COMPONENT LIMITATIONS TO PHOTOSYNTHESIS OF COTTON

UNDER DROUGHT STRESS

by

SAID ENNAHLI

(Under the guidance of Hugh J. Earl)

ABSTRACT

A new type of chlorophyll fluorometer was evaluated, and a novel technique was developed for accurately estimating thylakoid electron transport rates with this instrument. Then, combined measurements of leaf gas exchange and chlorophyll fluorescence were used to investigate the effects of water stress on photosynthesis of cotton leaves. A computer-automated lysimeter was used to impose distinct levels of water stress on potted cotton plants. Under mild water stress, limitations to photosynthesis were entirely attributable to stomatal closure, which reduced leaf internal CO_2 concentrations. Under moderate or severe water stress, non-stomatal limitations to photosynthesis were also observed; these were evident as a reduction in the net CO_2 assimilation rate for a given CO_2 concentration within the chloroplast. After re-watering severely water stressed plants, stomatal limitations to photosynthesis returned to control levels within 24 hours. However, non-stomatal limitations remained elevated even 48 hours after re-watering, indicating lasting injury to the photosynthetic apparatus.

INDEX WORDS: chlorophyll fluorescence, CO_2 assimilation, cotton, drought stress, *Gossypium hirsutum*, lysimeter, photorespiration, photosynthesis, photosystem II, stomatal limitation, thylakoid electron transport, water stress

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 - Water deficit and crop productivity.

Soil water deficits constitute the single greatest limitation to productivity of field crops in the southeastern United States. In this region much of the cropland is without irrigation, but even on irrigated land water supplies and irrigation equipment are insufficient to prevent the development of some level of water stress in a typical year. As productive capacity in the southeast increases, there is evidence that conflicts over access to available water supplies are intensifying.

In Georgia, future restrictions on water use for irrigation of field crops, or the imposition of fees for surface water or groundwater extraction, may increase the prevalence of dryland cropping systems. This in turn will necessitate the development of crop cultivars with acceptable performance under water deficit conditions, meaning increased drought tolerance (ability to produce high yields under conditions of limited water supply) and/or water use efficiency (WUE, the yield or total biomass produced per unit of soil water transpired). Using classical breeding techniques to produce more drought tolerant cultivars has generally met with limited success, primarily because drought (or indeed any yield-affecting stress) reduces yield heritability in field tests (Blum, 1988; Sneller and Dombek, 1997).

In addition to breeding better-adapted cultivars, water use efficiency in crop production systems may be improved by refining strategies for irrigation scheduling. The best strategies would prevent water deficits from developing during the most critical crop phenological stages, and would regulate the duration and severity of water stress throughout the growing season such

that the productive capacity of the crop could be maximized, within the constraints of the available water supply. For maximally efficient use of water, late season irrigation should also be regulated to ensure complete utilization of available soil water, so that the soil water storage capacity for off-season rainfall is ensured (Bordovsky and Lyle, 1996). Clearly, such an approach depends upon the ability to accurately predict the effect on final yield of any particular water deficit episode during the growing season. The information needed to support such predictions will be specific to the crop species concerned, and may be regionally specific as well. Such models are likely to be most robust if they are based on a sound understanding of the crop's physiological responses to its aerial environment, rather than simple empirical relationships between crop performance and meteorological data.

Thus, an enhanced understanding of the effects of drought stress on crop growth could contribute significantly to the development of more effective irrigation management strategies, as well as efforts to breed more water-efficient crop cultivars and to develop more accurate crop growth models. It is widely acknowledged that one of the primary effects of drought stress is to restrict rates of photosynthetic CO_2 assimilation, thereby limiting crop dry matter accumulation. At the level of the whole canopy, the effects of water deficits on leaf area expansion and absorption of photosynthetically active radiation (PAR) are easily measured, and have been well documented (Turner et al., 1986; Puech-Sanchez et al., 1989; Ball et al., 1994). However, the effects of soil water deficit on photosynthetic processes occurring at the individual leaf level and the cell level are still poorly understood.

1.2 -Traditional Approaches for Determining Physiological Restrictions to Leaf Photosynthesis under Drought Stress.

The effects of water stress on CO_2 assimilation by leaves have classically been divided into two categories: "stomatal limitations", whereby stomatal closure leads to reduced internal

CO_2 partial pressure (C_i), and "non-stomatal limitations", which include all effects limiting CO_2 assimilation at a given level of C_i . There is considerable debate as to the relative importance of stomatal vs. non-stomatal effects of drought in limiting photosynthesis *in vivo.* Since the early 1980s, many experimenters have measured leaf gas exchange parameters in open, closed, or null balance systems, and used some variation of the technique developed by von Caemmerer and Farquhar (1981) to calculate C_i. This approach requires accurate measurements of the leaf net CO_2 assimilation rate (A_N), the transpiration rate (E), the leaf temperature (t_l) and the ambient (i.e., leaf chamber) partial pressures of water vapor and CO_2, as well as an estimate of the leaf boundary layer conductance to water vapor. With this information, it is possible to estimate (in order) leaf-internal vapor pressure, stomatal conductance to water vapor diffusion, stomatal and boundary layer conductance to CO_2 diffusion, and C_i (Figure 1.1). Experimentally, curves of A_N *versus* C_i can be constructed by altering the ambient CO_2 partial pressure within the measurement chamber, while PAR incident on the leaf is held constant. Many experimenters have reported suppression of the response of A_N to C_i under drought stress in the field, thus implicating non-stomatal factors (e.g. Chen et al., 1993; Ephrath et al., 1993; Leidi et al., 1993).

However, many such results are suspect, because of important artifacts that can invalidate the gas exchange-based estimates of C_i in drought-stressed leaves. Specifically:

1. Most models of leaf gas exchange indicate that C_i will be overestimated if drought stress results in "patchy" (ie., heterogeneous) stomatal closure (Laisk et al., 1980; Farquhar et al., 1987; Downton et al., 1988a; Terashima et al., 1988; Raschke et al., 1990; Mott, 1995; but see also Cheeseman, 1991 and Buckley et al., 1997), thus calling into question any conclusions regarding the involvement of non-stomatal limitations to photosynthesis (Terashima et al., 1988; Downtown et al., 1988a, 1988b). Some authors have reported that such patchy stomatal closure is uncommon, so long as drought stress is imposed slowly, and that this phenomenon is therefore unlikely to affect estimates of C_i in either

field experiments or properly designed controlled environment experiments (Wise et al., 1992; Lawson et al., 1998). However, there is also evidence to the contrary (e.g., Dai et al., 1992; Lal et al., 1996), and so the issue remains unresolved.

2. The gas exchange equations of Von Caemmerer and Farquahar (1981) rely on the assumption that the diffusive pathways for water vapor and CO_2 are the same, and that essentially all such diffusion occurs through the stomata. However, when stomatal conductance is very low (for example, during drought stress), a significant portion of total water vapor exchange may occur directly through the leaf cuticle (Jones, 1992). Since resistance to diffusion via the cuticular pathway is typically more than an order of magnitude greater for CO_2 than for water vapor (Figure 1.1) (Boyer et al., 1997), C_i calculated from gas exchange measurements will be drastically overestimated when cuticular transpiration is significant (Kirschbaum and Pearcy, 1988; Meyer and Genty, 1998). Because traditional gas exchange techniques provide no independent estimate of cuticular transpiration, it is not possible to accurately account for this effect in field experiments.
3. Manufacturers and users of field-portable leaf gas exchange measurement instrumentation have only recently become aware of the possible significance of leaf chamber gasket permeability to CO_2 in invalidating A_N/C_i curves. For instance, maintaining very low CO_2 concentration in the leaf chamber for determination of the CO_2 compensation point creates a strong CO_2 gradient between the inside of the chamber and the ambient air, resulting in inward diffusion of CO_2, underestimation of the true A_N, and overestimation of the CO_2 compensation point. Even when gaps between chamber gaskets are completely sealed, diffusion of CO_2 down the concentration gradient occurs through the gasket material itself. The rate of such diffusion is usually very low, but can

become a significant portion of total net CO_2 exchange when the leaf in the chamber is operating near its compensation point.

There is a little doubt that one of the primary effects of drought stress under field conditions is to induce stomatal closure, thus reducing the CO_2 concentration at the carboxylation site. However, because of the general unreliability of A_N/C_i curves measured in the field using traditional techniques, it is still uncertain whether or not non-stomatal limitations also play an important role.

Recently new techniques have become available which allow this fundamental question to be addressed. These techniques, which combine improved gas exchange measurement techniques with independent measurements of photosynthetic electron transport and well-established biochemical models of C_3 photosynthesis, avoid the artifacts that have hampered previous investigations.

1.3 - Combining Gas Exchange Measurements and Chlorophyll Fluorometry to Estimate Stomatal and Non-Stomatal Resistances to Photosynthesis

By combining leaf gas exchange and chlorophyll fluorescence measurements, it is possible to estimate the concentration of CO_2 in the chloroplasts of C_3 leaves (C_c) *in vivo*. This approach does not rely on an estimate of C_i from leaf gas exchange, and is thus not susceptible to artifacts (1) and (2) described above.

The thylakoid electron transport rate (ETR) can be estimated in intact leaves under normal actinic light using pulse amplitude modulated (PAM) chlorophyll fluorometery (Genty et al., 1989). This technique requires measurement of the photosynthetic photon flux density (PPFD) incident normal to the leaf, and the quantum efficiency of photosystem II activity (Φ_{II}). These are now easily measured with commercially available quantum sensors and chlorophyll fluorometers. The value of ETR is calculated as:

$$ETR = \alpha f_{II} PPFD \Phi_{II} \qquad (1)$$

where α is leaf fractional absorption of incident PPFD and f_{II} is the fraction of absorbed PPFD which is absorbed by the antennae of photosystem II. The value of α may be a) determined with an integrating sphere, b) estimated using some alternate technique (e.g., Earl and Tollenear, 1997), or c) assumed. The value of f_{II} is most often assumed to be 0.5 for C_3 plants, although experimental evidence suggests that it may in fact range from 0.42 to 0.60 (Laisk and Loreto, 1996). Assuming four electrons are required per oxygenation or carboxylation reaction catalyzed by RubisCO,

$$ETR = 4v_C + 4v_0 \qquad (2)$$

Where v_C and v_0 are the rates of ribulose bisphosphate carboxylation and oxygenation, respectively. Since 0.5 CO_2 are evolved per oxygenation reaction,

$$A_G = v_C - 0.5v_0 \qquad (3)$$

where A_G is the gross assimilation rate, estimated as the sum of the measured net CO_2 assimilation rate (A_N) and the dark respiration rate (R_D). By combining equations (2) and (3), it is possible to estimate the ratio of v_C to v_0.

$$\frac{v_C}{v_O} = \frac{0.5ETR + 4A_G}{ETR - 4A_G} \qquad (4)$$

The value of C_c may then be calculated according to Lal et al. (1996) as:

$$C_C = \frac{O_C}{K_S} \cdot \frac{v_C}{v_0} \qquad (5)$$

where O_C is the O_2 concentration at the carboxylation site, and K_S is the CO_2/O_2 specifity ratio of RubiscCO. O_C is calculated by assuming no O_2 concentration gradient between the ambient air and the carboxylation site (Gerband and André, 1987), and adjusting for the effect of the leaf temperature on O_2 solubility. K_S is also estimated as a function of leaf temperature (Jorden and Ogren, 1984; Brooks and Farquhar, 1985).

Thus, whether or not a reliable estimate of C_i is available, leaf CO_2 exchange and chlorophyll fluorescence can be used to determine the relationship between A_N and C_c non-destructively *in vivo*. Under controlled environment conditions, this technique (or simplified versions of it) has been used to distinguish between inhibition of photosynthesis caused by reduced C_C (for example, due to stomatal closure), and inhibition attributable to "biochemical " factors, such as down-regulation of the light reactions or reduced activity of Calvin cycle enzymes (Dai et al., 1992; Lal et al., 1996; Sanchez-Rodriguez et al., 1999). The estimates of C_C and A_G can also be used to calculate the maximum carboxylation rate using the model of Harley and Sharkey (1991). If RubisCO activity is assumed to be the biochemical factor limiting the maximum carboxylation rate, which is likely the case under high PPFD, then RubisCO activity of the leaf can also be estimated without the need for a tissue extraction and *in vitro* assay (Loreto et al., 1994).

As stated above, estimates of C_C using these techniques are not susceptible to artifacts caused by heterogeneous stomatal closure or cuticular transpiration. However, they still rely on accurate determination of A_N, and can thus be invalidated by diffusion of CO_2 into the leaf chamber when the chamber CO_2 concentration is much lower than that of the surrounding air. For open flow gas exchange systems, one means of correcting for this effect is as follows: First, the conductivity of the sealed chamber to CO_2 diffusion is quantified in calibration experiments.

Then, if the concentration of the air surrounding the chamber is known during measurements (e.g., estimate approximately 350 ppm for measurements made outdoors), inward diffusion of CO_2 can be calculated based on simple diffusion theory, and the measured values of leaf A_N can be adjusted accordingly.

