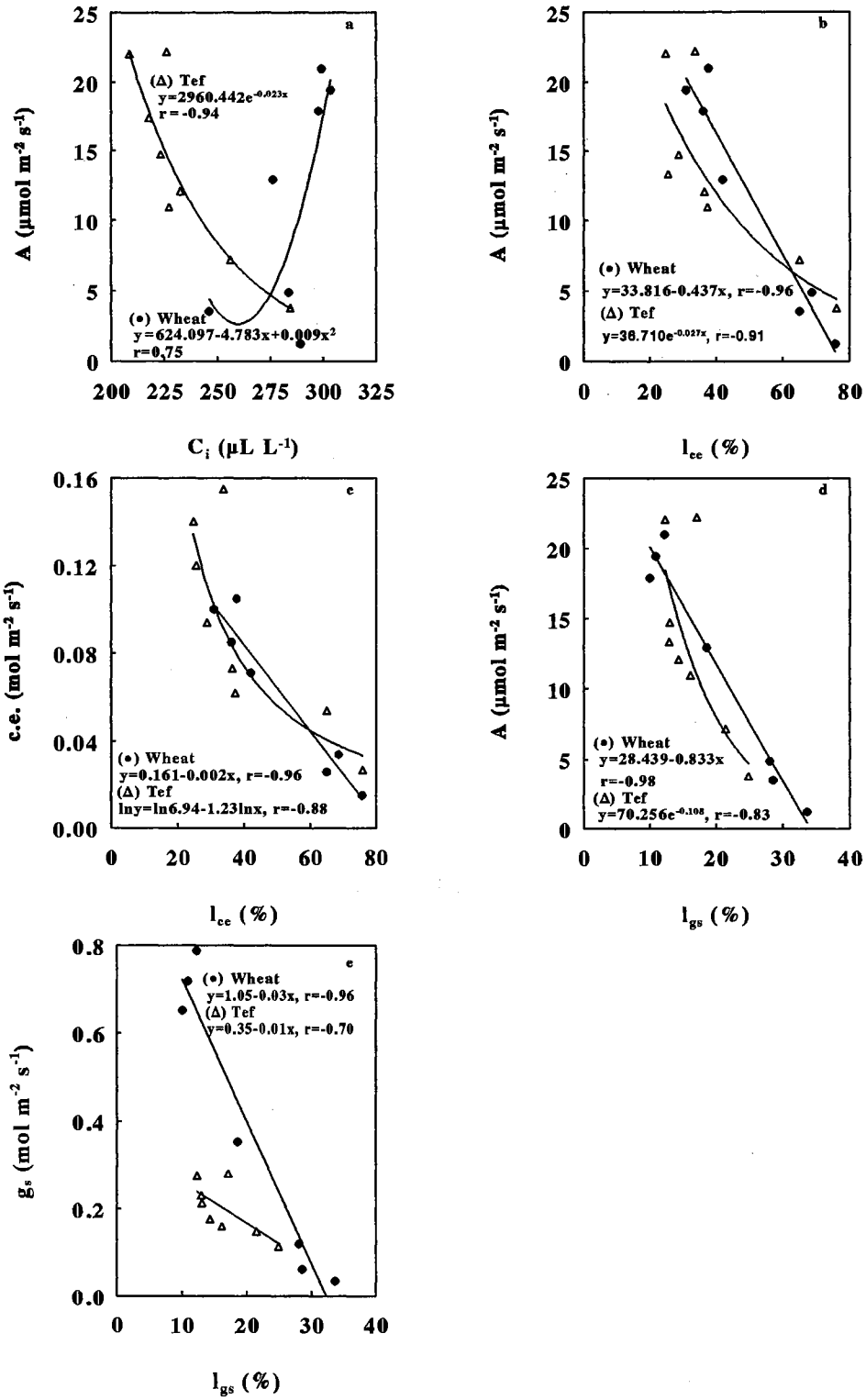

Figure 2. Dependence of A on C_i (a), A on l_{ce} (b), c.e. on l_{ce} (c), A on l_{gs} (d), and g_s on l_{gs} (e) in wheat and tef. Calculations of l_{gs} and l_{ce} were based on the methods of Farquhar and Sharkey (1982) and Martin and Ruiz-Torres (1992), respectively.

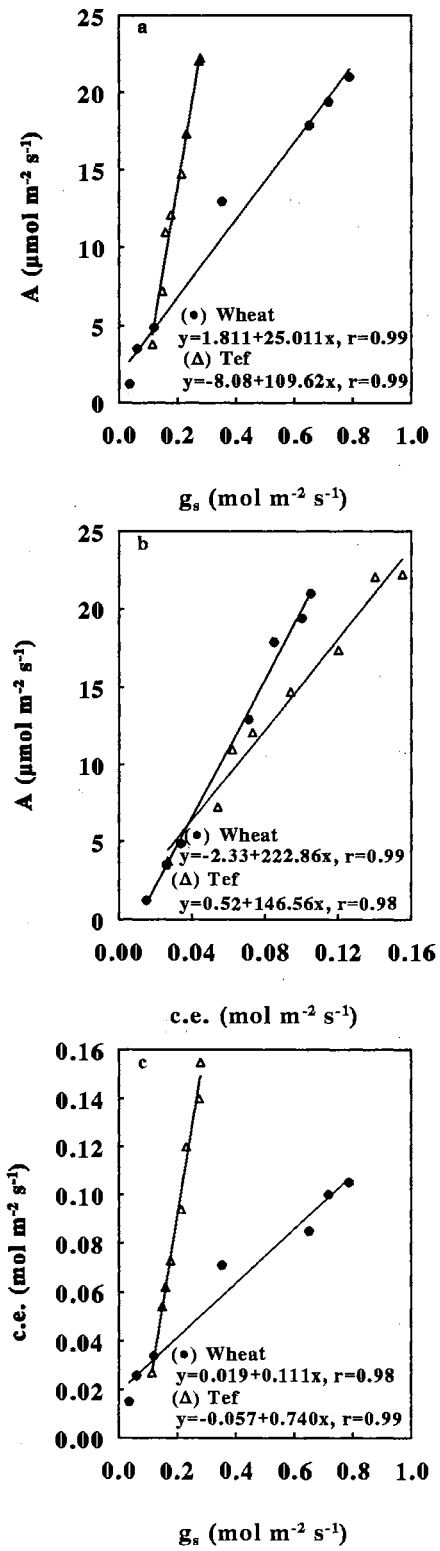

Figure 3. Relationship between A and g_s (a), A and c.e. (b), and c.e. and g_s (c) in water stressed wheat and tef. For experimental details see Fig. 1.

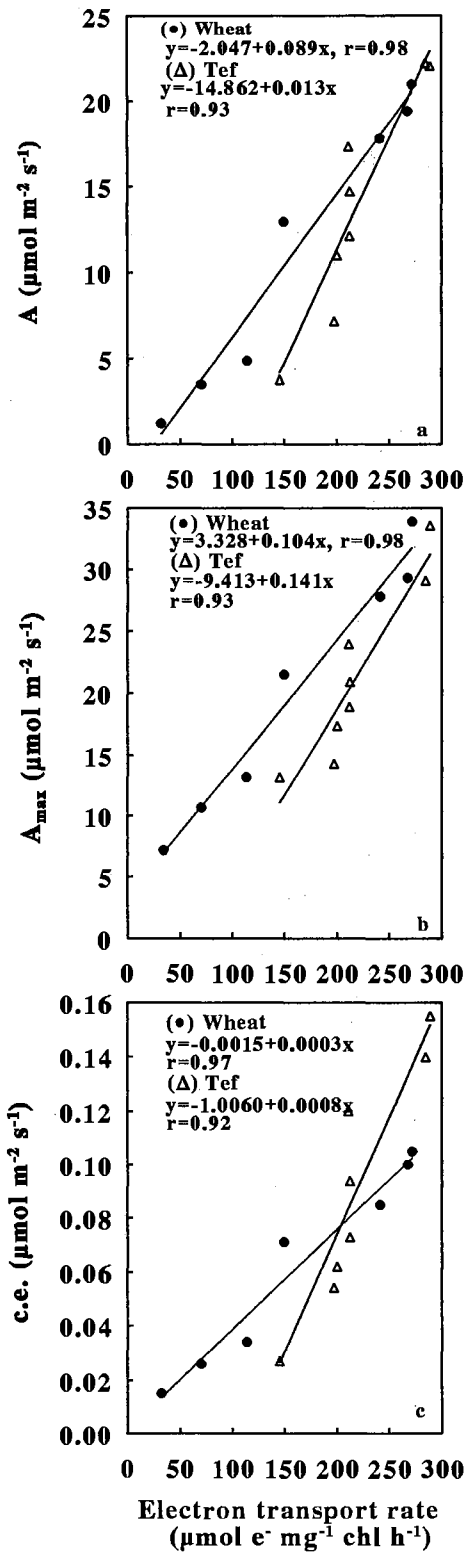

Figure 4. Relationship between PSII electron transport rate and A (a), A_{max} (b), and c.e. (c) in water stressed wheat and tef.

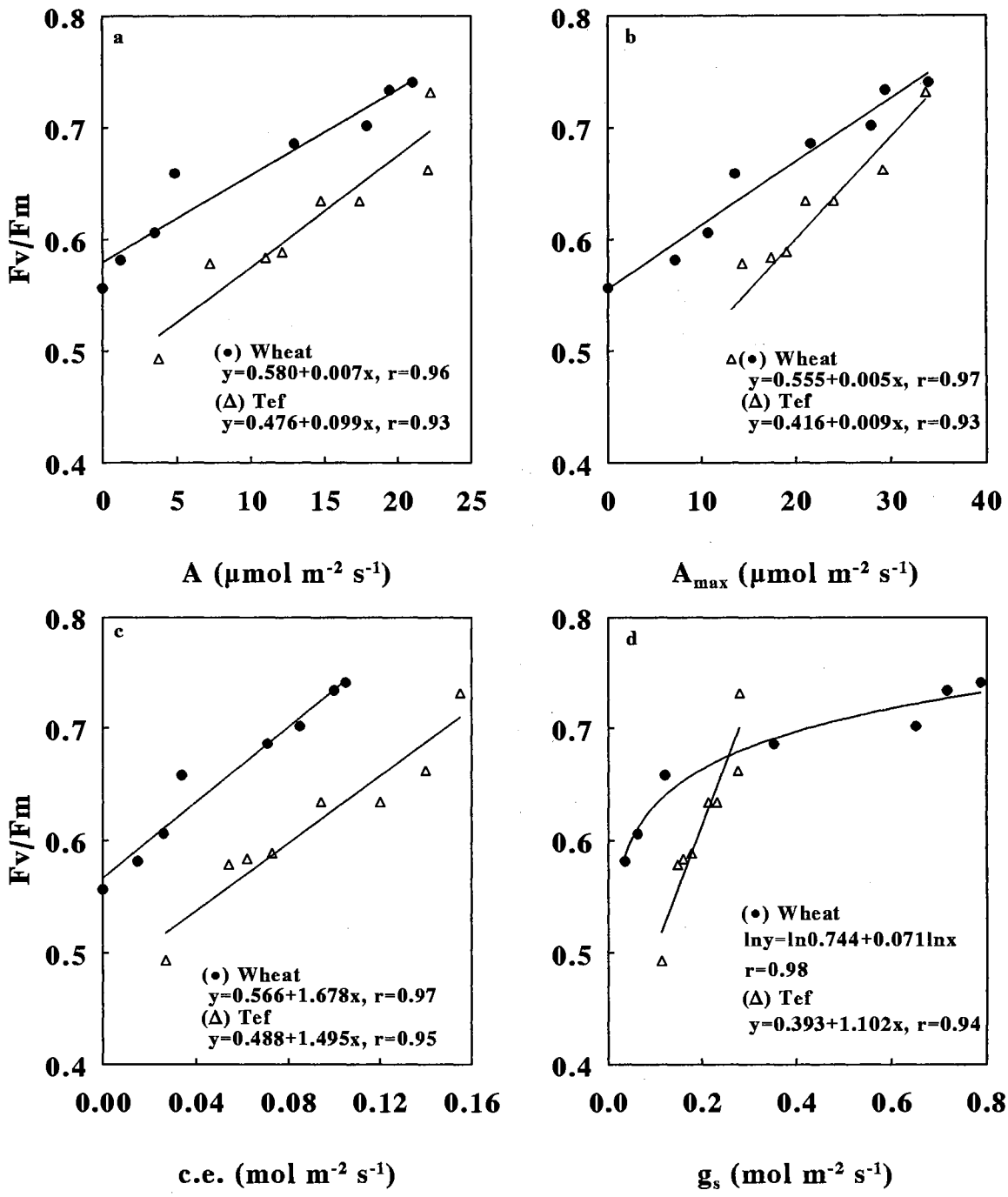

Figure 5. Relationship between Fv/Fm and A (a), A_{max} (b), c.e. (c), and g_s (d) in water stressed wheat and tef leaves. Fv/Fm was determined on leaves that were dark adapted for 10 min prior to a 5 s exposure to an excitation light intensity of 100 μmol m^{-2} s^{-1} PAR.

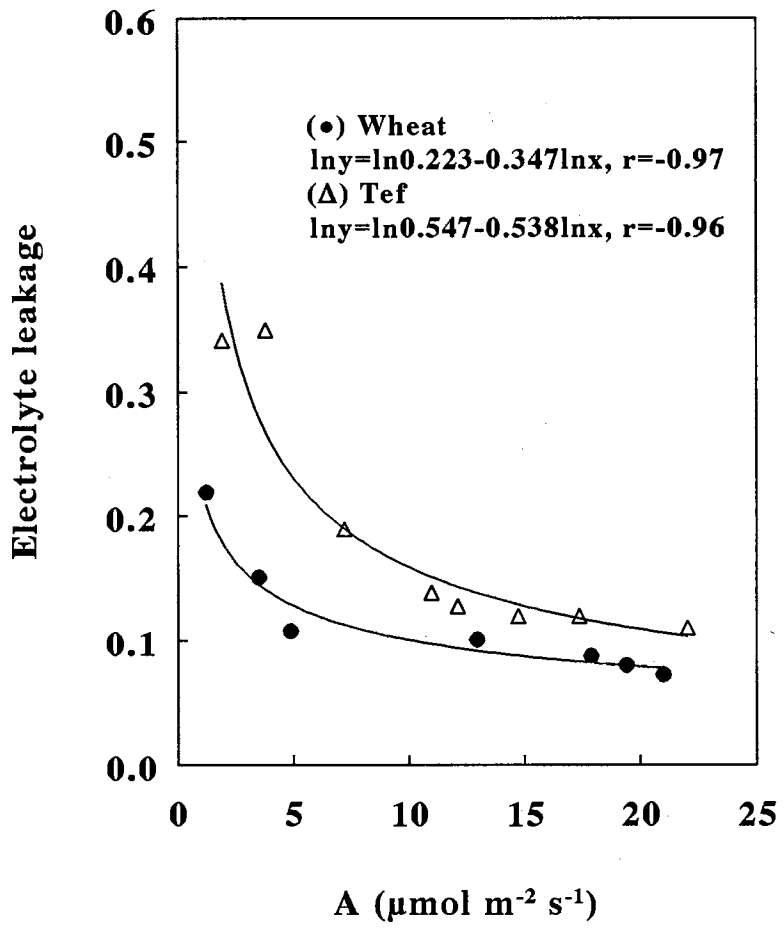

Figure 6. Relationship between A and electrolyte leakage in water stressed wheat and tef leaves. Symbols represent mean values of electrolyte leakage and A on consecutive days without watering.

Table I. Effects of water deficit stress in I_{gs} and I_{ce} of wheat and tef. Experiments were repeated 2 to 3 times with four replications each time. Means ± SE are presented.

