



## Measures of leaf-level water-use efficiency in drought stressed endophyte infected and non-infected tall fescue grasses

Debbie Swarthout\*, Emily Harper, Stephanie Judd, David Gonthier, Rebekah Shyne, Timothy Stowe, Thomas Bultman

Biology Department, Hope College, 35E 12 Street, Holland, MI 49423, United States

### ARTICLE INFO

#### Article history:

Received 7 November 2008

Accepted 4 December 2008

#### Keywords:

Tall fescue

Endophyte

Water-use efficiency

Photosynthesis

Conductance

### ABSTRACT

*Neotyphodium coenophialum* [Morgan-Jones and Gams], grows in the above-ground parts of tall fescue [*Lolium arundinaceum* (Schreb.) Darbysh.]. It is an asexual fungus that is transmitted through seed of its host plant. This grass/endophyte association is enhanced by the protection of the host from herbivory and improved drought stress. We investigated how a decline in leaf-level stomatal conductance impacts the instantaneous water-use efficiency (WUE), in endophyte-infected (E+) versus non-infected (E−) Kentucky-31 tall fescue grasses grown in a controlled environmental chamber over a 10-week period. Grasses were cut at 6 weeks after germination and allowed to regrow under high and low soil moisture availability. One week after cutting, soil moisture was allowed to decline in the low water treatment for 2 weeks until severe stress was demonstrated through a decline in stomatal conductance to less than  $100 \text{ mmol m}^{-2} \text{ s}^{-1}$ . We found no differences in WUE between E+ and E− plants when water was not limiting while higher WUE was exhibited in E+ plants relative to E− plants under severe drought stress. The E− plants showed an 18-fold reduction in mean WUE and a 70-fold reduction in photosynthesis under drought stress, while there was no change in WUE and only a fourfold decline in photosynthesis between well-watered and drought stressed E+ plants at 21 days. While there were no differences in the rates of transpiration between E+ and E− plants under severe drought stress, differences in WUE can be attributed mainly to higher photosynthetic rates of E+ than E− plants. The difference in photosynthetic rates between E+ and E− plants under drought conditions could not be explained by differences in stomatal conductance and Rubisco (EC 4.1.1.39) activities.

© 2008 Elsevier B.V. All rights reserved.

### 1. Introduction

Water-use efficiency (WUE) is the measurement of how much  $\text{CO}_2$  is used for photosynthesis per unit of water transpired. This efficiency index determines whether a plant leaf is optimizing the ratio of carbon gain to water loss as environmental conditions vary. Stomatal aperture size and WUE typically varies in response to changes in light intensity, saturation deficit of ambient water vapor and soil moisture availability (Wong et al., 1985; Assmann and Shimazaki, 1999; Mott and Buckley, 2000; Schroeder et al., 2001). Most plant species exhibit increases in WUE as soil moisture availability declines under drought conditions because the reduction in the rate of photosynthesis is smaller than the rate of transpiration for any given reduction in stomatal conductance (Jones, 1993; Earl, 2002). Some plant species may experience photoinhibition of their photosynthetic machinery under severe drought stress (Baker,

1993). Under these conditions, WUE decreases as stomatal conductance declines when the reduction in photosynthesis becomes larger than the reduction in transpiration. A plant with higher WUE under drought becomes more drought-tolerant because it can maintain a net carbon gain, even at a very low stomatal aperture when the supply rate of  $\text{CO}_2$  becomes limiting. In contrast, plants unable to maintain a net carbon gain at low stomatal conductance will not be water-use efficient as drought-induced damage to the photosynthetic machinery proceeds.

The drought adaptive physiological mechanisms of the mutualistic association between the endophyte, *N. coenophialum* and the  $\text{C}_3$  cool season grass, *L. arundinaceum* (tall fescue) have intrigued plant physiologists for some time (for review, see West, 1994; Schardl et al., 2004; Malinowski and Belesky, 2006). Past studies have shown that the fungal endophyte allows the plant to withstand drought stress primarily by causing early stomatal closure and adjusting osmotic potentials by increasing polyol concentrations in the grass (Bates and Joost, 1990; Richardson et al., 1990, 1993; Bacon, 1993; West, 1994; Elmi and West, 1995; Elbersen and West, 1996; Malinowski and Belesky, 2000, 2006). Because of

\* Corresponding author. Tel.: +1 616 395 7355; fax: +1 616 395 7125.  
E-mail address: [swarthout@hope.edu](mailto:swarthout@hope.edu) (D. Swarthout).

these water-conserving mechanisms, West (1994) predicted that endophyte-infected tall fescue grasses would be able to prolong net carbon assimilation or recover growth after short-term drought. It is expected then that endophytic tall fescues (E+) should exhibit increased WUE under drought conditions relative to plants without the fungus (E–). Research in this area however, has been inconclusive because studies have been performed on different cultivars of Kentucky-31 tall fescue host genotypes and under a range of environmental stress conditions.

Bates and Joost (1990) demonstrated that E+ tall fescue cultivars had lower WUE relative to E– plants at high soil water potentials. WUE declined more rapidly in E– tall fescue cultivars as soil water potentials declined until no differences were detected between E+ and E– plants. Johnson and Tieszen (1993) observed no significant difference in WUE based on estimates of the mean discrimination against the naturally occurring  $^{13}\text{C}$  isotope in leaf tissue of E– plants relative to E+ in field grown cultivars of Kentucky-31 tall fescue. Similarly, no differences in WUE were found between E+ and E– tall fescue cultivars under a range of moisture availability (Richardson et al., 1990). The lack of an impact of *N. coenophialum* on WUE under a range of soil moisture availability shown in past studies may have been confounded by variations in the interactions of WUE with plant age, environmental factors and fungal symbiont interactions with host genotypes (Richardson et al., 1990; Schardl et al., 2004; West et al., 2007).

