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Dissertation

Drought tolerance in tough lovegrass (*Eragrostis plana*) and their implications on competitiveness relations with *Paspalum notatum* and chemical control

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To my parents, Vicente and Ivonia

I DEDICATED MY DISSERTATION

“Never be sure of anything

because wisdom begins with doubt”

(Sigmund Freud)

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Resumo

BASTIANI, Marlon Ouriques. **Tolerância ao estresse hídrico em capim-annoni (*Eragrostis plana*) e suas implicações nas relações competitivas com *Paspalum notatum* e no controle químico.** 2019. 108f. Tese (Doutorado) - Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas, Brasil.

Eragrostis plana Nees (capim-annoni) é uma planta daninha em áreas de pastagens nativas da América do Sul. Apresenta baixa qualidade forrageira se comparada com *Paspalum notatum*, citada como uma das mais importantes forrageiras distribuídas nos campos nativos do sul do Brasil. *E. plana* possui características comuns de espécies invasivas, as quais lhe conferem vantagem competitiva em áreas degradadas, incluindo rápido crescimento, longa fase reprodutiva, potencial efeito alelopático, banco de sementes persistente, tolerância à geadas e déficit hídrico. Considerando essas características, as atuais e esperadas mudanças nos padrões de precipitação pluviométrica, os objetivos deste trabalho foram: determinar o estágio de desenvolvimento onde *E. plana* e *P. notatum* exibem a maior e menor tolerância ao déficit hídrico, quantificando as mudanças morfológicas, fisiológicas e bioquímicas das espécies em resposta ao estresse (Capítulo I); determinar se as condições de seca aumentariam a competitividade de *E. plana* com *P. notatum* (Capítulo II) e; investigar o efeito do estresse hídrico na eficiência do controle químico com diferentes sais de glifosato, através de análise da associação com sulfato de amônio como adjuvante, na absorção e translocação do herbicida em *E. plana* (Capítulo III). Os resultados demonstram que ambas espécies exibiram maior tolerância ao déficit hídrico no estágio de perfilhamento comparado com o estágio reprodutivo e; a regulação estomática inicial foi fator chave para evitar perdas de água em *P. notatum*, enquanto que plantas de *E. plana* apresentaram fechamento estomático mais tardio, porém tolerando baixo teor relativo de água na folha por mais tempo. As duas espécies usaram mecanismos para dissipar o excesso de energia dos fotossistemas sob déficit hídrico, associados a um eficiente sistema antioxidante e à biossíntese de osmólitos compatíveis. As espécies apresentaram mecanismos similares para tolerar o déficit hídrico, entretanto a taxa de desidratação/reidratação foi relativamente diferente (Capítulo I). Mesmo apresentando reduções de crescimento, tanto E. plana quanto *P. notatum* sobreviveram a ciclos contínuos de déficit hídrico. No entanto, *E. plana* sobrepôs o crescimento da Poaceae nativa *P. notatum*, tanto em condições de níveis adequados de água no solo, quanto em déficit hídrico (Capítulo II). Não houveram diferenças entre os sais de glifosato na eficácia do controle de *E. plana*, independentemente do estádio de desenvolvimento, sendo o estágio de perfilhamento mais tolerante ao herbicida. Além disso, o déficit hídrico causou redução na eficácia de glifosato no controle de *E. plana*, onde a adição de sulfato de amônio nas aplicações do herbicida superou o efeito antagonista do estresse. A melhoria na eficácia do glifosato com adição de sulfato de amônio pode ser explicada pelo aumento na translocação do herbicida na planta. Em síntese, os resultados indicam que *E. plana* e *P. notatum* são tolerantes à seca, independentemente do estágio de crescimento, e a tolerância é devida à ativação de um conjunto de mecanismos morfológicos, fisiológicos e bioquímicos, os quais provavelmente melhoram a habilidade competitiva de *E. plana* com *P. notatum*. O estresse hídrico também reduz a eficácia do glifosato, no entanto, a adição de sulfato de amônio como adjuvante ao glifosato pode superar os efeitos negativos do déficit hídrico, devido ao aumento na translocação do herbicida.

Palavras-chave: potencial invasivo, seca, eficácia de glifosato, sulfato de amônio, invasora de pastagem.

Abstract

BASTIANI, Marlon Ouriques. **Drought tolerance in tough lovegrass (*Eragrostis plana*) and their implications on competitiveness relations with *Paspalum notatum* and chemical control.** 2019. 108p. Dissertation (Doctor in Plant Protection) - Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas, Brasil.