1.4 -Drought stress simulation

Different methods have been used to simulate drought stress in greenhouse and other controlled environment experiments. Such a method ideally should maintain uniform soil water content around the roots, and allow the rate at which stress develops to be precisely controlled, regardless of differences in plant size and variation in environmental conditions. Also, it should allow a range of distinct and clearly definable levels of stress to be imposed.

Few traditional methods of simulating water stress in pot experiments meet the above criteria. Simply withholding water is not satisfactory, since usually with this method water deficit develops much more quickly than is typical under field conditions, which affects the types of physiological responses observed (Cornic et al., 1987; Saccady et al., 1996; Farrant et al., 1999). Also, a rapid decrease in soil water content may lead to a heterogeneous stomatal response (patchy stomatal closure) (Wise et al., 1992). There are two methods for preventing water stress from developing quickly. One consists of increasing the total plant-available water, either by using large containers (Allen et al., 1994) or by using a rooting medium with an unusually high water holding capacity (Pennypacker, 1990; Nissanka et al., 1997). However, the actual rate at which water stress develops still depends on environmental conditions (radiation load, air temperature, humidity). The other alternative consists of determination of water loss from each pot gravimetrically by frequent recording of pot weights, and replacing part of the transpired water to control the rate of soil dry-down (Sinclair and Ludlow, 1986; Ekanayake et

al., 1993; Ray and Sinclair 1997, 1998); however, this method is labor intensive and therefore rarely adopted.

A precise gravimetric method for simulating drought stress in pot experiments (Earl 2003; an adaptation of the Hunter and Tonks (1979) technique) was adopted for our experiments as a useful approach for drought stress simulation under greenhouse conditions. This method permits maintenance of stable levels of soil water deficit, with uniform water distribution throughout the rooting volume. Also, the relative soil water content determined gravimetrically can be used as an indicator of soil water deficit experienced by roots in such experiments.

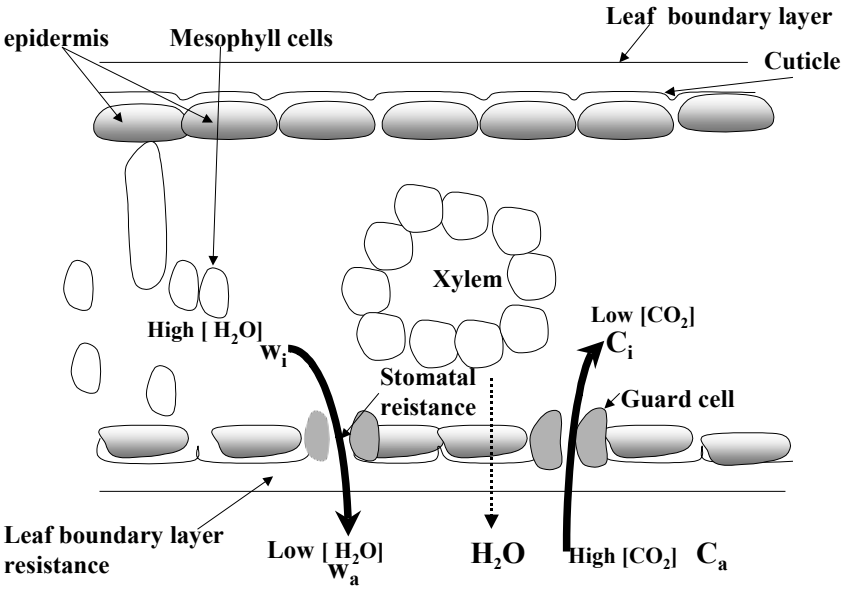
1.5 - Objectives:

The objectives of the present work were:

1. To test the ability of a newly introduced Leaf Chamber Fluorometer, which provides complete control over the actinic PPFD, to accurately measure Φ_{II}.
2. To quantify stomatal and non-stomatal restrictions to cotton leaf photosynthesis during drought stress events of different severities and durations, and after recovery from drought stress, under greenhouse conditions. Investigations conducted under this objective were designed to provide basic information about the physiological effects of drought stress on carbon assimilation by cotton. Specific research questions addressed in relation to this objective were:
    a. Does drought stress induce non-stomatal limitations to photosynthesis in cotton leaves, measurable as a change in the relationship of A_G to C_C at a given incident PPFD?
    b. What severity (leaf water potential or soil water content) and duration of water stress is required to induce non stomatal-limitations to photosynthesis?

c. How quickly and completely are measured stomatal and non-stomatal restrictions to photosynthesis relieved upon return to non-limiting soil water content?

Figure 1.1. Schematic cross-section of leaf showing the diffusion pathways of CO_2 and H_2O. C_i, C_a: internal and ambient CO_2 concentration; w_i, w_a: internal and ambient water vapor concentration.


Leaf boundary layer
epidermis
Mesophyll cells
Cuticle
Xylem
Low [CO2]
Ci
High [H2O]
Wi
Stomatal
reistance
Guard cell
Leaf boundary layer
resistance
Low [H2O]
Wa
H2O
High [CO2] Ca

CHAPTER 2

ACCURATE ESTIMATION OF PHOTOSYNTHETIC ELECTRON TRANSPORT WITH A NEW TYPE OF CHLOROPHYLL FLUOROMETER

2.1 - Abstract

Combined measurements of leaf gas exchange and chlorophyll fluorescence are useful in plant stress physiology research. We evaluated the ability of a commercially available field portable chlorophyll fluorescence / leaf gas exchange measurement system to accurately measure the photosynthetic electron transport rate (ETR) in maize (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) leaves. Gross assimilation (A_G) and ETR were estimated at nine photosynthetic photon flux density (PPFD) levels ranging from 150 to 2400 μmol m^{-2} s^{-1}. Contrary to expectations, the relationship between measured ETR and A_G was not linear in maize leaves, especially at high PPFD levels, where the ratio ETR/A_G was lower (4.07 ± 0.22) than predicted by theory. This appeared to be due to underestimation of the quantum efficiency of photosystem II (Φ_{II}) caused by insufficient intensity of the saturating pulse of light used to induce the maximum fluorescence signal (F_M'). Instead of estimating F_M' with a single saturating pulse, a series of pulses of different intensities was applied, and then the value of Φ_{II} expected with infinite pulse intensity was estimated from extrapolation of the linear regression of Φ_{II} on the inverse of the pulse intensity. When this estimate of Φ_{II} was used to calculate ETR, a near-linear relationship between ETR and A_G was observed, suggesting that the estimate of ETR had been improved. As with maize, ETR at high PPFD appeared to be underestimated in cotton when Φ_{II} was estimated simply using the highest available saturating pulse intensity, as

compared to the intercept method. Also, under non-photorespiratory conditions (2% O_2) the expected linear relationship between ETR and A_G was observed in cotton when the intercept method was used and also the ETR/A_G ratio was not as expected.

2.2 - Introduction

The thylakoid electron flux can be estimated in intact leaves under illumination with normal actinic light using pulse amplitude modulated (PAM) chlorophyll fluorometery (Genty et al., 1989). This technique requires measurement of the photosynthetic photon flux density (PPFD) incident normal to the leaf, and also the quantum efficiency of photosystem II activity (Φ_{II}), which can be estimated *in vivo* as:

$$\Phi_{II} = \frac{(F_M' - F_S)}{F_M'}, \qquad (1)$$

where F_S is the steady state fluorescence signal from an illuminated leaf, and F_M' is the maximum signal during a subsequent pulse of saturating light. Incident PPFD and Φ_{II} are now easily measured with commercially available quantum sensors and chlorophyll fluorometers. The non-cyclic electron transport rate (ETR) in the thylakoid membrane can be calculated as:

$$\text{ETR} = \alpha\, f_{II}\, \text{PPFD}\, \Phi_{II} \qquad (2)$$

where α is leaf fractional absorption of incident PPFD and f_{II} is the fraction of absorbed PPFD which is absorbed by the antennae of photosystem II (Genty et al., 1989). The value of α may be a) determined with an integrating sphere, b) estimated using some alternate technique (e.g., Earl and Tollenaar, 1997), or c) assumed. The value of f_{II} is most often assumed to equal 0.5 in C_3 plants (Loreto et al., 1992, 1994; Brestic et al., 1995) and 0.4 for C_4 plants (Edwards

and Baker, 1993; Lal and Edwards, 1996), although it may in fact range from 0.42 to 0.60 for C_3 plants (Laisk and Loreto, 1996).

Four electrons from photosynthetic electron transport are required to fix one molecule of CO_2 in C_3 plants; however, other alternative sinks, most importantly photorespiration, compete for these electrons. The rate of photorespiration relative to photosynthesis is variable, tending to increase at higher leaf temperatures, and under conditions that reduce the concentration of CO_2 at the carboxylation site. Other alternative electron sinks include nitrite and O_2 photoreduction, and pseudocyclic electron flow (Badger, 1985; Robinson, 1988).

Because photorespiration both consumes electrons from thylakoid electron transport and releases CO_2, the ratio of ETR to gross assimilation (A_G) increases as photorespiration increases. When photorespiration is inhibited (for example by reducing the oxygen concentration around the leaf) the relationship between ETR and A_G becomes linear (Harbinson et al., 1990; Oberhunber et al., 1993; He and Edwards, 1996), and the ratio decreases to very near 4.0. (Lal and Edwards, 1995; Edwards and Baker, 1993). Since four is the theoretical minimum if all electrons from thylakoid electron transport are consumed in CO_2 reduction, this result suggests that alternative electron sinks other than photorespiration are normally negligible.

By contrast, in C_4 plants the carboxylation of ribulose bisphosphate is enhanced by a very high CO_2 concentration at the site of RubisCO, and so oxygenation is mostly suppressed and photorespiration is minimal. However the ratio of ETR / A_G observed is around 4.6 (Earl and Tollenaar, 1998; Krall and Edwards, 1990; Edwards and Baker, 1993) - greater than 4.0 due mainly to the higher minimum energy requirement for CO_2 fixation in C_4 species (5 ATP and 2 NADPH per CO_2 fixed, as compared to only 3 ATP and 2 NADPH in C_3 species) (Krall and Edwards, 1990; Edwards and Baker, 1993). In C_4 plants, the observed ratio is insensitive to environmental conditions. Because of this consistent linear relationship between ETR and A_G

(Genty et al., 1989; Krall and Edwards, 1990, 1991; Edwards and Baker, 1993; Oberhuber and Edwards, 1993; Earl and Tollenaar, 1998), ETR can be used as a surrogate for CO_2 assimilation measurements for these species.

With simultaneous measurements of leaf gas exchange and chlorophyll fluorescence in C_3 plants, it is theoretically possible to calculate a wide range of parameters relating to the physics and biochemistry of leaf photosynthesis. These include the CO_2/O_2 specificity of ribulose-1,5-biphosphate carboxylase/oxygenase (Peterson 1989, 1990; Laisk et al., 1996), the dark respiration in the light, the alternative electron transport rate to acceptors other than bisphosphoglycerate (Loreto et al., 1994), the mesophyll resistance to CO_2 diffusion in the liquid phase, and the chloroplast CO_2 concentration (Di Marco et al., 1990; Loreto et al., 1992, 1994; Epron et al., 1995; Laisk et al., 1996).

In recent years, portable instrumentation has become commercially available that should permit the simultaneous measurements of ETR and CO_2 assimilation in field studies, while also providing control over important aspects of the leaf microenvironment (temperature, CO_2 concentration, incident PPFD). The objective of the present work was to evaluate the ability of one such instrument to provide accurate measurements of ETR. The expected linear relationship between ETR and gross CO_2 assimilation rate was not observed in C_4 (maize) leaves, nor in C_3 (cotton) leaves under non-photorespiratory condition. The current findings suggest that the lack of a linear trend at higher PPFD levels is due principally to an underestimation of Φ_{II}. This may be caused by insufficient intensity of the saturating pulse of light used to induce the maximum fluorescence signal. An alternate technique was developed, which uses multiple pulses of varying intensity to estimate Φ_{II}. When such estimates of Φ_{II} were used in the calculation of ETR, the relationship between ETR and A_G conformed much more closely to what has been observed in previous studies.

2.3 - Material and Methods

2.3.1 Plant Material

Both cotton (cv. Delta pearl) and maize (hybrid cv. Pioneer 3245) were grown in a greenhouse in Athens, Georgia, USA (34°N, 84°W). Plants were grown in 5-L plastic pots with drainage holes, filled with commercial potting soil (Brown Earth Potting Mix, Carven, Inc., Commerce, GA).

Maize and cotton seeds were sown four or five to a pot and immediately after, fertilized with 100 ml of a 0.8% (w/v) solution of 20-20-20 fertilizer plus micronutrients (Miller Greenhouse Special, Miller Chemical and Fertilizer Co. Corp., Hanover, PA). After germination, plants were thinned to one per pot, and fertilized twice weekly. Greenhouse temperature was maintained at 27± 4°C during the day and 20± 2°C during the night. Photoperiod was extended to 16 h with overhead 400-W metal halide lamps that produced a supplemental PPFD of approximately 230 μmol m^{-2} s^{-1} at the tops of the plants.

At 3-4 weeks (maize) or 4-5 weeks (cotton) after planting, plants were selected for gas exchange/chlorophyll fluorescence measurements youngest. Fully expanded mainstem leaves. were selected for measurements in all experiments. Eleven repetitions were conducted for maize under ambient air. Seven repetitions were conducted for cotton, with measurements on each leaf made under both under ambient air and under non-photorespiratory (2% O_2) conditions.

