

Effect of Endophyte Association with *Brachiaria* Species on Shoot and Root Morpho-physiological Responses under Drought Stress

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Abstract

A greenhouse experiment was conducted at the International Centre for Tropical Agriculture in Colombia to evaluate effects of the fungal endophyte, *Acremonium implicatum*, on growth and physiological responses of five *Brachiaria* cultivars. Plants were grown under well-watered (WW) and drought-stressed (DS) conditions, with (E+) and without (E-) endophyte; and their morpho-physiological responses were determined. Significant two-way and three-way interactions produced variable effects on leaf area, number of tillers, shoot elongation, shoot biomass, total root diameter, diameter of cortex, area of stele and diameter of xylem vessel. Main effect of endophyte significantly increased leaf stomatal conductance and reduced diameter of xylem. Smaller leaf area was found in endophyte-infected than control plants of three cultivars, both under WW and DS conditions, which indicates a cost of endophyte infection to the host cultivars. Large root diameter and area of stele under WW conditions, as well as small diameter of xylem vessels in some cultivars suggests that endophyte may improve efficiency for water uptake and use under different water regimes. Less Root Cortical Aerenchyma (RCA) was observed in endophyte-infected plants of Tully and Cayman than the control, which may influence plant capacity for resource acquisition in *Brachiaria*. Genotype-specific variation among hosts generally segregated the cultivars in terms of their shoot and root responses, based on presence (E+) or absence (E-) of endophyte. However, future studies should examine how association of *A. implicatum* with *Brachiaria* grass affects capacity for water uptake and carbon accumulation, and the role of RCA in these processes.

Keywords: *Acremonium implicatum*; Aerenchyma; *Brachiaria*; Drought stress; Fungal endophyte

Introduction

Prolonged and intermittent drought episodes present a major limitation to forage productivity in sub-Saharan Africa, which negatively affect feed availability in livestock production systems. Although *Brachiaria* grass has a promising potential to fill this forage gap, it is predominantly cultivated in South America [1]. Therefore, widening its cultivation requires research to evaluate and select cultivars with capacity to survive and perform better under severe drought conditions experienced in sub-Saharan Africa.

Apart from their well-known role in plant protection against invertebrate herbivores and pathogens, fungal endophytes that form symbiotic associations with grass species have been shown to enhance growth and persistence under drought conditions [2-5]. Beneficial effects of endophytes on shoot traits such as tiller number leaf expansion, and shoot biomass have been reported [6-8]. Mutualistic associations of grass roots with endophytic fungi have also been reported to increase capacity for water and nutrient uptake, particularly under stress conditions [9].

Perennial grass infected with *Neotyphodium* endophytes in temperate turf grasses is reported to alter host grass physiology, root morphology and function, including increased root growth and biomass, longer root hairs and decreased root diameter [10-12]. Due to

interactions of several factors, some cultivars may benefit from the symbiotic associations while others may not experience benefit under different environmental conditions. Such factors may include limited water and low nutrient availability when Photosynthate is limiting, or when the host is also in association with certain strains of mycorrhizae [13-15].

Although several *Acremonium* species have been reported to improve drought stress resistance in cool-season grasses, there is little information on the role of endophytic colonization of tropical grasses. Tropical forage grasses are grown on marginal lands with limited or no agricultural inputs and their growth and survival depends on a wide range of environmental stresses [16]. Previous studies have investigated the role of *A. implicatum* mainly for its biocontrol property against pathogens such as *Drechslera* fungal pathogen in *Brachiaria* and against *Meloidogyne incognita* in tomato [1,17,18]. There has not been any detailed study on the effects of *A. implicatum* on morpho-physiological responses of *Brachiaria* under drought stress conditions. The objective of this study was to evaluate the effects of *A. implicatum* endophyte on shoot and root growth and physiological responses in selected cultivars of *Brachiaria* grass under drought stress.

Materials and Methods

Plant material, treatments and growth conditions

Before transplanting, pre-germinated seedlings of five selected *Brachiaria* cultivars (Basilisk, Tully, Marandu, Cayman and Mulato II) were soaked in a solution of Tebuconazole (Folicur) fungicide at a concentration of 0.6 mL/L (250 g a.i./L) for 6 hours [19]. Efficacy of disinfection with the fungicide to eliminate natural endophytes was evaluated by microscopic examination of plant leaves.

A total number of 60 seedlings of uniform sizes were selected and transplanted in a greenhouse in transparent plastic cylinders covered with PVC tubes (100 mm diameter and 800 mm length) containing 7 kg Oxisol. The soil was mixed at a 2:1 ratio of soil: sand (w/w), along with six blank cylinders (bare soil without plants) for estimating water losses by surface evaporation. The soil was fertilized to supply adequate level of nutrients for *Brachiaria* grass as recommended by Rao et al. [20]. Plants were grown under 12 hours daylight, maximum photon flux density of 1200 $\mu\text{mol m}^{-2}\text{s}^{-1}$, mean temperatures of 19°C (night) and 31°C (day), relative humidity of ~48% low and 94% maximum. Two weeks after establishment, half (30) of the plants in the greenhouse were inoculated with solution of *A. implicatum* (i.e., E+ plants) using a combination of foliar spray and soil drenching; while the other half (30) were left as control (endophyte-free, E-) plants.