Eragrostis plana Nees (Tough lovegrass) is an invasive weedy grass in several native pasture areas of South America. It has low forage quality compared with *Paspalum notatum,* which is one of most important native forage distributed in livestock areas in South of Brazil. *E. plana* has traits of an invasive grass that confer a competitive advantage under degraded areas, including rapid growth, a long reproductive phase, a potential allelopathic effect, a persistent seed bank and tolerance to freezing and drought conditions. Thus, considering this plant traits and currently global changes on rainfall patterns, the objective of this research were: to determine the growth stage where *E. plana* and *P. notatum* exhibit the highest and lowest drought tolerance and then quantifing changes in morphological, physiological and biochemical traits of *E. plana* and *P. notatum* in response to water deficit (Chapter I); to determine whether drought conditions would increase *Eragrostis plana* competitiveness with the forage grass *Paspalum notatum* (Chapter II); to investigate the effect of water stress in the efficacy of chemical control with different glyphosate salts, through analyses of ammonium sulfate association as adjuvant on absorption and translocation of glyphosate herbicide in *E. plana* (Chapter III). The results showed that both species exhibited the highest drought tolerance at tillering stage compared to reproductive growth stage and; the early stomatal regulation was the key factor to avoid water loses in *P. notatum*, while *E. plana* plants had late stomatal closure, however tolerating low leaf relative water content for a longer time. The two species used mechanisms to dissipate energy excess from photosystems under drought, associated with an efficient antioxidant system also linked to biosynthesis of compatible osmolytes. The species shared similar mechanisms to tolerate water deficit, however the rate of dehydration/rehydration was quite different (Chapter I). Even suffering fitness penalties on growth, both *E. plana* or *P. notatum* survived to continuous cycles of drought. However, *E. plana* had overcome the native grass *P. notatum* either in well-watered or drought conditions (Chapter II). There were no relevant differences among salts of glyphosate in the efficacy of *E. plana* control regardless of stage, being the full tillering the most glyphosate-tolerant growth stage. Besides, water shortage caused severe reduction on efficacy of glyphosate in *E. plana* control, where the addition of ammonium sulphate in the herbicide applications could overcome antagonist effect of drought. This enhanced performance of glyphosate efficacy when adding ammonium sulfate was explained by increased translocation of the herbicide in the whole plant. Overall, the results suggest that *E. plana* and *P. notatum* are tolerant to drought regardless of growth stage and the tolerance is due to activation of a set of several morphological, physiological and biochemical mechanisms, where these seem to improve *E. plana* competitive ability with *P. notatum*. Besides that, drought stress reduces glyphosate efficacy, however adding ammonium sulfate as an adjuvant in the tank mixture can overcome the negative effects of water shortage, by increasing herbicide translocation.

Key-words: invasive potential, water stress, glyphosate efficacy, ammonium sulfate, weed pasture.

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Summary

1. INTRODUCTION

The area occupied with natural pastures is around 7,519,347 hectares representing about 27% of Rio Grande do Sul (RS) state (IBGE, 2017) mainly concentrated in the Pampa Biome. There is a high diversity of species in these areas where it has been reported approximately 520 species of Poaceae family, 600 of Asteraceae and 250 Fabaceae species (BOLDRINI, 2009), among other families which represent the main feed source for cattle and sheep.

The extensive livestock is an activity extremely important to the Brazilian economy, although the lack or inadequate management has resulted in a degradation of native pastures, mainly due to overgrazing. The native pasture degradation, composed by several species and mainly represented by *Paspalum notatum* Flügge according to the present study area, resulted in decreasing the frequency and richness of species, resulting in an open space to weeds like tough lovegrass.

The tough lovegrass (*Eragrostis plana* Nees) was introduced accidentally in Brazil, in 50's years, as a seed contaminant of *Chloris gayana* Kunth, which also was spread as a forage species in the RS. Just a few years later tough lovegrass was diagnosed as a forage of low quality to feed animals (REIS, 1993). According to the last report, it is estimated that *E. plana* is spread over 2 millions of hectares in the state, where the species is the main weed in natural pastures under grazing or degraded (MEDEIROS; FOCHT, 2007; ZENNI; ZILLER, 2011), and the invasion continues steadily towards the neighboring countries, as identified by Barbosa et al (2016), where there are an optimum climatic conditions for the species development across the South America, and also predicted changes on precipitation patterns including drought events is likely to favor the invasion by *E. plana* (BARBOSA et al., 2016).