2.3.2 Gas Exchange and Fluorescence Measurements

Fluorescence measurements were made on youngest fully expanded mainstem leaves with two Leaf Chamber Fluorometers (Model 6400-40, LICOR Inc., Lincoln NE) attached to LI-6400 Portable Photosynthesis Systems (LICOR). The 6400-40 is a pulse-amplitude modulated (PAM) fluorometer used to make measurements on both darkened and light-adapted

samples. When used in conjunction with the LI-6400, fluorescence readings and leaf gas exchange are measured across the same circular 2-cm^2 leaf area exposed in the gas exchange chamber. Actinic illumination and also saturating pulses of light are provided by a mixture of red and blue light emitting diodes (LEDs) mounted above the leaf surface. Two additional red LEDs, modulated at high frequency, provide the weak pulses used to induce the modulated fluorescence signal, similar to the method described by Schreiber et al. (1986).

At the beginning of each measuring day the infrared gas analyzers of the LI-6400 were fully calibrated as per the manufacturer's instructions, and the fluorometer signal offset was zeroed. The flow rate of air through the sample chamber was set at 350 μmol s^{-1}, and leaf temperature was maintained at 27°C using the chamber thermoelectric coolers. The sample chamber CO_2 concentration was adjusted to 360 ppm using the system's CO_2 injector (model 6400-01, LI-COR).

The modulation frequency of the measuring light was 0.25 kHz on darkened samples, 10 kHz under actinic illumination, and increased to 20 kHz during saturating pulses. The measuring light intensity was set to its maximum level (level 10). For determination of F_S, the sampling rate was 0.5 Hz, but this was increased to 20 Hz for determination of F_M' during saturating pulses. Pulse duration was 0.8 s, which was determined in preliminary experiments to be adequate to maximize the measured F_M'.

Each leaf was dark adapted for 20-45 minutes before introducing it into the measurement chamber. Once inside the chamber under the same dark condictions, additional time was allowed so that leaf temperature, stomatal conductance, and leaf CO_2 exchange reached steady state. At this point, net CO_2 efflux from the leaf was recorded as an estimate of dark respiration (R_D). Then, the first fluorescence measurement was taken (see below). Following the dark measurements, eight levels of PPFD were applied in descending order (approximately 2500, 2000, 1600, 1200, 800, 600, 400, 250 μmol m^{-2} s^{-1}). Approximately 90%

of this actinic light was provided by the red LEDs and 10% by the blue LEDs. At each PPFD level, sufficient time was allowed for the plant to reach a steady state net CO_2 assimilation (A_N), then Fs and F_M' were determined six times, using six different saturating pulse intensity settings (1, 2, 4, 6, 8, and 10) applied in random order. The actual PPFD during the saturating pulses was recorded by the LCF's internal quantum sensor. The apparent quantum efficiency of photosystem II (Φ_{II}) for each pulse was calculated according to equation (1).

For cotton, the measurements were taken under normal atmospheric O_2 concentration (approximately 21%), and also under non-photorespiratory conditions. This was achieved by feeding the air intake of the LI-6400 with a gas mixture of 2% O_2 / 98% N_2. Flow of this air from a pressurized tank was regulated to about 1.5 times the flow rate required by the intake pump of the LI-6400, and was passed through a T-junction that allowed the excess to vent to atmosphere. The CO_2 concentration of the airstream was adjusted to 360 ppm by the CO_2 injector as normal.

For calculation of ETR (equation 2), f_{II} was assumed to be 0.4 for maize and 0.5 for cotton. The value of α was determined for each leaf with an 1800-12S external integrating sphere (LICOR) and LI-1800 portable spectroradiometer (LICOR). Leaf transmittance and reflectance measurements were made at 5 nm intervals between 400 and 700 nm, and the fractional absorptance of the actinic light in the chamber was calculated as described by Earl and Tollenaar (1997).

2.3.3 Calculations and Data Analysis

Respiration of illuminated leaf samples was assumed to be equal to R_D, and gross assimilation rate (A_G) was therefore calculated as $A_N + R_D$. The ETR was calculated as per equation (2), but in each case using two different estimates of Φ_{II}. The first estimate of Φ_{II} was simply the one calculated when the highest saturating pulse intensity (level 10) was used to

measure F_M'. The other method consisted of regressing the six estimates of Φ_{II} on the inverse of the pulse intensity. By extrapolating the linear regression to the y-axis, Φ_{II} that would be measured with infinite saturating pulse intensity was estimated (Figure 2.1). Linear regression of ETR on A_G was carried out using the REG procedure in SAS, and analysis of variance was performed using the GLM procedure (SAS Institute, 1985).

2.4 - Results and Discussion

A strong linear relationship between the apparent Φ_{II} and the inverse of the saturating pulse intensity was observed (Figure 2.1), with $r^2 > 0.99$ for all levels of actinic PPFD used in this experiment. As PPFD increased, Φ_{II} decreased as expected (due probably to a decrease in the proportion of open reaction centers, and reduced efficiency of transfer of energy from the antenna to the reaction centers (Krall and Edwards, 1991)). According to Genty et al. (1989), the efficiency of energy transfer from antenna complexes to the reaction center declines as PPFD increases, due to an increase in non-photochemical quenching**.**

The relationship between ETR and A_G for maize is shown for a single leaf in Figure 2.2 and for the eleven replications combined in Figure 2.3. The estimate of ETR using the single pulse method always tended to be lower than the estimate using the intercept method, but this difference became greater at high A_G (i.e., at higher PPFD). The ratio of ETR / A_G was significantly ($P < 0.05$) greater using the intercept method as compared to the single pulse method at every PPFD level (Figure 2.4).

When the regression of ETR on A_G through the origin was performed for each leaf individually, the average slope was 4.07 ± 0.22 electrons per CO_2 (n=11) with the intercept method, which is similar to the slope of the regression using the combined data (4.08, Figure 2.3). With the single pulse method, the average slope was 3.17 ± 0.22 (n=11), which is below the theoretical minimum if both ETR and A_G are measured accurately. Since the difference

between the two methods was most pronounced at high PPFD (Figure 2.4), this suggests that the saturating pulse used to measure F_M' was of insufficient intensity to provide an accurate estimate of Φ_{II} when Φ_{II} was low.

With either method, the ratio of ETR / A_G was lower than the value of 4.6 reported previously (Earl and Tollenaar, 1998). This may indicate that even with the intercept method Φ_{II} was underestimated in this experiment under some conditions. Another possibility is that the value of f_{II} was not 0.4 as assumed; this factor may be different in the present work than in the work of Earl and Tollenaar (1998), due to the very different spectral photon distribution of the actinic light (red / blue LED source here, as opposed to the broad-spectrum source used by them). The expected mean slope of 4.6 would have been calculated here if a f_{II} estimate of 0.45 had been used. Also, the ratio of ETR / A_G was less stable across PPFD levels than previously observed (Edwards and Baker, 1993; Earl and Tollenaar, 1998). In Figure 2.4, the mean ratio varies from 3.70 to 4.25, or about 13%. Across a similar range of PPFD, the ratio varied by only 7% in the work of Earl and Tollenaar (1998). Also, in that study the ratio tended to decline at high PPFD; in the present work it increased at higher PPFD.

In cotton, under non-photorespiratory conditions, the relationship between ETR and A_G appeared to be approximately linear when the intercept method was used, with a slope of 4.68 electrons per CO_2 (Figure 2.5). Similar to the results with maize, the ratio of ETR to A_G was lower when the single pulse method was used, and this difference increased at higher A_G (i.e., at high PPFD). As expected, the ratio of ETR to A_G was lower under non-photorespiratory conditions than at 21% O_2(Figure 2.5). This occurred for two reasons, both consistent with a higher rate of photorespiration at 21% O_2 as compared to 2% O_2. First, under 21% O_2 the ETR was higher than under 2% O_2, but only at higher PPFD levels (Figure 2.6). At PPFD below 500 $\mu mol\ m^{-2}\ s^{-1}$ the ETR was PPFD-limited, and did not differ between the treatments (linear portion of the ETR / PPFD curve; Figure 2.6). At higher PPFD the ETR was sink-limited, and

so was reduced by the removal of photorespiration as an electron sink at 2% O_2. Second, A_N was lower under 21% O_2 than under 2% O_2, consistent with the suppression of photorespiratory CO_2 evolution. This difference in A_N between the treatments was significant at all PPFD levels (Figure 2.7).

Under reduced photorespiration, the linear regression between the ETR and A_G had a mean slope of 4.77 ± 0.25 (n=7). Using the single pulse method, the mean slope was 3.30 ± 0.32 (n=7), which is below the theoretical minimum of 4.0 electrons per CO_2. Also, polynomial regression revealed a significant ($p < 0.0001$) non-linear component (downward curvature) of the relationship between ETR and A_G for the single pulse method. For the intercept method, the second-order component was not statistically significant ($p = 0.52$); i.e., there was no statistical evidence of non-linearity (data not shown).

This linear relationship between ETR and A_G under putative non-photorespiratory conditions is in accordance with previous results (Genty et al., 1989; Harbinson et al., 1990; Keiller and Walker 1990; Krall and Edwards 1990; Seaton and Walker 1990), but the slope is higher than the expected value of 4.0, in contrast to the maize experiment, where the ratio was lower than expected.

The reason for the unexpectedly high ratio of ETR / A_G in cotton under photorespiration cannot be determined with certainty from the present data. However, several possibilities exist. First, it is possible that reducing the oxygen concentration from 21% to 2% was insufficient to achieve complete suppression of photorespiration. Previous work has often used 1% O_2 for this purpose (Shimada et al.,1988; Genty et al., 1989; Edwards and Baker, 1993). However, other studies have used 2% O_2 (Oberhuber et al., 1993; Krall and Edwards, 1990; Laisk and Loreto, 1996; Loreto et al., 1994). Secondly, it is possible that an incorrect assumption about the value of f_{II} resulted in an overestimation of ETR using equation (1). An f_{II} value of 0.42 (rather than 0.5) would have resulted in the expected ETR / A_G ratio of 4.0. Laisk and Loreto (1996)

reported that this value may range from 0.36 to 0.60 in C_3 leaves, but did not provide an estimate for cotton. Also, the ratio is affected by the spectral composition of the actinic light, as mentioned above. Third, it is also possible that the intercept method of determining Φ_{II} somewhat overestimated the true Φ_{II} in this study.

In wheat, Krall and Edwards (1990) found that the quantum yield for PSII electron transport was linearly related to the quantum efficiency of CO_2 assimilation (Φ_{CO2}) across a broad range of C_i under 2% oxygen. The slope was near 8.0 mol m^{-2} s^{-1}, indicating complete suppression of photorespiration. Under 21% O_2, the relationship was non-linear, and the ratio of Φ_{II} to (Φ_{CO2}) was much higher. As proposed by Genty et al. (1989), changes in the various sinks for electrons (CO_2, O_2, NO_2^-,etc) will cause a deviation from linearity.

2.5 - Conclusion

The following major conclusions can be drawn from the results presented here.

1. Using the single pulse method, the relationship between ETR and A_G is near-linear at low PPFD, but deviates from linearity at higher PPFD levels both in C_4 leaves and in C_3 leaves under non-photorespiratory conditions. Present results indicate that this deviation is partially due to insufficient intensity of the saturating pulse used to induce Fm' in the 6400-40 leaf chamber fluorometer. However, his problem can be avoided by employing the multiple-pulse method presented here, which provides an estimate of Φ_{II} at infinite pulse intensity.
2. Despite the improved linearity of the ETR / A_G relationship when using the intercept method, the observed ratio between ETR and A_G was 12% lower than predicted by theory for maize, and 15% higher than predicted by theory for cotton under non-photorespiratory conditions. The reasons for these disparities can not be deduced from the present data, and should be investigated further.

Figure 2.1. Relationship between apparent Φ_{II} and the inverse of the saturating pulse intensities for a maize leaf at two levels of PPFD (2400 and 1600 μmol m^{-2} s^{-1}) . In each case, the true Φ_{II} is taken as the y-intercept of the linear regression.