Plants were grown inside a greenhouse for four more weeks after endophyte inoculation. A completely randomized block design was used with three replicates for each of the treatment combinations (endophyte-well-watered, E+_WW, no endophyte-well-watered, E-_WW; endophyte-drought stress, E+_DS, no endophyte-drought stress, E-_DS). After a total of six weeks of growth under WW conditions (i.e., 50% field capacity), DS was imposed on half (30) of the plants by stopping addition of water to DS plants for three weeks (21 days), while the other half (WW plants) were maintained at field capacity by regular supply of water.

Plant growth and morpho-physiological characteristics, including number of tillers, shoot elongation (length), leaf area (using Leaf area meter model LI-3000, LI-COR, NE, USA), leaf stomatal conductance (using leaf porometer, Decagon SC-1), and biomass, were determined. Leaf stomatal conductance was determined on the most recent expanded leaf. Measurements were performed daily between 10:30-12:00.

Microscopic detection of endophyte presence in plants and analysis of roots

On harvest, the PVC tubes were removed and differences in root growth along soil columns were visually analysed for the five cultivars. Both shoot and roots were separated for microscopic analysis of endophytes and root structural characteristics, as well as for biomass determination. Plants were washed with water and four young roots

were cut 10 cm above the apex and then dipped in sterile distilled water. Roots were cleared for 4 hours in 10% KOH at 60°C and transferred into 70% ethanol overnight. The roots were then cleared in 2.5% NaOCl.

Thin root sections were made by free-hand using a sharp entomological razor and stained with a Toluidine blue overnight. The root sections were observed under a Microscope (Model: Carl Zeiss, Göttingen, Germany) fitted with Axiocam ERc5 at $\times 400$ magnification. By appropriate scaling, area of the cortex and stele, and diameter of root xylem vessels were determined in transverse sections.

Statistical analyses

A multivariate three-way ANOVA using General linear model (GLM) was used to determine effects of endophyte (E), water regimes (W), cultivar (C) and their interactions. Post Hoc tests were performed for multiple comparisons of means ($p=0.05$) using SPSS software version 21.

Results and Discussion

Effects of interactions

The study found a significant three-way interaction effects (E \times C \times W) for variable traits, such as leaf area, root diameter, diameter of cortex and area of stele, as shown by the ANOVA in Table 1. Endophyte infection significantly reduced leaf area in three cultivars (Tully, Marandu and Cayman) under WW and DS conditions (Figure 1a and 1b). In Tully, endophyte-infected plants had 11% and 12% smaller leaf area under WW and DS conditions, respectively compared with the control ($p<0.05$). For Marandu, leaf area reduced due to endophyte by 4% under WW conditions, and this effect doubled (8%) under DS conditions. Meanwhile, endophyte-infected plants of Cayman had 11% smaller leaf area than in the control under WW conditions ($p<0.05$); and no significant differences existed under DS conditions ($p>0.05$).

Total root diameter in endophyte-infected plants of Basilisk was 6% greater than in the control under WW conditions, but no significant differences existed under DS conditions (Figure 1c and 1d). In Tully, no significant difference in effect of endophyte was found under WW conditions ($p>0.05$); whereas under DS, endophyte infection increased total root diameter by 12% relative to the control ($p<0.05$). While in Marandu, root diameter was 5% greater in endophyte-infected plants than in the control under WW conditions, and no significant differences occurred under DS conditions. Total root diameter of Cayman was not significantly affected by endophyte infection under WW conditions, but root diameter in endophyte-infected plants was 4% smaller in the control ($p<0.05$) under DS. In Mulato II, total root diameter increased due to endophyte by 6% under WW conditions, but decreased by 4% under DS compared with the control ($p<0.05$).

Source	df	Number of tillers	Stomatal conductance ($\text{mmol m}^{-2}\text{s}^{-1}$)	Shoot elongation (cm)	Leaf area (cm^2)	Shoot biomass (g)
E	1	35(0.022)	143(0.037)	89(ns)	269(<0.0001)	0.77(ns)
C	4	881(<0.0001)	8204(<0.0001)	4784(<0.0001)	8062(<0.0001)	83(<0.0001)
W	1	154(<0.0001)	472(<0.0001)	357(0.002)	724 (<0.0001)	1595(<0.0001)