Species	Ψ (MPa)	I_{gs}†	I_{ce}†	I_{gs}/I_{ce}
		%		
Wheat	-0.50±0.04	12.2±0.7	37.7±1.6	0.325±0.015
	-0.66±0.03	10.8±0.5	30.9±1.2	0.351±0.013
	-0.72±0.02	10.0±1.1	36.1±2.3	0.275±0.016
	-0.80±0.04	18.6±1.1	41.9±2.5	0.446±0.034
	-1.02±0.01	28.0±1.6	68.6±4.6	0.418±0.049
	-1.85±0.04	28.5±0.0	65.1±0.9	0.439±0.006
	-2.29±0.01	33.6±1.0	75.8±1.3	0.445±0.021
Tef	-0.57±0.05	17.1±0.9	33.6±1.5	0.507±0.006
	-0.78±0.08	12.3±1.0	24.6±1.1	0.500±0.032
	-0.89±0.04	12.9±0.9	25.5±2.7	0.520±0.060
	-1.10±0.09	13.0±1.3	28.7±1.4	0.479±0.027
	-1.15±0.09	14.3±0.4	36.4±0.6	0.392±0.004
	-1.38±0.05	16.1±0.9	37.4±4.5	0.437±0.025
	-1.65±0.10	21.4±1.5	64.9±3.9	0.330±0.017
	-2.37±0.07	24.9±0.7	76.0±3.9	0.328±0.007

† Calculations were made as described by Farquhar and Sharkey (1982) and Martin and Ruiz-Torres (1992)

DISCUSSION

In an effort to study the effect of water stress on photosynthetic gas exchange of wheat and tef A, A_{max}, g_s, c.e., and C_i were measured. All parameters were less susceptible to water stress in tef than in wheat except for c.e. for which the relative susceptibility to water stress was rather similar. Under non-stressed conditions wheat and tef showed similar A vs. C_i response curves (data not shown), except that A of tef became saturated at lower C_i and that C_i at ambient CO_2 in the air was lower in tef (Fig. 1e). Nie et al. (1992) also reported higher C_i in C_3 than in C_4 plants. According to Edwards and Walker (1983), the CO_2 concentrating mechanism of C_4 plants keeps their C_i below that of C_3 plants. Photosynthesis in C_4 plants operates at lower C_i than in C_3 plants because the initial carboxylase of C_4 plants, PEP-carboxylase, has much greater affinity for CO_2 than rubisco (Collatz et al. 1992). Rubisco fixes CO_2 in the photosynthetic carbon reduction pathway in both C_3 and C_4 plants. In C_3 photosynthesis, CO_2 fixed by rubisco is obtained directly from the intercellular spaces of the leaf by diffusion. In C_4 plants CO_2 is first fixed in the mesophyll cells by PEP-carboxylase and delivered to rubisco, which is localized in the bundle sheath chloroplasts, by a metabolic pump that concentrates CO_2 (Krall and Edwards, 1990).

In addition to having low C_i, control leaves of tef showed lower g_s (about 2.8 fold) than wheat (Fig. 1c). The lower g_s value of tef than wheat agrees with reports of Collatz et al. (1992), and Dai et al. (1993) on maize and other C_4 plants. Plants with

C_4 photosynthesis in general exhibit lower g_s than C_3 plants, and this difference is thought to help C_4 plants restrict water loss to a minimum and thereby increase their water use efficiency relative to C_3 plants (Leegood, 1993; Dai et al., 1993). In healthy C_3 and C_4 plants with identical g_s C_4 plants have lower C_i due to the great affinity for CO_2 of PEP-carboxylase. Whereas transpiration rate (E) will be the same in both types of plant, C_4 plants will have greater A because of the steeper CO_2 gradient between air and leaf interior. As a result, C_4 plants will operate at greater leaf water-use efficiency (A/E) than C_3 plants.

Our results show that allowing Ψ of wheat and tef plants to decrease from -0.5 MPa to -2.3 MPa led to significant decreases in A, A_{max}, g_s, and c.e (Fig. 1, a-d). The data reported here are consistent with many earlier studies on water stress (Johnson et al., 1987; Martin and Ruiz-Torres, 1992; Ni and Pallardy, 1992; Kicheva et al., 1994). In wheat, water stress reduced A and g_s (photosynthetic supply function, Farquhar and Sharkey, 1982) equally. However, in tef decreasing Ψ caused a smaller decline in g_s than in A and c.e. (Fig. 1a; Fig. 1, c and d). The effects of water stress on A, A_{max}, g_s, and c.e. were different in tef and wheat. Under water stress A appeared to be controlled by the stomata in wheat and by the Calvin Cycle in tef. Farquhar and Sharkey (1982), Farquhar et al. (1987) and Kicheva et al. (1994) suggested that the decrease in C_3 photosynthesis caused by moderate water stress resulted from reduced g_s, but that this phase is followed by inhibition of RuBP regeneration (A_{max}) and inhibition of the Calvin cycle (c.e.) at more severe stress. Our gas exchange measurements showed concerted declines in g_s, c.e., and A_{max} with declining A, but the magnitude of the decline in g_s in the C_4 species tef was much smaller than in the C_3 species wheat.

According to the model of von Caemmerer and Farquhar (1981) developed for C_3 plants, A_{max} is controlled by ribulose-1,5-bisphosphate (RuBP) regeneration. Thus, the reduction in A_{max} with declining Ψ (Fig. 1b) should be an indication of suppressed capacity of RuBP regeneration (Farquhar et al., 1987). Declining RuBP level is thought to stem from insufficient photosynthetic electron transport from H_2O to $NADP^+$ and/or ATP supply (photophosphorylation). Sharkey and Badger (1982) and Gimenez et al. (1992) reported a decrease in RuBP content by approximately 71% and 44-65% as Ψ decreased from -0.3 MPa to -2.8 MPa and -0.6 MPa to -1.6 MPa, respectively. In the present study PSII electron transport of isolated thylakoids was strongly inhibited by water deficit stress in the same range of Ψ. Chloroplast activities may have been inhibited by increased internal solute concentration (particularly Mg^{2+}) (Boyer and Younis 1984). PSII electron transport in isolated thylakoids was as sensitive to low Ψ as A_{max} of intact leaves. It is yet not known whether decreased electron transport and A_{max} under water stress are the cause of lowered A. In fact, our gas exchange measurements suggest that A_{max} is in greater excess of A at low Ψ than at high Ψ but Masojidek and Hall (1992) suggested that electron transport limited A in sorghum. Inhibited photophosphorylation as a reason for declining RuBP regeneration has also been proposed (Mayoral et al., 1981; Sharkey and Badger, 1982) in water stressed leaves. If true, RuBP synthesis depends on the rate of supply of ATP to the photosynthetic carbon reduction cycle as described by Boyer and Younis (1984). Younis et al. (1979) suggested that loss of photophosphorylation was caused by a change in conformation of the coupling factor (CF_1) in chloroplasts of stressed leaves, possibly because of increased ion concentration in the chloroplast stroma.

Von Caemmerer and Farquhar (1981) reported a linear correlation between whole chain electron transport and A_{max}. In our study the decline in PSII electron transport activity was not only linearly correlated with A_{max} (Figs. 4b) but also with A (Figs. 4a). Thus, the present study suggests that RuBP regeneration (electron transport/ photophosphorylation) is correlated with A and a site of direct or indirect inhibition by low Ψ (Gimenez et al., 1992). There appears to be multiple sites of declining activity within the photosynthetic apparatus (but not necessarily multiple sites of rate limitation of A since reductions may be by downregulation) caused by water stress ranging from photochemistry (primary reactions) to the dark reaction (Kaiser 1984).

It has been previously indicated that c.e. of C_4 plants, whether at 21% or 2% O_2, is higher (about 1.4 fold) than in C_3 plants (Krall and Edwards, 1990, Krall et al., 1991). In control plants we noticed 1.5 fold higher c.e. in tef than in wheat (Fig. 1d). The higher c.e. of tef than wheat is attributed to the high affinity of PEP-carboxylase for CO_2 and lack of O_2 sensitivity (Edwards and Walker, 1983). Water stress decreased c.e. of both wheat and tef to the same degree. The inhibition of c.e. supported the findings of Krieg and Hutmacher (1986) on sorghum and Martin and Ruiz-Torres (1992) and Kicheva et al. (1994) on wheat. The initial slope of the A/C_i curve of C_3 plants is a function of the Calvin cycle activity, of which rubisco (a chloroplast enzyme catalyzing the initial CO_2 fixation step in C_3 plants and the second carboxylation step in C_4 plants) is the major component (von Caemmerer and Farquhar 1981; Farquhar et al. 1987). Martin and Ruiz-Torres (1994) and others (Mayoral et al., 1981; Kicheva et al., 1994) have shown that Rubisco is not as sensitive to water stress as A or A_{max}. Krall et al. (1991) relate the O_2 inhibition of c.e. to

decreased C_i through competitive inhibition of RuBP carboxylase with respect to CO_2 particularly in mesophyll cells. Perhaps O_2 increases the state of activation of Rubisco in wheat (Kobza and Edwards 1987), and this may have an effect on O_2 inhibition of A. In tef, however, since C_i increased at low Ψ, the greater decline in c.e. at atmospheric O_2 may reflect depletion of RuBP (Kobza and Edwards, 1987), HCO_3^{-1} (substrate for PEP-carboxylase, O'Leary, 1982; Leegood, 1993) or involvement of other factors such as stromal acidification (Berkowitz and Gibbs, 1983; Chaves, 1991). Comparing the results of c.e. in our study with rubisco activity/content reported elsewhere, it appears rubisco is not as sensitive to water stress as c.e., A or A_{max}. The biochemical limitation must reside in some other part of the Calvin cycle as suggested by Sharkey and Seemann (1989) and Cornic et al. (1992).

Regression analysis showed that relationships between c.e. and A (Fig. 3b), c.e and A_{max} (data not shown) and c.e. and g_s (Fig. 3c) were linear, indicating that both stomatal and biochemical components were influenced by water stress. Although the mechanism is not clear, it is apparent there is a remarkable dependence between c.e. and g_s in wheat and tef plants subjected to water stress (Fig. 3c). This relationship may be due to the dependency of guard cells on the mesophyll for its reduced carbon as suggested by Farquhar and Sharkey (1982). An alternative explanation is downregulation of mesophyll biochemistry to maintain balance between the stomatal supply and the mesophyll demand capacities.

Changes in C_i are attributable either to effects on stomatal conductance or chloroplast activity, or both. In wheat exposed to moderately low Ψ the calculated C_i decreased, but it increased again at very low Ψ (Fig. 1e). Assuming homogenous stomatal closure (Gunasekera and Berkowitz, 1991; Martin and Ruiz-Torres, 1992) we

conclude that increased stomatal limitation first controlled A but that at severe stress (below Ψ -1.8 MPa) a substantial mesophyll limitation became the dominant factor. Cornic and Briantais (1991) reported that water stress causing no more than 35% leaf water deficit induced stomatal closure leading to decreased C_i. Boyer and Younis (1984) estimated that 32% water was lost from stressed sunflower leaves in the range of -0.25 MPa to -1.00 MPa. In our case 32% water loss was adequate to decrease C_i in wheat by 19% (data not shown). The associated decline in A was much greater (77%). In contrast Legg et al. (1979) reported that stomatal closure in spring barley accounted for only 6% of the reduction in A during water stress. Thus, discrepancies exist regarding the explanation of reduced A at Ψ above -1 MPa. In severely stressed plants there is undoubtedly a considerable nonstomatal limitation that likely resides in the Calvin cycle. An earlier study in two wheat cultivars by Johnson et al. (1987) found similar C_i values as we observed.

In tef on the other hand, C_i increased with decreasing Ψ over the entire range of Ψ values (Fig. 1e). This suggests a dominant mesophyll inhibition causing reduced A. This is consistent with reports from other laboratories (Matthews and Boyer, 1984; Ni and Pallardy, 1992). Electron transport and chlorophyll fluorescence measurements also clearly showed inhibition of chloroplast activities. Kobza and Edwards (1987) relate the increase in C_i with decreased RuBP level and suggested that the capacity to regenerate assimilatory power and/or the enzymatic capacity to regenerate RuBP may limit A. Due to the high affinity of PEP-carboxylase for CO_2, C_4 plants are expected to be less affected by stomatal properties than C_3 plants.

The main effect of water deficit stress on chlorophyll fluorescence was a reduction in Fv/Fm. In both wheat and tef the decline in A, A_{max}, c.e. and g_s was

accompanied by decreases in Fv/Fm (Fig. 5) confirming the relative sensitivity of PSII photochemistry to water stress. As previously noted by Jones et al. (1990), gas exchange was affected proportionally more than Fv/Fm by water stress. Nevertheless, since there were good correlations between Fv/Fm and gas exchange properties, both types of measurements were diagnostic of water stress. Martiniello and Blum (1990) found a linear correlation between Fm and A in different wheat genotypes exposed to water stress suggesting loss of PSII photochemistry. However, Castonguay and Markhart (1991) and Kicheva et al. (1994) noted no significant relationship between photosynthetic O_2 evolution or A and Fv/Fm. Such discrepancies might be attributed to different age of plants, species, methodology and level of water stress.

In wheat electrolyte leakage increased only little until it reached 0.22 when A had dropped to 1.14 μmol m^{-2} s^{-1} at about -2.4 MPa. At even lower Ψ there was a large increase in leakage. Chetal et al. (1983) showed that water stress decreased phosphatidylglycerol (PG) and increased phosphatidylcholine (PC) contents. PG is a major constituent of photosynthetic membranes of higher plants and its glycerol component provides a carbon reservoir for hexose synthesis. Thus, under condition of stress low PG may reduce hexose synthesis and starve the cells. Our results agree with the report of Kaiser (1987) that plasma membrane damage develops first at Ψ sufficiently low (< -2.0 MPa) to cause severe inhibition of A.

In conclusion, A was severely reduced by water deficit stress in both wheat and tef. In wheat, first stomata and later also the Calvin cycle contributed to the loss of CO_2 fixation. Inhibition of A in tef appeared dominated by the Calvin Cycle alone.

Our findings support the contention that changes in membrane permeability are not early signs of water stress, but perhaps indicate irreversible membrane breakdown.

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CHAPTER V

PHOTOSYNTHETIC GAS EXCHANGE OF LEAVES OF CHILLED AND HEAT STRESSED WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.)

SENAYET ASSEFA AND BJORN MARTIN

Abbreviations: A, rate of net CO_2 fixation in ambient air; A_{max}, light and CO_2 saturated rate of A; c.e., initial slope of A/C_i curve (carboxylation efficiency); C_i, CO_2 concentration in the intercellular air spaces; C_a, CO_2 concentration in the air; chl, chlorophyll; DAD, diaminodurene (2,3,5,6-tetramethyl-*p*-phenylenediamine); DHQ, durohydroquinone (tetramethyl-*p*-hydroquinone); e^-, electrons; Fo, initial fluorescence; Fm, maximum fluorescence; Fv, variable fluorescence (Fv = Fm - Fo); g_s, stomatal conductance for water vapor; MV, methyl viologen; PAR, photosynthetically active radiation; PSI and PSII, photosystem I and II.

ABSTRACT

Agricultural crops are damaged by both high and low temperatures. Here we use an open gas exchange system to compare the net photosynthetic assmilation rate (A) of the temperate C_3 cereal wheat (*Triticum aestivum* L.) and the subtropical C_4 cereal tef (*Eragrostis tef* Z.) subjected to high (35°C, 40°C, and 45°C) and chilling low (2°C, 7°C, and 12°C) low temperatures. Exposure to heat and chilling stresses for as little as 24 h led to inhibition of A, carboxylation efficiency (c.e.) and light and CO_2 saturated photosynthesis rate (A_{max}) except in wheat that was less susceptible to chilling stress. Prolonged exposure, however, caused a gradual reduction so that A after 168 h at 2°C was only 50% and c.e. and stomatal conductance (g_s) 60% of the values measured at 25°C in wheat. Heat and chilling stresses caused gradual increases in the intercellular CO_2 concentration (C_i), and small declines in g_s except in chilled wheat. This suggests that the nonstomatal limitation to A became increasingly dominant by exposure to high and low temperature stress. PSII and whole chain electron transport, and Fv/Fm were inhibited in proportion to A, A_{max}, and c.e. Heat stressed wheat leaves revealed loss of PSII activity at the same temperature and duration at which A and A_{max} ceased. In chilled and heat stressed tef inhibition of A and A_{max} preceded inhibition of PSII activity. A, A_{max}, c.e. and PSII electron transport was closely correlated with Fv/Fm. PSI and chlorophyll content were not significantly influenced by any of the stresses although there was a tendency to small reductions

at very long exposure times. Enhanced membrane leakage coincided with more severe temperature stress than what initiated heat or chill inhibition of A. Decreased enzymatic activity but not electron transport, appeared to cause the major chill and heat-induced inhibition of A of tef, whereas in heat stressed wheat both electron transport and Calvin cycle activity showed proportional decline and may have co-limited A.

INTRODUCTION

Depending on the intensity and duration, heat and chilling stresses impair numerous physiological, biochemical and photochemical functions. Direct comparisons of photosynthesis and photosynthetic components of susceptible plants subjected to heat and chilling low temperature stresses have received limited attention.