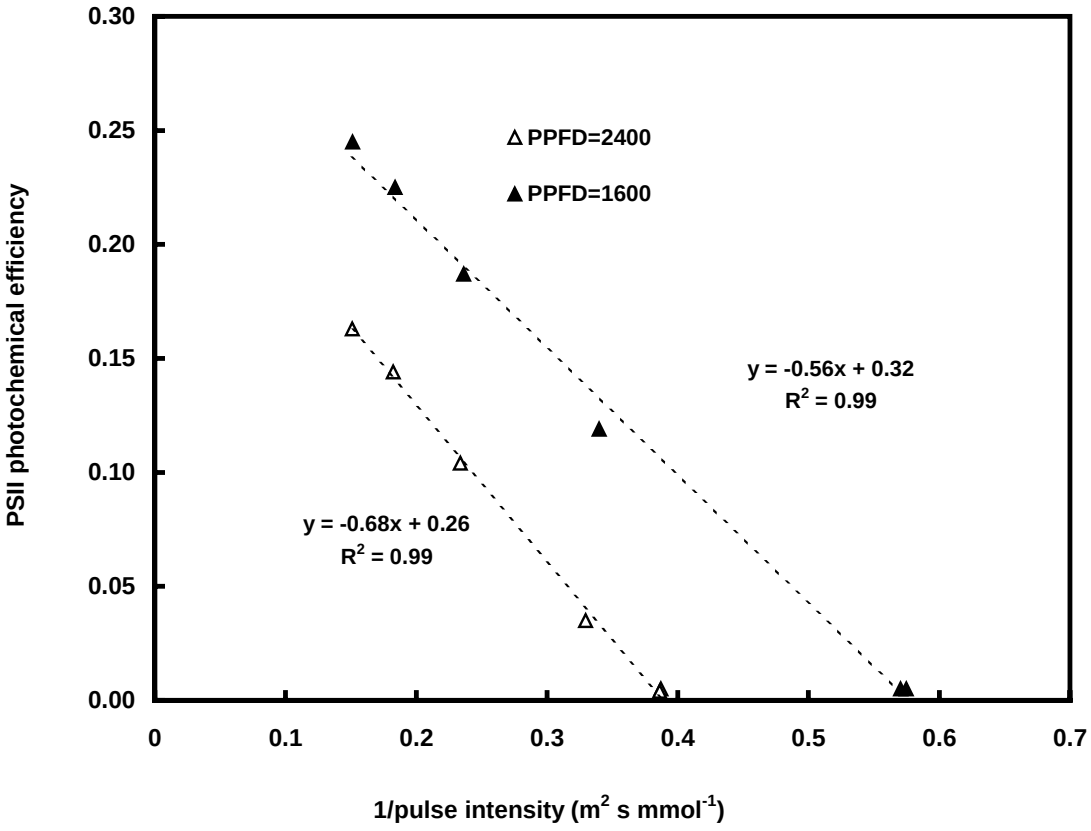

0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
PSII photochemical efficiency
1/pulse intensity (m2 s mmol-1)
△ PPFD=2400
▲ PPFD=1600
y = -0.56x + 0.32
R2 = 0.99
y = -0.68x + 0.26
R2 = 0.99

Figure 2.2: Relationship between the electron transport rate (ETR) and gross CO_2 assimilation rate (A_G) for a single maize leaf. Closed symbols – using the intercept technique in estimating the true Φ_{II}; open symbols - direct measurement of Φ_{II} using the highest saturating pulse intensity. Line is the best-fit linear regression through the origin, fit to the closed symbols only. Data are for eight different PPFD levels on a typical maize leaf.

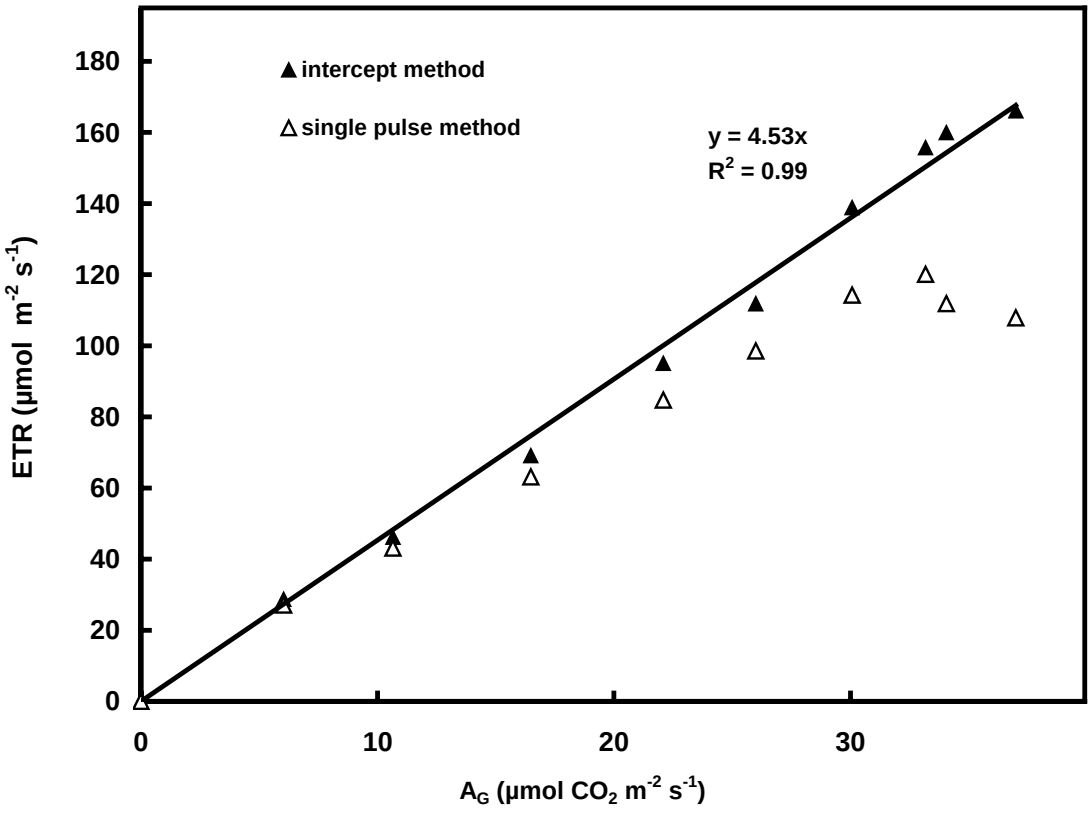

▲intercept method
△single pulse method
y = 4.53x
R2 = 0.99
ETR (µmol m-2 s-1)
AG (µmol CO2 m-2 s-1)
0
20
40
60
80
100
120
140
160
180
0
10
20
30

Figure 2.3: Relationship between the electron transport rate (ETR) and gross CO_2 assimilation rate (A_G) for maize. Closed symbols - using the intercept technique in estimating the true Φ_{II}; open symbols - direct measurement of Φ_{II} using the highest saturating pulse intensity. Line is the best-fit linear regression through the origin, fit to the closed symbols only. Data are for eight different PPFD levels on each of eleven different leaves.

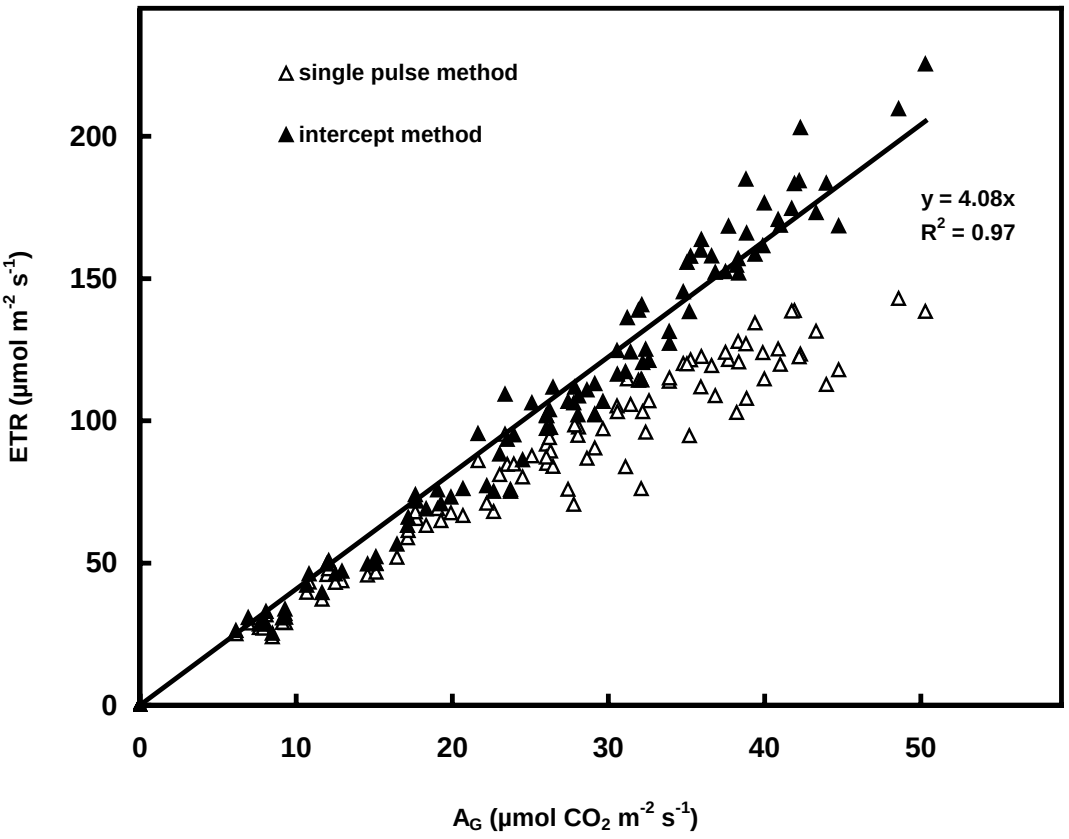

△ single pulse method
▲ intercept method
y = 4.08x
R2 = 0.97
ETR (µmol m-2 s-1)
AG (µmol CO2 m-2 s-1)
0
50
100
150
200
0
10
20
30
40
50

Figure 2.4: The relationship between the ratio ETR/A_G and PPFD for maize. Closed symbols - using the intercept technique in estimating the true Φ_{II}; open symbols - direct measurement of Φ_{II} using the highest saturating pulse intensity. Each point is the mean of 11 replications, each using a different plant. The ETR / A_G ratio was significantly ($p < 0.05$) different between the methods at every PPFD level, according to an LSD test.

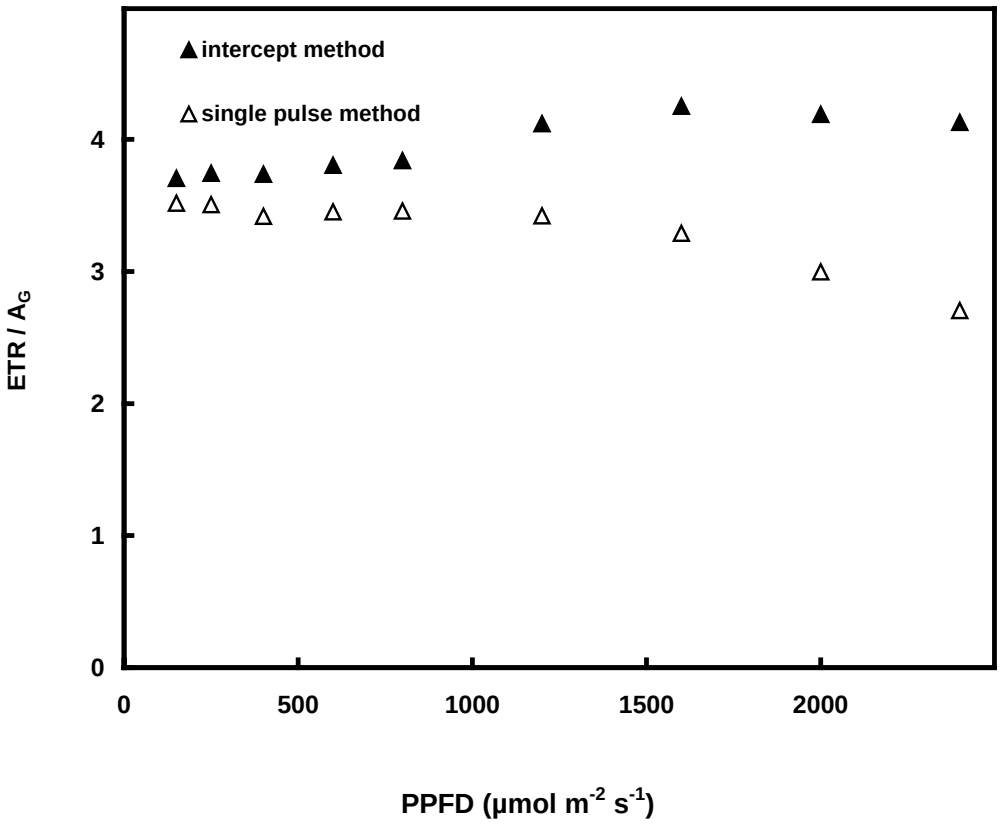

intercept method
single pulse method
ETR / A_G
0
1
2
3
4
0
500
1000
1500
2000
PPFD (µmol m^{-2} s^{-1})

Figure 2.5. The relationship between ETR and A_G for cotton under ambient air (open diamonds) and 2% oxygen (closed triangles) using the intercept method in both cases. Open triangles – 2% O_2, ETR estimated using the single pulse method. Line is best fit linear regression through the origin, fit to the closed triangles only. Combined data for eight PPFD levels, applied to each of seven different leaves.