E × C	4	31(0.002)	174(ns)	147(0.004)	33(0.016)	28(0.041)
E × W	1	3(ns)	57(ns)	130(ns)	2(ns)	11(ns)
C × W	4	12(ns)	83(<0.0001)	21(ns)	69(0.012)	6(ns)
E × C × W	4	8(ns)	71(ns)	56(ns)	57(0.027)	5(ns)
Error		6.3	40	32.7	18.6	11.4
Source	df	Root diameter (mm)	Diameter of cortex (mm)	Area of stele (mm²)	Diameter of xylem vessels (mm)	Total biomass (g)
E	1	0.012(0.019)	<0.0001(ns)	451(ns)	<0.0001(0.015)	0.92(ns)
C	4	0.044(<0.0001)	0.049(<0.0001)	32110(<0.0001)	0.001(<0.0001)	107(<0.0001)
W	1	0.031(<0.0001)	0.001(ns)	7463(0.001)	2.33 × 10 ⁻⁰⁰⁶ (ns)	1815(<0.0001)
E × C	4	0.010(0.002)	0.013(<0.0001)	917(ns)	2.26 × 10 ⁻⁰⁰⁵ (ns)	19(ns)
E × W	1	0.008(0.047)	0.023(<0.0001)	15435(<0.0001)	<0.0001(ns)	14(ns)
C × W	4	0.006(0.0027)	0.009(0.001)	5680(<0.0001)	<0.0001(<0.0001)	26(0.037)
E × C × W	4	0.019(<0.0001)	0.010(<0.0001)	1997(0.021)	5.88 × 10 ⁻⁰⁰⁶ (ns)	10(ns)
Error		0.002	0.001	611	1.54 × 10 ⁻⁰⁰⁵	13

Data presented are mean squares (MS) with p-values (in Parentheses). Significant level: p<0.05; ns=non-significant values.

Table 1: Results of three-way ANOVA for effects of *Brachiaria* cultivars (C), endophyte treatments (E), water regimes (W) and their interactions.

Diameter of root cortex (Figure 1e and 1f) in Basilisk was not significantly affected by endophyte infection under WW conditions ($p>0.05$), while under DS, endophyte-infected plants had 17% greater diameter of cortex than the control ($p<0.05$). In Tully, endophyte reduced diameter of cortex by 7% under WW conditions, but significantly increased diameter of cortex under DS conditions by 22% relative to the control. Diameter of cortex did not differ between endophyte-infected and control plants of Marandu, both under WW and DS conditions ($p>0.05$). However, endophyte induced significant reduction in diameter of cortex of Cayman under WW and DS conditions by 20% and 10%, respectively compared with the control. Meanwhile, diameter of root cortex in endophyte-infected plants for Mulato II was 8% greater than the control under WW conditions ($p<0.05$), with no significant differences found under DS conditions ($p>0.05$).

Endophyte infection increased area of stele in roots of Basilisk under WW conditions by 6%, which was doubled (12%) under DS conditions compared with the control ($p<0.05$; Figure 1g and 1h). In Tully, area of stele in endophyte-infected plants was 11% greater than the control under WW conditions, while no significant differences existed under DS conditions. Area of stele in roots of Marandu was not significantly affected by endophyte both under WW and DS conditions ($p>0.05$). In Cayman, area of stele was 5% and 11% greater in

endophyte-infected plants than the control under WW and DS conditions, respectively ($p<0.05$). Meanwhile, in Mulato II, no significant differences were found under WW conditions, whereas area of stele was 15% greater in endophyte-infected plants than the control under DS.

Significant two-way interactions also existed for several response variables. Cultivar × endophyte interaction produced 12% ($p=0.001$) and 9% ($p=0.005$) greater number of tillers in endophyte-infected plants of Tully and Marandu, respectively than their control (Figure 2a). Shoot elongation of endophyte-infected plants of Tully was 3% higher than control ($p=0.005$), while endophyte-infected plants of Marandu had 6% lower shoot elongation than the control ($p<0.0001$) (Figure 2b). Increase in number of tillers and shoot elongation in endophyte-infected Tully corresponded with 8% more shoot biomass than the control (Figure 2c).

Interaction of cultivar × water regimes resulted into significant differences in leaf area ($p=0.012$), stomatal conductance ($p<0.0001$), area of stele ($p<0.0001$), diameter of xylem vessels ($p<0.0001$) and total biomass ($p<0.05$). Under DS, leaf area in Tully, Marandu, Cayman and Mulato II significantly reduced by 10%, 9%, 6% and 5% respectively, compared with under WW conditions; while no significant differences existed in Basilisk under WW and DS conditions (Figure 3a).