Analysis of the photosynthetic response to the supply of  $\text{CO}_2$  as a function of stomatal conductance provides insight about the biochemical integrity of the photosynthetic machinery as  $\text{CO}_2$  becomes limiting as stomata close. When reanalyzing data from the literature using light-saturated stomatal conductance as a parameter indicative of water deficits, Flexas and Medrano (2002) reported that many photosynthetic parameters including electron transport rates, carboxylation capacity, intrinsic water-use efficiency and respiration in the light showed a higher dependency with stomatal conductance than with plant relative water content or water potential. It was suggested that stomatal conductance should be used as a reliable indicator of drought stress because it is more integrative of both external and internal factors relating to drought including soil water status, leaf vapor pressure deficit, ABA signals, xylem conductivity and leaf water status (Medrano et al., 2002).

Previous investigations on WUE in E+ and E– cool season grasses have not investigated how WUE responds to changes in stomatal conductance as water availability declines during drought stress (Bates and Joost, 1990; Richardson et al., 1990). It is also not known whether the biochemical limitations to photosynthesis are influenced by the fungus as diffusion to  $\text{CO}_2$  becomes limiting as stomata close in response to drought. In this study, we tested whether there was an endophyte effect on WUE over a range of soil moisture availability and stomatal conductances in individuals of Kentucky-31 tall fescue grasses. We also measured initial *in vitro* activities of Rubisco in E+ and E– plants under drought conditions to investigate if this potential biochemical regulation on photosynthesis was impacted by the endophyte as photosynthesis was significantly reduced at severe stress levels of stomatal conductance.

## 2. Materials and methods

Leaf-level rates of photosynthesis, transpiration, WUE and stomatal conductance were studied in *L. arundinaceum* (Kentucky-31 cultivar) plants that were uninfected (E–) or infected with (E+) the fungal endophyte, *N. coenophialum*. The experiment took place in a controlled environmental growth chamber (Convion, Canada) during September to November, 2005 at Hope College, MI, USA.

### 2.1. Plant material growth conditions

Infected and uninfected seeds of Kentucky-31 were obtained from Dr. Henry Fribourg at the University of Tennessee in 2001. Half of the seeds, which originated from infected plants collected in Kentucky, USA, had been heat-treated to kill the endophyte (Nott and Latch, 1993), and then increased in the field at the Plant Science farm in Tennessee, USA, for six generations. Both E+ and E– plants came from the same maternal line from the field population in Kentucky. Seeds were stored at  $-20^\circ\text{C}$  until planting. Two seeds of E+ and E– *L. arundinaceum* were planted into pots (14 cm diameter at top, 11 cm diameter at bottom, 14 cm height) with drainage holes, containing Sunshine LC1 mix (Sun Gro Horticulture, Vancouver, British Columbia, Canada). Pots were placed into a controlled environmental growth chamber (Convion, Canada) on 13 September 2005 until 23 November 2005. Half of 40 pots contained either E+ or E– seeds and these were further assigned to either a high or a low water treatment. This design reflected a 2X2 factorial with 10 replicates in each treatment. Pots were randomly assigned positions in the chamber and were saturated with water three times a week. Pots were fertilized with 100 mL of  $1.5\text{ g L}^{-1}$  Peter's solution (20:20:20 NPK; Scotts-Sierra Horticultural products, Marysville, OH, USA) when randomizing each pot position once a week throughout the experiment. Plants were grown in environmental chambers with 14:10 day/night light cycle,  $400\ \mu\text{mol mol}^{-1}\ \text{CO}_2$ , 45–50% relative humidity, 20/15  $^\circ\text{C}$  day/night temperature regime and a day-time photon flux density (PPFD) of  $600\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ . At 4 weeks after germination, one of the seedlings was removed from each pot so that only one seedling was left in each experimental pot. Some seedlings that were removed were transplanted into pots in the same treatment that did not have any seedlings germinate.

### 2.2. Water treatments

At 6 weeks, plants (5–7 tillers) were cut 3 cm above the soil and newly developing leaf blades were allowed to grow under two water treatments. Water availability in the pots was measured gravimetrically throughout the experiment and compared with leaf stomatal conductance to determine plant physiological drought stress as water availability declined over time (Earl, 1999). Pots of well-watered plants were watered to 1000 g (saturated mass) three times a week after cutting and this included the application of  $100\text{ mL } 1.5\text{ g L}^{-1}$  of Peters solution (20:20:20 NPK; Scotts-Sierra Horticultural products, Marysville, OH, USA) fertilizer at 11 and 18 days after cutting. Pots of low water plants were watered to 600 g during the first 7 days after cutting. During the remaining 14 days of the drought experiment, only the 100 mL fertilizer was added to all the low water pots once at 18 days after cutting.