The long periods of selective grazing and/or animal load in areas of native pasture has decreased the competitive capacity of native species with high forage quality and consequently replacing these species to species of low forage quality (INOUE et al., 2012), like tough lovegrass. Besides that, the tolerance to drought may increase the competitive ability of tough lovegrass with native species when exposed to low water availability environments (ABICHEQUER et al., 2009).

In the Pampa Biome, lower-growing rhizomatous and stoloniferous grasses, such as *P. notatum* or *Axonopus affinis* have high dominance, at least under higher grazing pressure (OVERBECK et al., 2007). *P. notatum* is reported to be resistant to animal trampling, intensive grazing (OVERBECK et al., 2018), to cold and freezing, drought and to the capacity of growing in low nutritional soils (CHEN et al., 2019).

According to predictions of the Intergovernmental Panel on Climate Change (IPCC, 2014), ranging the region to region, it is suggested that several areas of the planet would exhibit increases on temperature and periods of drought in the summer. Besides that, it is known that water scarcity is currently observed over the last years in the medium south, where the rain events are less frequent if compared to the north side of the state (MATZENAUER et al., 2007). As a result of that south medium has become favorable to invasion by tough lovegrass in the last years; firstly, due to climate conditions suitable to tough lovegrass spread; and secondly in this region are located substantial areas of native pastures, normally subjected to overgrazing or under degradation process.

Tough lovegrass have several traits which confers adaptation to a vast range of environments, including: high rate of seed production (REIS, 1993), deep root system (ABICHEQUER et al., 2009), medium tolerant to low land soils (KOTZE; O'CONNOR, 2000), biosynthesis of allelopathic compounds (FAVARETTO et al., 2019), herbicide tolerance (GOULART et al., 2012), long period of seed viability in soil (MEDEIROS et al., 2014), among others mechanisms which contribute to invasive potential of this species.

Plants of tough lovegrass possess basal architecture with constant renovation of basal shoots, dense and compact leaves in tiller bases, high tillering rate and biomass allocation to roots (SCHEFFER-BASSO et al., 2012), which confer a roots system deep and aggressive (ABICHEQUER et al., 2009). These traits can be indicative of superior competitive ability compared to native species, as well as it may result in better efficiency of water uptake in the soil.

The water shortage impacts directly over the species growth and development, resulting in alterations on the physiological, biochemical and molecular process

(GOMES; PRADO, 2007). The plants have developed over the decades mechanisms to tolerate water stress, which involve several integrated events ranging from perception and tradition of signals to gene regulation and metabolic plant changes (SÁNCHEZ-MARTÍN et al., 2015).

Plants dispose of efficient system of antioxidative defense, that include the activity of several enzymes or non-enzymatic oxidant which avoid or decreasing damages caused by oxidative stress (LI; PENG; MA, 2013). Also plants can alter their morphology under drought conditions, as well as, increasing wax content, lignification of foliar lamina, increasing on cell wall hardness, among other strategies that help to keep cell turgor (BALSAMO et al., 2006).

Some of strategies to control tough lovegrass at native pastures has been the non-selective herbicide spray. Herbicide efficacy can be compromised by water shortage where target plants are growing, which have a direct effect on herbicide absorption, translocation, and metabolism (ABBOTT; STERLING, 2006). It is important highlights that absorption is limited by herbicide amount crossing the cuticle, as well as due to characteristics of herbicide and the weed growth stage (DEUBERT, 1992).

The failure of herbicides to control tough lovegrass has been reported over the years, even when using grass herbicides, like inhibitors of acetyl-CoA carboxylase (ACCase) (GONZAGA; GONÇALVES, 1999; GOULART et al., 2012; GOULART et al., 2009). The low level of control could be associated with innate traits of the weed to tolerate herbicide and/or due to environmental influence like water shortage during the operation of herbicide spray.

Periods of water shortage affect directly the growth of native species leaving opened spaces to invasive plants like tough lovegrass. In this context, there is a lack of researches related to mechanisms that give to tough lovegrass tolerance to drought, so aiming to elucidate what is the strategies used by invasive species responsible to be more competitive than native species. Besides, the knowledge of main factors that affect the herbicide absorption and translocation, associated to the adequate growth stage has the potential to optimize herbicide use to control tough lovegrass in native pasture, reducing environmental impact and the processes of invasion of this important weed of native pastures.