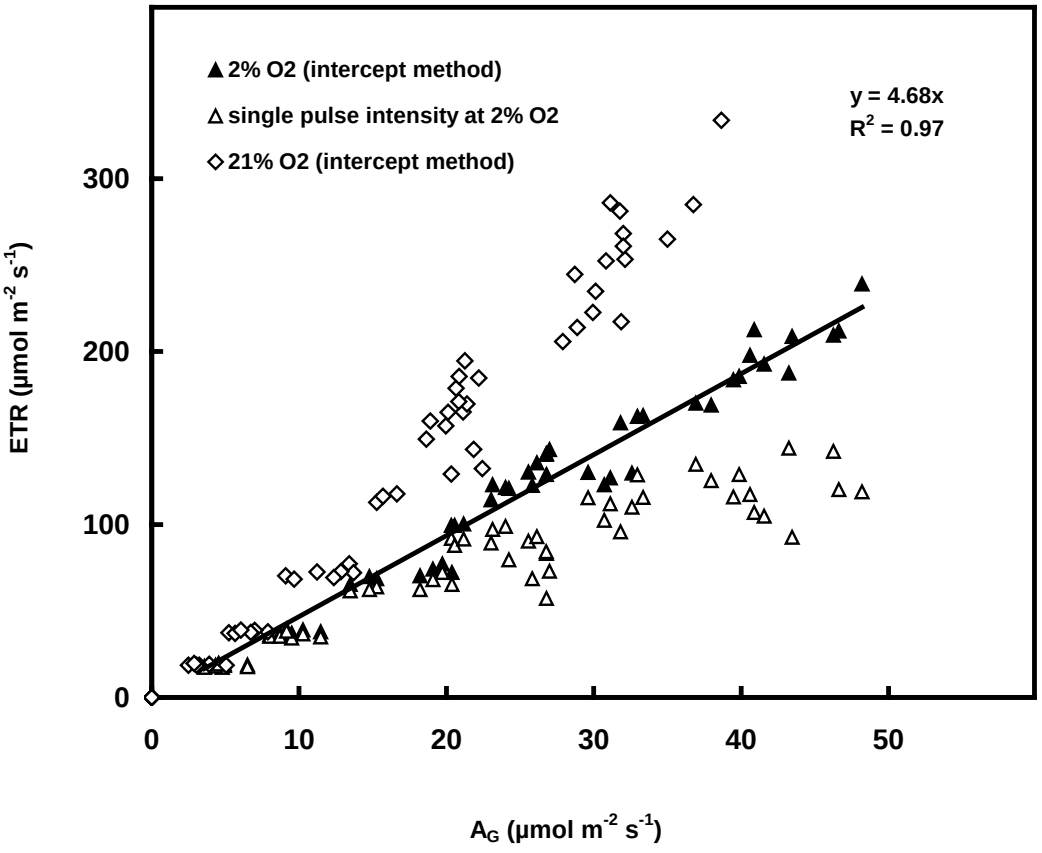

▲2% O2 (intercept method)
△single pulse intensity at 2% O2
◇21% O2 (intercept method)
y = 4.68x
R2 = 0.97
ETR (µmol m-2 s-1)
AG (µmol m-2 s-1)
0
100
200
300
0
10
20
30
40
50

Figure 2.6. The relationship between ETR and PPFD for cotton under ambient air (closed symbols) and 2% oxygen (open symbols). ETR was estimated using Φ_{II} from the intercept method. There were no significant difference in ETR between the oxygen levels at low PPFD (lower than 500 µmol m^{-2} s^{-1}). The two treatments differed significantly ($p < 0.05$) at all PPFD levels equal to or greater than 500 µmol m^{-2} s^{-1}. n = 7.Where the symbols for the 21% O_2 treatment are not visible, they lie directly beneath the symbols for the 2% O_2 treatment.

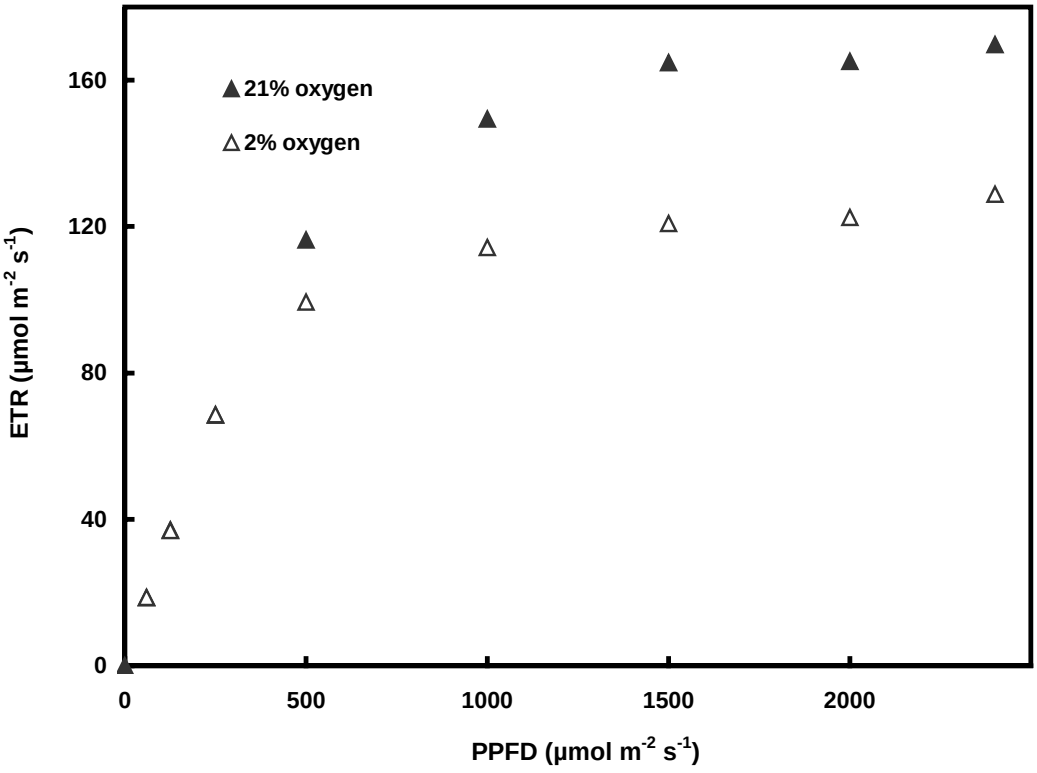
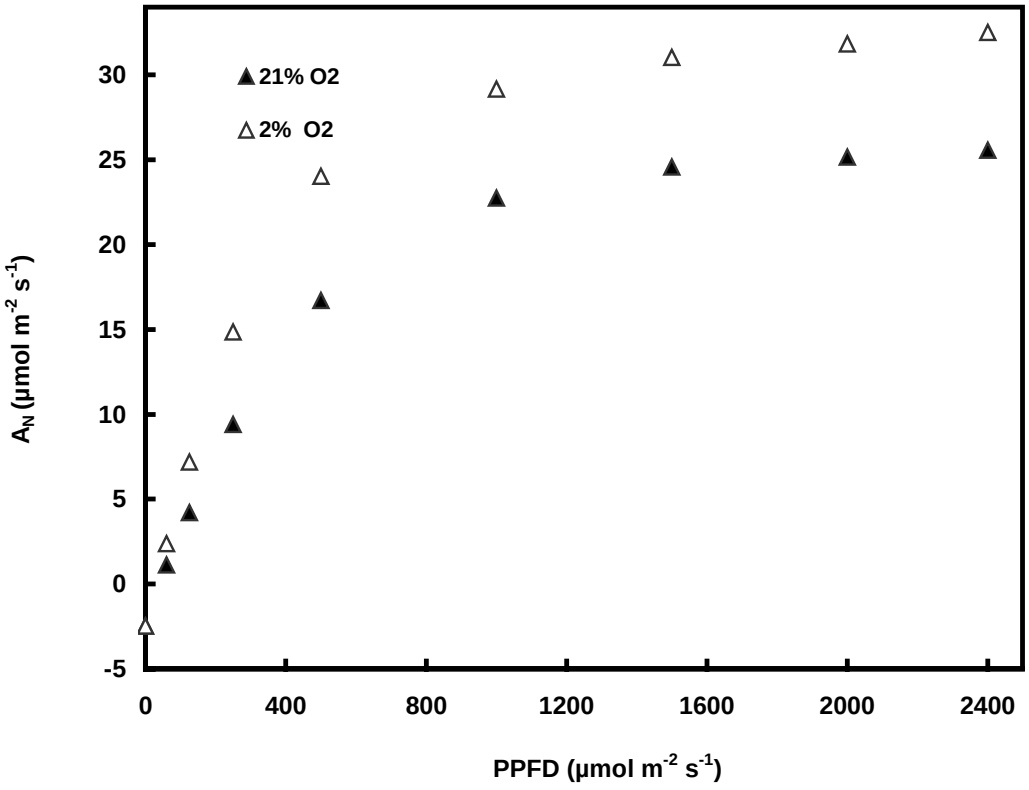

21% oxygen
2% oxygen
ETR (µmol m-2 s-1)
PPFD (µmol m-2 s-1)
0
40
80
120
160
0
500
1000
1500
2000

Figure 2.7. The relationship between A_N and PPFD for cotton under ambient air (closed symbols) and 2% oxygen (open symbols). A_N differed significantly ($p < 0.05$) between the treatments at every PPFD level other than 0. $n = 7$.


21% O2
2% O2
30
25
20
15
10
5
0
-5
0
400
800
1200
1600
2000
2400
A_N (µmol m^-2 s^-1)
PPFD (µmol m^-2 s^-1)

CHAPTER 3

STOMATAL AND NON-STOMATAL RESTRICTIONS TO PHOTOSYNTHESIS IN COTTON UNDER DROUGHT STRESS

3.1-Abstract

Reduction of photosynthetic capacity under water stress is a function of both an increase in stomatal resistance and inhibited chloroplast metabolism. The distinction between the stomatal and non-stomatal photosynthetic restriction has been facilitated by recently developed gas exchange and fluorescence techniques. Under water stress, the mesophyll limitation cannot be quantified precisely with CO_2 exchange measurements because of inaccurate estimation of leaf internal CO_2 concentration (C_i) when stomatal conductance and leaf net carbon assimilation (A_N) are very low. In the present work, we used measurements of A_N from gas exchange in combination with estimates of photosynthetic electron transport from chlorophyll fluorescence to calculate CO_2 concentration at the carboxylation site (C_c) as an alternative to C_i. Cotton plants were exposed to three distinct levels of water stress and a control treatment and measurements were made on youngest fully expanded leaves. Estimates of C_i were not reliable under severe water stress, but whenever A_N was greater than approximately 1.0 μmol m^{-2} s^{-1}, the relationship between A_N and C_c was still a smooth curve. A decrease in the slope of the initial (linear) part of this curve was taken as evidence of increased restrictions to A_N occurring within the chloroplast. Water stress significantly reduced C_c for all three stress levels, and reduced the slope of A_N / C_c at the two most severe stress levels, indicating that the reduction in A_N had a significant mesophyll (chloroplast-level) component under severe but not under mild water stress. When severely water stressed cotton plants werere-watered and allowed to recover for 24

or 48 h, we observed a partial recovery in A_N and other physiological parameters, but the slope of A_N / C_C remained significantly depressed relative to the control, indicating lasting effects of the water stress at the chloroplast level.

3.2-Introduction

Water stress is considered a limiting factor for a wide range of physiological process in plants (Cornic, 1994; McDonald and Davis, 1996). It is often accompanied by other limiting factors such as high temperature, high leaf to air vapor pressure deficit, nutrient depletion and high levels of solar irradiation; when combined they may cause a photoinhibition which limits the photosynthetic capacity of a plant (Bjorkman and Powels, 1984; Valladares and Pearcy, 1997).

At the whole plant level, the effect of the stress is usually manifested as a decrease in photosynthesis and therefore plant growth, and associated with alteration in carbon and nitrogen metabolism (Cornic and Massacci, 1996; Mwanamenge et al., 1999). The plant response is complex because it reflects the integration over space and time of stress effects and responses at all underlying levels of organization (Blum, 1996). Water stress results in stomatal closure and reduced transpiration rate. Stomatal control of water loss has been identified as an early event in plant response to water deficit under field conditions leading to limitation of carbon uptake by the leaves (Chaves, 1991; Cornic and Massacci, 1996). Other effects of water stress on plants include a decrease in tissue water potential, accumulation of abscisic acid (ABA), proline, manitol, and sorbitol, and formation of scavenging compounds (ascorbate, glutathione etc.) (Yrodanov et al., 2003). Also, several enzymes involved in photosynthetic carbon metabolism may be inhibited due to the increase in the cellular concentration of solutes as leaf tissue dehydrates (Kaiser, 1987). The relative importance of stomatal *versus* non-stomatal limitations

to photosynthesis under drought stress is an area of ongoing debate in the plant physiology literature.

Stomatal limitation

One of the most important components that limits photosynthesis under drought stress is stomatal limitation. The relative part of stomatal limitation of photosynthesis depends on the severity of water deficit - under mild stress it is a primary event, which is then followed by changes in photosynthesis reactions (Cronic and Briantais, 1991). According to Maroco et al. (1997), stomatal closure is a response either to a decline in leaf turgor and /or water potential, or to a low-humidity atmosphere (Maroco et al., 1997). However, stomatal closure may be more linked to soil moisture than to a leaf water status *per se*, which suggests that stomata are responding to chemical signals (e.g. ABA) produced by dehydrating roots (Davies and Zhang, 1991).