Photosynthesis is heat and chilling labile. In chill susceptible plants, injury develops at temperatures below 15°C (Long et al., 1983). Advances have been made in defining and identifying biochemical, photochemical and ultrastructural changes related to chilling (Martin and Ort, 1981; Huner and Hopkins, 1984; Wise and Ort, 1989; Terashima et al., 1989; Nie and Baker, 1991) and heat (Weis, 1981; Kobza and Edwards, 1987; Sabat et al. 1991) stress damage. Martin et al. (1981) estimated the relative roles in photosynthesis inhibition of chill-induced stomatal closure and chloroplast impairment in tomato. The inhibition of chloroplast activity dominated over the chill-induced reduction in g_s. Chilling under moderate light disrupted chloroplast protein composition (Nie and Baker, 1991; Bredenkamp et al., 1992). In particular, thylakoid proteins that are encoded by the chloroplast genome appear to be repressed. Membrane analysis by Western blotting and SDS-PAGE showed that the D1 (the 32 KD protein of PSII), cyt_f (34 KD), cyt b_6/f (17 KD), the α (58 KD) and β (58 KD) subunits of CF_1 (coupling factor), and the CC1 apoprotein (core complex of PSI,

65-70 KD) were deficient in chilled plants. The water splitting machinery (oxygen evolving enhancer, OEE 33 KD) and light harvesting chlorophyll-proteins I and II (LHC I and LHC II) were unaffected (Nie and Baker, 1991). In maize, inhibition of PSII activity (Nie and Baker, 1991) and the photochemical quantum yield of PSII accounted for the inhibition of CO_2 saturated photosynthesis at low temperature. However, in tomato, Kee et al. (1986) have shown that although electron transport activity was substantially reduced by chilling in the light, the activity that remained in chilled plants was more than sufficient to support the measured rates of light and CO_2 saturated net photosynthesis.

Low temperature-induced inhibition of C_4 photosynthesis has been reported by Long et al. (1983) and Bredenkamp et al. (1992). Long et al. (1983) reported 70% to 80% reduction of A in *Zea mays* L. from lowering the leaf temperature from 15°C to 5°C. Various potential causes have been suggested for the sharp decline. Cold lability of pyruvate phosphate dikinase (an enzyme of the C_4 photosynthetic pathway) has been proposed. Alternatively, phosphate depletion is responsible for the decline in A due to accumulation of phosphorylated soluble carbohydrates (fructose, glucose and sucrose) (Labate et al., 1990; Bruggemann et al., 1992; Paul et al., 1990; Bruggemann et al., 1992), or decreases in Rubisco (Bruggemann et al., 1992), fructose-1,6-bisphosphatase or NADP-glyceraldehyde-3-P-dehydrogenase activities (Maruyama et al., 1990) might be responsible for the chill-induced lowering of A.

Previous reports ascribed heat-inactivation of A to inhibition of thylakoid reactions rather than to inhibition of stromal enzymes (Berry and Bjorkman, 1980). The oxygen evolving complex on the oxidizing side of PSII (Smith and Low, 1989;

Thompson et al., 1989), PSII electron transport activity (Sabat et al., 1991; Yordanov, 1992), and photophosphorylation appeared susceptible, whereas PSI (Sabat et al., 1991) and the water soluble Calvin cycle enzymes were thought to be comparatively stable. Studies have linked inhibition by moderately high temperature stress to PSII and inactivation of photophosphorylation. However, loss of A due to heat-inactivation of the Calvin cycle (Rubisco activity) has been reported in spinach (20°C to 40°C, Weis 1981) and in wheat (15°C to 45°C, Kobza and Edwards, 1987). Studies using isolated mesophyll protoplasts from lettuce did not confirm that the initial depression of A caused by mild heat exposure was due to lowering of the Rubisco activity and photophosphorylation (Santarius et al., 1991). These discrepancies may stem from use of different species and experimental techniques.

Several recent studies indicate the usefulness of *in vivo* chlorophyll fluorescence measurements in combination with leaf gas exchange for detection of temperature effects on PSII electron transfer and CO_2 assimilation (Walker, 1992; Oberhuber and Edwards, 1994). Temperatures that affect the function of PSII also reduce the Fv/Fm ratio.

Increased membrane permeability has been reported in heat stressed leaves, isolated vacuoles, and protoplasts (Santarius et al., 1991). Chilling induced membrane phase transitions (Raison and Orr, 1986) were suggested as a possible cause of enhanced membrane permeability in sensitive species. Altered lipid composition and membrane order could lead to membrane dysfunction. However, the relationship between membrane leakage and inhibition of A in intact leaves is not yet clear.

Several studies have used a single leaf wrapped in wet tissue, or floated on chilled or heated water, usually for a few minutes or hours. Effects of extended heat and chilling stress on whole plant photosynthesis have less frequently studied. The objectives of the present work was to a) compare the responses of photosynthesis and its components of a C_3 and a C_4 plant subjected to different durations of heat and chilling low temperature stress, b) examine the relationship between leaf photosynthesis and its stomatal, biochemical and photochemical components, and c) examine leaf photosynthesis as it relates to chlorophyll fluorescence and membrane stability.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of the temperate C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101), and the subtropical C_4 cereal tef (*Eragrostis tef* Z. cv. DZ-01-354) belonging to the NAD-malic enzyme subgroup were grown in controlled environment growth chambers (Conviron CMP 3244, Winnipeg Ltd., Manitoba, Canada) in pots containing a peat moss:soil mix (1:1, v/v) (Metro Mix growing medium, Grace Sierra Horticultural Products Company, Milpitas, CA). Chambers for wheat and tef were maintained at 25°C/18°C and 30°C/23°C, respectively, day/night temperature and 14 h photoperiod. The irradiance at plant height was between 460 and 500 μmol m^{-2} s^{-1} PAR. The chambers were humidified to maintain at least 50% RH. For more detailed growth conditions see chapter IV.

Heat and Chilling Low Temperature Stress Exposure

Thirty days after seedling emergence plants were exposed for up to 168 h to high (35°C, 40°C, and 45°C) or low (2°C, 7°C, and 12°C) temperatures. Control plants remained at the original temperature. Every 24 h plants were removed from the growth chamber for measurements of gas exchange, chlorophyll fluorescence, and chlorophyll content. They were returned to the growth chamber with the appropriate temperature following each measurement. Thus, whole plants were subjected to the stress treatments, and measurements were made on the same plants throughout the

experiment. Consistent with electron transport measurements on isolated thylakoids, no rewarming or recooling periods were given prior to measurements.

Gas Exchange Measurements

Measurements were carried out on young, fully expanded, attached leaves held horizontally in an open gas exchange system previously described by Johnson et al. (1987). The CO_2 concentrations were measured using an infrared gas analyzer (Horiba PIR 2000 R, Horiba Instrument Inc., Irvine, CA) and chamber humidity with a dew point hygrometer (System 1100DP, General Eastern Instruments Corporation, Watertown, MA). Dry, CO_2-free air from a compressed air cylinder mixed in various ratios with dry air from another cylinder containing 1700 $\mu L\ L^{-1}$ CO_2 was used to generate air ranging from 0 to 1700 $\mu L\ L^{-1}$ CO_2. Air temperature was maintained at 25°C and 30°C for wheat and tef, respectively (same as daytime growth temperatures). The irradiance incident on the leaf was 1800 $\mu mol\ m^{-2}\ s^{-1}$ PAR, measured with quantum sensor (LI-19OSB, Li-Cor Instruments, Lincoln, NE), provided by a 1000 W multivapor lamp (R1000, General Electric, Cleveland, OH). The RH in the assimilation chamber was maintained at 50% by adjusting the leaf area and air flow rate. Ambient photosynthesis rate (A) was measured at a CO_2 concentration in the air (C_a) of 350 $\mu L\ L^{-1}$. A_{max} was measured at 1600 to 1700 $\mu L\ L^{-1}$ CO_2 in the air. Calculations of A, g_s and C_i followed the methods of von Caemmerer and Farquhar (1981). Leaf area was determined using a leaf area meter (LI-3000A Portable Area Meter combined with LI-3050A Transparent Belt Conveyer, Li-Cor Instruments, Lincoln, NE). See Chapter IV for details.

Chlorophyll Fluorescence

Measurements of chlorophyll fluorescence were made with a portable

fluorometer (Polar Tech, Umea, Sweden) (Oquist and Wass, 1988). Fully expanded leaves were dark adapted for 10 min prior to a 5 s exposure to excitation light with an intensity of 100 μmol m^{-2} s^{-1} PAR. Fo, Fm, Fv, Fv/Fm, and $t_{1/2}$ were measured.

Chloroplast Isolation

Immediately after plants had been chilled or heat stressed, chloroplasts were isolated from leaves of 30-day-old wheat and tef plants using the procedure of Kee et al. (1986). Chlorophyll contents of the chloroplast suspensions were determined spectrophotometrically (Spectronic 1201, Milton Roy, Rochester, NY) according to Arnon (1949).

To determine the leaf chlorophyll content sixteen 0.39 cm^2 disks were punched out from the middle portion of 16 different leaves and ground in a tissue homogenizer (Pyrex, Corning 7727-7, UK) containing 2 mL 80% acetone (v/v). The extract was centrifuged at full speed in a table-top centrifuge (Clay Adams Model 0005, Parsippany, NJ) for 2 to 3 min. The absorbance of the extract was read at 663 nm (A_{663}), 645 nm (A_{645}) and 750 nm (A_{750}). The contents of chl a, chl b, and chl (a+b) (mg L^{-1}) was calculated using the equations below from Arnon (1949) modified by using A_{750} as zero absorbance base line:

$$\text{chl a} = ((A_{663} - A_{750}) \times 12.7) - (A_{645} - A_{750}) \times 2.69))$$

$$\text{chl b} = ((A_{645} - A_{750}) \times 22.9) - (A_{663} - A_{750}) \times 4.68))$$

$$\text{chl (a+b)} = ((A_{663} - A_{750}) \times 8.02) + (A_{645} - A_{750}) \times 20.2))$$

Chlorophyll contents in units of mass were converted to molar units using molecular weights of 893.5, 907.5, and 900 for chl a, chl b and chl (a+b), respectively (Nobel, 1991).

Photosynthetic Electron Transport

Electron transport activities of isolated thylakoids were measured by monitoring O_2 evolution or consumptions with a temperature controlled Clark-type oxygen electrode assembly (Model LD-2, Hansatech, Ltd., King's Lynn, Norfolk, UK) at 25°C and saturating light from a 100 W projector lamp. Thylakoids were assayed for whole chain ($H_2O \rightarrow MV$), PSII ($H_2O \rightarrow DAD_{ox}$) and PSI ($DHQ \rightarrow MV$) electron transport rates as described by Kee et al. (1986) for tomato, and Allen and Holmes (1986) for spinach and pea were used with slight modifications. See Chapters I and II for details.

Electrolyte Leakage

Test tubes containing 0.7 g leaf material and 20 mL deionized double distilled water (ddH_2O) were vacuum infiltrated at 120 to 140 mm Hg for 15 min. Prior to vacuum infiltration, sample leaves were gently washed for approximately 90 min with 3 changes of ddH_2O and cut into 1 cm pieces. The initial electrical conductance (C_1) of the bathing solution was measured (Cole-Parmer conductivity meter, Model 1481-60, Chicago, IL). Leaf samples were then autoclaved for 20 min at 121°C, agitated for 1 h, and the electrical conductance (C_2) measured again. Electrolyte leakage was expressed as the C_1/C_2 ratio.

All measurements were repeated at least twice, and for each treatment four replicate measurements were made. A completely randomized design was used.

RESULTS

I. Chilling Stress

Leaf Gas Exchange

Figure 1 shows the chilling low temperature dependencies of A, A_{max}, g_s, c.e., and C_i of tef at different exposure times. The severity of inhibition increased with the duration of chilling. Exposing tef plants to 2°C, 7°C, and 12°C for 24 h resulted in reduction of A by 66%, 57%, and 38% of the control values (Fig. 1a). Complete inhibition of A occurred after 72 h at 2°C, whereas plants at 7°C and 12°C maintained substantial rates of A for up to 96 h of chilling (Fig. 1a).

A_{max} was proportionally less inhibited than A. A_{max} decreased by 45%, 38%, and 25%, when tef plants were exposed to 2°C, 7°C, and 12°C for 24 h (Fig. 1b). The 7°C and 12°C exposure for 96 h resulted in 67% and 51% reduction of A_{max} (Fig. 1b).

Stomatal conductance declined with decreasing temperature (Fig. 1c). After 24 h at 2°C g_s was almost halved. The 7°C and 12°C exposures for 24 h reduced g_s by 40% and 15%, respectively. The g_s was slightly lowered by extending the exposure time up to 96 h, but at 120 h g_s was zero at all temperature treatments.

Carboxylation efficiency decreased greatly with decreasing temperature (Fig. 1d). The decline in c.e. after 24 h of exposure to 2°C, 7°C, and 12°C amounted to 71%, 56%, and 36%, respectively, (Fig. 1d). The magnitude of reduction in c.e. was similar to the reduction in A and larger than the reduction in A_{max}.

Figure 1e shows the response of C_i in tef to chilling low temperature. At all three temperature treatments C_i gradually increased up to 135% (2°C), 133% (7°C), and 129% (12°C) of the values measured at 30°C by extending the chilling duration up to 120 h. At the 96 h exposure time C_i increased from 234 $\mu L\ L^{-1}$ to 320 $\mu L\ L^{-1}$ as temperature dropped from 30°C to 2°C (Fig. 1e).

Gas exchange was less responsive to chilling in wheat (Fig. 2) than in tef. A and the various photosynthetic components of chilled wheat were little affected during the first 72 h. With time, however, 2°C, 7°C and 12°C reduced A (Fig. 2a), g_s (Fig. 2c) and c.e. (Fig. 2d) were reduced. After 168 h at 12°C, 7°C, and 2°C A had declined by 45% to 50% (Fig. 2a), g_s by 30% to 42% (Fig. 2c), and c.e. by 40% to 45% (Fig. 2d), whereas A_{max} (Fig. 2b), and C_i (Fig. 2e) showed little or no effects.

Regression analysis shows that there were linear correlations between A and g_s (Fig. 3a), c.e. (Fig. 3b), and A_{max} ($r=0.95$, data not shown) in chilled tef leaves, showing that both biochemical and stomatal components were simultaneously influenced by chilling low temperature stress. In tef C_i increased with declining A (Fig. 3c) and declining g_s (Fig. 3d), whereas C_i of wheat remained unaffected as A and g_s fell.

PSII ($H_2O \rightarrow DAD_{ox}$) and whole chain ($H_2O \rightarrow MV$) electron transport rates and Fv/Fm of tef (and wheat to some extent) leaves were affected by chilling stress, whereas PSI was largely unaffected (see Chapter I). As was the case with A and A_{max}, the activities declined progressively with the duration of chilling, but the magnitude of inhibition of A was greater than the inhibition of electron transport rates or Fv/Fm. In tef A and A_{max} declined to zero at briefer exposure times than PSII, was needed to eliminate and whole chain electron transport. Regression analysis revealed that the

relationship between A or A_{max} and PSII electron transport in tef (Fig. 4, a and b) were linear, suggesting a possible link between the inhibition of PSII and CO_2 assimilation. Carboxylation efficiency also declined together with electron transport (Fig. 4c). In wheat there was much more variability, and electron transport was not significantly correlated with gas exchange. The relationships between Fv/Fm and A, A_{max}, and c.e. were also linear (Fig. 5, a-c). This indicates an association between the fluorescence-estimated quantum efficiency of PSII and carbon assimilation. Membrane leakage was negatively correlated with A in tef (Fig. 6). A similar trend that was not statistically significant was observed in wheat.

Chlorophyll Content

In contrast to gas exchange the chlorophyll content of chilled tef and wheat leaves was largely unaffected (Table I) suggesting that the light harvesting potential remained relatively intact. No statistical difference was observed in the contents of chl a, chl b and chl (a+b) between chilled and unchilled tef leaves until the end of 120 h of chilling. After that a 10% to 20 % reduction was observed in the chlorophyll content but there was no change in the chl a/b ratio (Table I).

II. Heat stress

Leaf Gas Exchange

Increasing the temperature of wheat from 25°C to 45°C and tef from 30°C to 45°C, respectively, had large effects on photosynthetic gas exchange. A (Fig. 7, and b) and A_{max} (Fig. 7, c and d) greatly declined at high temperature. A and A_{max} of wheat and A of tef decreased by 35% to 37% when exposed to 35°C for 24 h (Fig. 7, a-c). With increasing temperature and duration A decreased proportionally more than A_{max}, however, and both A and A_{max} decreased more prominently in wheat than in tef.

Exposing wheat and tef plants to 40°C for 24 h decreased A by 88% and 73% (Fig. 7, a and b), and A_{max} by 64% and 52% (Fig. 7, c and d). Maintaining wheat plants at this temperature for longer than 24 h lead to complete inhibition of A (Fig. 7a) and A_{max} (Fig. 7c), whereas substantial rates were retained by tef up to 48 h (Fig. 7, b and d). The 45°C treatment was very damaging and reduced A and A_{max} in wheat to zero after 24 h. In tef A and A_{max} was reduced by 91% and 67% by this treatment.

Stomatal conductance also declined in response to increasing temperature although not as much as A (data not shown). The decline was greater in wheat than in tef for the first 24 h at 35°C.

Carboxylation efficiency decreased significantly during high temperature exposure in both wheat (Fig. 7e) and tef (Fig. 7f). The reduction in c.e. was similar in magnitude to the decline in A.