E. plana is considered the main weed of pastures in Pampa biome, with competitive characteristics that stand out over local species, where currently is spread over the state of Rio Grande do Sul. Here, the chosen species were expected to have different mechanisms of drought adaptations, where some mechanisms of *P. notatum* have been described in the literature before, although the strategies of drought tolerance in *E. plana* are still unknown. Considering *E. plana* as an weed originated from dry regions of South Africa and due to the success of establishment across the Southern Brazilian grasslands (some areas also subjected to dry seasons), we hypothesize that *E. plana* has evolved mechanisms of faster responses to deal with water stress compared to the *P. notatum*, where these mechanisms would improve the competitive ability of the weed over the native forage. Also, we hypothesize that *E. plana* exhibit the highest tolerance to drought stress at vegetative stage and also during this stage the plants is likely to be more tolerant to glyphosate, which could be even less efficient under conditions of water scarcity. These assessments may provide clues about the distribution and spread of the *E. plana* as well the currently and future permanence of the native species *P. notatum* under drought environment in Southern Brazilian grasslands.

2. CHAPTER I – Analytical approach of drought tolerance mechanisms in *Eragrostis plana* and *Paspalum notatum*

2.1 Introduction

Eragrostis plana Nees known as tough lovegrass or South African lovegrass (capim-annoni in Brazil) is a C_4 perennial grass native to South Africa (KISSMANN, 2007). This species was accidentally introduced in Brazil in 1957 (REIS, 1993), and currently, tough lovegrass is spread in more than two million of hectares in RS (MEDEIROS; FOCHT, 2007; ZENNI; ZILLER, 2011) being considered as one of the most important weed species in South Brazilian natural grasslands (GUIDO et al., 2016). Among some others traits of invasiveness, the tolerance to drought assumes an important predictor of how *E. plana* is successful distributed in Southern Brazil (ABICHEQUER et al., 2009).

Drought stress occurs in plants either when the water supply to roots becomes limited/unavailable or when the transpiration rate becomes high (ANJUM et al., 2011). Water supply depends on rainfall events, while transpiration rate is dependent on interactions of some climatic variables like air temperature, relative humidity, wind, soil water, plant traits, etc. Gene expression profiling and a cluster analysis suggested that transcriptomic regulatory mechanisms controlling water stress responses in *Setaria italica* were greatly affected by genotype *versus* environment interactions (TANG et al., 2017). Periods of soil water deficit are relatively frequent in Southern Brazil (mainly in the Pampa Biome), as well as future climate changes will exacerbate this problem. According to the last report of IPCC (2014), changes in precipitation and air temperature are expected to happen. This will not be uniform across the world, where several mid-latitude and subtropical dry regions, mean precipitation is likely to decrease, where at the same time rising air temperature and heat waves might occur simultaneously with a higher frequency and longer duration.

Water shortage can alter a number of biological aspects of invasive weeds including germination, plant size, seed production, and the distribution of waterborne seeds (ZISKA et al., 2011). These aspects can directly impact the invasive potential of tough lovegrass with native species. In general, natural grasslands are characterized by having a high number of grass species with different growth strategies (MARQUES et al., 2017), like the genus *Paspalum* L. especially the native species *Paspalum notatum* Flügge (popularly known as Bahiagrass or grama-forquilha in Brazil) (BOLDRINI, 2009). Bahiagrass is a C_4 grass considered as high-quality forage and high resistance to grazing in native pastures of Southern Brazil. Also, *P. notatum* has been reported to have large and a deep fibrous root system (ACUÑA et al., 2010) which could be associated with plant ability to recover water from deep soil layers and thereby expression of drought tolerance (BENERAGAMA et al., 2018).