### 2.3. Measurements of photosynthesis, stomatal conductance, transpiration and water-use efficiency

Gas exchange measurements were undertaken on the 15-day and 21-day old leaf blades. The second fully emerged leaf blade on a tiller leaf was measured per pot in order to make comparative measurements on all the individuals in the four treatments on a given sampling day. Conditions in the leaf cuvette were held at a flow rate ranging between 200 and  $400\ \mu\text{mol s}^{-1}$ , depending on the rate of photosynthesis of the leaf. The reference air temperature was kept constant at an ambient growth chamber air temperature of  $20^\circ\text{C}$ , while the reference relative humidity was maintained at ambient growth chamber humidity of 45–50%. Leaf vapor pressure deficit ranged between 1 and 1.2 kPa during the sampling periods. PPFD inside the cuvette was kept constant at  $800\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$  and the reference  $\text{CO}_2$  entering the chamber was constant at  $400\ \mu\text{mol mol}^{-1}$  for all measurements. Rates of photosynthesis,

stomatal conductance, transpiration internal to external leaf CO<sub>2</sub> concentrations (*c<sub>i</sub>/c<sub>a</sub>*) and water-use efficiency were measured after the leaf stabilized under the cuvette conditions after 2–3 min. Measurements were made between 10h00 and 16h00 on the respective dates.

2.4. Rubisco activity

Rubisco (EC 4.1.1.39) activity was measured only on the low water plants at 21 days of plant regrowth. Enzyme activity was assayed spectrometrically by the continuous measurement of 3-PGA-dependent NADH oxidation in a coupled enzyme system modified from Sharkey et al. (1991). Fresh leaves of all but two tillers of E+ and E- *L. arundinaceum*, were collected and frozen in liquid nitrogen while in the growth chamber. Some of this material was used immediately for initial Rubisco activity measurements while the rest was stored at -80 °C for soluble protein analysis. The fresh leaves on the remaining two tillers were used to determine the ratios of fresh to dry mass of the tissues.

The frozen leaf segments (1 g) were ground in 3 mL of extraction buffer and 50 mg PVP and centrifuged for 120 s for initial Rubisco activity measurements. The extraction buffer (pH 8.0) contained 100 mM Bicine, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol and 0.02% (w/v) bovine serum albumin. The supernatant was collected in a fresh tube and 100 μL was used for each assay. The assay mixture, adjusted to pH 8.0, contained 50 mM Bicine, 1 mM EDTA, 15 mM MgCl<sub>2</sub>, 20 mM KCl, 10 mM dithiothreitol, 0.6 mM RuBP, 10 mM NaHCO<sub>3</sub>, 0.2 mM B-NADH, 5 mM ATP, 5 mM phosphocreatine, 5 units mL<sup>-1</sup> glyceraldehyde 3-phosphate dehydrogenase, 5 units mL<sup>-1</sup> creatine phosphokinase, and 5 units mL<sup>-1</sup> 3-phosphoglyceric acid kinase. The reactions were started by the addition of 100 μL of plant extract to 3 mL of assay mixture. Rubisco activity was calculated from the change in absorbance following the oxidation of NADH at 340 nm for 60 s. The oxidation was monitored repeatedly during the first 7 min of incubation in order to obtain the maximum oxidation rate as the sample warmed up to room temperature (25 °C). Soluble protein concentrations were determined on 100 mg of tissue, that was frozen in liquid nitrogen and stored at -80 °C, using a Bio-Rad assay (Bio-Rad Laboratories, Inc., CA) with BSA as a standard.

2.5. Immunoblotting detection of endophyte

Immunoblotting (Hiatt et al., 1999) was used to determine the infection status of the E+ plants on 2 December 2005. A single stem was cut about 2.5 cm from the base of the tiller and the sap from the cut stem was placed onto a nylon membrane. Two tillers were sampled in this way from each of the E+ plants. The sap was exposed to a mycelial antibody (obtained from Dr. Chris Schardl at the University of Kentucky) at a concentration of 1:1000 in Tris-buffered saline Tween-20 (TBST buffer, pH 7.8) for 2 h after blocking the nylon membrane with dried milk powder dissolved in TBST buffer for 2 h. The membrane was rinsed three times with TBST buffer before exposing the primary antibody mycelial protein complex to the secondary anti-rabbit alkaline phosphatase antibody at a concentration of 1:2000 in TBST buffer for 1 h. After rinsing three times with TBST, the primary-secondary antibody complex was reacted with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets (BCIP/NBT, Sigma USA) dissolved in 10 mL of ultra pure water. Two E- plants were included in the initial immunoblot run to serve as a control for the technique because all E- plants should not contain the fungus after the E- seed had been heat-treated to effectively kill the fungus (Nott and Latch, 1993). The immunoblot tissue-printing was repeated on all the tillers of E+ plants that tested negative in the first immunoblot analysis.

2.6. Statistical analysis

The immunoblot revealed that forty percent of the E+ plants were not infected and these plants were not included in the data analysis. A two-way ANOVA was used to determine the effects of endophyte and water treatment on the gas exchange variables at 15 and 21 days after cutting. Simple linear regressions were performed on physiological variables that were predicted to be interdependent. In the case where significant regressions between two physiological variables were found, an Analysis of Covariance was performed to compare the slopes of the best-fit regression lines to investigate whether E+ and E- physiological responses were different.