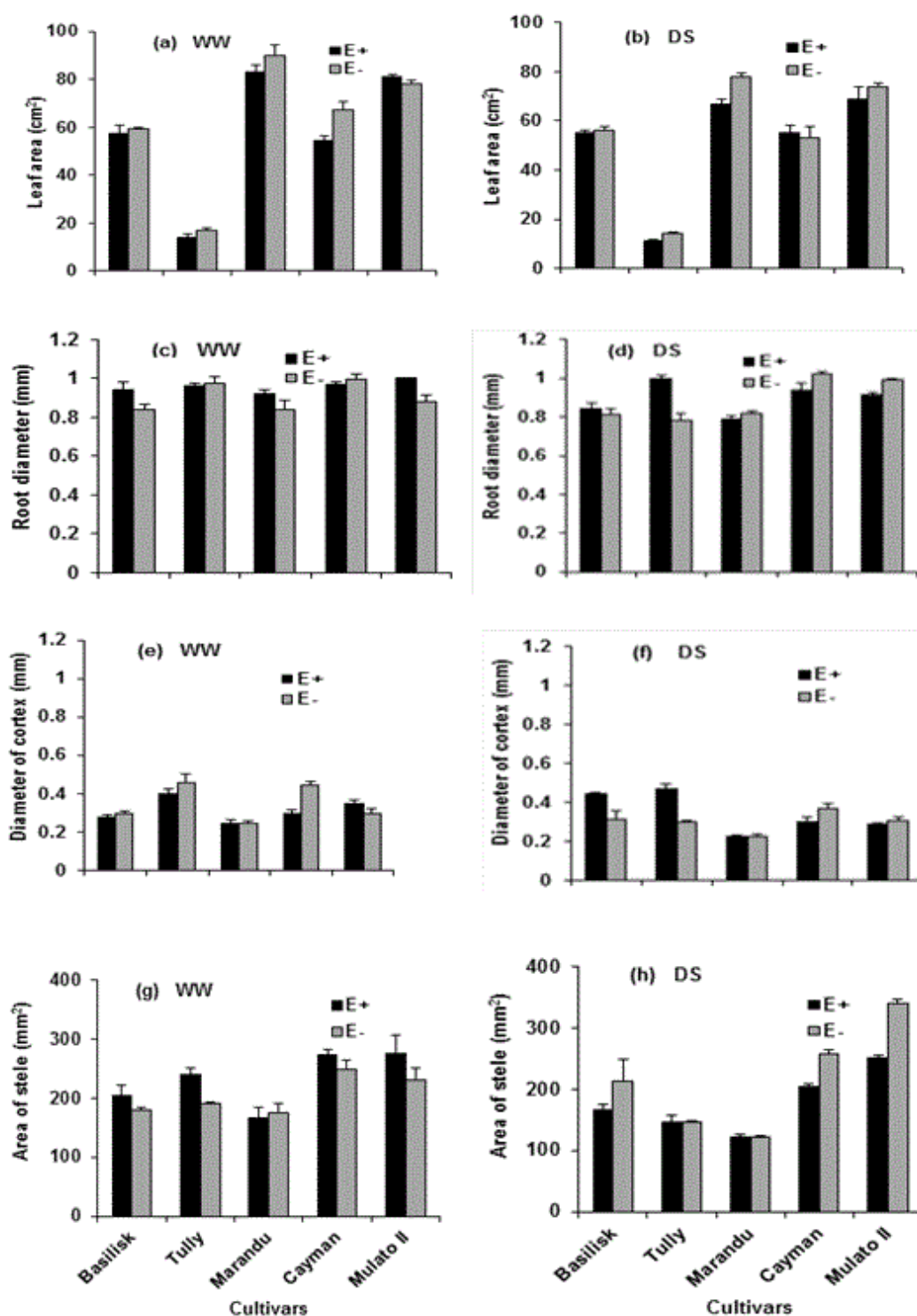


Figure 1: Three-way interaction effects (E×W×C) on leaf area (a and b), root diameter (c and d), diameter of cortex (e and f), and area of stele (g and h). E+= Endophyte treatment, E-= No endophyte treatment; WW= well-watered plants, DS=drought stressed plants Error bars are SE of the means (n=3).