C_i gradually increased at elevated temperature in both wheat and tef (Figs. 7, g and h). The increase was more pronounced in tef (from 220 to 325 $\mu L\ L^{-1}$) than in wheat (from 300 $\mu L\ L^{-1}$ to 334 $\mu L\ L^{-1}$). At high temperature and long exposure time the difference in C_i between the two species became small (Fig. 7, g and h).

Figure 8 shows dependence of A on g_s (Fig. 8a), c.e. (Fig. 8b) and C_i (Fig. 8c). A was positively correlated with g_s and c.e. but negatively correlated with C_i. C_i increased as g_s declined (Fig. 8d).

Concomitant with the decrease in A and A_{max}, heat stress caused severe reductions in PSII ($H_2O \rightarrow DAD_{ox}$) and whole chain ($H_2O \rightarrow MV$) electron transport rates and Fv/Fm. PSI (DHQ → MV) remained unaltered (see Chapter II). Complete elimination of electron transport of wheat occurred at the same temperature and duration that caused loss of A and A_{max}, whereas in tef A and A_{max} were inhibited more

than PSII and whole chain electron transport. The relationships between PSII electron transport rates and A or A_{max} (Fig. 9, a and b) and c.e. (Fig. 9c) were linear. The negative y-intercept of A, A_{max}, and c.e. as functions of electron transport of tef demonstrates presence of some electron transport capacity when no Carbon reduction capacity remains.

Fv/Fm of wheat and tef decreased in a curvilinear fashion with decreasing A, A_{max}, and c.e. (Fig. 10). Heat stress led to increased membrane permeability in both species. However, leakage occurred at higher temperatures and/or prolonged exposures compared to heat-inactivation of A (Fig. 7, a and b). A of wheat and tef were inversely correlated with electrolyte leakage (Fig. 11).

Chlorophyll content

Small losses of chl a, chl b, and chl (a+b) were observed after 96 h of exposure at 35°C in both wheat and tef (Table II). In wheat, but not in tef, the chlorophyll loss was associated with reduced chl a/b ratio (Table II). Heat stressed wheat leaves that had been exposed to 35°C lost 45% to 70% of chl a from 96 to 168 h, whilst the content of chl b was reduced by 35% to 40%.

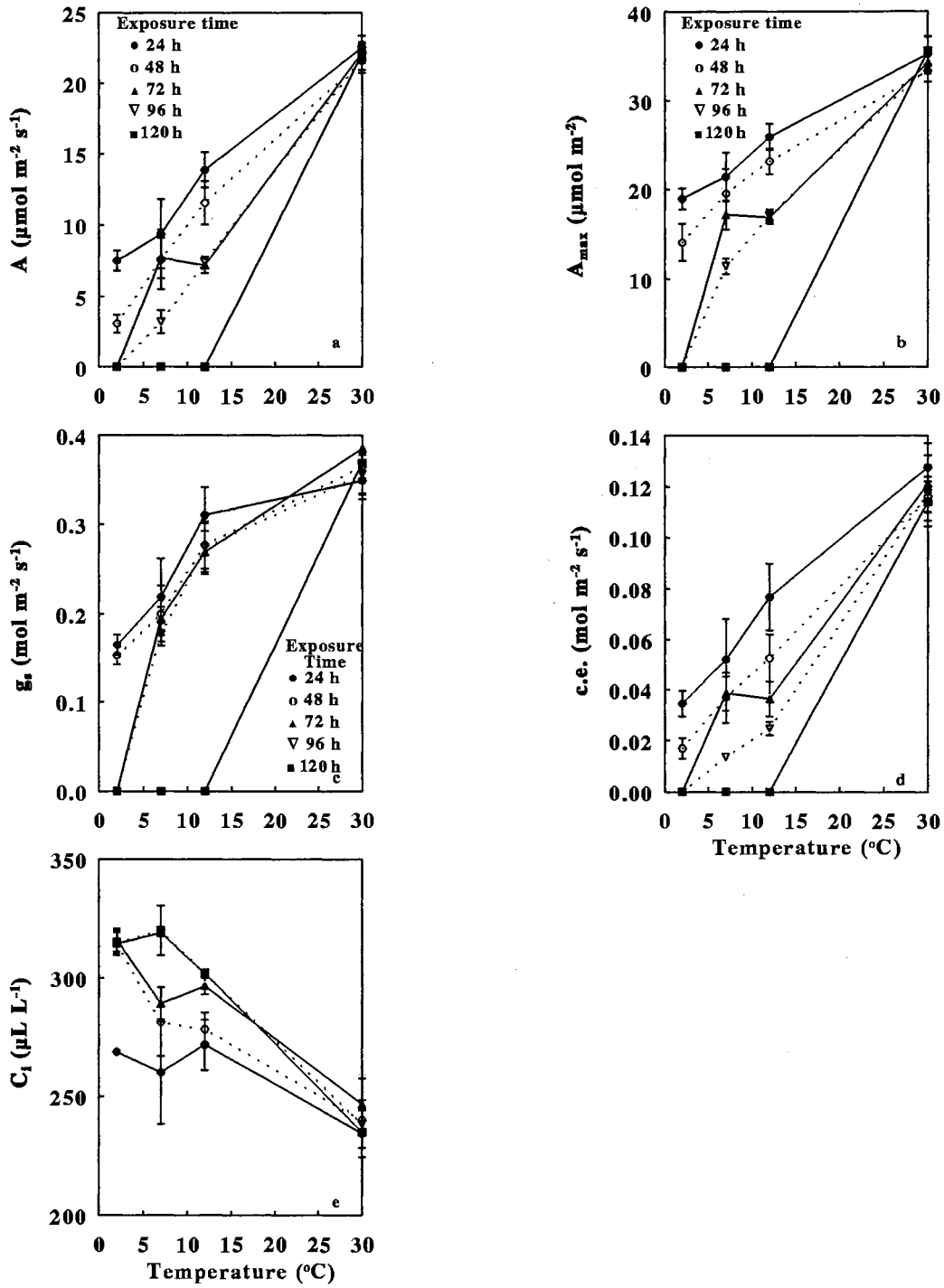

Figure 1. Dependence on chilling low temperature of A (a), A_{max} (b), g_s (c), c.e. (d), and C_i (e) of tef leaves at different exposure times. Measurement were made at an irradiance of 1800 µmol m^{-2} s^{-1} PAR, 50% RH and an air temperature of 30°C. Bars indicate ± SE. The experiment was repeated two times with four replications for each treatment.

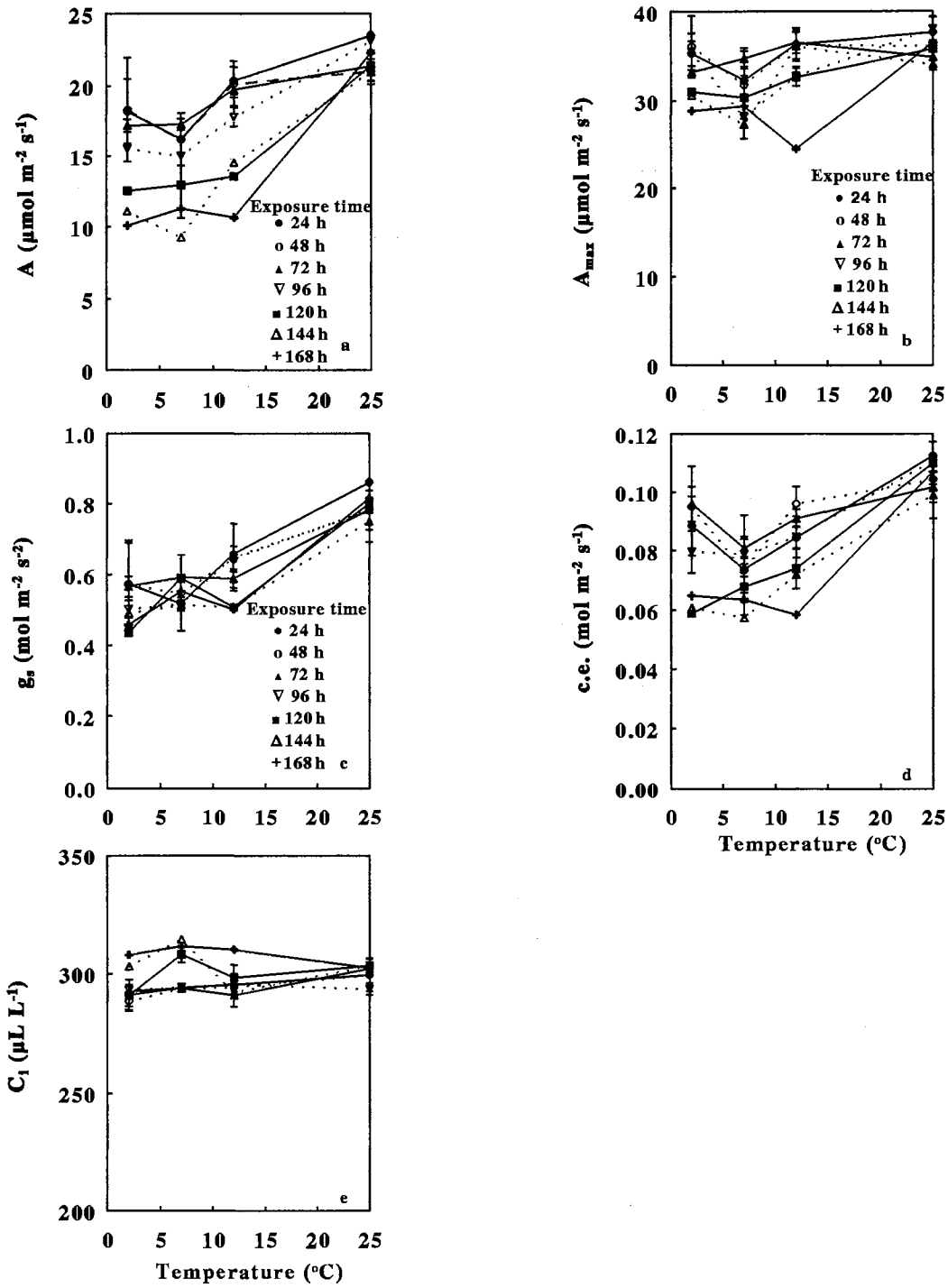

Figure 2. Dependence on chilling low temperature of A (a), A_{max} (b), g_s (c), c.e. (d), and C_i (e) of wheat leaves at different exposure times. Measurements were made at an irradiance of 1800 µmol m^{-2} s^{-1} PAR, 50% RH and an air temperature of 25°C. Bars indicate ± SE. The experiment was repeated two times with four replications for each treatment.

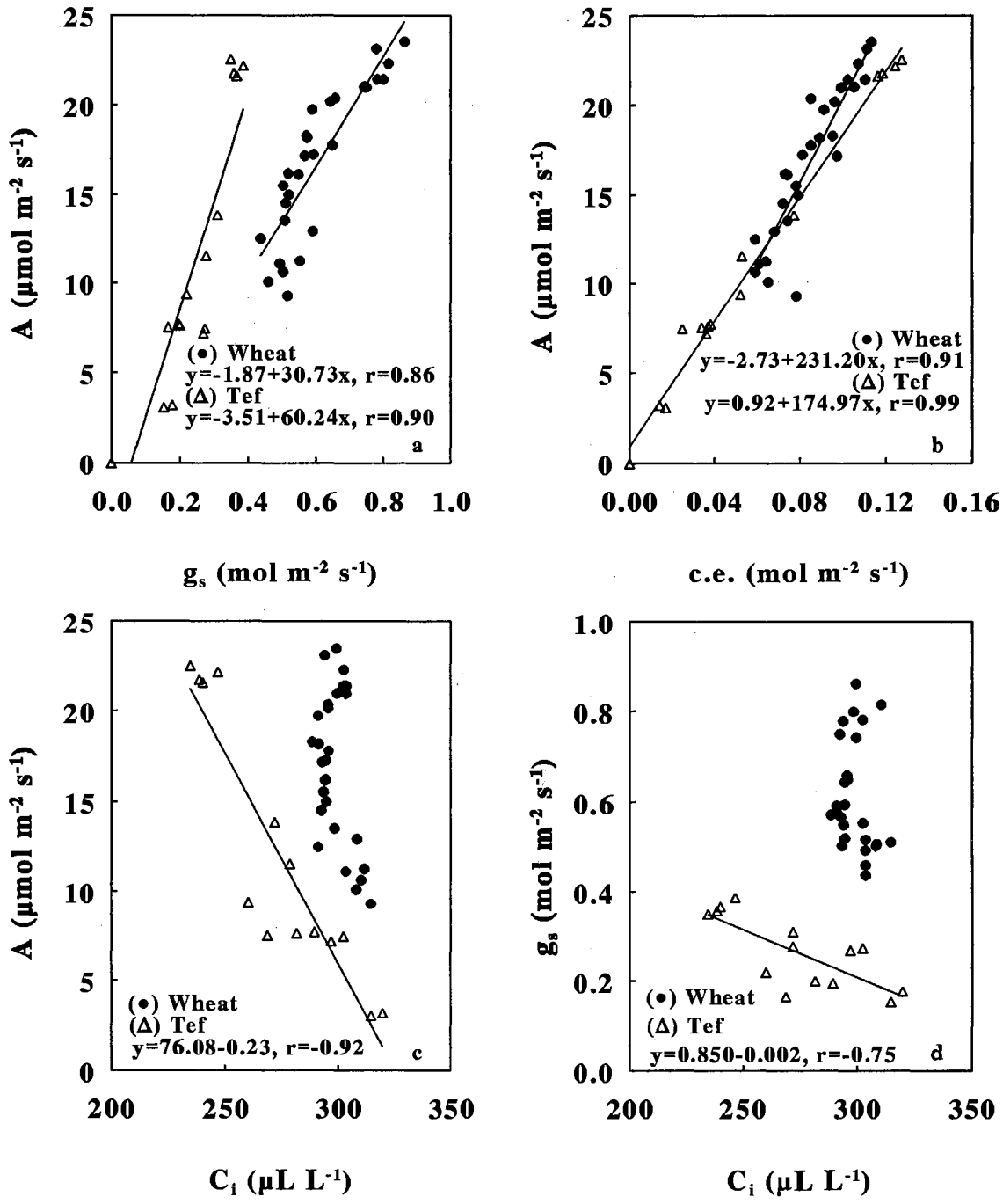

Figure 3. Dependence of A on g_s (a), c.e. (b), and C_i (c), and dependence of g_s on C_i (d) in tef and wheat exposed to chilling low temperature. Each data point represents a specific temperature and duration. For experimental details see Figs. 1 and 2. Figures 3c and 3d are for tef data only.

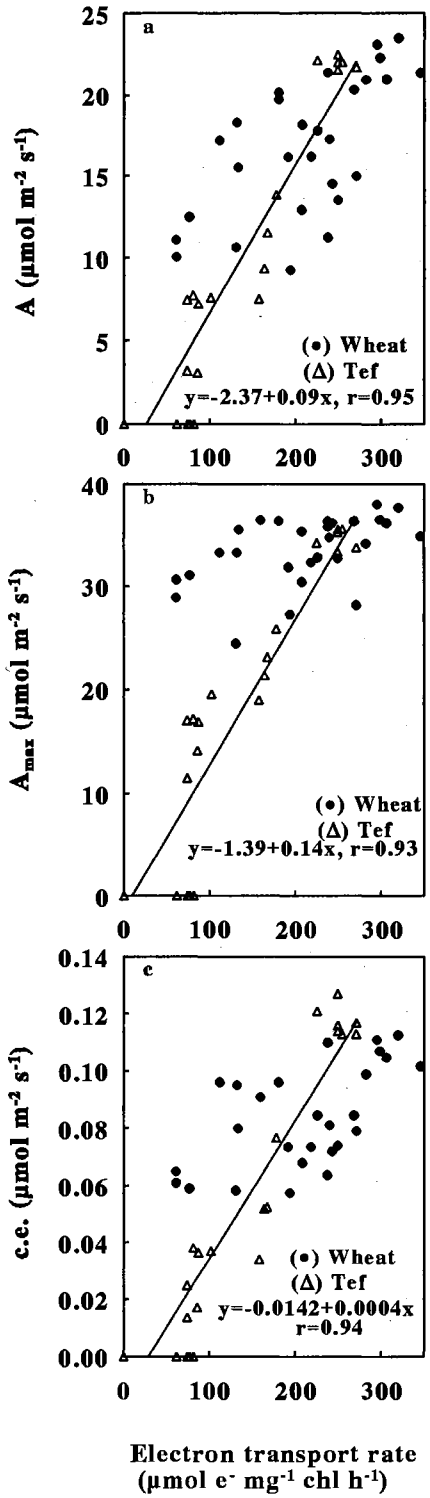

Figure 4. Association between PSII electron transport rate and A (a), A_{max} (b), and c.e. (c) of tef and wheat exposed to chilling temperatures for up to 120 h in tef and 168 h in wheat. Regressions are for tef data only.