3. Results

3.1. Water availability and physiological variables

The low water pots were allowed to dry out to 29.5% the amount of water available to the well-watered pots at 21 days (Fig. 1). There was no significant difference in water availability in the pots of E+ versus E- plants in both water treatments. When growing tall fescue grasses under well-watered conditions, there was no endophyte effect on the mean rates of leaf-level photosynthesis, stomatal conductance, transpiration, WUE and *c<sub>i</sub>/c<sub>a</sub>* ratios at 15 days after plants were cut and allowed to regrow (Table 1). In contrast, when growing plants under drought conditions, E+ plants maintained a similar WUE to well-watered plants while E- plants showed a significant decline in WUE as stomatal conductance decreased below 100 mmol m<sup>-2</sup> s<sup>-1</sup> in the drought treatment at 21 days (Fig. 2, Table 2). When comparing the linear response of photosynthesis to stomatal conductance, there was a much greater reduction in photosynthesis as stomatal conductance declined below 200 mmol m<sup>-2</sup> s<sup>-1</sup> in E- plants relative to E+ plants under drought conditions (ANCOVA, *F*<sub>1,23</sub> = 1.89, *p* < 0.05, Fig. 3). In contrast, there was no significant difference between E- and E+ plants in the decline in transpiration with stomatal conductance (ANCOVA, *F*<sub>1,23</sub> = 0.10, *p* > 0.05, Fig. 4). The E- plants showed an 18-fold reduction in mean WUE and a 70-fold reduction in photosynthesis, while there was no change in WUE and only a fourfold decline in photosynthesis in E+ plants between well-watered and drought stressed tall fescue grasses at 21 days. There

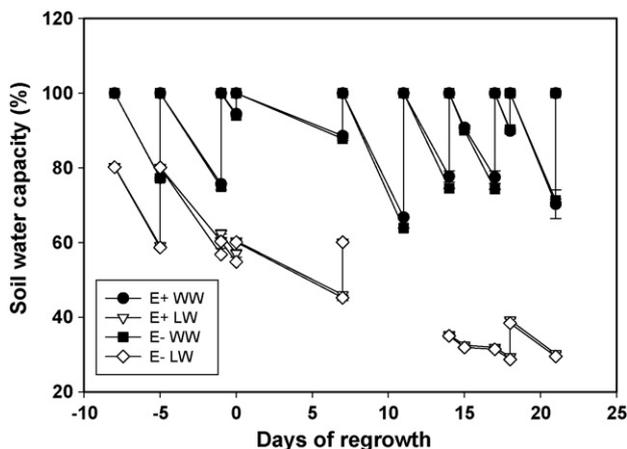


Fig. 1. Mean pot water capacity (±S.E.M.) in the four treatments of tall fescue grasses in the growth chamber experiment during 2005. Water capacity is calculated at pot mass expressed as a percentage of saturated pot mass. Days of regrowth are expressed relative to time before and after cutting. WW, well-watered treatment and LW, low water treatment.

**Table 1**

Mean physiological variables and pot moisture availability with (S.E.M.) values for E– and E+ tall fescue plants in well-watered (WW) and low-watered (LW) pots measured at 15 days during regrowth. A, photosynthesis, E, transpiration, g, stomatal conductance, WUE, water-use efficiency and  $c_i/c_a$ , ratio of internal to external leaf CO<sub>2</sub> concentrations.

Treatment	A ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	E ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	g ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	WUE ( $\mu\text{mol mmol}^{-1}$ )	$c_i/c_a$
WW E+ (n=5)	17 ± 0.8	4 ± 0.5	455 ± 68	4.3 ± 0.4	0.78 ± 0.02
WW E– (n=9)	16 ± 1.0	5 ± 0.3	477 ± 4	3.7 ± 0.3	0.80 ± 0.01
LW E+ (n=6)	6 ± 1.3	2 ± 0.2	116 ± 20	4.1 ± 0.6	0.75 ± 0.03
LW E– (n=8)	7 ± 1.8	2 ± 0.3	130 ± 22	4.2 ± 0.8	0.75 ± 0.04
Endophyte	* $F_{1,24} = 0.4 p > 0.05$	* $F_{1,24} = 0.8 p > 0.05$	* $F_{1,24} = 0.2 p > 0.05$	* $F_{1,24} = 0.2 p > 0.05$	* $F_{1,24} = 0.04 p > 0.05$
Water	* $F_{1,24} = 49.4 p < 0.05$	* $F_{1,24} = 65.8 p < 0.05$	* $F_{1,24} = 76.6 p < 0.05$	* $F_{1,24} = 0.1 p > 0.05$	* $F_{1,24} = 1.4 p > 0.05$
Interaction	* $F_{1,24} = 0.32 p > 0.05$	* $F_{1,24} = 0.1 p > 0.05$	* $F_{1,24} = 0.01 p > 0.05$	* $F_{1,24} = 0.3 p > 0.05$	* $F_{1,24} = 0.21 p > 0.05$

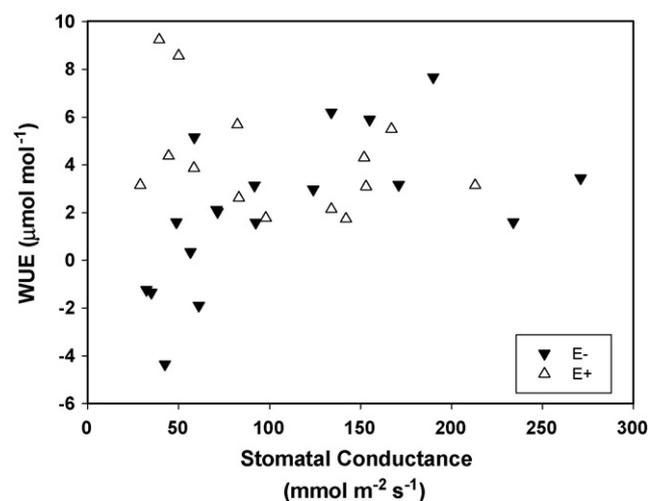
\* F values with degrees of freedom and probabilities are given for the results of a Two-Way analysis of variance for each physiological parameter.

