Effects of *Epichloë festucae* Fungal Endophyte Infection on Drought and Heat Stress Responses of Strong Creeping Red Fescue

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**Abstract.** Strong creeping red fescue (*Festuca rubra* ssp. *rubra*) is an important cool season turfgrass species. Cultivars are often infected with the fungal endophyte *Epichloë festucae*. Endophyte infection is known to confer insect and disease resistance to the plants. The effect of endophyte infection on drought or heat stress tolerance of strong creeping red fescue is not yet established. The objectives of this controlled-environment study were to determine if endophyte infection had any effect on physiological parameters associated with plant tolerance to drought or heat stress or the combination of the two stresses. In this study, endophyte status had no effect on turf quality (TQ), relative water content (RWC), photochemical efficiency, chlorophyll content, electrolyte leakage (EL), or malondialdehyde (MDA) content of the plants under any of the stress treatments. Our results suggested that *E. festucae* infection had no physiological effects on improving drought, heat or the combined stress tolerance in strong creeping red fescue.

*Epichloë* species are fungal endophytes of many cool season grass species. The fungi exist exclusively in association with their plant hosts and the associations are considered to be mutualistic. Forty-three unique *Epichloë* taxa have been described, infecting numerous grass species (Leuchtmann et al., 2014). Likely additional species will be discovered in the future. The benefits of endophyte infection are often generalized as applying to all endophyte species–grass species associations, although there are many examples demonstrating that is not the case.

The plants provide nutrients to the endophytes and the endophytes provide tolerance to biotic and abiotic stresses to the plants, although the specific benefits vary among the many grass species/endophyte species combinations (Kuldau and Bacon, 2008). The best-characterized benefit to the plants is protection from predation by insects and mammals due to the production of toxic fungal alkaloids (Schardl et al., 2013). Endophyte-mediated disease resistance in tall fescue (*Festuca arundinacea*) and disease resistance in strong creeping red fescue and chewings fescue (*F. rubra* ssp. *commutata*) are also well established (Bonos et al., 2005; Clarke et al., 2006; Malinowski and Belesky, 2000) although the underlying physiological mechanisms are not as well established.

Drought and heat are the most widespread abiotic stresses encountered by plants. Numerous studies have demonstrated that tall fescue infected with the fungal endophyte *Epichloë coenophiala* exhibits improved drought tolerance relative to endophyte-free plants (Kuldau and Bacon, 2008; Malinowski and Belesky, 2000, 2006; West, 1994). Both drought avoidance and drought tolerance mechanisms have been implicated in the effect of endophyte infection on tall fescue (Malinowski and Belesky, 2000). In response to water stress, endophyte-infected tall fescue plants were found to accumulate higher levels of several compatible solutes than the endophyte-free plants, supporting osmotic adjustment as a component of the observed drought tolerance (Nagabhyru et al., 2013).

Although endophyte-mediated enhanced drought-tolerance is well established in tall fescue, reports on endophyte effects in other grass species are variable. In perennial ryegrass (*Lolium perenne*), infection with the endophyte *E. festucae* var. *lolii* has been reported to have no effect on vegetative tissues under drought conditions (Barker et al., 1997; Briggs et al., 2013; Cheplick et al., 2000; Hesse et al., 2005). Other studies have reported endophyte infection to be beneficial to perennial ryegrass under drought stress conditions (Amalric et al., 1999; Hahn et al., 2008; He et al., 2013; Ravel et al., 1997). Endophyte infection by an *Epichloë* species was reported to be beneficial to Arizona fescue (*Festuca arizonica*) and grove bluegrass (*Poa alsodes*) under conditions of low water availability (Kannadan and Rudgers, 2008; Morse et al., 2002). There are no studies on the effect of fungal endophyte infection on the responses of the host grass to sustained heat stress conditions in any species. In addition, little is known of endophyte effects on the combination of drought and heat stress, which often occurs during summer months in many areas.