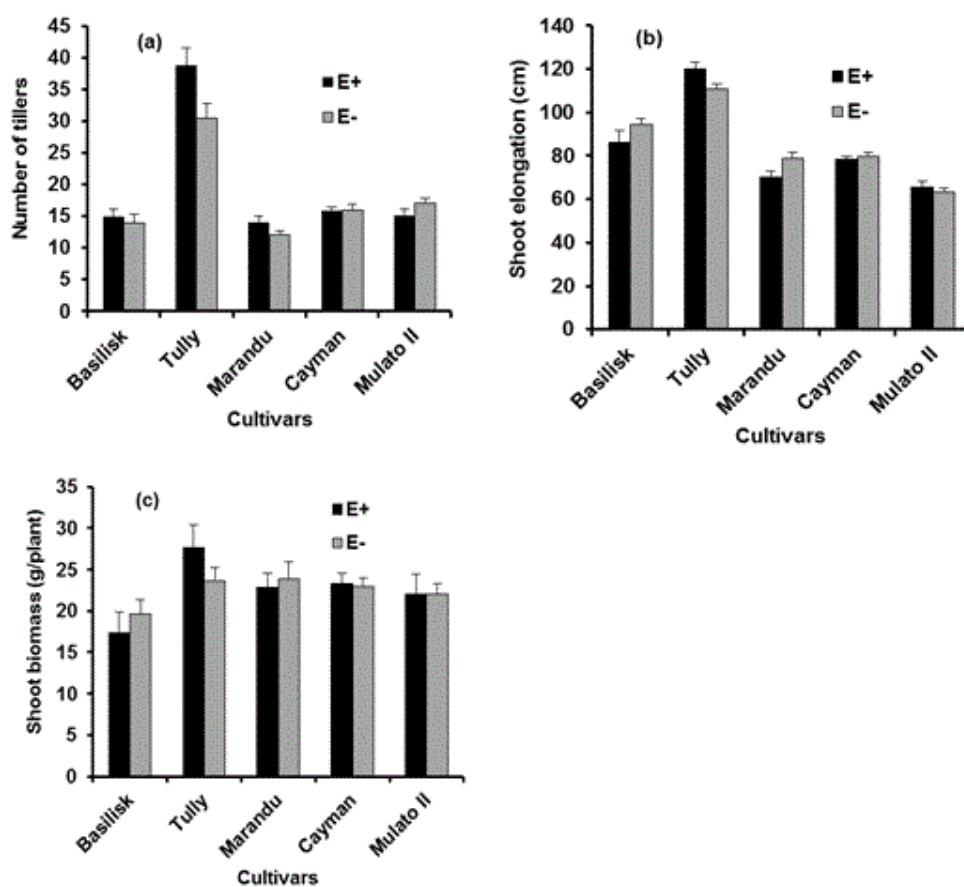


Figure 2: Cultivar \times endophyte interaction effects on number of tillers (a), shoot elongation (b), and shoot biomass (c). E+= Endophyte treatment; E-= No endophyte treatment. Data presented are mean values (n=6) across water regimes. Error bars are SE of the mean.

Stomatal conductance significantly reduced due to DS in all cultivars (Figure 3b), with greater reduction being observed in Basilisk (26%), Tully (39%) and Marandu (31%) than in Cayman (22%) and Mulato II (11%). Area of stele was greater in in three cultivars (Tully by 19%, Marandu by 17% and Cayman by 6%) under WW than DS conditions (Figure 3c). However, area of stele in Mulato II was 8% smaller under WW than under DS conditions; while in Basilisk remained unaffected. Diameter of xylem vessels was significantly smaller under WW conditions in Cayman (by 9%) and Mulato II (by 7%) than under DS conditions (Figure 3d). Total biomass significantly

reduced under DS conditions in all cultivars compared with WW plants, where Mulato II showed the smallest reduction (by 13%).

Microscopic (visual) analysis of Root Cortical Aerenchyma (RCA) showed less RCA in endophyte-infected plants of Tully and Cayman, both under WW and DS conditions (Figure 4). In spite of the observed differences in RCA endophyte-infected and control plants of Tully and Cayman, no observable differences were detected in Basilisk, Marandu, and Mulato II (data not shown).