**Table 2**

Mean physiological variables and pot moisture availability with SEM values for E– and E+ tall fescue plants in well-watered (WW) and low-watered pots (LW) measured at 21 days during regrowth. A, photosynthesis; E, transpiration, g, stomatal conductance; WUE, water-use efficiency and  $c_i/c_a$ , ratio of internal to external leaf CO<sub>2</sub> concentrations.

Treatment	A ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	E ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	g ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	WUE ( $\mu\text{mol mmol}^{-1}$ )	$c_i/c_a$
WW E+ (n=5)	15 ± 1.2	4 ± 0.4	400 ± 66	4.6 ± 0.7	0.76 ± 0.03
WW E– (n=9)	18 ± 1.0	4 ± 0.3	437 ± 6	5.0 ± 0.3	0.76 ± 0.01
LW E+ (n=6)	4 ± 0.7	1 ± 0.2	80 ± 20	4.6 ± 1.4	0.76 ± 0.08
LW E– (n=8)	0.3 ± 0.7	1 ± 0.1	58 ± 8	–0.3 ± 0.9	0.99 ± 0.04
Endophyte	* $F_{1,24} = 0.3 p > 0.05$	* $F_{1,24} = 0.1 p > 0.05$	* $F_{1,24} = 0.2 p > 0.05$	* $F_{1,24} = 7.1 p < 0.05$	* $F_{1,24} = 8.9 p < 0.05$
Water	* $F_{1,24} = 272.9 p < 0.05$	* $F_{1,24} = 98.4 p < 0.05$	* $F_{1,24} = 59.3 p < 0.05$	* $F_{1,24} = 9.7 p < 0.05$	* $F_{1,24} = 5.2 p < 0.05$
Interaction	* $F_{1,24} = 9.6 p < 0.05$	* $F_{1,24} = 0.5 p > 0.05$	* $F_{1,24} = 0.4 p > 0.05$	* $F_{1,24} = 9.6 p < 0.05$	* $F_{1,24} = 8.1 p < 0.05$

\* F values with degrees of freedom and probabilities are given for results of a two-way analysis of variance for each physiological parameter.



**Fig. 2.** WUE versus stomatal conductance for E+ and E– plants tall fescue plants grown under drought conditions at 15 and 21 days during regrowth in the growth chamber experiment during 2005.

was no difference in mean stomatal conductance between E+ and E– plants at 21 days under drought (Table 2). Initial specific Rubisco activities and soluble protein concentrations were also not significantly different between E+ and E– plants under drought at 21 days (Table 3) and cannot explain the large differences in mean photosynthetic rates in E– plants relative to E+ plants under these conditions.

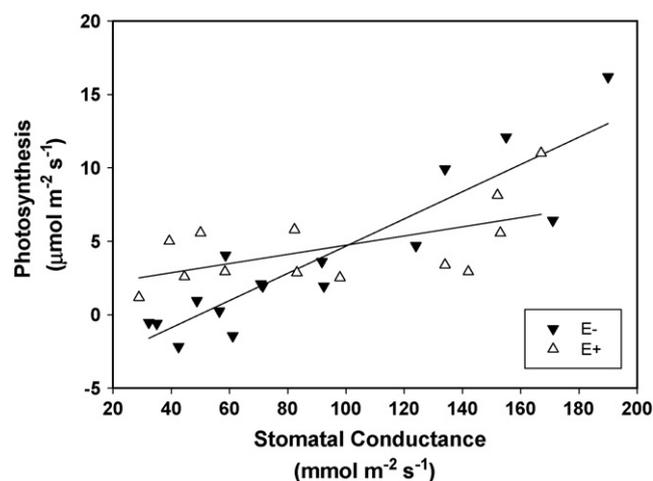
**Table 3**

Initial specific rubisco activities and soluble protein concentrations expressed per gram dry mass (DM) under drought conditions at 21 days during regrowth. Means of three E+ and four E– plants with standard errors are provided.

Endophyte status	Specific rubisco activity ( $\mu\text{mol mg protein}^{-1} \text{min}^{-1}$ )	Soluble protein (% DM)
E+	1.32 ± 0.24	2.15 ± 0.27
E–	1.31 ± 0.22	1.65 ± 0.36

#### 4. Discussion

Our study confirms that there is no difference in WUE between E+ and E– plants when water is not limiting. As water stress was imposed on plants, presence of the fungus allowed plants to maintain WUE when conductance declined to severe stress levels ( $<100 \text{ mmol m}^{-2} \text{ s}^{-1}$ ), whereas E– plants showed a significant decrease in WUE under these conditions. The response of the E– plants followed the predicted reduction in WUE that would be observed when biochemical damage to the photosynthetic machinery occurs under severe drought stress (Flexas and Medrano, 2002; Zhou et al., 2007). A reduction in the biochemical efficiency in photosynthesis can be influenced by numerous control points in the photosynthetic biochemical pathway. These include reduced photochemistry as oxidative stress increases under drought, as well as a reduction in production of ATP and enzyme activities of Rubisco and sucrose phosphate synthase during the gradual decline of moisture availability (Vassey and Sharkey, 1989; Demmig et al., 1988;



**Fig. 3.** Photosynthesis versus stomatal conductance for E+ and E– tall fescue plants under drought conditions at 15 and 21 days during regrowth in the growth chamber experiment during 2005. The slopes of the regression lines are significantly different between E+ and E– plants (ANCOVA,  $F_{1,23} = 1.89, p < 0.05$ ).