Strong creeping red fescue is an important low maintenance turfgrass species (*Ruemmele et al., 2003*) and plants are often naturally infected with the fungal endophyte *E. festucae* (Schardl, 2001; Scott et al., 2012). Endophyte infection is preferred in strong creeping red fescue cultivar development because of the well-documented insect and disease resistance conferred on the host grass (Bonos et al., 2005; Clarke et al., 2006; Saha et al., 1987; Yue et al., 2000). Whether endophyte infection has a beneficial effect in strong creeping red fescue in responses to drought or heat stress has not been established. Therefore, the objectives of this study were to determine whether *E. festucae* has effects on strong...
creeping red fescue under heat stress, drought stress, and the combination of the two.

Materials and Methods

PLANT MATERIALS AND EXPERIMENTAL DESIGN. The development of the plants used in this study was described previously (Johnson-Cicalese et al., 2000). These endophyte-infected plants have been stably maintained in the greenhouse through clonal propagation for over 15 years. Endophyte status was confirmed microscopically (Saha et al., 1988) before initiation of this study. The plants used were a strong creeping red fescue not infected with *E. festucae* (designated E-) and plants infected with two different isolates of *E. festucae*. The endophyte-infected plants were the E- plant genotype, designated S1139 (Johnson-Cicalese et al., 2000), inoculated with either the Rose City (RC) isolate or the Delaware (DE) isolate of *E. festucae*. The RC isolate was obtained from an endophyte-infected strong creeping red fescue plant and the DE isolate was obtained from an endophyte-infected chewings fescue. The endophyte-infected plants are thus the same plant genotype as the endophyte-free plant infected with two different isolates of *E. festucae*. Although strong creeping red fescue and chewings fescue are subspecies of *F. rubra*, they do not naturally hybridize (Ruemmele et al., 2003). In an amplified fragment length polymorphism analysis of many *Epichloë* isolates, the *E. festucae* isolates from strong creeping red fescue and chewings fescue grouped into separate clades, indicating the endophytes from these two distinct grass species are also distinct from each other (Tredway et al., 1999).

On 11 Mar. 2014, mature sod pieces from the greenhouse grown strong creeping red fescue plants were washed with water, and transplanted into 12 plastic boxes (30 cm length × 44 cm width × 30 cm height) filled with fritted clay (Surface Green Grade; Profile Products, Buffalo Grove, IL). Each box was divided into six equal spaces to separate the plant types. Six samples of 5 cm diameter were arranged randomly in one divider box. Plants were watered every other day, fertilized every week with half-strength Hoagland’s solution, and trimmed every 3 weeks to 5 cm canopy height. The plants were maintained for 50 d in a greenhouse with an average temperature of 22/17 °C (day/night), 700 μmol·m⁻²·s⁻¹ photosynthetically active radiation (PAR) from natural sunlight, and 65% relative humidity to establish root and canopy systems. On 29 Apr. 2014, three boxes were moved into each of four controlled-climate growth chambers (Environmental Growth Chamber, Charrgin Falls, OH) set to 22/17 °C (day/night) temperature, 60% relative humidity, 650 μmol·m⁻²·s⁻¹ PAR, and a 14-h photoperiod. Soil moisture was measured everyday to maintain soil water status using time domain reflectometry (6050X1 Trase System; Soilmoisture Equipment Corp., Santa Barbara, CA). The plants were watered every other day for 6 d until the day before treatments started.

On 5 May 2014, the treatments were initiated. In each growth chamber there were three boxes. Each box contained a total of six plants, two samples of each of the three plant types, yielding a total of six replicates of each plant type for each treatment. Plants were exposed to four treatments applied in the different growth chambers: 1) in the control treatment, plants were well-watered, while the temperature of the growth chamber was kept 22/17 °C; 2) in the heat stress treatment, plants were well-watered, but the temperature of the growth chamber was kept 35/30 °C; 3) in the drought stress treatment, water was withheld and the temperature was kept 22/17 °C; 4) in the combination of heat and drought stress treatment, water was withheld and the temperature was kept 35/30 °C.

Beginning on 2 June 2014, after 28 d of stress treatments, the plants were allowed to recover by returning the temperature to 22/17 °C (day/night) for all growth chambers and plants were watered every other day for 14 d.