Cell growth is considered one of the most drought-sensitive physiological processes due to the reduction in turgor pressure (ANJUM et al., 2011). Water stress caused impaired mitosis; cell elongation and expansion resulted in reduced growth and yield traits (HUSSAIN et al., 2018). Initially, roots produce abscisic acid (ABA) and then translocate it to the shoots *via* xylem causing stomatal closure under water deficit. Thus, decreased photosynthetic activity could be explained by reduced biochemical processes of CO_2 fixation caused by different factors, ranging from “stomatal” limitations for CO_2 entry to “non-stomatal" mechanisms, like mesophyll conductance, limited synthesis of ribulose-1,5-bisphosphate (RuBP), enzymes inhibition of the Calvin cycle (BERTOLLI; RAPCHAN; SOUZA, 2012), disturbances in transport and distribution of assimilates, reduced chlorophyll synthesis, functional and structural changes in chloroplasts (ANJUM et al., 2011). In this situation, excess of energy for CO_2 fixation results in surplus energy that must be dissipated; however, the observed increase in the nonphotochemical quenching (NPQ) is often inadequate, resulting in the production of reactive oxygen species (ROS) that can damage the ATP synthase and the photosystems (particularly the D1 protein of PSII) leading to a reduction of electron transport and photophosphorylation (BERTOLLI; RAPCHAN; SOUZA, 2012).

Reactive oxygen species (ROS) can seriously damage plants by increasing lipid peroxidation, protein degradation, DNA fragmentation and ultimately cell death (MITTLER, 2017). As a result, overproduction of ROS increases the content of malondialdehyde (MDA) which is a suitable marker for membrane lipid peroxidation (ANJUM et al., 2011). To minimize the effects of oxidative stress, plants have evolved a complex enzymatic and non-enzymatic antioxidant system, such as low-molecular-mass antioxidants (glutathione, ascorbate, carotenoids) and ROS scavenging enzymes (superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) (MITTLER, 2017).

Plants show different physiological strategies to “escape”, “avoid” or “tolerate” drought stress under a short time-scale (measurement of tissue organ or whole-plant individual) (VOLAIRE, 2018). Some strategies including efficient stomatal regulation to maintain internal water status, osmotic regulation, enzymatic and non-enzymatic antioxidant system (ASHRAF; FOOLAD, 2007; BERTOLLI; RAPCHAN; SOUZA, 2012; FOYER, 2018; HUSSAIN et al., 2018; MERILO et al., 2018; PER et al., 2017; VELIKOVA; YORDANOV; EDREVA, 2000). Relative water content (RWC) is considered a measure of plant water status, reflecting the metabolic activity in tissues and used as the most meaningful index for dehydration tolerance (ANJUM et al., 2011). RWC is related to water uptake by the roots as well as water loss by transpiration (ANJUM et al., 2011). Under low RWC, plants accumulate different types of organic and inorganic solutes in the cytosol to lower osmotic potential thereby maintaining cell turgor, among them proline, sucrose, soluble carbohydrates, glycine betaine as a mechanism of stress tolerance (IQBAL et al., 2014; PER et al., 2017). Also, decreasing on aerial biomass play an important role in reducing total plant water losses, ensuring cell turgor and therefore in the survival of many species especially under drought, where it has been considered as a dehydration avoidance mechanism (GEPSTEIN, 2004; MUNNÉ-BOSCH; ALEGRE, 2004; VOLAIRE, 2018).

In this study, we assessed the changes in photosynthesis, chlorophyll fluorescence, water relations and morphological parameters under water stress in *E. plana* and *P. notatum*. The chosen species were expected to have different mechanisms of drought adaptations. Some mechanisms of *P. notatum* have been described in the literature before, whereas the strategies of drought tolerance in *E. plana* are still unknown. Considering *E. plana* as an invasive weed originated from dry regions of South Africa and due to the success of establishment across the Southern Brazilian grasslands (some areas also subjected to dry seasons), we hypothesize that *E. plana* has evolved mechanisms of faster responses to deal with water stress compared to the *P. notatum*. These assessments may provide clues about the distribution and spread of the invasive species *E. plana* as well the permanence of the native species *P. notatum* under drought environment in Southern Brazilian grasslands. Thus, to test our hypothesis, this paper aims (i) to determine the growth stage where *E. plana* and *P. notatum* exhibit the highest and lowest drought tolerance and (ii) to quantify the changes in morphological, physiological and biochemical traits of *E. plana* and *P. notatum* in response to water deficit.

2.2 Material and Methods

Two experiments (Experiment I and II) were carried out in order to perform an analytical approach of drought tolerance mechanisms in *Eragrostis plana* and *Paspalum notatum (*cv. Pensacola). Seeds of *E. plana* were collected in a native pasture area of Federal University of Pelotas, Capão do Leão, RS, Brazil (31°80”33.94’ latitude S; 52°49”49.78” longitude W).

The first experiment aimed to identify the growth stage more tolerant to the water stress for the chosen species. The second experiment targeted quantify the changes in physiological and biochemical traits of *E. plana* and *P. notatum* under water deficit.