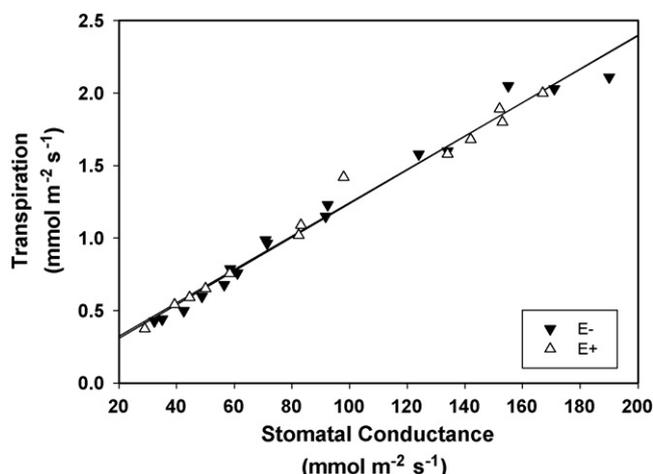


Fig. 4. Transpiration versus stomatal conductance for E+ and E- tall fescue plants under drought conditions at 15 and 21 days during regrowth in the growth chamber experiment during 2005.

Ort, 2001; Flexas and Medrano, 2002; Lawlor, 2002; Parry et al., 2002; Ghannoum et al., 2003). In this study, *in vitro* enzyme analysis showed that initial Rubisco activity was not higher in E+ than E- plants while photosynthetic rates of E- plants were 14-fold lower than in E+ plants, when stomatal conductances declined to 58 and 80  $\text{mmol m}^{-2} \text{s}^{-1}$ , respectively. It was similarly reported that E+ Arizona fescue plants (*Festuca arizonica*) had significantly higher rates of photosynthesis than E- plant under severe water stress, while no differences were found in carboxylation efficiency estimated from  $\text{CO}_2$ -response curves (Morse et al., 2002). While the effect of water stress on rubisco activities may be species specific, it is commonly found that both total and initial Rubisco activities decrease with water stress. Reports have shown that decreases in Rubisco amount, as well as increased inactivation of Rubisco by inhibitors, are important regulatory factors of Rubisco activity under water stress (Sharkey and Seeman, 1989; Flexas and Medrano, 2002; Parry et al., 2002). Although the results for our water-stressed tall fescue plants suggest that the initial activities were not different between E+ and E- plants under water stress, the regulatory mechanisms of the initial activities remain unknown and could be influenced by the presence of the fungus in the above-ground tissues of tall fescue plants.

Although we did not measure the fluorescence of PSII as an indicator of the level of oxidative damage, our results imply that there may have been less biochemical damage to the photosynthetic machinery in E+ plants relative to E- plants as drought stress increased over time. The observation of a simultaneous rise in internal leaf  $\text{CO}_2$  concentration ( $c_i$ ) with a decline in stomatal conductance and photosynthesis in E- plants suggests that the biochemical efficiency of the photosynthetic machinery declined under severe drought stress in E- plants. However, we are cautious about this interpretation because estimates of  $c_i$  are erroneously overestimated when the proportional contribution of cuticular conductance starts to increase as stomata close (Boyer et al., 1997) or when there is non-homogenous stomatal closure under stress (Terashima et al., 1988). We did not measure cuticular conductance in this study and we cannot assume that it was the same between E+ and E- as the leaf tissues dehydrated over time.

The difference between the slopes of photosynthesis versus stomatal conductance under water stress for E- versus E+ plants suggests that changes in photosynthesis may have been influenced by something more than stomatal limitation in E- plants. The similar relationships between transpiration and stomatal conductance for E+ and E- plants under drought suggest E+ plants did not lose less water vapor at a given stomatal conductance relative to

E- plants. This suggests that the rate of water loss may not have been impeded by potential differences in osmotic adjustment, as previously suggested (West, 1994; Elmi and West, 1995; Elbersen and West, 1996).

Studies on tall fescue and creeping bentgrass (*Agrostis palustris*) have shown that the antioxidant,  $\alpha$ -tocopherol, typically increases under drought conditions and is thought to be involved with scavenging oxygen radicals and protecting the photosynthetic proteins (Zhang and Schmidt, 2000). Increased expression of dehydrin proteins and polyphenolic compounds in tall fescue cultivars have been suggested to play a role in preventing membrane degradation under drought stress (Malinowski and Belesky, 2006). In two tall fescue cultivars, dehydrin expression increased with an associated decrease in dehydration damage under drought conditions but no endophyte effects were investigated (Jiang and Huang, 2002). Recently, Guerber et al. (2007) investigated the effects of endophyte on dehydrin expression in two genotypes of tall fescue under well-watered and drought conditions. They found no endophyte effect on dehydrin expression in the xeric genotype which produced thicker cuticles whereas dehydrin expression increased in the mesic genotype with thinner cuticles, substantiating the additional complexity of the interaction of the host genotype with the effects of the fungal symbiont.