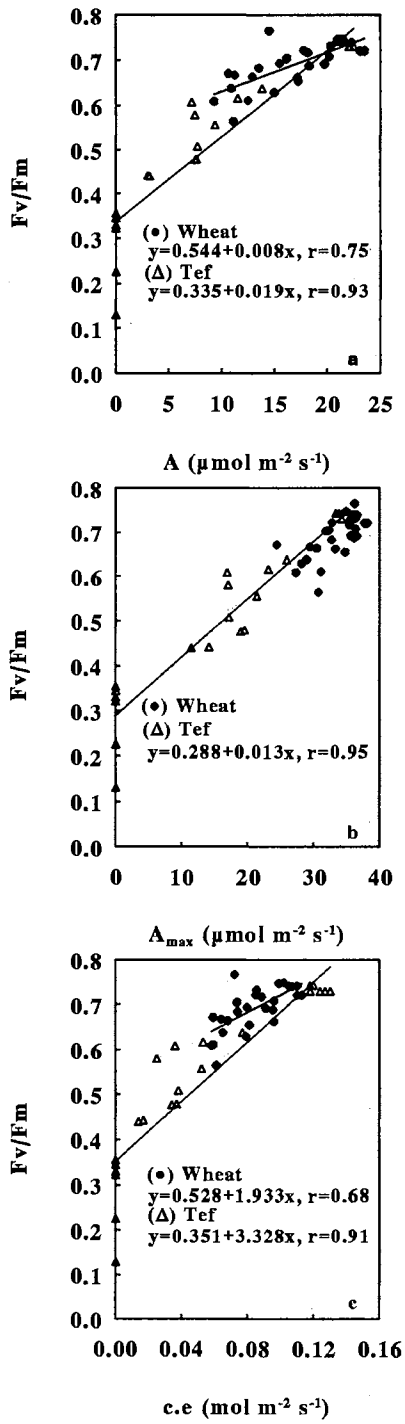

Figure 5. Relationship between Fv/Fm and A (a), A_{max} (b) and, c.e. (c) of chilled tef and wheat leaves. Fv/Fm of chilled leaves were measured in intact leaves that had been dark adapted for 10 min prior to a 5 s exposure to 100 μmol m^{-2} s^{-1} PAR excitation light. Data represent mean values for each day and temperature treatment.Regression in Fig. 5b is for tef data only.

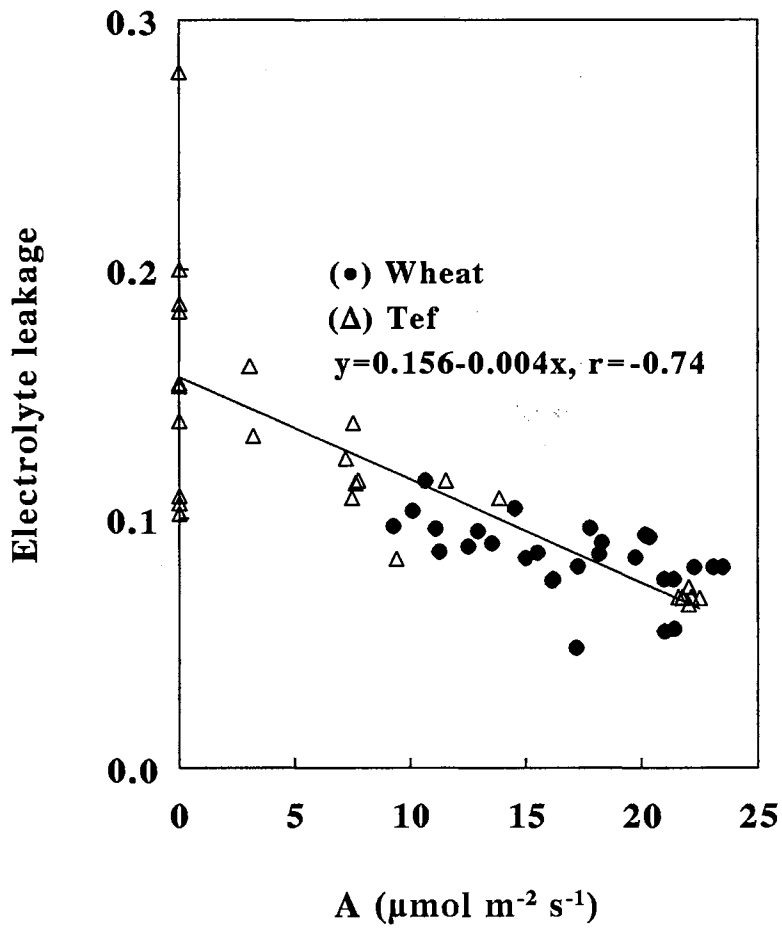

Figure 6. Relationship between electrolyte leakage and A of chilled tef and wheat leaves. Symbols represent mean values for each day and temperature treatment. Regression is for tef.

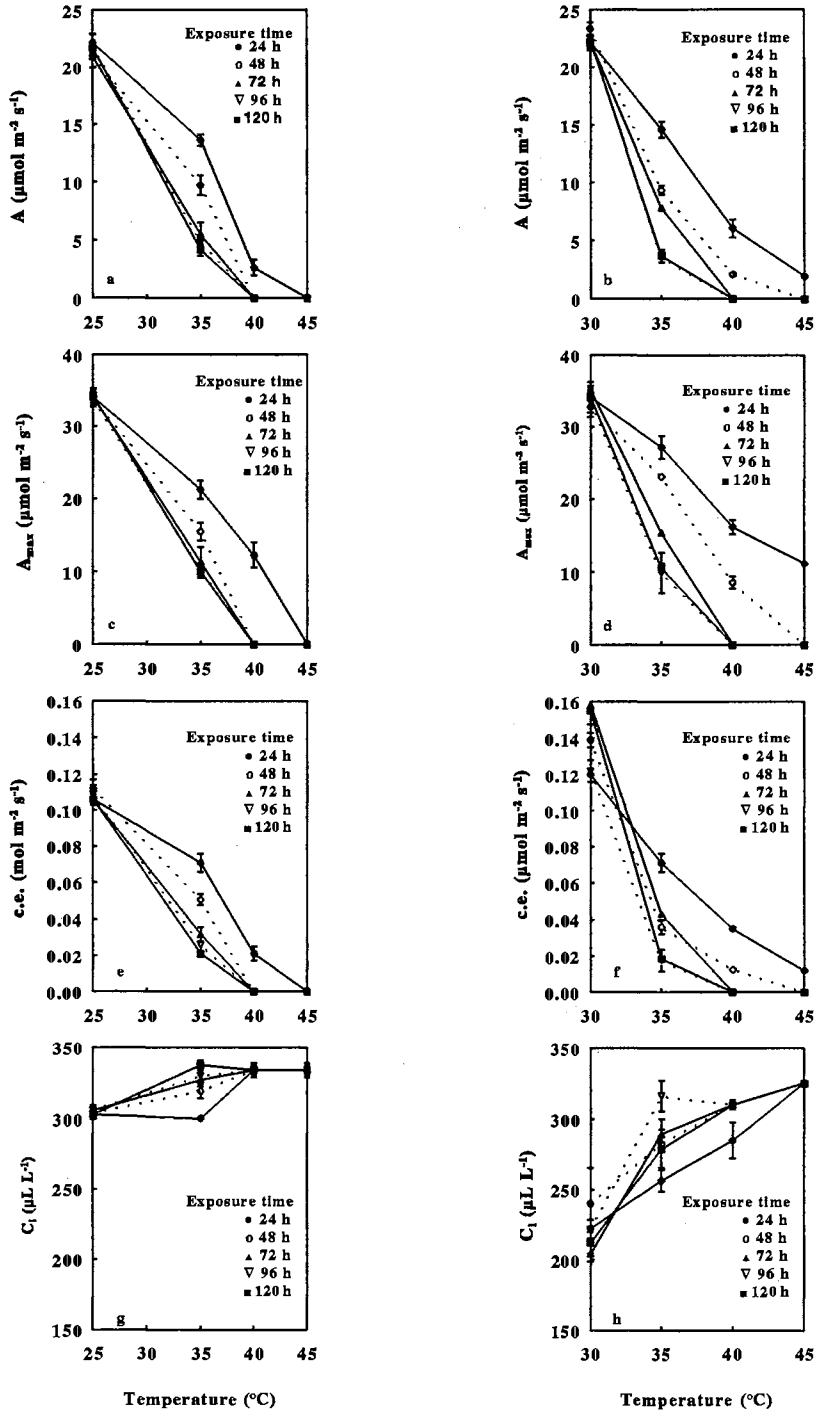

Figure 7. Dependence on high temperature exposure of A in wheat (a), A in tef (b), A_{max} in wheat (c), A_{max} in tef (d), c.e in wheat (e), c.e. in tef (f), C_i in wheat (g), and C_i in tef (h) leaves at different exposure times. Measurements were performed at an irradiance of 1800 µmol m^{-2} s^{-1} PAR, 50% RH and an air temperature of 25°C and 30°C, respectively. Bars indicate ± SE. Experiments were repeated two to three times with four replications for each treatment.

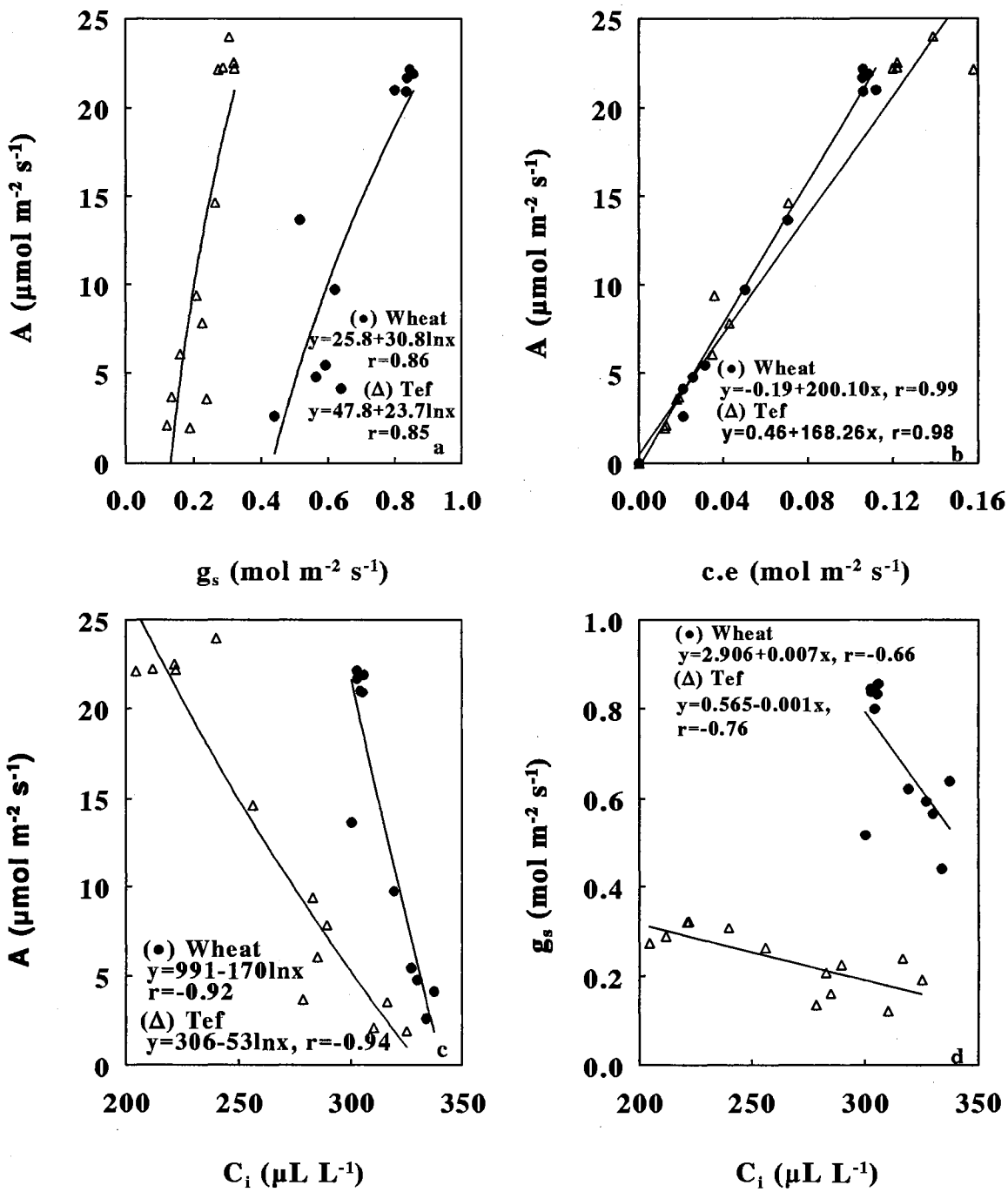

Figure 8. Dependence of A on g_s (a), c.e (b), and C_i (c), and dependence of g_s on C_i on (d) in wheat and tef exposed to heat stress. Each data point represents the mean value for a day and temperature treatment. For experimental details see Fig. 7.

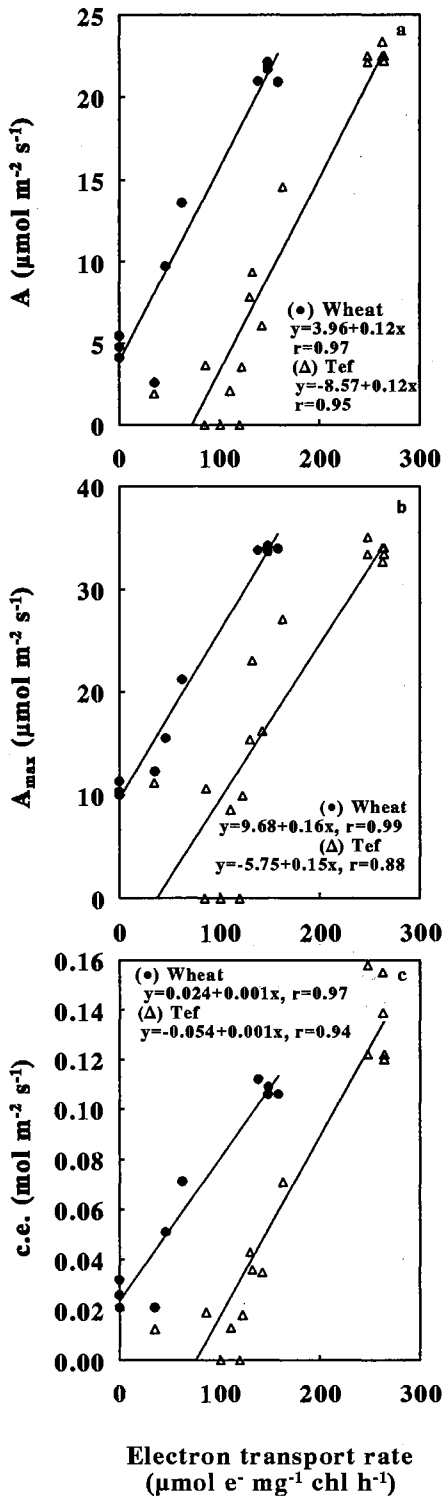

Figure 9. Association between PSII electron transport rate and A (a), A_{max} (b), and c.e. (c) of wheat and tef exposed to heat stress. Each data point represents the mean value for a temperature and duration.

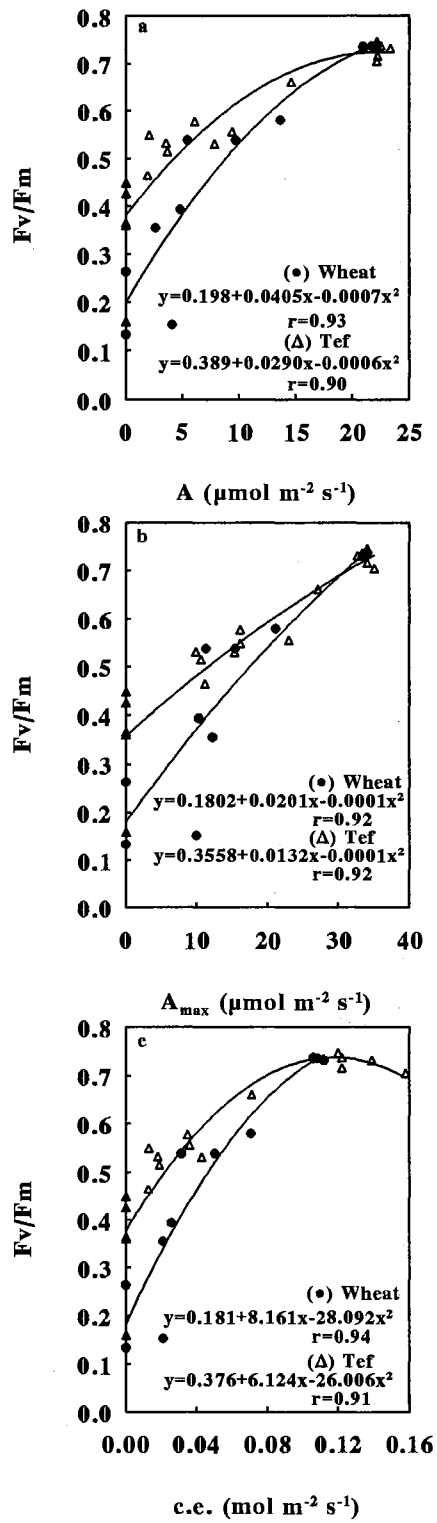

Figure 10. Relationship between Fv/Fm and A (a), A_{max} (b), and c.e. (c) in heat stressed wheat and tef leaves. Each data point represents a mean value for a duration and temperature treatment. For experimental details see Fig. 5.