2.2.1 Experiment I – Growth stage effect on drought tolerance

Plant material and growth conditions

Seeds of *E. plana* and *P. notatum* were sown under greenhouse conditions in pots containing 8kg of sandy loam soil. The pots were watered daily until be established four plants per pot. The establishment was done by sown the seeds in different periods in order to have the following growth stages: 5-6 leaves (January 21st, 2016), tillering (December 21st, 2015), panicle initiation (November 21st, 2015) and grain filling (November 5th, 2015).

Water-Stress Treatment

The plants were maintained at field capacity until the beginning of the water-deficit treatments when they reached the growth stages aforementioned (March 10th, 2016). The plants were subjected to water deficit by maintaining the water content in the soil at 25% and 100% of the total water storage capacity of the pot (C_w). The C_w was determined using the fresh mass of the soil after water saturation (C_{fm}) and the dry mass (C_{dm}) after soil drying for 24 h at 105 °C and applied in the equation $C_w = (C_{fm} - C_{dm})/C_{fm} \times 100$ as described by Santos et al. (2015). At the beginning of water treatment all pots were saturated by water, drained and weighted. Then, the pots were weighted every day by replacing the water lost by evapotranspiration assuming the maintenance of C_w at 25 and 100% and considering 1mL = 1g. The plants were kept under these conditions for 30 days.

The following parameters were measured after 30 days period of water restriction (April 10th, 2016) under each irrigation regime: relative water content (RWC), stomatal conductance (g_s) and biometric parameters (tiller number, panicle/spikelet number per plant, root volume, root and shoot dry mass).

Relative water content (RWC)

The RWC was determined as described by Barrs and Weatherley (1962) following the equation: RWC (%) = [(fresh mass - dry mass)/(water saturated mass - dry mass)] X 100. Leaf sections (±10cm length) were taken from the last fully expanded leaf in one plant for each replicate. Then, these segments were weighted (fresh mass) and immediately placed on a plastic box (11 x 11 x 3.5cm) fully with deionized water for 24h to obtain the water saturated mass. To determine the dry mass, saturated leaf segments were dried at 60°C until the constant weight was reached.

Stomatal conductance

The stomatal conductance (g_s) was performed by using a steady state porometer (Decagon SC1) (Decagon Devices, Inc., Pullman, Washington) between 9 – 11h am at the last totally expanded leaf.

Plant growth analysis

The biometric analysis was performed at the end of the experiment by measuring tiller number, panicle number, root volume, root and shoot dry mass. Root volume was determined according to the water displacement technique where the roots were immersed in a graduate cylinder fully with water and the overflow water volume was measured. The root and shoot dry mass was obtained by weighting the biomass after drying at 65°C until the constant weight was reached.

Data analysis

Data were analyses *via* one-way ANOVA considering the experiment as a completely randomized design (CRD) with two treatments (water conditions). The Student's t-test ($p \leq 0.05$) was used to compare the two water conditions within of each growth stage for *E. plana* and *P. notatum*, by using the software SAS System version 9.0. Data are mean of six replications ± SE (standard error).

A multivariate analysis was performed aiming to identify which growth stage was supposed to be more tolerant to the water stress. To perform the analysis, we took account the entire data set *via* principal component analysis (PCA) by using the software PC-ORD 4.10.

2.2.2 Experiment II – Physiological and biochemical traits of drought tolerance

Plant material and growth conditions

This experiment was carried out in similar way as previously described for Experiment I. However, after determined the lowest tolerant stage to water deficit, the Experiment II was conducted only at “panicle initiation” growth stage of *E. plana* and *P. notatum*. Seeds of *E. plana* and *P. notatum* were sown under greenhouse conditions in pots containing 8kg of sandy loam soil in December 15th, 2016.

Water-Stress Treatment

The plants were maintained at field capacity until the beginning of the water-deficit treatments when the plants reached panicle initiation growth stage (March 18th, 2017). Here, we performed a slow water-deficit experiment where the water stress was induced by suspending irrigation under greenhouse conditions until reach a reduction of less than 75% in stomatal conductance (g_s) in relation to well-watered treatment (Figure 1). At this point, the plants were rehydrated by the maintenance of field capacity until the full recovery of stomatal conductance (100% in relation to well-watered treatment).