Little is known about the endophyte effects on upregulating biochemical pathways involved in minimizing oxidative damage under drought stress. Increases in activity of superoxide dismutase, ascorbate peroxidase and glutathione reductase were observed during the initial stages of drought in tall fescue and Kentucky bluegrass (*Poa pratensis*), but no endophyte effects were investigated (Jiang and Huang, 2001). Similarly, tall fescue grasses exhibited less damage to chloroplast membranes under heat stress relative to perennial ryegrass (*Lolium perenne*), but no endophyte effects were investigated (Xu et al., 2005). Even though Marks and Clay (1996) demonstrated that the presence of the endophyte resulted in higher photosynthetic rates in tall fescue grasses subjected to heat stress, the mechanism giving rise to improved photosynthetic rates remains unknown. In a similar manner, dehydrin expression patterns and the biochemical regulation of oxidative scavenging pathways have not been investigated simultaneously with gas exchange measurements in E+ and E- plants during soil moisture dehydration experiments in any of the *Neotyphodium* associations with cool season grasses.

## 5. Conclusions

This study shows that when using stomatal conductance as an index of water stress, distinguishable effects of the fungus on WUE and photosynthesis are observed in Kentucky-31 tall fescue grasses under drought. Future physiological studies on this endophytic mutualistic system should concentrate on discovering how the endophyte impacts the mechanistic role that antioxidants play in maintaining the biochemical efficiency of the photosynthetic apparatus under heat and soil moisture stress conditions.

## Acknowledgements

H. Fribourg, the University of Tennessee, kindly supplied the Kentucky-31 tall fescue seeds. This study was funded by the NSF-CRUI grant (#0330840) and NSF-MRI grant (#0319938). We are grateful to Chris Schardl (University of Kentucky) for providing us with the fungal antibody.

## References

- Assmann, S.M., Shimazaki, K., 1999. The multisensory guard cell. Stomatal responses to blue light and abscisic acid. *Plant Physiol.* 119, 809–815.