PHYSIOLOGICAL MEASUREMENTS. Turf quality, based on leaf color, density, and uniformity of turf, was rated on a 1–9 scale (1 = dead; 9 = fully turgid, green, and dense).

Leaf RWC was measured to evaluate leaf water status. To determine RWC, 0.1 g leaf tissues cut into 1-cm segments were collected and weighed immediately for fresh weight (FW). They were then soaked in deionized water for 24 h in 50-millilitre centrifuge tubes, the tissues were then blotted dry with paper towels, and the turgid weight (TW) was measured. The samples were then dried in the oven at 80 °C for 72 h and dry weight (DW) of tissues was measured. Relative water content was calculated as (FW – DW)/(TW – DW) × 100 (Barrs and Weatherley, 1962).

Leaf photochemical efficiency was determined as an indication of photosynthetic activity. It was expressed as the ratio of variable fluorescence to maximum fluorescence (Fv/Fm), which was measured with a fluorescence induction monitor (BioScientific, Hoddesdon, UK).

Leaf chlorophyll content was measured to assess level of leaf chlorosis or senescence. To determine chlorophyll concentration, leaf tissue (0.1 g) was cut into 1-cm segments and soaked in 10 mL dimethyl sulfoxide (DMSO) in the dark for at least 72 h (Hiscox and Israelteh, 1979). A 0.5-mL aliquot of the extract was mixed with 1 mL of DMSO and the absorbance at 645 and 663 nm was measured (Spectronic Instruments, Rochester, NY). Chlorophyll concentration was calculated as described by Arnon (1949).

Leaf EL was determined as the indication of cell membrane stability (Blum and Ebercon, 1981). For EL determinations, fresh leaf tissue (0.2 g) was cut into 1-cm segments, rinsed with deionized water and shaken in 40 mL deionized water on a conical flask shaker (Laboratory-Line Instruments, Melrose Park, IL) for 15 h. The initial conductivity (C₁) was measured with a conductivity meter (model 32; YSI, Yellow Springs, OH). The leaves were then killed by autoclaving at 121 °C for 20 min. The solution was shaken for 15 h and then the final conductivity (C₂) was measured. EL was calculated as C₂/C₁ × 100.

Leaf membrane lipid peroxidation was evaluated by measuring the content of MDA, which is the final product of lipid peroxidation, following the procedure described in Dhindsa et al. (1981). For determination of MDA content, frozen leaf tissue (0.25 g) was ground into a powder with liquid nitrogen and placed into 2 mL 0.1% trichloroacetic acid (TCA). The samples were centrifuged at 12,000 g for 15 min and 1 mL of the supernatant was mixed with 4 mL reaction solution containing TCA and thiobarbituric acid (40:1, v/v). The samples were heated in an incubator at 95 °C for 30 min then cooled quickly in an ice water bath. The samples were again centrifuged at 12,000 g for 15 min. The absorbance of the supernatant at 532 and 600 nm was
measured. The value for the non-
specific absorption at 600 nm was
subtracted from the 523 nm read-
ning. The MDA concentration was
calculated using the extinction co-
efficient of 155 mM$^{-1}$cm$^{-1}$ (Heath
and Packer, 1968).

**Statistical analysis**. This ex-
periment was a completely ran-
domized block design. Each of the
four growth chambers was consid-
ered a single block. Statistical sig-
nificance was tested using the
analysis of variance procedure
(ANOVA) in SAS (version 9.3;
SAS Institute, Cary, NC). Differ-
ences between treatments were sep-
parated by Fisher’s protected least
significance difference (LSD) test
at the 0.05 $P$ level. Bonferroni
posttests were carried out on the
data for each treatment at each
rating date to compare the data
from each of the endophyte-
infected plants to that of the
endophyte-free plants by using the
PRISM 4 program (GraphPad Soft-
ware, San Diego, CA).