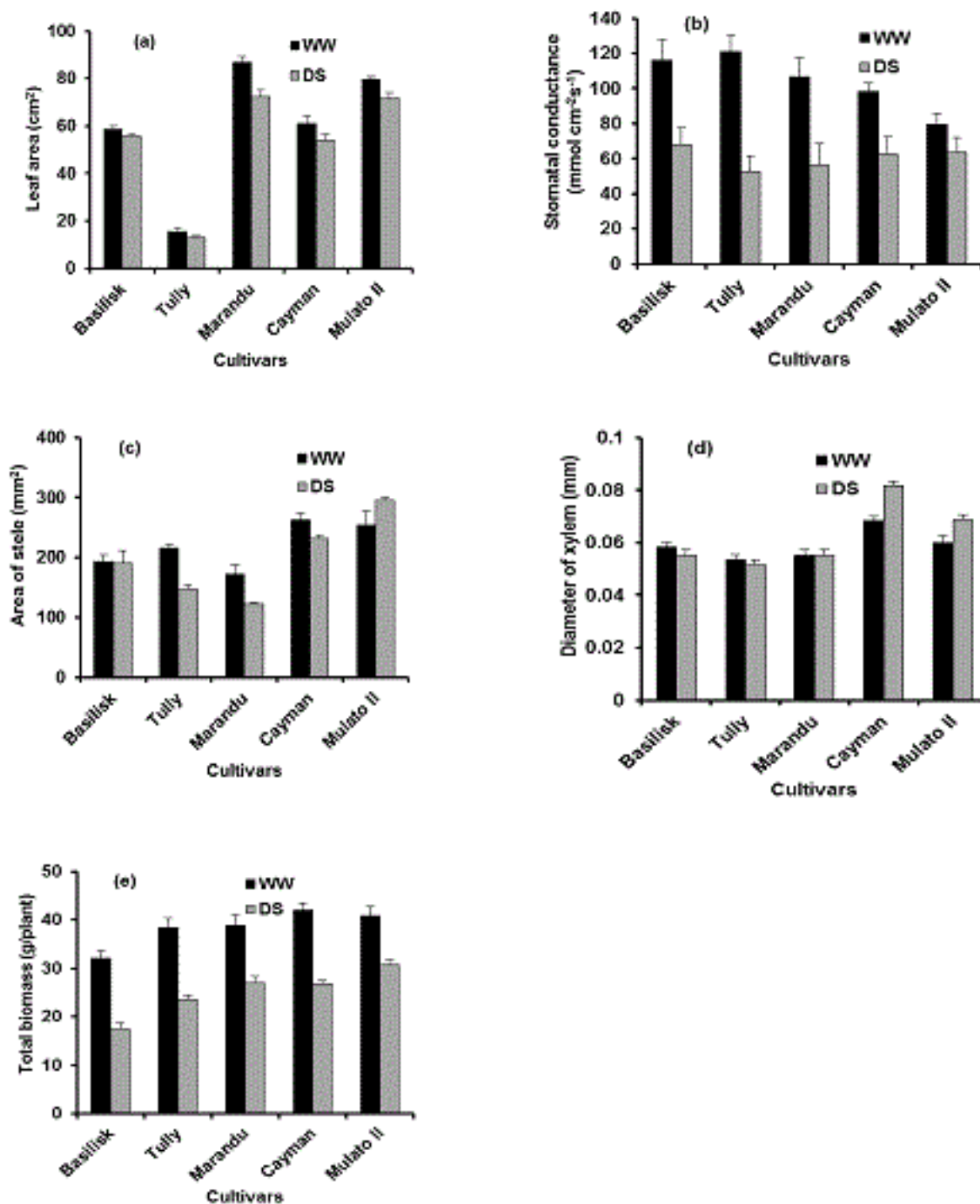


Figure 3: Interaction effects of cultivar × water regimes on leaf area (a), stomatal conductance (b), area of stele (c), diameter of xylem vessel (d), and total biomass (e). WW= well-watered plants; DS= drought stressed plants. Data presented are mean values (n=6) across endophyte treatments. Error bars are SE of the mean.