- Bacon, C.W., 1993. Abiotic stress tolerances (moisture, nutrients) and photosynthesis in endophytic-infected tall fescue. *Agricult. Ecosys. Environ.* 44, 123–141.
- Baker, N.R., 1993. Light-use efficiency and photoinhibition of photosynthesis under environmental stress. In: Smith, J.A.C., Griffiths, H. (Eds.), *Water Deficits: Plant Responses from Cell to Community*. Bios Scientific, Oxford, UK, pp. 221–236.
- Bates, G.E., Joost, R.E., 1990. The effect of *Acremonium coenophialum* on the physiological responses of tall fescue to drought stress. In: Quisenberry, S.S., Joost, E. (Eds.), *Proceedings of the International Symposium on Acremonium/Grass Interactions*. Baton Rouge, LA, pp. 121–124.
- Boyer, J.S., Wong, S.C., Farquhar, G.D., 1997. CO<sub>2</sub> and water vapor exchange across leaf cuticle (Epidermis) at various water potentials. *Plant. Physiol.* 114, 185–191.
- Demmig, B., Winter, K., Kruger, A., Czygan, F.-C., 1988. Zeaxanthin and the heat dissipation of excess light energy in *Nerium oleander* exposed to a combination of high light and water stress. *Plant Physiol.* 87, 17–24.
- Earl, H.J., 1999. A precise gravimetric method for simulating drought stress in pot experiments. *Crop Sci.* 44, 1868–1873.
- Earl, H.J., 2002. Stomatal and non-stomatal restrictions to carbon assimilation in soy bean (*Glycine max*) lines differing in water-use efficiency. *Environ. Exp. Bot.* 48, 237–246.
- Elmi, A.A., West, C.P., 1995. Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of tall fescue. *New Phytol.* 131, 61–67.
- Elbersen, H.W., West, C.P., 1996. Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. *Grass Forage Sci.* 51, 333–342.
- Flexas, J., Medrano, H., 2002. Drought-inhibition of photosynthesis in C<sub>3</sub> plants: stomatal and non-stomatal limitations revisited. *Ann. Bot.* 89, 183–189.
- Guerber, C.A., West, C.P., Carson, R.D., Havelly, A.M., 2007. Dehydrin expression in drought-stressed tall fescue. In: *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses*. Grassland Research and Practice Series No. 13, Christchurch, New Zealand, pp. 225–227.
- Ghannoum, O., Conroy, J.P., Driscoll, S.P., Paul, M.J., Foyer, C.H., Lawlor, D.W., 2003. Nonstomatal limitations are responsible for drought-induced inhibition in four C<sub>4</sub> grasses. *New Phytol.* 159, 599–608.
- Hiatt III, E.E., Hill, N.S., Bouton, J.H., Stuedemann, J.A., 1999. Tall fescue endophyte detection: commercial immunoblot test kit compared with microscopic analysis. *Crop Sci.* 39, 796–799.
- Jiang, Y., Huang, B., 2001. Drought and heat stress injury to two cool-season turfgrasses in relation to antioxidant metabolism and lipid peroxidation. *Crop Sci.* 41, 436–442.
- Jiang, Y., Huang, B., 2002. Protein alterations in tall fescue in response to drought stress and abscisic acid. *Crop Sci.* 42, 202–207.
- Johnson, R.C., Tieszen, L.L., 1993. Carbon isotope discrimination, water relations, and gas exchange in temperate grass species and accessions. In: Ehleringer, J.R., Hall, A.E., Farquhar, G.D. (Eds.), *Stable Isotopes and Plant Carbon-water Relations*. Academic Press Inc., San Diego, CA, pp. 281–292.
- Jones, H.G., 1993. Drought tolerance and water-use efficiency. In: Smith, J.A.C., Griffiths, H. (Eds.), *Water Deficits: Plant Responses from Cell to Community*. Bios Scientific, Oxford, UK, pp. 193–204.
- Lawlor, D.W., 2002. Limitation to photosynthesis in water-stressed leaves: stomata vs. metabolism and the role of ATP. *Ann. Bot.* 89, 871–885.
- Malinowski, D.P., Belesky, D.P., 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Sci.* 40, 923–940.
- Malinowski, D.P., Belesky, D.P., 2006. Ecological importance of *Neotyphodium* spp. grass endophytes in agroecosystems. *Grassl. Sci.* 52, 1–14.
- Marks, S., Clay, K., 1996. Physiological responses of *Festuca arundinacea* to fungal endophyte infection. *New Phytol.* 133, 727–733.
- Medrano, H., Escalona, J.M., Bota, J., Gulias, J., Flexas, J., 2002. Regulation of photosynthesis of C<sub>3</sub> plants in response to progressive drought: stomatal conductance as a reference parameter. *Ann. Bot.* 89, 895–905.
- Morse, L.J., Day, T.A., Faeth, S.H., 2002. Effect of *Neotyphodium* endophyte infection on growth and leaf gas exchange of Arizona fescue under contrasting water availability regimes. *Environ. Exp. Bot.* 48, 257–268.
- Mott, K.A., Buckley, T.N., 2000. Stomatal heterogeneity. *J. Exp. Bot.* 49, 407–417.
- Nott, H.M., Latch, G.C.M., 1993. A simple method of killing endophyte in ryegrass seed. In: Hume, D.E., Latch, G.C.M., Easton, H.S. (Eds.), *Proceedings of the Second International Symposium on Acremonium/Grass Interactions*. AgResearch, Palmerston North, New Zealand, pp. 14–15.
- Ort, D.R., 2001. When there is too much light? *Plant Physiol.* 125, 29–32.
- Parry, M.A., Andralojc, P.J., Khan, S., Lea, P.J., Keys, A.J., 2002. Rubisco activity: effects of drought stress. *Ann. Bot.* 89, 833–839.
- Richardson, M.D., Bacon, C.W., Hoveland, C.S., 1990. The effect of endophyte removal on gas exchange in tall fescue. In: Quisenberry, S.S., Joost, R.E. (Eds.), *Proceedings of the International Symposium on Acremonium/Grass Interactions*. Baton Rouge, LA, pp. 189–193.
- Richardson, M.D., Hoveland, C.S., Bacon, C.W., 1993. Photosynthesis and stomatal conductance of symbiotic and nonsymbiotic tall fescue. *Crop Sci.* 33, 145–149.
- Schardl, C.L., Leuchtmann, A., Spiering, M.J., 2004. Symbiosis of grasses with seed-borne fungal endophytes. *Annu. Rev. Plant Biol.* 55, 315–340.
- Schroeder, J., Allen, I., Hugouvieux, G.J., Kwak, J.M., Waner, D., 2001. Guard cell signal transduction. *Annu. Rev. Plant Physiol. Mol. Biol.* 52, 627–658.
- Sharkey, T.D., Seeman, J.R., 1989. Mild water stress effects on carbon-reduction-cycle-intermediates, ribulose biphosphate carboxylase activity, and spatial homogeneity of photosynthesis in intact leaves. *Plant Physiol.* 89, 1060–1065.
- Sharkey, T.D., Savitch, L.V., Butz, N.D., 1991. Photometric method for routine determination of  $k_{cat}$  and carbamylation of rubisco. *Photosynth. Res.* 28, 41–48.
- Terashima, I., Wong, S.C., Osmond, C.B., Farquhar, G.D., 1988. Characterization of non-uniform photosynthesis induced by abscisic acid having different mesophyll anatomies. *Plant Cell Physiol.* 29, 385–394.
- Vassey, T.L., Sharkey, T.D., 1989. Mild water stress leads to reduced extractable sucrose-phosphate synthase activity in leaves of *Phaseolus vulgaris* L. *Plant Physiol.* 89, 1066–1070.
- West, C.P., 1994. Physiology and drought tolerance of endophyte-infected grasses. In: Bacon, C.W., White, J.F. (Eds.), *Biotechnology of Endophytic Fungi of Grasses*. CRC Press, Boca Raton, FL, pp. 87–99.
- West, C.P., Volaire, F., Lelievre, F., 2007. Tiller survival after drought of “Grasslands Flecha” tall fescue as influenced by endophyte. In: *Proceedings of the 6th International Symposium on Fungal Endophytes of Grasses*. Grassland Research and Practice Series No. 13, Christchurch, New Zealand, pp. 267–269.
- Wong, S.C., Cowan, I.R., Farquhar, G.D., 1985. Leaf conductance in relations to rate of CO<sub>2</sub> assimilation. III. Effects of water stress and photoinhibition. *Plant Physiol.* 78, 830–834.
- Xu, S., Li, J., Zhang, X., Wei, H., Cui, L., 2005. Effect of heat acclimation pretreatment on changes in membrane lipid peroxidation, antioxidant metabolites, and ultrastructures of chloroplasts in two cool-season turfgrass species under heat stress. *Environ. Exp. Bot.* 56, 274–285.
- Zhang, X., Schmidt, R.E., 2000. Hormone-containing products' impact an antioxidant status of tall fescue and creeping bentgrass subjected to drought. *Crop Sci.* 40, 1344–1349.
- Zhou, Y., Lam, H.M., Zhang, J., 2007. Inhibition of photosynthesis and energy dissipation induced by water and high light stresses in rice. *J. Exp. Bot.* 58, 1207–1217.