**Results**

There were similar trends in turf appearance, TQ ratings, and
RWC among the endophyte-free, RC endophyte-infected,
and DE endophyte-infected plants in response to heat, drought,
and heat + drought stresses (Table 1; Figs. 1–3). None of the
data for TQ and RWC of the endophyte-infected plants was
significantly different from the endophyte-free plants except
the 14 d RWC, which was lower for the endophyte-infected
plants. The stress treatments had similar effects on all plant
types. Heat stress had no significant effect on overall appear-
ance, TQ rating, and RWC. Drought and the combination of
heat and drought resulted in reductions in appearance, TQ
rating and RWC. The appearance, TQ and the RWC of both
heat-stressed and drought-stressed plants recovered when
returned to control conditions. However, heat + drought-
stressed plants did not recover by one week after cessation of
the stress.

Endophyte infection had no significant effects on leaf Fv/Fm
in the responses to the stress treatments. Fv/Fm was unaffected
by heat or drought stress treatments for any of the plant types
and was maintained at about 0.8 throughout the stress treatment
(Fig. 4). However, Fv/Fm was severely affected by the
combination of heat + drought stress, which declined to below
0.1 at 28 d of stress in all three plant types.

Leaf chlorophyll levels showed similar responses to the
stresses in all three plant types (Fig. 5). Only the 21 d level in
the DE endophyte-infected plant was significantly different
from the endophyte-free plants. The chlorophyll levels of the
heat + drought-stressed plants of all three plant types were most
severely affected and did not recover after cessation of the
stress.

None of the data for EL of the endophyte-infected plants was
significantly different from the endophyte-free plants except
the 14-d measurement in response to the heat + drought stress,
which was higher for the endophyte-infected plants. EL was
unaffected by heat stress or drought stress in all three plant
types (Fig. 6). However, the combination of heat + drought
stress resulted in a large increase in EL, up to about 80% by 28 d
of stress in all three plant types. After 7 d of recovery from the
stress, EL was reduced from its peak, but was still at about 65%.

Similar trends of MDA content were found in all three plant
types in response to the stresses and there were no significant
differences in MDA content between the endophyte free plants
and endophyte-infected plants under drought, heat, or the
combined heat + drought stress (Fig. 7). The endophyte
significant difference detected by the ANOVA analysis was
the difference between the RC and DE endophyte-infected

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**Table 1. Analysis of variance of effect of *Epichloë festucae* fungal endophyte infection on strong creeping red fescue responses to the heat, drought, and heat + drought stress treatments.**

| Treatment            | Turf quality | Relative water content | Fv/Fm$^*$ | Chlorophyll content | Electrolyte leakage | MDA content$^*$ |
|----------------------|--------------|------------------------|-----------|---------------------|---------------------|-----------------|
| Endophyte            | NS           | *                      | NS        | *                   | ***                 | *               |
| Treatment            | ***          | *                      | NS        | ***                 | ***                 | ***             |
| Endophyte $\times$ treatment | NS           | NS                     | NS        | NS                  | NS                  | NS              |

$^*$Fv/Fm = photochemical efficiency; MDA = malondialdehyde.
NS, *, **, *** = nonsignificant or significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

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Fig. 1. (A) Endophyte-free strong creeping red fescue (E-), Rose City (RC), and Delaware (DE) isolates of *Epichloë festucae* fungal endophyte-infected strong creeping red fescue plants under control conditions and after 28 d of heat, drought, and heat + drought stress conditions. (B) Plants after 14 d of recovery from the stress conditions.

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plants at 21 d of heat + drought stress. Heat-stressed plants had similar MDA contents as the control plants throughout the duration of the test. Drought-stressed plants had increased MDA levels at 28 d of stress, whereas the heat + drought-stressed plants began to show increased MDA levels by 14 d of stress. The MDA levels of the drought-stressed plants returned to control levels by 7 d of recovery but those of the heat + drought-stressed plants did not.

Fig. 2. Turf quality ratings (1 = dead; 9 = fully turgid, green, and dense) of endophyte-free strong creeping red fescue (E-), Rose City (RC), and Delaware (DE) isolates of *Epichloë festucae* fungal endophyte-infected strong creeping red fescue plants in response to heat, drought, and heat + drought stress. Vertical bars are least significant difference values (*P* ≤ 0.05) for treatment comparisons of each plant type at a given day of treatment.