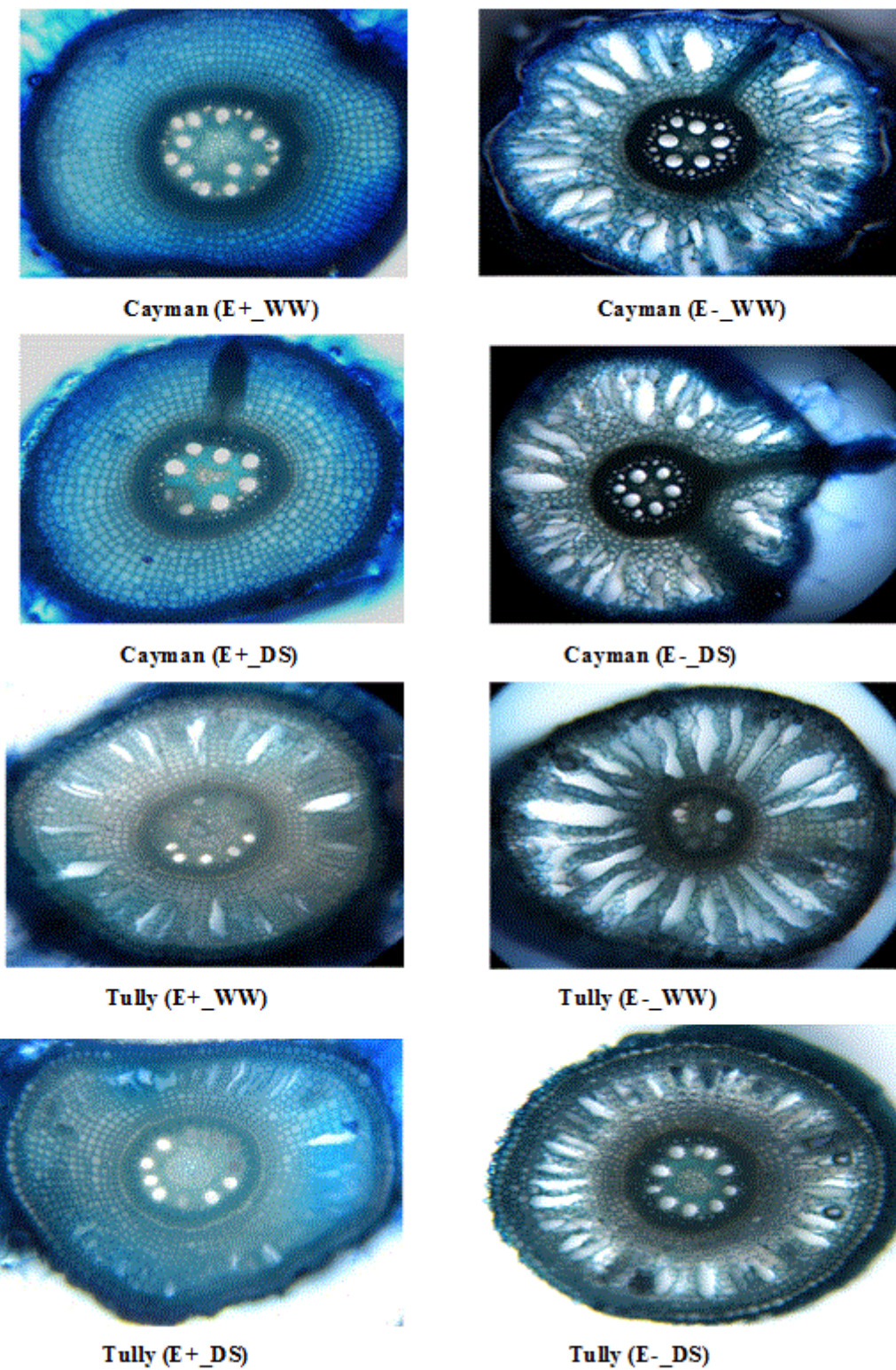


Figure 4: Root Cortical Aerenchyma (RCA) development in endophyte-infected (E+) and control (E-) plants of Cayman and Tully under well-watered (WW) and drought stress (DS) conditions.

Main effects of endophyte, cultivar and water regimes

Main effect of endophyte infection resulted into 7% greater leaf stomatal conductance than the control (Table 2). At the same time, endophyte significantly reduced diameter of root xylem vessel by 3% compared to the control ($p < 0.05$). Leaf area was smallest in Tully with 71% smaller leaf area than that of Marandu, which had the largest leaf area (Table 3). According to area of stele, main effects of cultivar separated the cultivars into two groups, with Cayman and Mulato II having statistically similar but larger area of stele than Basilisk, Tully and Marandu. Under DS, number of tillers, shoot elongation and shoot biomass reduced by 7% ($p < 0.0001$), 5% ($p = 0.002$), and 23% respectively compared with those in WW plants (Table 4).

Endophyte status	Stomatal conductance (mmol m ⁻² s ⁻¹)	Diameter of xylem (mm)
Endophyte (E+)	89.9	0.058
No endophyte (E-)	78.9	0.061
p-value ($p < 0.05$)	0.037	0.015

Data presented are means (n=30) across cultivar and water regimes. Means are statistically significant at $p = 0.05$.

Table 2: Main effects of endophyte on stomatal conductance and diameter of root xylem.

Cultivar	Leaf area (cm ²)	Area of stele (mm ²)
Basilisk	55.4 ^b	191.7 ^b
Tully	13.7 ^a	181.2 ^b
Marandu	79.5 ^c	146.5 ^a
Cayman	54.1 ^b	246.5 ^c
Mulato II	65.6 ^c	274.7 ^c

Data presented are mean values (n=12) for each trait. Superscripts with similar letters are not significantly different (Tukey HSD tests of significance).

Table 3: Main effects of cultivar on leaf area and area of stele.

Water regimes	Number of tillers	Shoot elongation (cm)	Shoot biomass (g/plant)
Well-watered (WW)	19.8	89.1	27.6
Drought stress (DS)	17.4	81.4	17.4
p-value (< 0.05)	< 0.0001	0.002	< 0.0001

Values presented are overall means (n=30) across cultivars and endophyte treatment.

Table 4: Main effects of water regimes on shoot traits.

Variable effects of endophytes are related to high dependence of host-endophyte associations on environmental conditions and genetics of both host and endophyte [21-24]. In the present study, significant three-way and two-way interactions influenced several traits, including leaf area, total root diameter, diameter of cortex, area of stele, number

of tillers, shoot elongation, stomatal conductance, shoot biomass, and diameter of xylem vessels ($p < 0.05$).

A significant three-way interaction denotes that variation in the phenotypic responses of specific *Brachiaria* cultivars was influenced by endophyte presence under different water regimes [22]. Due to strong cultivar-endophyte interaction under WW and DS conditions, some sorting of cultivars may occur since discrimination by natural selection would not simply depend on plant genotype as expected, but also on the presence or absence of endophyte in the host [25]. Consequently, genotypic variation in *Brachiaria* generally segregated the cultivars in terms of their shoot and root responses, based on presence (E+) or absence (E-) of endophyte.

The interactions are usually characterized by both benefits and costs of endophyte infection to host plants [22]. For example, in some cultivars, endophyte association increased number of tillers (in Tully and Marandu), shoot elongation (in Tully and Marandu) and shoot biomass (only in Tully), but reduced leaf area and diameter of xylem in comparison with control plants ($p < 0.05$). Establishment of new tillers is essential toward biomass production, as well as for the perennation of *Brachiaria* and sustainable production of tropical grasslands. Growth of new tillers is controlled by several interacting physiological and environmental variables within individual tillers [26,27]. Endophytic ability to stimulate osmotic adjustment in host plants was proposed to partly explain how endophytes enhance tiller growth and number, and increase in stomatal conductance [28]. This is also demonstrated by the main effect of endophyte infection producing significant increase in stomatal conductance, which could contribute to increased photosynthetic carbon assimilation per unit leaf area [29,30]. Allocation of photosynthates from source to sinks could therefore stimulate growth of new tillers [31,32].