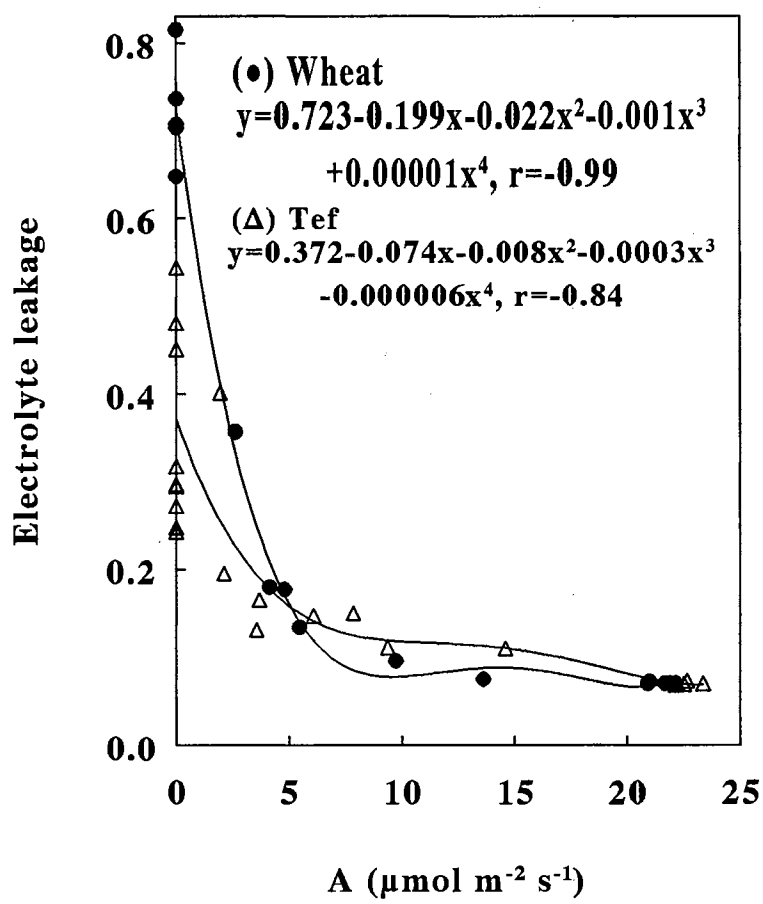

Figure 11. Relationship between electrolyte leakage and A of heat stressed wheat and tef leaves. Data represent mean values for each day and temperature treatment.

Table I. Effects of chilling low temperature stress on the contents of chl a, chl b and chl (a+b), and on the chl a/b ratio of tef and wheat. Chlorophyll contents were measured spectrophotometrically following extraction of leaf tissue in 80% acetone. The values were calculated using Arnon's (1949) extinction coefficients with modification and converted to molar values using molecular weights of 893.5, 907.5 and 900, for chl a, chl b, and chl (a+b), respectively. The experiment was repeated three times and for each treatment four replicate measurements were made. Means ± SE are reported.

Species	Temp (°C)	Time (h)	chl a	chl b	chl (a+b)	chl a/b
			(μmol chl m^{-2})			
Tef	30 control	0	268 ± 7	76 ± 4	344 ± 9	3.95 ± 0.06
	12	24	284 ± 10	79 ± 3	362 ± 13	3.60 ± 0.03
		48	268 ± 9	72 ± 2	340 ± 12	3.70 ± 0.04
		72	229 ± 4	56 ± 1	285 ± 6	4.07 ± 0.05
		96	236 ± 12	66 ± 4	312 ± 16	3.64 ± 0.16
		120	182 ± 7	47 ± 2	229 ± 8	3.91 ± 0.25
		144	267 ± 10	71 ± 3	338 ± 13	3.73 ± 0.05
		168	300 ± 11	80 ± 3	380 ± 14	3.77 ± 0.05
	7	24	281 ± 8	73 ± 2	353 ± 11	3.83 ± 0.03
		48	255 ± 11	63 ± 7	318 ± 11	4.05 ± 0.25
		72	259 ± 9	65 ± 3	323 ± 12	3.97 ± 0.05
		96	260 ± 9	70 ± 3	329 ± 12	3.76 ± 0.12
		120	246 ± 14	63 ± 3	309 ± 17	3.94 ± 0.09
		144	242 ± 7	65 ± 3	307 ± 9	3.74 ± 0.16
		168	200 ± 8	58 ± 2	257 ± 10	3.42 ± 0.04

Table I. cont.

	2		24	271 ± 12	74 ± 3	345 ± 15	3.65 ± 0.08
			48	251 ± 8	70 ± 2	320 ± 11	3.61 ± 0.34
			72	281 ± 13	74 ± 5	354 ± 18	3.97 ± 0.37
			96	283 ± 8	83 ± 2	366 ± 10	3.40 ± 0.03
			120	265 ± 8	72 ± 2	337 ± 10	3.67 ± 0.03
			144	215 ± 6	57 ± 3	272 ± 10	3.78 ± 0.13
			168	239 ± 9	62 ± 6	300 ± 11	3.85 ± 0.33
Wheat	25	control	0	351 ± 20	103 ± 5	452 ± 22	3.51 ± 0.04
	12		24	284 ± 11	80 ± 4	364 ± 14	3.56 ± 0.15
			48	285 ± 11	83 ± 4	369 ± 15	3.43 ± 0.07
			72	269 ± 9	86 ± 8	356 ± 14	3.13 ± 0.15
			96	299 ± 11	89 ± 4	390 ± 19	3.34 ± 0.03
			120	281 ± 12	85 ± 4	366 ± 16	3.29 ± 0.07
			144	249 ± 8	75 ± 2	324 ± 10	3.32 ± 0.05
			168	253 ± 8	81 ± 5	334 ± 12	3.17 ± 0.12
	7		24	346 ± 9	97 ± 7	444 ± 12	3.91 ± 0.76
			48	295 ± 13	88 ± 4	384 ± 17	3.34 ± 0.04
			72	312 ± 13	97 ± 3	410 ± 14	3.23 ± 0.14
			96	341 ± 11	98 ± 3	439 ± 14	3.51 ± 0.06
			120	301 ± 8	86 ± 3	387 ± 10	3.52 ± 0.02
			144	339 ± 14	100 ± 4	440 ± 17	3.36 ± 0.06
			168	322 ± 13	92 ± 3	416 ± 16	3.47 ± 0.06
	2		24	391 ± 14	113 ± 4	504 ± 17	3.47 ± 0.08

Table I. cont.

48	373 ± 32	112 ± 7	485 ± 31	3.41 ± 0.26
72	332 ± 14	96 ± 4	429 ± 18	3.43 ± 0.02
96	362 ± 14	102 ± 4	464 ± 19	3.55 ± 0.04
120	335 ± 13	96 ± 4	432 ± 17	3.48 ± 0.05
144	335 ± 14	92 ± 4	427 ± 16	3.62 ± 0.07
168	335 ± 14	93 ± 4	427 ± 16	3.62 ± 0.07

Table II. Effects of heat stress on the contents of chl a, chl b and chl (a+b), and the chl a/b ratio of wheat and tef. For experimental details see Table I.

Species	Temp (°C)	Time (h)	chl a	chl b	chl (a+b)	chl a/b
			(μmol chl m^{-2})			
Wheat	25 control	0	352 ± 13	110 ± 8	462 ± 17	3.29 ± 0.04
	35	24	314 ± 11	90 ± 8	404 ± 17	3.60 ± 0.25
		48	255 ± 10	77 ± 3	332 ± 15	3.31 ± 0.09
		72	253 ± 16	73 ± 8	325 ± 22	3.48 ± 0.22
		96	159 ± 9	60 ± 2	214 ± 12	2.84 ± 0.09
		120	130 ± 9	71 ± 9	202 ± 16	1.90 ± 0.13
		144	102 ± 13	45 ± 7	147 ± 19	2.36 ± 0.24
	40	24	275 ± 11	96 ± 3	371 ± 14	2.86 ± 0.04
		48	269 ± 11	103 ± 5	372 ± 15	2.62 ± 0.05
		72	267 ± 16	123 ± 7	390 ± 23	2.17 ± 0.07
	45	24	272 ± 21	111 ± 7	383 ± 28	2.42 ± 0.05
		48	243 ± 9	102 ± 4	345 ± 13	2.37 ± 0.03
Tef	30 control	0	274 ± 7	78 ± 4	352 ± 10	3.56 ± 0.09
	35	24	237 ± 7	68 ± 3	305 ± 10	3.50 ± 0.04
		48	184 ± 6	50 ± 4	234 ± 7	3.51 ± 0.43
		72	182 ± 11	49 ± 4	231 ± 14	3.80 ± 0.19
		96	196 ± 8	47 ± 6	243 ± 11	4.13 ± 1.89
		120	161 ± 12	43 ± 3	204 ± 14	3.74 ± 0.10
		144	137 ± 9	43 ± 4	180 ± 12	3.28 ± 0.13

Table II. Cont.					
	168	140 ± 10	41 ± 5	180 ± 10	3.99 ± 0.24
40	24	306 ± 9	78 ± 4	385 ± 12	3.94 ± 0.15
	48	253 ± 12	64 ± 4	317 ± 16	3.97 ± 0.05
	72	246 ± 8	69 ± 2	315 ± 11	3.56 ± 0.02
	96	198 ± 6	64 ± 9	262 ± 12	3.27 ± 0.18
	120	223 ± 21	63 ± 9	286 ± 28	3.55 ± 0.97
	144	168 ± 10	54 ± 3	222 ± 13	3.16 ± 0.05
	168	218 ± 9	65 ± 3	283 ± 12	3.35 ± 0.04
45	24	264 ± 19	76 ± 6	339 ± 24	3.49 ± 0.12
	48	266 ± 9	68 ± 3	334 ± 12	3.93 ± 0.06
	72	226 ± 8	72 ± 2	298 ± 10	3.16 ± 0.04

DISCUSSION

One objective of this study was to evaluate effects of heat and chilling low temperature stresses at moderate light on A, A_{max}, g_s, c.e., and C_i. The temperature-optimum for *Eragrostis tef* in its natural habitat is around 30°C. In the present study photosynthesis of tef was severely at chilling low as well as high temperatures. Under optimum growth temperature, the assembly of thylakoid proteins *in vivo* are subject to the complex organization of the chloroplast photosynthetic membranes. The assembly is potentially vulnerable to chill and heat inactivation, possibly in part due to alteration of the thylakoid membrane as detected by electron transport and fluorescence induction measurements.

Wheat (*Triticum aestivum* L.), which better withstands chilling low temperatures, also showed some chill-induced reductions of A, g_s and c.e. over time. Stomata as well as the mesophyll tissue might have caused the low temperature change in A. Janacek and Prasil (1992) reported chill-induced decreases in A in non-acclimated winter wheat plants growing at temperatures below 15°C. Berry and Bjorkman (1980) and Kobza and Edwards (1987) suggested reduced photosynthetic enzyme activity and Pi depletion, respectively, as possible causes for the declining A. The same factors might have co-limited A in our wheat experiments. However, whether chill-inhibition of A in wheat at long exposure times is the result of stomatal or mesophyll inhibition, or both, is not yet clear.

Photosynthetic gas exchange of tef leaves was rapidly inhibited by chilling. Classifying this species among the thermophilic plants is not unexpected since most subtropical C_4 grasses fall into this category (Miedema, 1982; Long et al., 1983). Chilling stress did not reduce g_s in tef fully as much as it reduced A (Figs. 1, a and c). The negative y-intercept of A regressed on g_s (Fig. 3a) shows that A decreased to zero before the stomata were fully closed. The g_s first declined greatly but as the exposure time increased the further reduction in g_s became smaller until at 120 h g_s reached zero in all three low temperature treatments. C_i in tef increased with decreasing temperature and increasing chilling time (Fig. 1e), which has also been observed in maize (Long et al., 1983) and *Betula* species (Hallgren et al. 1982) exposed to chilling temperatures. Long et al. (1983) and Hallgren et al. (1982) concluded that in neither species the stomatal limitation determined the decrease in A. Thus, the reduction in g_s might not be the major reason for chilling inhibition of A in tef. Rather, the concurrent decrease with decreasing temperature of A (Fig. 1a) and c.e. (Figs. 1d, 3d) and the elevation of C_i (Fig. 1e) imply that impairment of photosynthetic enzyme activities becomes the dominant limitation to A. Bruggemann et al. (1992) reported a large and irreversible decrease of *in vitro* Rubisco activity as tomato plants experienced long-term chilling. They concluded it played a role in the inhibition of A. The decline of c.e. in tef may reflect a reduction in the amount or activity of carboxylase and/or declining supply of substrate for carboxylation. The biochemical limitation may also reside in some other part of the enzymatic processes as suggested by Maruyama et al. (1990) in rice.

Chill-exposure resulted in large decreases in A_{max} (RuBP regeneration, von Caemmerer and Farquhar, 1981). Berry and Bjorkman (1980) and Farquhar et al.

(1987) suggested the decline in A_{max} may indicate suppressed capacity of RuBP regeneration resulting from inadequate electron transport, ATP supply, or photochemistry. In the present study we measured electron transport rates of isolated thylakoids and chlorophyll fluorescence of dark adapted leaves. These measurements showed significant inhibition of PSII and whole chain electron transport rates and reduction of Fv/Fm. The underlying cause of the decline in electron transport activity may be retarded chloroplast development, possibly deficient chloroplast encoded gene products (in particular the D1 protein) as described by Nie and Baker (1991) and Bredenkamp and Baker (1994). The large decrease in electron transport rate in this study demonstrates that PSII electron transport is sensitive to chilling temperatures and decreases in parallel with A_{max}. However, electron transport, although greatly inhibited by chilling, showed substantial rates when A was reduced to zero (Fig. 4a). This is consistent with pervious findings in tomato (Kee et al. 1986). Thus, other biochemical factors in the chloroplast, such as the Calvin cycle, may be responsible for the chill-inhibition of A.

Although there are differences between the two species, heat stress exerts significant inhibition of A in both wheat and tef. In tef, noticeable rates of A were observed at temperatures where A of wheat was completely abolished. The wheat data are in agreement with other reports on reduction by high temperature stress of A, c.e., g_s, and A_{max} (Kobza and Edwards, 1987; Santarius et al., 1991). In both wheat and tef heat stress impaired A and the two biochemical components A_{max} and c.e. to similar extents (Fig. 7, a - c; Fig. 7, e and f) except in tef where A_{max} (Fig. 7d) was slightly less reduced. The coincident decrease of A_{max} and c.e. shows a concerted decline in two distinctly different components of chloroplast function. Whether any

one, or both, are direct targets of heat stress, or whether downregulation is involved, yet remains uncertain.

There were large inhibitions of PSII and whole chain electron transport rates and reduced Fv/Fm at temperatures above 35°C. The effect was greater in wheat than in tef. Chloroplast activities may have been inactivated at these temperatures at the site of oxygen evolution (Thompson et al., 1989) due to loss of Cl^- (Critchley and Chopra, 1988; Coleman et al., 1988) or Mn^{2+} (Nash et al., 1985). It is likely, therefore, that the decline in electron transport by heat stress caused reduced RuBP regeneration and lowered A. This conclusion is consistent with the report by Berry and Bjorkman (1980) that heat stress reduces A_{max} and RuBP regeneration in the chloroplasts. In tef, however, the reduction of whole chain electron transport activity (data not shown) after each duration at high temperature was nearly two fold smaller than the reduction of A or A_{max}. Seventy-two h at 40°C induced 55% inactivation of PSII and 48% reduction of the whole chain electron transport activity (data not shown), whereas A (Fig. 9a) and A_{max} (Fig. 9b) were completely abolished. Therefore, it is uncertain whether electron transport limited A. Berry and Bjorkman (1980) reported a strong correlation between whole chain electron transport and photosynthetic CO_2 assimilation, which was also the case in our study (Fig. 9, a and b).