Both plant species were daily monitored by measurement of stomatal conductance (g_s) and relative water content (RWC), according to Experiment I. The g_s reduction was calculated based on the values of well-watered treatment (Figure 1). The physiological and biochemical parameters were determined when plants attained g_s reduction of less than 50% (moderate water deficit) and 75% (severe water deficit) along the dehydration phase; and when g_s recovered more than 50% (medium recovery) and 100 (full recovery) during the water rehydration period (as indicated by the references lines in Figure1). At harvest time for biochemical evaluations, six biological replicates of leaves were removed from each treatment and kept frozen (-80°C) until analysis be performed.

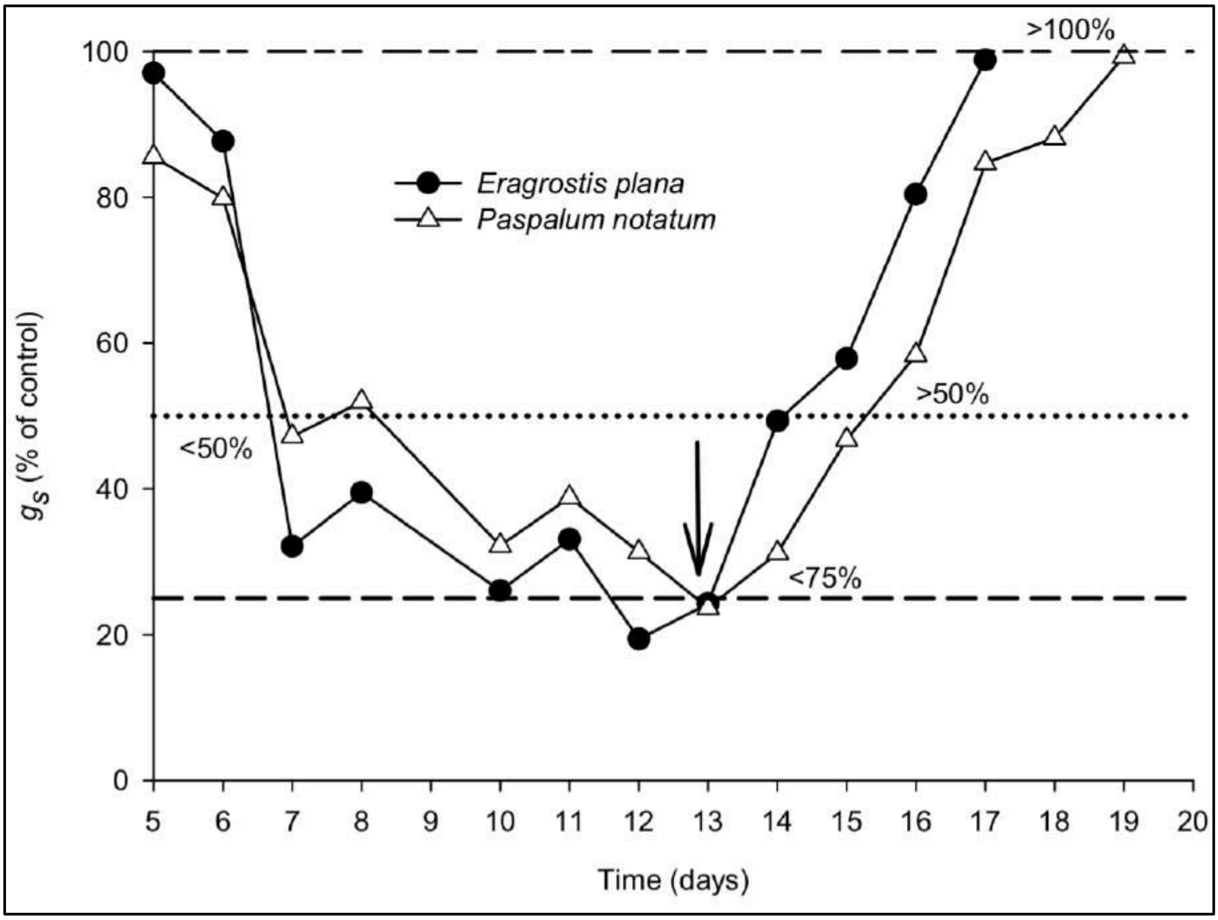


Figure 1 – Stomatal conductance (g_s) during the induction of water deficit (5 to 13 days) and after water recovery (13 to 19 days). The values were measured every day from the 5th day of suspension of irrigation in *Eragrostis plana* and *Paspalum notatum*. The reference lines (<50%, <75%, >50% and >100% of g_s in relation to well-watered treatment) indicate the time when the physiological and biochemical evaluations were done. The black arrow indicates the time when the rehydration period began. Means, *n* = 6.