Several studies have also reported greater number of tillers in endophyte-infected than endophyte-free plants of some genotypes in [6,18,25,33,34]. In contrast, Cheplick [35] found less number of tillers, leaf area and biomass in some endophyte-infected genotypes of perennial ryegrass than in endophyte-free plants under both irrigated and drought conditions. Decrease in leaf area due to endophyte infection in some cultivars could be ascribed to either resource allocation to tiller base and root or to endophyte metabolic use of photosynthates supplied by the host [21,36-38].

Reduction in leaf area of Tully, Marandu and Cayman both under WW and DS conditions therefore indicates a cost of endophyte infection to these host cultivars; while Basilisk and Mulato II remained unaffected. Similarly, Cheplick and Cho [25] reported that four genotypes of perennial ryegrass (*Lolium perene*) had less leaf area when infected with *Neotyphodium lolii* while two genotypes were unaffected. Such variation has been suggested to arise due to strong influence of host genotypes on the concentration and distribution of endophytic hyphae within leaves [39]. Therefore, genotypic variation among *Brachiaria* cultivars, in relation to their evolutionary ecology and response to endophyte infection under WW and DS conditions, probably accounted for the different responses observed [22].

Larger root diameter and area of stele found in endophyte-infected plants of four cultivars under WW conditions implies greater root hydraulic than in the control. In addition, a large root system in *Brachiaria* has been suggested to maximize carbon assimilation under conditions of available soil water [40-43]. However, this may not be so under DS because, increased efficiency for maintaining water acquisition and plant productivity under DS occurs for small root

diameter and small diameter of xylem vessels [41,44,45]. In addition to significant main effect of endophyte in reducing diameter of xylem vessel, reduction in root diameter (in Cayman and Mulato II), diameter of cortex (Cayman), and area of stele (in Basilisk, Cayman and Mulato II) under DS indicates an adaptation for conservative water use under DS conditions [40,41,46-48].

Respective decrease and increase in diameter of cortex due to endophyte in some cultivars under WW and DS conditions may be due to changes in cellular osmotic conditions. Low cellular osmotic adjustments in endophyte-infected plants under WW conditions might induce less turgor and less cell expansion within the cortex. This is because high osmotic adjustments under DS could be associated with increased cell turgor and more cell wall expansion and larger diameter of cortex in endophyte-infected plants (of Basilisk and Tully) than in the control under DS. However, this may not be so for all cultivars as demonstrated in the results [49].

Significant development of RCA in nodal roots of Tully has been reported both under well-drained and waterlogged conditions [50]. Previous study suggested that RCA increases nutrient and water acquisition, and therefore improves plant performance by reducing metabolic (carbon) cost of soil exploration under DS in maize [52]. However, Yang et al. [53] reported that RCA impeded radial movement of water through root cortex and reduced water uptake in rice under DS. Despite the conflicting results, previous studies did not assess how RCA formation might be affected by endophyte infection. In the present study, low RCA development in endophyte-infected plants of Tully and Cayman may affect capacity of plants toward water and nutrient extraction for plant growth. However, it is necessary that future studies examine how endophyte association could affect capacity for water uptake and carbon accumulation in relation to RCA formation in *Brachiaria* grass.

The study shows that DS had a profound effect on stomatal conductance. Significant reduction in leaf stomatal conductance under DS substantially hinders carbon assimilation [30]. This could be responsible for the significant decrease in total biomass in all cultivars under DS. Large size of root anatomical features accounts for difference in water extraction ability in *Brachiaria* under water stress [43,54,55]. This implies that Cayman and Mulato II have superior water extraction ability than other cultivars, as demonstrated by their larger area of stele compared to other cultivars.

Conclusion

The present study showed significant two-way and three-way interactions on several response variables. Interactions of endophyte with specific cultivars increased number of tillers in two cultivars and increased shoot biomass in one cultivar. However, most traits were mirrored by interaction of endophyte × water regime × cultivar; which generally reduced leaf area in endophyte-infected plants of two cultivars both under WW and DS conditions. Main effect of endophyte significantly increased leaf stomatal conductance and reduced diameter of xylem vessels. Total root diameter was larger under WW but smaller under DS in endophyte-infected plants of some cultivars compared to control plants. Large root diameter and area of stele under WW conditions, as well as small diameter of xylem vessels under DS observed in the study may be associated with endophyte-regulated adaptation toward efficient water uptake and use under WW and DS conditions, respectively. Low RCA observed in endophyte-infected plants of two cultivars (Tully and Cayman) may affect plant

potential toward water and nutrient acquisition for plant growth. It is proposed that significant benefits from the endophyte might be negated by endophyte metabolic demand for photosynthate supplied by the host. However, it is necessary that future studies examine how endophyte association affects capacity for water uptake and carbon accumulation in relation to RCA formation in *Brachiaria* grass.

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Conflict of Interest

This study involved no conflict of interest from any party.

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