Carboxylation efficiency of both wheat and tef was much reduced by heat stress (Fig. 7, e and f). Farquhar et al. (1987) suggested that the decrease in c.e. of C_3 plants was caused by depressed activity of Rubisco. Weis (1981) and Kobza and Edwards (1987) showed that the *in vitro* activity of Rubisco is as sensitive to temperatures between 20°C and 45°C as is A and concluded that inactivation of Rubisco causes the inhibition of A. Heat perturbation of thylakoid membranes may

cause adverse changes in the stroma. Reduced pH or ionic composition (Mg^{2+} concentration) may play a role in the inhibition of enzymes.

In both species a close positive relationship between c.e. and electron transport (Fig. 9c) and Fv/Fm (Fig. 10c) was found, suggesting that Fv/Fm acts as an intrinsic probe of the thylakoid membrane and responds to temperature in the same way as photosynthetic enzymes.

In both wheat and tef changes in C_i are at least in part attributable to the chloroplast. Reduced electron transport rate, Fv/Fm, and c.e. clearly showed that inhibition of A was linked to chloroplast activities rather than to stomata. Our results on C_i agree with previous findings of Kobża and Edwards (1987) and Wolf et al. (1990) who reported higher C_i values in wheat and potato exposed to elevated temperatures.

Electrolyte leakage was small until more than 50% of A was lost by high (Fig. 11) or chilling low (Fig. 6) temperature stress. At even greater heat or chilling stress there was a large increase in membrane leakage suggesting disintegration of cellular membrane(s). It is important to note that massive membrane leakiness resulted only from extreme temperatures or long exposures, suggesting that increased membrane leakage was possibly a result rather than a cause of chilling and heat injury. Santarius et al. (1991) came to the same conclusion in studies on lettuce protoplasts. However, they detected no significant increase in electrolyte leakage below 55°C at which temperature CO_2-dependent O_2 evolution was completely inhibited. The discrepancy could be due to use of different procedures, for example the extended periods of heat stress used in our experiments.

In neither species did exposure to moderate heat and chilling temperatures decrease chlorophyll content (Table I, II). Nie and Baker (1991) and Bredenkamp et

al. (1992) in maize and Yordanov (1992) in barley reported no effect of low and high temperature stresses on the contents of light harvesting chlorophyll protein complexes (LHC I and LHC II). Their findings support our chlorophyll data and the conclusion that the light harvesting potential remained intact at moderate stress, a condition under which the photosynthetic CO_2 fixation had already been considerably inhibited (Table I, II). Thus, in neither species did chlorophyll content control the decrease of A in heat or chilling stressed plants. At more severe heat stress, however, and in wheat but not in tef, the chl a/b ratio was lowered to about 1.9 from the normally high ratio of 3.3 to 3.6 measured at 25°C (Table II). An earlier study in spinach by Sundby et al. (1986) on isolated thylakoids came to the same (1.9) chl a/b ratio at 40°C. Sundby et al. (1986) suggested a relative increase in LHC II content and a decrease in the chlorophyll a protein complex of PSII as a cause for thermally induced changes in the chl a/b ratio.

In conclusion, we show comparative responses of leaf photosynthesis of two cereal species to temperature stress. The relatively lower sensitivity of wheat to chilling and tef to heat stress is likely linked to adaptation to their respective native thermal environments. Photosynthesis of chilled and heat stressed tef was primarily limited by enzymatic processes whereas in heat stressed wheat both electron transport and the Calvin cycle may have co-limited A. Membrane leakage and chlorophyll contents were affected only by extended exposure times or severe temperatures and more by heat than by chilling stress. Thus, plasmalemma permeability and chlorophyll content are not early detectors of temperature stress.

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CHAPTER VI

SHORT-TERM CHILLING AND HEATING RESPONSE IN WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.) MEASURED BY CHLOROPHYLL FLUORESCENCE

SENAYET ASSEFA AND BJORN MARTIN

Abbreviations: Fo, initial fluorescence; Fm, maximum fluorescence; Fv, variable fluorescence (Fv = Fm - Fo); $t_{1/2}$, time required for fluorescence to rise from Fo to half of its maximum value.

ABSTRACT

In vivo chlorophyll fluorescence measurements were employed to evaluate short term chilling and heating sensitivity of chloroplast membranes. Wheat (*Triticum aestivum* L.) and tef (*Eragrostis tef* Z.) were exposed to high (35°C, 40°C, and 45°C) or chilling low (2°C, 7°C, and 12°C) temperatures in moderate light. Brief exposure to high temperature greatly affected Fv/Fm, Fv, Fm and $t_{1/2}$ of wheat more than it affected tef. Increased Fo was seen in wheat within 30 min of exposure to 45°C, whereas about 120 min of exposure was needed to prolong the time required for fluorescence to rise from Fo to half its maximum value ($t_{1/2}$). In tef $t_{1/2}$ increased within 60 min of exposure to 45°C. Heat stress had no consistent effect on Fo in this species. In general Fv/Fm (and Fv and Fm) is greatly reduced by heating. Exposure to chilling low temperature for shorter duration than 60 min showed little or no damage in tef. Considerable reductions in Fv/Fm, Fv, and Fm were seen at exposure times between 330 min and 390 min at 2°C, 7°C and 12°C, whereas Fo was largely unaffected. The $t_{1/2}$ was greatly increased at 2°C suggesting limitation in PSII electron transfer. This study revealed that both high and low temperature stress eventually inactivated Photosystem II function in wheat and tef.

INTRODUCTION

Chlorophyll fluorescence measurements are frequently used to asses high and low temperature injury to plants, both at the leaf and isolated chloroplast levels (Neuner and Larcher, 1990; Krause and Weis, 1991). At room temperature chlorophyll a fluorescence is emitted exclusively from photosystem II. The nature and extent of changes of chlorophyll fluorescence can indicate effects on *in vivo* photosynthesis of plants influenced by chilling or high temperature stresses. When dark-adapted leaves are exposed to light, the intensity of chlorophyll a fluorescence undergoes changes due to the onset of different photosynthetic processes (Krause and Weis, 1984). The important features of the chlorophyll fluorescence induction curve of dark-adapted leaves at room temperature, known as the Kautsky effect (for review see Lichtenthaler 1992), are described by Papageorgiou (1975). The terminology and nomenclature used by Krause and Weis (1984) and van Kooten and Snel (1990) are adopted here.

Earlier studies (see Chapters I and II) showed that the quantum efficiency of PSII photochemistry (Fv/Fm) and *in vitro* electron transport inhibition were impaired during extended (24-168 h) temperature stress treatments. Here we investigate whether photochemical events are affected by short term (minutes to hours) chilling and heat exposure of two cereal crops.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Exposure

Seeds of the C_3 cereal wheat (*Triticum aestivum* L. cv. TAM W-101), and the C_4 cereal tef (*Eragrostis tef* Z. cv. DZ-01-354) were planted in controlled environment growth chambers in 2 L pots containing a modified peat moss:soil mix (1:1 v/v) soil (Metro Mix growing medium, Grace Sierra Horticultural Products Company, Milpitas, CA). Wheat and tef were maintained at 25°C/18°C and 30°C/23°C, respectively, day/night temperature and 14 h photoperiod. For details see Chapters I and II.

Heat and Chilling Low Temperature Stress Exposure

Thirty days after emergence, plants were transferred to another temperature controlled growth chamber (Burrows Scientific Equipment Co., Model 1848, Evanston, IL) for high or low temperature treatment. This chamber was humidified at 50% RH and plants were exposed for periods of up to 480 min to 35°C, 40°C, and 45°C or 2°C, 7°C, and 12°C. The pot temperature was constantly checked with a thermometer. Measurements were initiated when the pots had reached the required temperature. Every 30 min over the next 480 min 16 fully expanded leaves (4 leaves/pot x 4 pots) were removed for measurements of chlorophyll fluorescence parameters. Four control pots remained at the original temperatures (25°C and 30°C for wheat and tef, respectively) and were measured in the same way as leaves exposed to heat or chilling temperature.

Chlorophyll fluorescence

Measurements of chlorophyll fluorescence were made with a portable fluorometer (Polar Tech, Umea, Sweden) (Oquist and Wass, 1988). Four fully expanded leaves were sampled from each pot and placed in black plastic sleaves with holes to guide the measuring probe. The leaves were dark adapted for 10 min prior to a 5 s exposure to excitation light with an intensity of 100 μmol m^{-2} s^{-1} PAR. Fo, Fm, Fv, Fv/Fm, and $t_{1/2}$ were measured. Both chilling and heat stress experiments were repeated three times with four replicate measurements per treatment. A completely randomized design was used.

RESULTS

Heat Stress

Figure 1 shows time dependencies of Fv/Fm, Fm, Fo, and $t_{1/2}$ of dark adapted wheat and tef leaves after exposure to elevated temperatures. In wheat not only the temperature but also the time of exposure determined the extent of changes of chlorophyll fluorescence, whereas the later factor was more prominent in tef. In wheat even the moderate exposure to 35°C caused considerable changes. A 30 min exposure to 35°C and 40°C caused inhibition of Fv/Fm by 21% and 33% in wheat (Fig. 1a). In tef 30 min exposure to 35°C and 40°C reduced Fv/Fm by 8% and 22%, respectively (Fig. 1b). The 45°C treatment for 30 min decreased Fv/Fm of both species by 33-36% (Figs. 1, a and b). By the end of 8 h at 45°C Fv/Fm had a decrease from 0.733 to 0.273 in wheat (Fig. 1a), which was substantially more than in tef (0.733 to 0.487, Fig. 1b).

In both species there were concomitant decreases in Fm (Figs. 1, c and d) and Fv (data not shown) with increasing temperature up to 40°C. Like in previous studies wheat leaves exposed to high temperature for short periods showed an increase of Fo (Fig. 1e) and $t_{1/2}$ (Fig. 1g). At 45°C an increase in Fo was detected within 30 min of exposure in wheat. In tef no consistent pattern was seen in Fo as time and temperature exposure increased.

Wheat leaves maintained more or less constant $t_{1/2}$ values until 120 min of exposure to high temperature (Fig. 1g). Beyond this time significant increases were observed at 35°C and 40°C followed by declines again at the longest times. In tef $t_{1/2}$ was increased by the 45°C treatment only, and the effect was much more rapid than seen in wheat (Figs. 1, g and h). In tef time of exposure in general appeared less critical than in wheat.

Dependencies of Fv/Fm on Fo, Fv and Fm are shown in Figure 2. The Fv/Fm of wheat decreased as Fo increased, whereas no significant trend was noted in tef (Fig. 2a). Fv/Fm was positively correlated with Fv (Fig. 2b) and Fm (Fig. 2c) in both species.

Chilling Stress

Figure 3 illustrates the time-dependence of Fv/Fm, Fm, Fv and $t_{1/2}$ of tef exposed to chilling low temperature treatments. All chlorophyll fluorescence parameters were significantly modified by temperature and there was a time-temperature dependence. However, time by itself had little or no effect. Following 60 min of exposure to 2°C, 7°C, and 12°C Fv/Fm of tef leaves was considerably reduced compared to unstressed leaves at 30°C (Fig. 3a). Shortly after five and a half hours of chilling at 2°C to 7°C, Fv/Fm had decreased by 30%. At the time Fm and Fv fell proportionally (Figs. 3, b and c), and $t_{1/2}$ increased to a maximum (Fig. 1d). Thus, decreased Fv/Fm (Fig. 1a), Fm (Fig. 3b) and Fv (Fig. 3c), and increased $t_{1/2}$ (Fig. 3d) were the salient features of chilling exposure in the present study, whereas Fo remained unchanged (data not shown).

Figure 4 shows the linear dependence of Fv/Fm on Fm, Fv, and $t_{1/2}$ in chilled tef leaves. The correlation between Fv/Fm and Fv ($r=0.92$, $P \leq 0.05$), and Fv/Fm and $t_{1/2}$ ($r=0.80$, $P \leq 0.05$) appeared to be high at all temperatures and exposure times.

In Chapter I Fv/Fm of wheat remained largely unaffected upon exposure to chilling low temperature. For this reason no study was made on short term chilling effects.

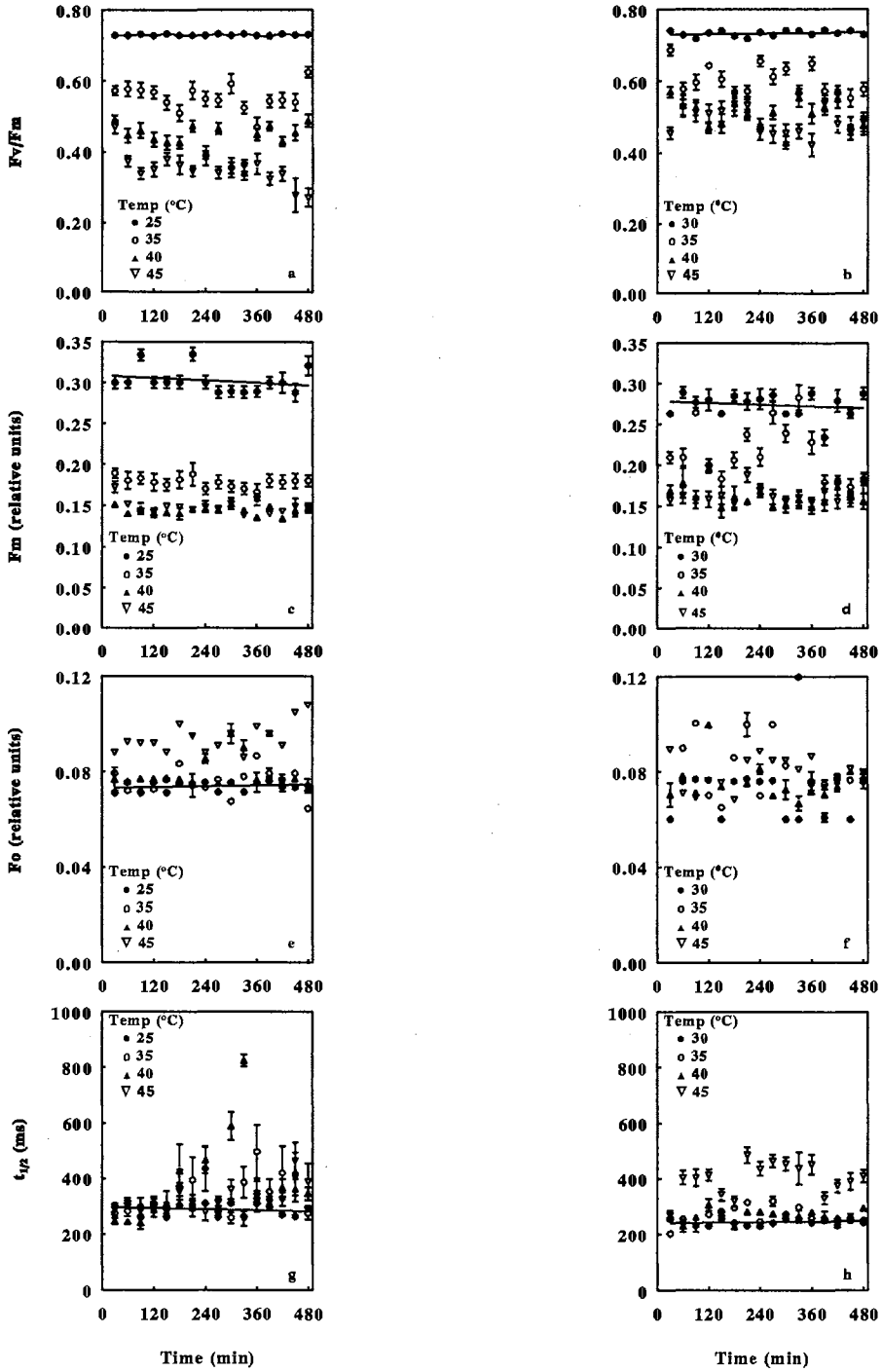

Figure 1. Dependence on time of Fv/Fm in wheat (a), Fv/Fm in tef (b), Fm in wheat (c), Fm in tef (d), Fo in wheat (e), Fo in tef (f), $t_{1/2}$ in wheat (g), and $t_{1/2}$ in tef leaves (h) exposed to high temperatures. Fluorescence measurements were performed on intact leaves that had been dark adapted for 10 min prior to a 5 s exposure to 100 μmol m^{-2} s^{-1} PAR excitation light. Bars indicate ± SE. The experiment was repeated three times with four replication for each treatment. Regression lines are for the control treatments (25°C in wheat and 30°C in tef).