In addition, stomatal closure occurs before inhibition of photosynthesis at the mesophyll level, by restricting CO_2 availability at the assimilation site in chloroplasts. When water stress is imposed slowly, as generally occurs under field conditions, a reduction in the biochemical capacity for carbon assimilation and utilization may occur along with restriction in gaseous diffusion. For instance, in grapevines grown in the field, CO_2 assimilation was limited to a great extent due to stomatal closure as summer drought progressed, but there were also proportional reductions in the activity of various enzymes of the reductive Calvin cycle (Maroco et al., 2002; Chaves et al., 2002).

Non-stomatal limitation

A direct mesophyll restriction to photosynthesis has been reported widely in literature (Gunasekera and Berkowitz, 1992; Gimenez et al., 1992, Rodriguez, 1999). When stomatal limitations are overcome by high CO_2 concentration, reduced maximum rates of carbon assimilation are often still observed (Quick et al., 1992; Brestic et al., 1995; Tourneaux and

Peltier, 1995). Other workers have identified mesophyll restrictions through combined measurements of leaf CO_2 exchange and by chlorophyll fluorescence (Epron et al., 1995; Flexas et al., 1999; Laisk et al., 1996; Loreto et al., 1992). A general conclusion is that the likelihood of observing significant mesophyll restrictions to photosynthesis increases with the severity of the water stress.

Numerous data sets where internal partial pressure of CO_2 remained unchanged in leaves of water-stressed plants, in spite of a decline in both stomatal conductance and CO_2 fixation (Wong et al., 1985; Raschke and Rosemann, 1986) seemed to confirm that mesophyll capacity of photosynthesis was affected by water stress. Also, measurements of photosynthetic O_2 evolution at high CO_2 concentration agreed with this view of important non-stomatal limitation of photosynthesis induced by water stress (Ben et al.,, 1987; Di Marco et al., 1988; Bjorkman and Shafer, 1989; Flexas et al., 1999).

Under severe stress, photosynthesis may be more controlled by the chloroplast's capacity to fix CO_2 than by the increased diffusive resistance (Faver et al., 1996; Herppich and Pechmann, 1997), however, it is not clear which chloroplast-level processes are most affected. PSII energy conversion and the activity of Rubisco are resistant to water deficit (Chaves, 1991; Dickson and Tomlinson, 1996; Congming and Zhang, 1998). The tight correlation between mesophyll photosynthesis and stomatal aperture may reflect a down-regulation of the photosynthetic apparatus by low C_i (Tourneaux and Peltier, 1995).

However, conventional methods of calculating the intercellular concentration of C_i from gas exchange data may result in an overestimation of the value of C_i if non-uniform closure of stomata occurs (Terashima, 1992). Another complication with stomatal closure is that the larger conductance of the epidermal (non-stomatal) diffusive pathway to water vapor than to CO_2 leads to overestimation of C_i when stomatal conductance is low and a significant fraction of total water vapor exchange occurs directly through the cuticle (Meyer and Genty, 1998).

One of the most important parameters used to estimate the leaf carboxylation efficiency is the slope of A_N/C_i, which is related directly to Rubisco activity (von Caemmerer and Farquhar, 1981). However, as mentioned, estimates of C_i can not be considered reliable under drought stress, where heterogeneous stomatal closure and cuticular conductance to water vapor invalidate the assumptions underlying its calculation (Laisk et al., 1980; Farquhar et al., 1987; Downton et al., 1988; Terashima et al., 1988; Raschke et al., 1990; Mott, 1995; Boyer et al., 1997; but see also Cheeseman, 1991 and Buckley et al., 1997), thus calling into question any conclusions regarding the involvement of non-stomatal limitations to photosynthesis (Terashima et al., 1988; Downtown et al., 1988a, 1988b).

By combining leaf gas exchange and chlorophyll fluorescence measurements, it is possible to estimate the concentration of CO_2 in the chloroplasts of C_3 leaves (C_c) *in vivo* as an alternative to $C_{i.}$ (Di Marco et al., 1990; Harley et al., 1992; Loreto et al., 1992; 1994; Epron et al., 1995). This permits differentiation between the stomatal limitation (stomatal closure leading to reduction in internal CO_2 partial pressure) and non-stomatal limitation (which includes all effects limiting CO_2 assimilation at a given level of leaf-internal [CO_2]), without relying on estimates of C_i.

The main objective of the present work was to measure stomatal and non-stomatal restrictions to cotton leaf photosynthesis during drought stress events of different severities, and during recovery from drought stress, under greenhouse conditions. Specific research questions to be addressed in relation to this objective were:

a. Does drought stress induce non-stomatal limitations to photosynthesis in cotton leaves, measurable as a change in the relationship of A_N to C_c at a given incident photosynthetic photon flux density (PPFD)?
b. What severity (leaf water potential or soil water content) is required to induce non- stomatal limitations to photosynthesis?

c. How quickly and completely are measured stomatal and non-stomatal restrictions to photosynthesis relieved upon return to non-limiting soil water content?

3.3 - Material and Methods

3.3.1 Plant Material

Two experiments were conducted between December 2002 and July 2003 in a greenhouse in Athens, Georgia, USA (34°N, 84°W). Cotton (cv. Delta pearl) plants were grown in 2.5-L plastic food containers (Berry Plastics Corp., Evansville, IN) with drainage holes added. The soil was a Pacolet sandy loam (a member of the clayey, Kaolinitic, thermic family of Typic Hapludults) amended with sand to a texture of 800 g kg^{-1} sand, 120 g kg^{-1} silt, and 80 g kg^{-1} clay. The pots were filled with 3300g of soil. Seeds were sown five to a pot and fertilized with 50 ml of a 8g/l of 20-20-20 (N-P-K) fertilizer plus micronutrients (Miller Greenhouse Special, Miller Chemical and Fertilizer Co. Corp., Hanover, PA). Cotyledons were expanded and horizontal at 10-12 days after planting (DAP); at this time, plants were thinned to one per pot, and an additional 50 ml of fertilizer solution was added twice weekly thereafter. Temperatures were maintained at 27 ± 4°C during the day and 20 ± 2°C during the night. Photoperiod was extended to 16 h with overhead 400-W metal halide lamps that produced a supplemental photosynthetic photon flux density (PPFD) of approximately 230µmol m^{-2} s^{-1} at the tops of the plants.

3.3.2 Relative Soil Water Content and Soil Water Holding Capacity

Before planting, soil water holding capacity was determined. In addition to the pots that were prepared for the cotton plants, two extra pots were filled, then watered to excess, capped with plastic lids, and allowed to drain until reaching a constant weight. The constant weight was the wet weight of the soil + pot + lid (W_W). A third soil-filled sample pot was emptied into a

pan and placed in an 80ºC forced air dryer until it had reached constant weight. The oven-dried soil weight + pot weight + lid weight (W_D) was subtracted from W_W to calculate the grams of water held by the soil at 100% pot capacity (maximum amount of water held after free drainage has stopped). This was calculated for each of the two pots, and the mean value was used as the soil water holding capacity estimate. To prevent water stress during the initial growth stages, water was maintained at between 65 and 75% of pot capacity by daily weighing and watering of the pots for approximately 35 to 45 days, and then they were placed on the lysimeter.

3.3.3 Lysimeter Design and Operation

The gravimetric lysimeter is described in detail by Earl (2003). It consisted of sixteen electronic balances with 6-kg maximum capacity and 0.1-g readability (Model XL-6100, Denver Instruments, Arvada, CO), connected to a personal computer that also operated sixteen mechanical relays. Each relay operated a normally closed two-way solenoid valve, each of which controlled water flow from a 20-L reservoir to one of the sixteen pots on the lysimeter balances. When a solenoid valve was activated by the computer, water was conducted by gravity flow from the reservoir to the watering hole in the lid of the appropriate pot, via vinyl tubing. The computer ran custom control software that was designed to read the weights from each balance approximately every 2 seconds, and then activate the appropriate solenoid valve to replace transpired water if any pot weight had fallen below the predetermined target weight for that balance. The lysimeter added water based on a 30-g threshold so that each pot was maintained between 15 g below and 15 g above its current target weight. The target weight (W_T) for each balance was calculated by the computer software as:

$W_T = W_D + W_P + RSWC\ (W_W - W_D)$, where

W_D is the dry weight of soil + pot + lid,

W_W is the wet weight of soil + pot + lid,

W_P is total plant fresh weight estimated from measuring the shoot and root weights of two extra pots for each run of the experiment, and

RSWC is the desired relative soil water content expressed as a fraction between 0 and 1.

New target RSWC values were entered manually on a daily basis as required by the experimental protocol. The control software included logic to reject anomalous data caused by occasional faulty communication, balance malfunction, tampering, etc., and posted error alerts whenever such events occurred. Pot weights were recorded every 10 minutes by the software, and each time water was added to a pot the amount added was also recorded in the data file.

3.3.4 Drought Stress Simulation

Before transferring plants to the lysimeter, each pot was capped with a plastic lid to reduce evaporation of water from the soil surface. Each lid had two holes - one to accommodate the plant stem, and another to permit water additions. Then the 16 plants and their pots were transferred to the automated lysimeter and watering system (day 0). In one experiment, four pots were randomly assigned to each of four treatments: well watered (75 ± 2.5% RSWC), mild water stress (25 ± 2.5%RSWC), moderate water stress (15 ± 2.5% RSWC), and severe water stress (5 ± 2.5% RSWC), permitting four complete replications per cycle of the experiment. Also previous work with cotton in this culture system had identified 25% RSWC as a stress level that induced stomatal closure, but did not induce wilting (H.J. Earl, unpublished data). The automated watering system was programmed to permit soil water to decline by a maximum of 10% RSWC per day due to transpiration; once a pot weight had declined by this amount in a single day, its weight was maintained within 2.5% RSWC of the target weight by frequent automatic watering for the rest of the day. Similarly, when a pot had reached its target final weight range, the watering system maintained it between the upper and lower limits of that

range. Once the four "severe water stress" pots reached their target weight (approximately day 7(Figure 3.1)), physiological parameters were measured.

Another experiment was conducted to investigate the time course of recovery of leaf physiological parameters upon relief of the water stress by re-watering. In this case, the four treatments were the control (75% RSWC), severe stress for one day (5% RSWC), and severe stress followed by one day or two days of recovery (re-watered to 75% RSWC 24 h or 48 h before physiological measurements were made, respectively).

For both experiments, within a replication the dry-down protocol for the different stress and recovery treatments were begun on different days so that the physiological measurements could be made on all four plants on the same day.

3.3.5 Gas Exchange and Fluorescence Measurements

Fluorescence measurements were made on youngest fully expanded mainstem leaves with two Leaf Chamber Fluorometers (Model 6400-40, LICOR Inc., Lincoln NE) attached to LI-6400 Portable Photosynthesis Systems (LICOR). The 6400-40 a pulse-amplitude modulated (PAM) Fluorometer can be used to take measurements on both darkened and light-adapted samples. When used in conjunction with the LI-6400, fluorescence readings and leaf gas exchange are measured across the same circular 2-cm^2 leaf area exposed in the gas exchange chamber. Actinic illumination and also saturating pulses of light are provided by a mixture of red and blue light emitting diodes (LEDs) mounted above the leaf surface. Two additional red LEDs, modulated at high frequency, provide the weak pulses used to induce the modulated fluorescence signal, similar to the method described by Schreiber et al. (1986).

At the beginning of each measuring day the infrared gas analyzers of the LI-6400 were fully calibrated as per the manufacturer's instructions, and the fluorometer signal offset was zeroed. The flow rate of air through the sample chamber was set at 250 μmol s^{-1}, and leaf

temperature was maintained at 28°C using the chamber thermoelectric coolers. The sample chamber CO_2 concentration was adjusted to 360 ppm using the system's CO_2 injector (model 6400-01, LI-COR).

The modulation frequency of the measuring light was 0.25 kHz on darkened samples, 10 kHz under actinic illumination, and increased to 20 kHz during saturating pulses. The measuring light intensity was set to its maximum level (level 10), and the gain setting was 20. For determination of F_S, the sampling rate was 0.5 Hz, but this was increased to 20 Hz for determination of F_M' during saturating pulses. Pulse duration was 0.8 s, which was determined in preliminary experiments to be adequate to achieve F_M'.

Each leaf was dark adapted for 20-45 minutes before introducing it into the measurement chamber. Once inside the chamber additional time in darkness was allowed so that leaf temperature, stomatal conductance, and leaf CO_2 exchange reached steady state. At this point, net CO_2 efflux from the leaf was recorded as an estimate of dark respiration (R_D). Then, the first fluorescence measurement was taken (see below). Following the dark measurements, incident PPFD was set to 1500 μmol m^{-2} s^{-1} (approximately 90% of this actinic light was provided by the red LEDs and 10% by the blue LEDs). The leaf was allowed to achieve steady state g_s and A_N at a chamber CO_2 level of 360 ppm (standard conditions) before making the first fluorescence measurements. Then, measurements were made at different levels of chamber CO_2 (produced by using reference side (incoming) CO_2 concentrations of 50, 100, 200, 400, 500, 700, 1000, 1500, 2000, and 2400 ppm). At each level of CO_2, sufficient time was allowed for the plant to reach a steady state net CO_2 assimilation, then Fs and F_M' were determined three times, using three saturating pulse intensity settings (4, 6, and 10) applied in random order.