Fig. 3. Leaf relative water content of endophyte-free strong creeping red fescue (E-), Rose City (RC), and Delaware (DE) isolates of *Epichloë festucae* fungal endophyte-infected strong creeping red fescue plants in response to heat, drought, and heat + drought stress. Significant differences between the RC and DE endophyte-infected plants, each relative to the endophyte free plants, are indicated by *** (*P* < 0.001) and * (*P* < 0.05). Vertical bars are least significant difference values (*P* ≤ 0.05) for treatment comparisons of each plant type at a given day of treatment.
Discussion

The beneficial effects of *E. festucae* fungal endophyte infection on strong creeping red fescue regarding insect and fungal disease tolerance are well established (Bonos et al., 2005; Clarke et al., 2006; Saha et al., 1987; Yue et al., 2000). However, the effect of *E. festucae* infection on abiotic stress tolerance of strong creeping red fescue has not been well studied. Only one previous study has addressed the effect of *E. festucae* infection of *F. rubra* during drought stress and concluded that endophyte infection did not increase drought tolerance (Vázquez-de-Aldana et al., 2013). Here we have...
determined the effect of drought stress, heat stress, and the combination of heat + drought stress on TQ, RWC, Fv/Fm, chlorophyll content, EL, and MDA content of endophyte-free and endophyte-infected strong creeping red fescue. Overall, in this study, endophyte status had no effect on the physiological responses of the plants to any of the stress conditions. The endophyte-mediated drought tolerance seen in some Epichloë species–grass species combinations, particularly tall fescue infected with *E. coenophiala*, was not observed in strong creeping red fescue infected with *E. festucae*.

Fig. 6. Electrolyte leakage of endophyte-free strong creeping red fescue (E-), Rose City (RC), and Delaware (DE) isolates of *Epichloë festucae* fungal endophyte-infected strong creeping red fescue plants in response to heat, drought, and heat + drought stress. Significant differences between the RC and DE endophyte-infected plants, each relative to the endophyte free plants, are indicated by *** ($P < 0.001$). Vertical bars are least significant difference values ($P \leq 0.05$) for treatment comparisons of each plant type at a given day of treatment.

Fig. 7. Malondialdehyde (MDA) content of endophyte-free strong creeping red fescue (E-), Rose City (RC), and Delaware (DE) isolates of *Epichloë festucae* fungal endophyte-infected strong creeping red fescue plants in response to heat, drought, and heat + drought stress. Vertical bars are least significant difference values ($P \leq 0.05$) for treatment comparisons of each plant type at a given day of treatment.
Strong creeping red fescue is generally considered to have good drought tolerance but to have poor heat tolerance (Grimshaw et al., 2014; Ruemmele et al., 2003). Our data indicate that drought tolerance of strong creeping red fescue is not related to endophyte infection. Even at 28 d of drought stress, the plants were just beginning to show visible symptoms of stress, such as leaf wilting, and on cessation of stress could fully recover. In contrast to the general perception that fine fescues have poor heat tolerance, under well-watered conditions the plants in this study showed good heat tolerance to 35 °C. Only slight wilting was observed, even after 28 d of high temperature. However, the combination of heat + drought stress caused severe damage to the plants and they were not able to fully recover on cessation of the stress conditions. A similar dramatic detrimental effect of the combined stresses of drought and heat was seen in kentucky bluegrass (Potan pratensis), tall fescue, and perennial ryegrass (Jiang and Huang, 2000, 2001; Yu et al., 2012). The results reported here suggest that the combined drought and heat stress was more detrimental than either drought or heat stress alone for strong creeping red fescue, and adequate irrigation of strong creeping red fescue during summer may be able to mitigate the severe damage observed with the combined stresses. In addition, endophyte infection provided no benefits of promoting plant tolerance to drought, heat, or the combined stress in strong creeping red fescue.

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