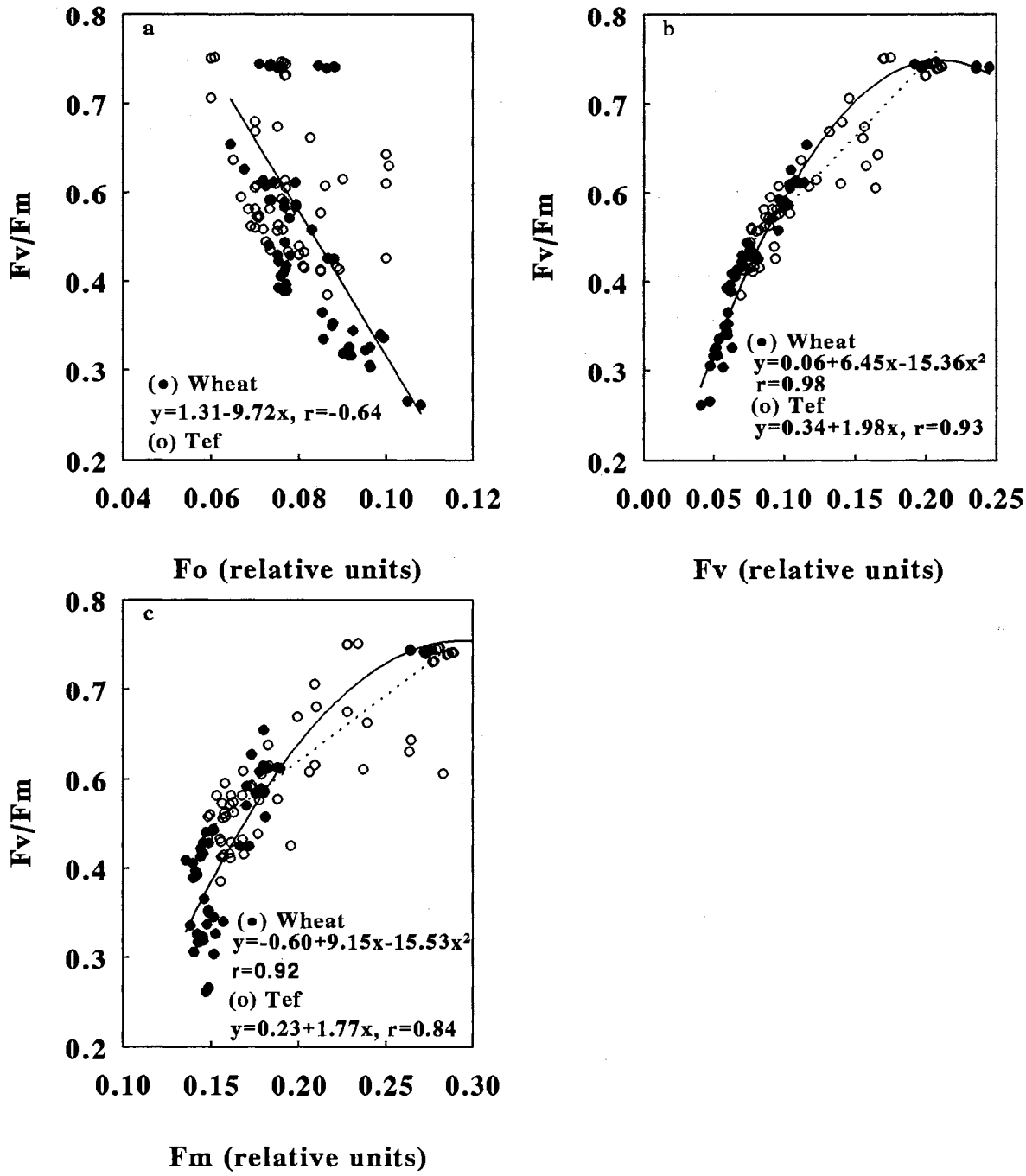

Figure 2. Dependence of Fv/Fm on Fo (a), Fv (b), and Fm (c) of heat stressed wheat and tef leaves. Symbols represent mean values of Fv/Fm and the various fluorescence components of plants exposed temperatures between 25°C and 45°C for periods up to 480 min. For experimental details see Fig. 1.

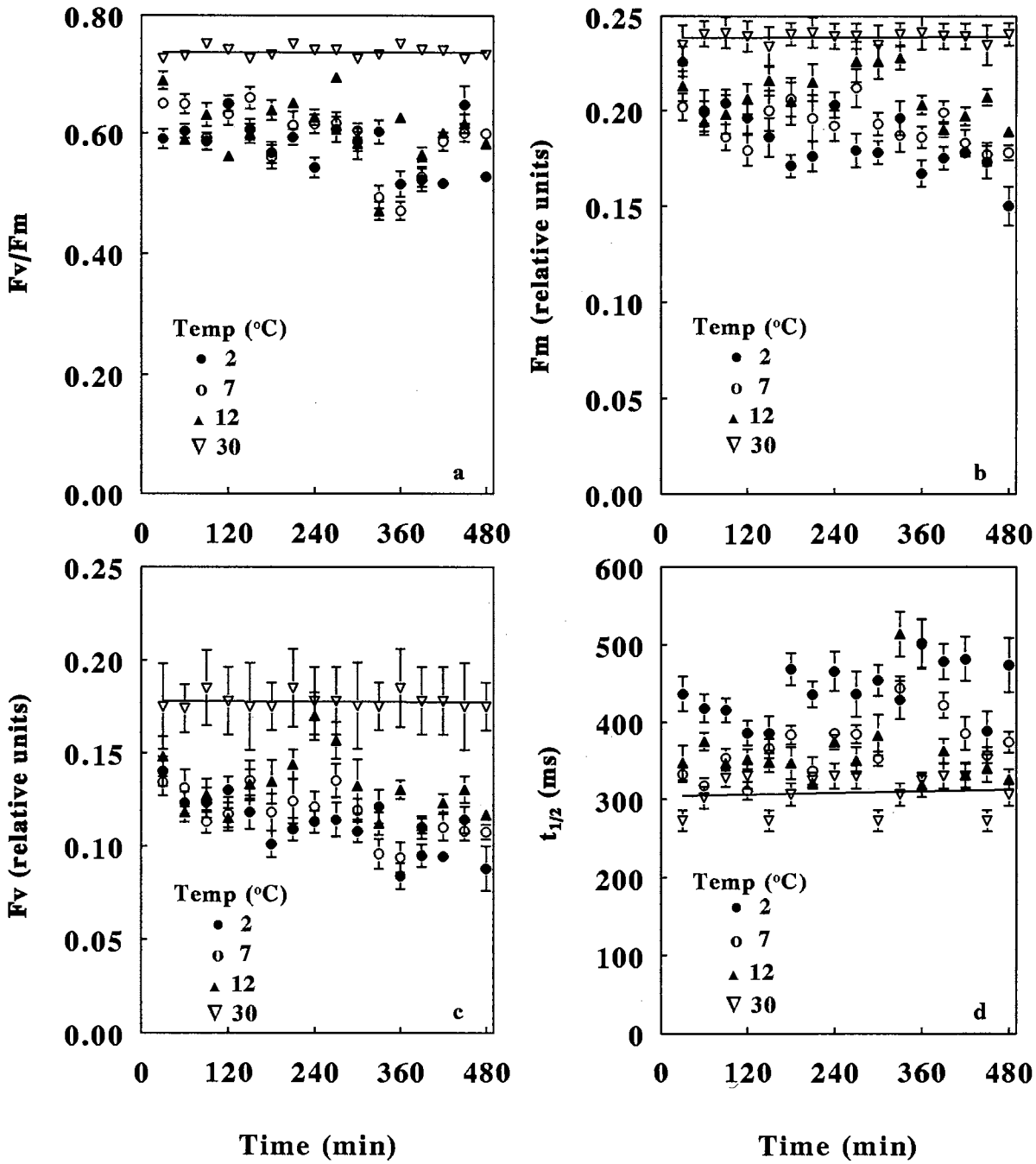

Figure 3. Dependence on time of Fv/Fm (a), Fm (b), Fv (c), and $t_{1/2}$ (d) of tef leaves exposed to chilling low temperatures. For experimental details see Fig. 1. Regression line is for the 30°C (control) treatment.

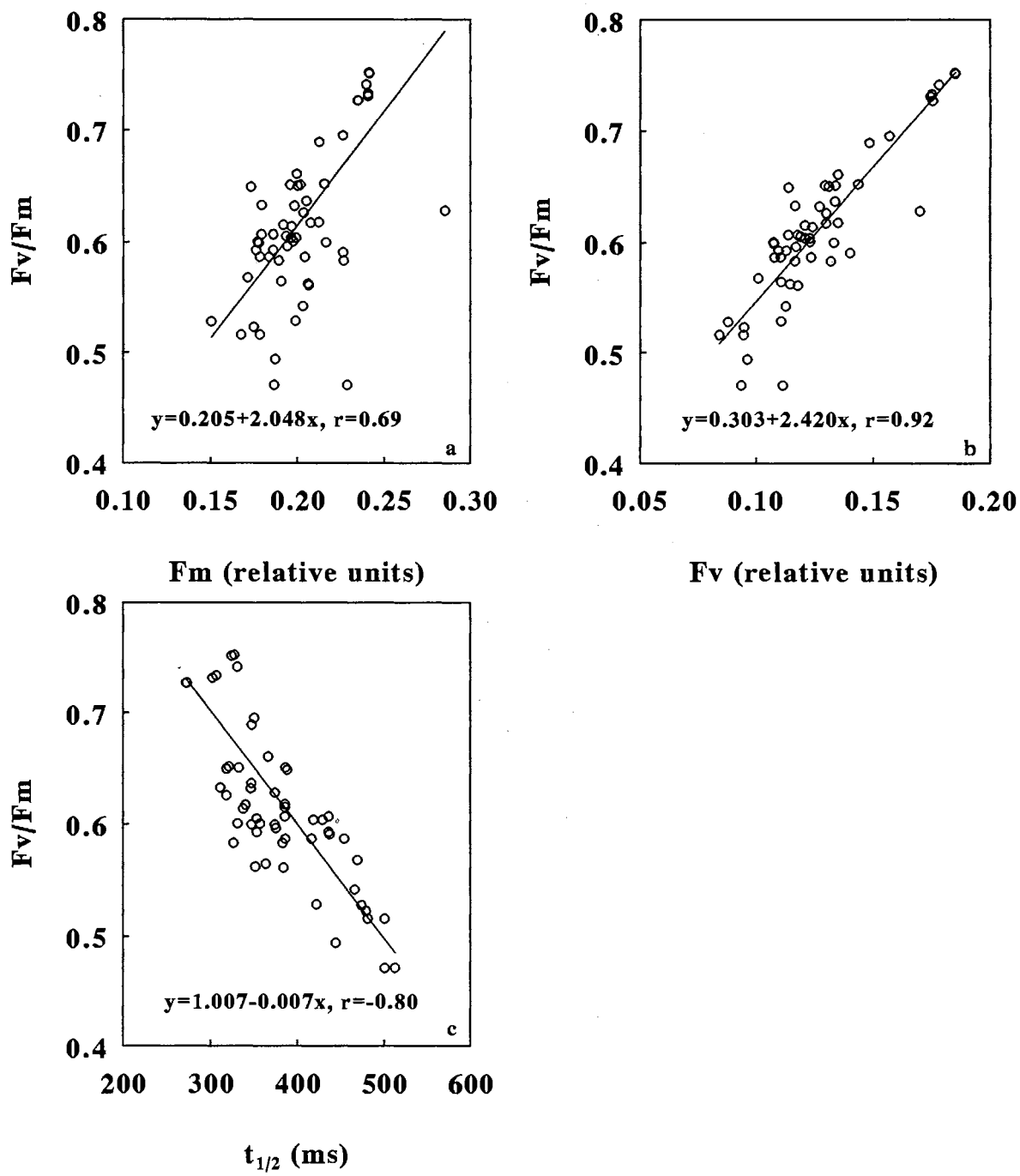

Figure 4. Dependence of Fv/Fm on Fm (a), Fv (b), and $t_{1/2}$ (c) of chilled tef leaves. Data represent mean value of Fv/Fm and the different fluorescence components of plants exposed to temperature between 2°C and 30°C for periods up to 480 min. For experimental details see Fig. 1.

DISCUSSION

The objectives of these experiments were to examine the time-temperature dependencies of photochemical properties of chloroplasts of two cereal crops exposed to short term heat and chilling exposure. Berry and Bjorkman (1980) proposed that the onset of heat inactivation of isolated thylakoid reactions occurs at nearly the same temperature that causes a sudden rise in the Fo level of chlorophyll a fluorescence. The coincident reduction in Fv/Fm and increase in Fo (Fig. 1e) in wheat agrees with this interpretation. The decrease in Fv/Fm has been proposed to indicate a reduction in the photochemical efficiency of photosystem II, presumably due to inefficient energy transfer to the reaction center (Bolhar-Nordenkampf et al., 1989) or due to slow rate of reoxidation of Q_A^- (Chylla and Whitmarsh 1989, Nedbal and Whitmarsh, 1992). The increase in Fo is indicative of damage to the reaction center of PSII (Bolhar-Nordenkampf et al., 1989). The data presented here support our previous conclusion that the most heat sensitive site of photosynthesis in wheat is on the oxidizing side of PSII. In tef, on the other hand, high temperature affected Fo less than Fv/Fm. This suggests that early damage to the photochemical activity in heat stressed tef plants lies beyond the donor side of PSII but perhaps before the plastoquinone pool. Conformational changes leading to destablization of thylakoid membranes (Smith and Low, 1989) might have limited electron transport from Q_A to

Q_B and subsequently to the plastoquinone pool. This would cause low values of Fm due to photochemical quenching.

Berry and Bjorkman (1980) suggested that exposure to chilling temperatures for only a few minutes is often not damaging, at least in part because chilling injury is a cumulative phenomenon. In our case chilling-induced inhibition was less damaging at exposure times shorter than 60 min. After five and a half h of chilling Fv/Fm markedly declined by about 30% (Fig. 3a). This was accompanied by decreased Fm and longer $t_{1/2}$ with no or little effect on Fo. A decrease in Fv/Fm and an increase in $t_{1/2}$ have been reported to result from chilling injury (Bolhar-Nordenkampf et al., 1989). The increase in $t_{1/2}$ suggests that reducing equivalents accumulate slower at Q_A. Thus, the chilling-induced changes in fluorescence suggest that chilling injury takes place in photosystem II. Csapo et al. (1991) measured chlorophyll fluorescence induction and photosynthetic activity of maize leaves. They observed altered fluorescence properties typical of inhibition of PSII electron transport (at the Q_B site) and inhibition of Q_A reduction. Also in our case the decrease in Fv/Fm may have been caused by limited electron transfer, possibly by degradation of the D_1 protein (Nie and Baker, 1991) or inefficient reduction of Q_A.

In conclusion, the *in vivo* chlorophyll fluorescence measurements showed that short term exposure to extreme temperatures caused impairment of chloroplast activity. The primary damage to wheat thylakoids isolated from short-term heat stressed leaves seemed to involve the PSII reaction center as revealed by Fo, whereas other components of PSII appeared more susceptible to heat and chilling-induced injury in tef. The effects of short-term sensitivity was in good agreement with effects of prolonged temperature stress treatments observed in our previous studies.

The *in vivo* chlorophyll fluorescence study reported here involved only the fast induction kinetics of dark adapted leaves. Although, these measurements need not be indicative of steady state photosynthesis, they detect and quantify rapidly developing effects of temperature stress on primary events of photosynthesis.

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VITA

Senayet Assefa

Candidate for the degree of

Doctor of Philosophy

Thesis: **PHOTOSYNTHETIC GAS EXCHANGE, ELECTRON TRANSPORT, FLUORESCENCE, AND ELECTROLYTE LEAKAGE OF WHEAT (*Triticum aestivum* L.) AND TEF (*Eragrostis tef* Z.) EXPOSED TO ENVIRONMENTAL STRESS**

Major Field: Crop Science/Stress Physiology

Biographical:

Personal Data: Born in Tigray, Adwa, Ethiopia, On June 3, 1959, the daughter of Assefa Fanta and Lemlem Haliu.

Education: Graduated from Queen Sheba Comprehensive High School, Adwa, Ethiopia in June 1977; received Bachelor of Science Degree in Plant Science from Addis Abeba University, College of Agriculture, Alemaya, Ethiopia, in July 1981; received Master of Science Degree in Agronomy from Addis Abeba University, School of Graduate Studies, Ethiopia in May, 1984; completed requirements for the Doctor of Philosophy degree in Crop Science from Oklahoma State University in Stillwater in December, 1995.

Professional Experience: Teaching and Research Assistant in Department of Plant Science, Addis Abeba University, Alemaya, Ethiopia, October 1981 to September 1982. Graduate Research and Teaching Assistant, Department of Plant Science, Addis Abeba University, Alemaya, Ethiopia October 1982 to May 1984. Lecturer for undergraduate Department of Plant Science, and Research Associate in Agronomy Alemaya University of Agriculture, Ethiopia, September 1984 to April 1990. Graduate Research Assistant, Department of Agronomy, Oklahoma State University, Fall 1990 to present.

Professional Membership: American Society of Plant Physiologists.