Leaf relative water content (RWC) was determined for the same leaves used for gas exchange / fluorescence measurements using the method of Catsky (1960). Three leaf disks 2-cm in diameter were extracted and their fresh weight was determined. They were then

submerged for 24 h in distilled water to determine their turgid weight, then dried over 24 hours at 80°C to determine their dry weight. RWC (%) was calculated as follows:

100 x (fresh weight - dry weight)/(turgid weight - dry weight).

An additional 5 leaf disks of 1cm diameter were used to determine leaf water potential according to Brown and Bartos, 1982, using thermocouple psychrometers (Wescor Inc, Wescor, Logan, UT).

Leaf absorptance was determined for each leaf with an LI-1800-12S external integrating sphere (LICOR) connected to a reflectance spectrometer (Unispec, PP Systems, Haverhill, MA). Leaf transmittance and reflectance measurements were made at 3.6-nm intervals between 400 and 700 nm, and the fractional absorptance of the actinic light in the chamber was calculated as described by Earl and Tollenaar (1997).

3.3.6 Calculations and Data Analysis

ETR was calculated according to Genty et al., (1989) as:

$$\text{ETR} = \alpha f_{II} \text{PPFD} \Phi_{II}$$

where α is the leaf absorptance of incident PPFD, and f_{II} is the fraction of absorbed PPFD absorbed by the antennae of photosystem II (assumed to be 0.5). C_c was calculated according to Lal et al. (1996) as:

$$C_C = \frac{O_C}{K_S} \cdot \frac{V_C}{V_O}$$

where O_C is the dissolved O_2 concentration at the carboxylation site, taken as 21% multiplied by the solubility coefficient for O_2 at 28°C (Gerbau and Andre, 1987), and K_S value is the CO_2/O_2 specificity ratio for RubisCO at 28°C (Jorden and Ogren, 1984).

Preliminary analysis revealed that the relationship between A_N and C_c remained linear up to a C_c value of 150 ppm for every leaf. The initial slope of this relationship was thus determined for each leaf via regression analysis, making use only of data for which C_c was below 150 ppm. Analysis of variance was used to determine significant treatment effects on RWC, leaf water potential, the initial slope of the A_N/C_c curve, leaf absorptance, and (at standard conditions and steady state), g_s, A_N, C_i, ETR and C_c.

3.4 – Results and Discussion

3.4.1 Drought Stress Severities Experiment

The four water stress severities treatments: well watered (75 ± 2.5% RSWC), mild water stress (25 ± 2.5 RSWC), moderate water stress (15 ± 2.5% RSWC) and severe water stress (5 ± 2.5% RSWC) were compared at steady state conditions. As water stress increased, net CO_2 assimilation rate decreased from 15.0 μmol m^{-2} s^{-1} in well-watered plants to 0.3 μmol m^{-2} s^{-1} in severely water stressed plants (Table 3.1). There was no significant difference between well watered and mildly water stressed plants; in contrast, a pronounced decline was observed under moderate stress and severe stress. Decreases in A_N and ETR were generally proportional, suggesting a close link between the photosynthetic processes.

Reduction of CO_2 assimilation was due at least in part to stress-induced stomatal closure (Table 3.1). This is generally assumed to be the main cause of drought-induced decreases in photosynthesis, since stomatal closure decreases CO_2 availability in the mesophyll (Chaves, 1991; Cornic and Massacci, 1996). According to Wong et al. (1985), variation in the leaf conductance is not autonomous from mesophyll activity. Changes in conductance can be induced, or at least modulated, by events occurring in mesophyll tissue. Whatever the mechanism involved, it is apparent that a remarkable concordance exists between leaf conductance and photosynthetic metabolism in plants subjected to water stress.

On the other hand, C_i initially decreased as stomatal conductance decreased with increasing water stress (from well watered to moderate stress), but the declining tendency disappeared under severe stress, where the estimation of internal C_i from gas exchange equations became unreliable, with, for example, large negative values sometimes calculated (data not shown). This inaccuracy of C_i estimation under severe stress may be partially attributed to instrument limitations - at very low CO_2 and water vapor exchange rates, the lower signal to noise ratio will decrease reliability of the measurements. Also, overestimation of C_i under severe stress may occur for two other reasons, as discussed above. First, the method used to calculate C_i from leaf gas exchange measurements relies on the assumption that cuticular transpiration is negligible relative to stomatal transpiration. This assumption is unlikely to be valid under severe water stress. Second, non-uniform stomatal closure under stress results in overestimation of C_i (e.g., Gunasekera and Berkowitz 1992; Terashima et al., 1988; Epron et al, 1995).

The relationship between A_N and C_i was a smooth curve for control plants (Figure 3.2), but the curve became less reliable as the level of water stress increased. Under severe stress (RSWC = 5%), estimates of C_i from gas exchange measurements were very inaccurate. By contrast, most plots of A_N on C_c gave smooth curves, regardless of stress level (Figure 3.3). The value of C_c showed a declining trend as the stress become more pronounced. There was no significant difference between the control and mild drought stress treatments, but C_c declined by more than 50% when RSWC reached 15 and 5 % (Table 3.1). This is consistent with a reduction in A_N under water stress caused by reduced CO_2 availability at the carboxylation site in the chloroplast.

As mentioned above, plots of A_N *versus* C_c gave smooth curves even under severe stress. The initial slope of this curve declined as the stress became more severe, with the moderate and severe stress treatments having significantly lower slopes than the control (Table

3.1). This result provides strong evidence that, under moderate and severe water stress, A_N was reduced in this experiment by chloroplast-level restrictions, in addition to the reduction in A_N caused by reduced CO_2 availability at the carboxylation site.

The stress treatments resulted in reduced leaf water potential and leaf RWC (although the reduction in RWC was significant only for the severe stress treatment; Table 3.1). Previous investigations have indicated that water stress begins to induce mesophyll-level restrictions to photosynthesis when leaf tissue RWC reaches between 50 and 70% (Kaiser, 1987; Robinson et al., 1988; Cornic et al., 1989). In the present work, a significant reduction in the initial slope of A_N / C_c indicated increased mesophyll restrictions both under moderate (RWC = 70%) and severe (RWC = 39%) stress (Table 3.1). It has been suggested that low leaf water potential may predispose the photosynthetic system to photoinhibition by a direct effect on the chloroplast (Boyer, 1976; Govindjee et al., 1981; Younis et al., 1979). In addition, the water stress may have a direct effect on carbon metabolism through its effect on the activity of the photosynthetic carbon reduction cycle (PCR cycle), perhaps by impairing enzyme activation, or due to a reduced RuBP regeneration (Gimenez et al., 1992; Gunasekera and Berkowitz, 1993; Lawlor, 1995; Escalona et al., 1999).

3.4.2 Recovery Experiment

In the second experiment, control plants had higher A_N but very similar C_i to their counterparts in the first experiment. This would appear to indicate a higher mesophyll-level photosynthetic capacity for plants in the second experiment, which is also reflected by a higher slope of A_N / C_C. Unexpectedly, leaf water potential and RWC of control plants were also much higher in the second experiment (Compare Tables 3.1 and 3.2). The reasons for these differences cannot be discerned from the present data.

In both experiments, A_N and g_s of plants exposed to the severe stress treatment were close to zero. As in the first experiment, the calculated value of C_c for the severe stress treatment was very low under standard conditions (ambient CO_2 concentration of 360 ppm), so part of the reduction in A_N could be attributed to reduced availability of CO_2 at the carboxylation site. Also consistent with the first experiment, the slope of A_N / C_c was greatly reduced under severe stress, indicating that chloroplast-level restrictions were increased by the stress treatment (Table 3.2).

One day after re-watering, A_N recovered substantially but was still reduced by 30% relative to control plants, thus indicating lasting effects of the stress treatment on leaf photosynthesis. Two pieces of evidence indicate that this lasting effect of stress on photosynthesis manifested itself at the mesophyll level. First, 24 h after rewatering, C_i and C_c were no longer lower in stressed plants than in control plants, but A_N remained suppressed. Second, the slope of A_N / C_C was significantly lower in the plants that had been stressed than in the control plants. Leaf water status, measured as either leaf water potential or RWC, recovered significantly in the first 24 h after re-watering. Both parameters recovered further during the second day after re-watering, although this change was not statistically significant in either case. No other measured parameter changed significantly between 24 and 48 h after re-watering, suggesting that no additional recovery of leaf photosynthetic capacity occurred after the first day (Table 3.2).

3.4.3 – Conclusions

Results of these experiments indicate that the suppression of leaf photosynthetic rates in cotton has both stomatal and non-stomatal components. Under mild stress, the suppression appears to be entirely stomatal in nature. However, under moderate or severe stress, non-stomatal limitations are also apparent as reductions in the net carbon assimilation rate for a

given CO_2 concentration within the chloroplast. Following a severe water stress, stomatal restrictions were completely relieved 24 h after re-watering. However, A_N did not recover completely even 48 h after re-watering, due to continued suppression of photosynthetic capacity at the level of the chloroplast.

The exact nature of the non-stomatal effects of water stress cannot be determined from these data. However, two possibilities may be ruled out. First, leaf absorptance incident PPFD was not affected by any of the treatments (Tables 3.1 and 3.2). Second, a change in the mesophyll diffusive resistance to CO_2 cannot be responsible, since conclusions regarding non-stomatal limitations were based on the photosynthetic response to CO_2 at the carboxylation site in the chloroplast.

Results of this experiment also point out the potential utility of C_c as an alternative to C_i under conditions when C_i calculated from gas exchange parameters is unreliable. For instance, curves of A_N / C_c remained smooth even under severe water stress, when accurate measurements of C_i were not possible with this instrumentation. However, it should be noted that part of this difference can be attributed to the different ways in which these two calculated parameters respond to certain types of measurement errors. Specifically, if instrument noise results in an overestimation of A_N, then the calculated value of C_i will be decreased. These two errors then combine to produce excessive scatter in the plot of A_N vs. C_i. By contrast, overestimation of A_N has the opposite effect on the calculated value of C_c; i.e., higher values of A_N produce higher C_c estimates. Thus, these errors tend to cancel one another, producing an artificially smooth plot of A_N / C_c.

Table 3.1. Effect of water stress severity on leaf parameters relating to photosynthesis and water status. The values of A_N, g_s, C_i, ETR and C_C are for steady-state conditions, with a CO_2 concentration of 360 ppm in the leaf chamber. Values within a column followed by the same letter do not differ according to a protected LSD test ($\alpha = 0.05$). Treatment effects on C_i were not statistically significant ($p > 0.05$), whether the 5% RSWC treatment was included or excluded. n = 4. The initial slope of A_N / C_C was determined for each leaf (linear portion of the curve, C_C < 150 ppm) by linear regression. Note that the RSWC values listed are target values; the 5% treatment usually did not reach 5% RSWC.

Treatment (% RSWC)	A_N (μmol m^{-2} s^{-1})	g_s (mol m^{-2} s^{-1})	C_i (ppm)	abs	ETR (μmol m^{-2} s^{-1})	Cc (ppm)	Ψ_{leaf} (MPa)	RWC (%)	Slope A_N/C_C (mol m^{-2} s^{-1})
75	**15.0 a**	**0.17 a**	**204**	**0.90 a**	**130 a**	**128 a**	**-1.10**	**79 a**	**0.18 a**
25	**13.0 a**	**0.13 a**	**186**	**0.91 a**	**136 a**	**100 a**	**-1.60 b**	**74 a**	**0.19 a**
15	**3.2 b**	**0.03 b**	**322**	**0.89 a**	**73 b**	**64 b**	**-1.84 b**	**70 a**	**0.13 b**
5	**0.3 c**	**0.000 b**	**389**	**0.90 a**	**37 c**	**50 b**	**-3.12 c**	**37 b**	**0.03 c**

Abbreviations: A_N = net CO_2 assimilation rate; g_s = stomatal conductance to water vapor; C_i = leaf internal CO_2 concentration; α = leaf fractional absorptance of incident photosynthetic photon flux density; ETR = thylakoid electron transport rate; C_c = CO_2 concentration at RubisCO; Ψ_{leaf} = leaf water potential; RWC = leaf relative water content.

Table 3.2. Effect of severe water stress and rewatering on leaf parameters relating to photosynthesis and water status. The values of A_N, g_s, C_i, ETR and C_c are for steady-state conditions, with a CO_2 concentration of 360 ppm in the leaf chamber. Values within a column followed by the same letter do not differ according to a protected LSD test ($\alpha = 0.05$). Treatment effects on g_s and C_i were not statistically significant ($p > 0.05$), whether the 5% RSWC treatment was included or excluded. n = 6. The initial slope of A_N / C_c was determined for each leaf (linear portion of the curve, $C_c < 150$ ppm) by linear regression. Note that the RSWC values listed are target values; the 5% treatment usually did not reach 5% RSWC.

Treatment	A_N (μmol m^{-2} s^{-1})	C_i (ppm)	g_s (mol m^{-2} s^{-1})	C_C (ppm)	abs	ETR (μmol m^{-2} s^{-1})	Ψ_{leaf} (MPa)	RWC (%)	Slope A_N/C_C (mol m^{-2} s^{-1})
control	**20.0 a**	**202**	**0.231**	**143 a**	**0.90 a**	**216 a**	**-0.44 a**	**87 a**	**0.24 a**
48 h recovery	**14.0 b**	**202**	**0.155**	**154 b**	**0.89 a**	**148 b**	**-0.74 ab**	**86 a**	**0.16 b**
24 h recovery	**14.0 b**	**211**	**0.186**	**154 b**	**0.90 a**	**154 b**	**-0.91 b**	**83 a**	**0.16 b**
5% RSWC	**0.3 c**	**-305**	**-0.005**	**68 c**	**0.90 a**	**63 c**	**-1.64 c**	**58 b**	**0.06 c**

Abbreviations: A_N = net CO_2 assimilation rate; g_s = stomatal conductance to water vapor; C_i = leaf internal CO_2 concentration; α = leaf fractional absorptance of incident photosynthetic photon flux density; ETR = thylakoid electron transport rate; C_c = CO_2 concentration at RubisCO; Ψ_{leaf} = leaf water potential; RWC = leaf relative water content.

Figure 3.1. Relative soil water content (RSWC) vs. time for the four water stress treatments in experiment one. Each line represents data from a single pot. Graph shows all data for a single replication of the experiment. Dashed line shows the approximate time when physiological measurements were made.

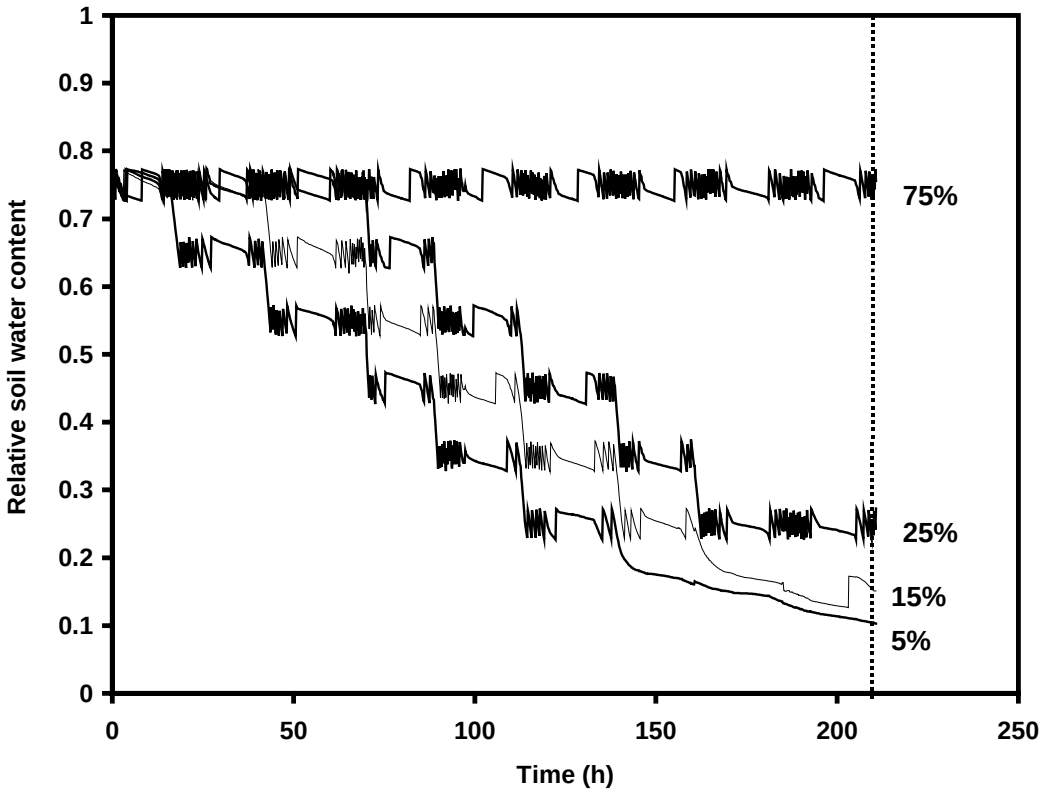

Relative soil water content
Time (h)
75%
25%
15%
5%
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0
50
100
150
200
250

Figure 3.2. Effect of water stress on the relationship between A_N and C_i. Data for the 5% RSWC treatment could not be graphed on these axes, since some estimates of C_i were negative, and some were greater than 800 ppm. Shown are example data for a single replication of the experiment.

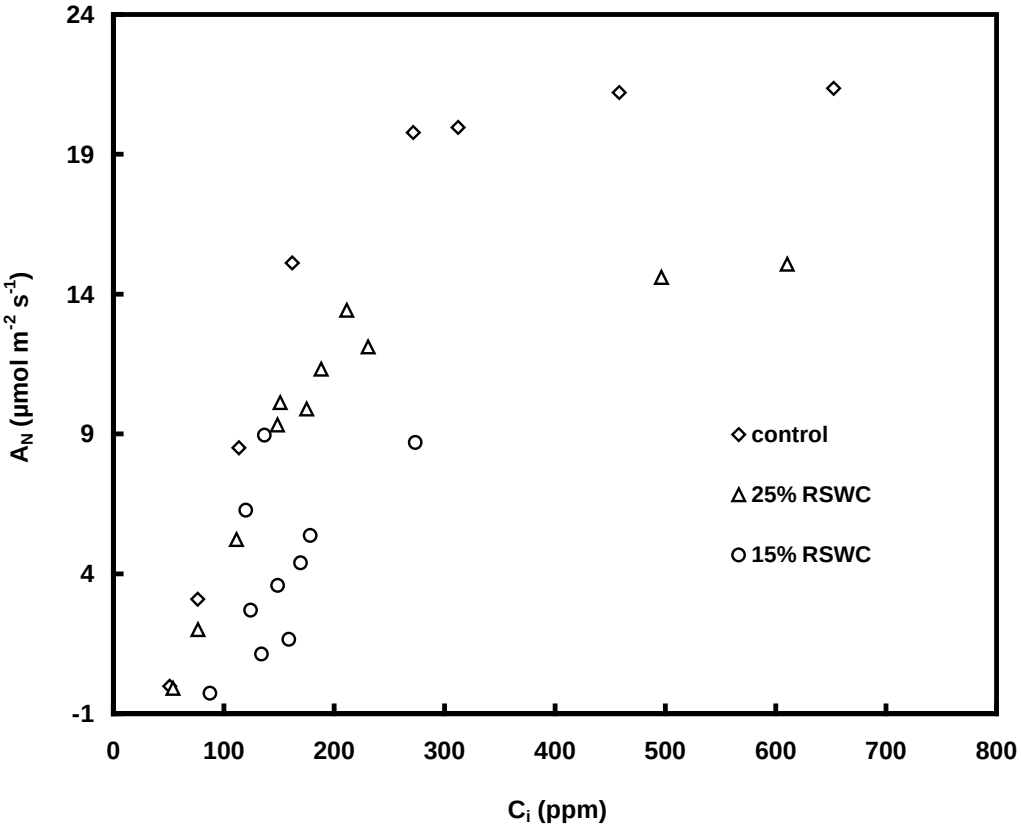

24
19
14
9
4
-1
0
100
200
300
400
500
600
700
800
A_N (µmol m^{-2} s^{-1})
C_i (ppm)
control
25% RSWC
15% RSWC

Figure 3.3. Effect of water stress on the relationship between A_N and C_c for experiment one. Shown are example data for a single replication of the experiment.

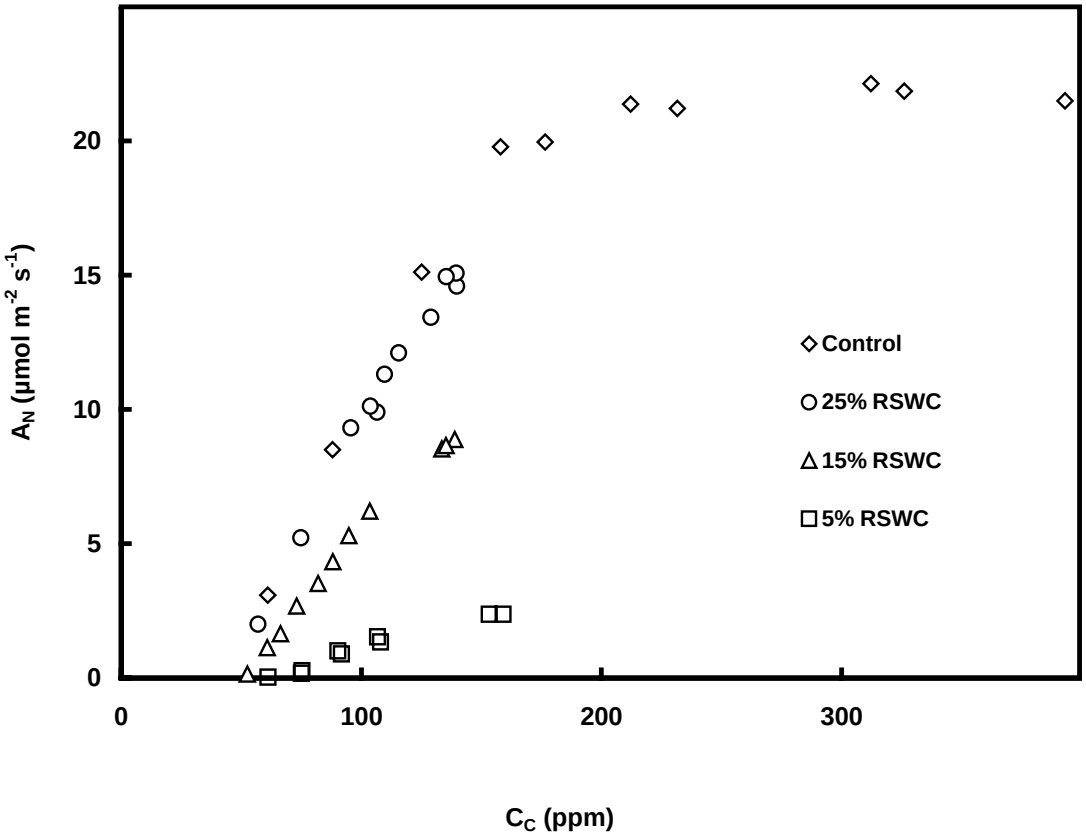
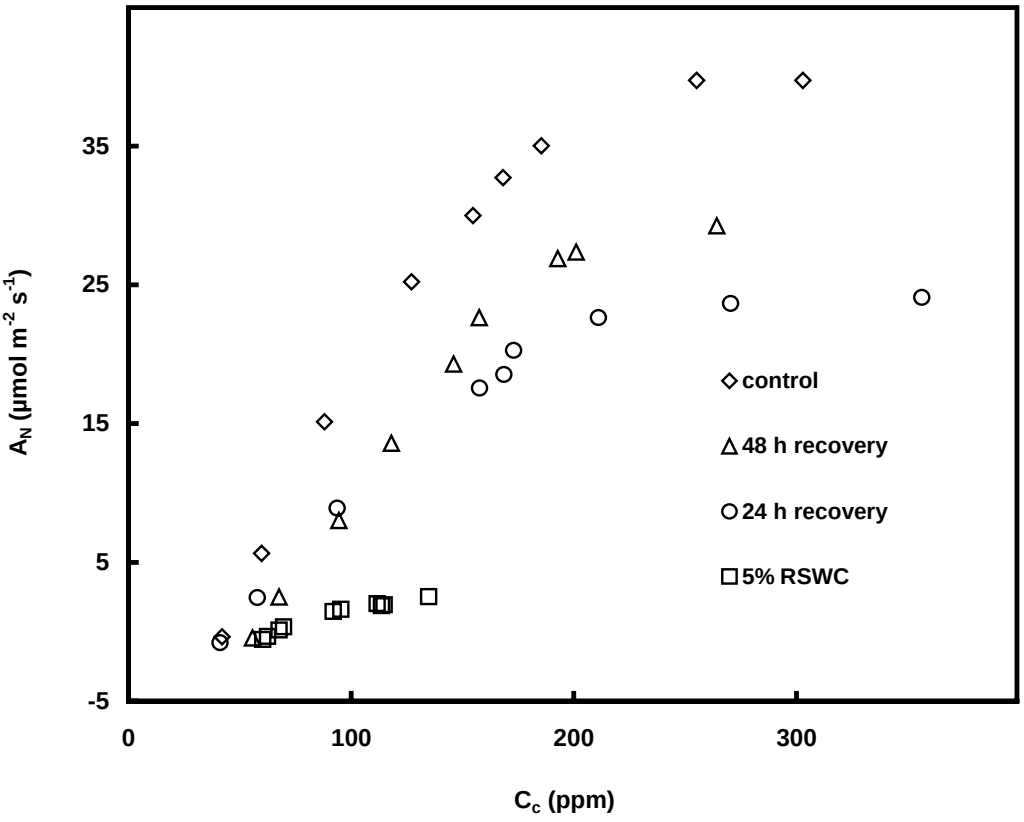

20
15
10
5
0
0
100
200
300
A_N (µmol m^{-2} s^{-1})
C_C (ppm)
Control
25% RSWC
15% RSWC
5% RSWC

Figure 3.4. Effect of water stress and recovery (re-watering) on the relationship between A_N and C_c for experiment two. Shown are example data for a single replication of the experiment.


35
25
15
5
-5
0
100
200
300
A_N (µmol m^{-2} s^{-1})
C_c (ppm)
control
48 h recovery
24 h recovery
5% RSWC

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