Physiological Parameters

Leaf chlorophyll content – Leaf chlorophyll content index was accessed using a chlorophyll-meter Dualex 4 Scientific (Dx 4) (FORCE-A, Orsay, France) by measuring one random spot on the adaxial side of four leaves per pot.

Chl a fluorescence analysis – The chlorophyll a fluorescence transient parameters were measured in the last fully expanded leaf between 9-11h am using a portable fluorometer (Handy PEA, Hansatech Instruments, King's Lynn, Norfolk, UK). Initially, the leaves were adapted for 30 minutes in dark to allow the complete oxidation of the photosynthetic electron transport system. Then, fluorescence emission was induced in a 4 mm diameter area by exposing the leaves to a saturating red actinic light at the intensity of 3.000μmol m^{-2} s^{-1}.

Biochemical Parameters

Determination of hydrogen peroxide and lipid peroxidation – Hydrogen peroxide (H_2O_2) content and lipid peroxidation were determined according to Velikova; Yordanov and Edreva (2000). Leaves (400mg) were ground in 0.1% (w:v) trichloroacetic acid (TCA). The homogenate was centrifuged (12,000 x g, 4°C, 20min) and the supernatant was used for the analyses. Then, an aliquot of the supernatant was added to 0.5mL 10 mM potassium phosphate buffer (pH 7.0) and 1mL 1M potassium iodide (KI) in order to determine H_2O_2. The absorbance was read at 390 nm in a spectrophotometer and H_2O_2 content was given on a standard curve. Thiobarbituric acid (TBA) test was used to quantify lipid peroxidation in leaves. This test determines malonyldialdehyde (MDA) as an end product of lipid peroxidation. The supernatant was added to 1mL 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in boiling water for 30min, and the reaction stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10,000 x g for 5min, and the absorbance of the supernatant was read at 532nm. The value for non-specific absorption at 600nm was subtracted. The amount of MDA–TBA complex was calculated from the extinction coefficient (ε =155 × 103 M^{-1} cm^{-1}).

Antioxidant enzymes activity – Leaves (± 400mg) were ground using liquid N_2 in porcelain mortars, containing 5% (w:v) polyvinylpolypyrrolidone (PVPP) and homogenized in 100mM potassium phosphate buffer, pH 7.8, containing 0.1mM ethylenediaminetetraacetic acid (EDTA) and 20mM sodium ascorbate. The homogenate was centrifuged at 12,000g (for 20min 4°C) and the supernatant obtained was used as crude enzyme extract. An aliquot of the extract was used to determine protein content according to Bradford (1976) using bovine serum albumin as standard. The superoxide dismutase activity (SOD; EC 1.15.1.1) was determined according to the method described by Giannopolitis and Ries (1977) and monitoring the inhibition of the nitroblue-tetrazolium (NBT) coloration at 560nm. The catalase activity (CAT; EC 1.11.1.6) was measured following Azevedo Neto et al. (2006) by monitoring hydrogen peroxide consumption measuring the decline in absorbance at 240nm. The ascorbate peroxidase activity (APX; EC 1.11.1.11) was determined according to the method described by Nakano and Asada (1981) measuring the rate of ascorbate oxidation at 290nm for 3min. The glutathione reductase activity (GR; EC 1.6.4.2) was measured according to Cakmak et al. (1993) by following the decrease in absorbance at 340nm due to NADPH oxidation. Measuring the activity of POD followed Chance and Maehly (1955) by monitoring the rate of the increase in the absorbance at 470nm for 2min due to guaiacol oxidation.

Determination of proline content – Proline was determined by the ninhydrin method, according to Bates; Waldren and Teare (1973) with some methodologic adaptations. This method resulted in the formation of two-phase, where 1mL of the upper phase (pink) was collected, which was then analyzed by a spectrophotometer at 520nm. The obtained absorbance was compared to the proline standard curve, and the results expressed in μmol g^{-1} fresh weight.

Determination of carbohydrates and total free amino acid content – For extraction of starch, total soluble sugar, and total free amino acid content, leaf tissues (500mg) were grounded and centrifugated to obtain an alcoholic extract. Then, the extract was evaporated and added distilled water to obtain an aqueous phase, which was used to determine soluble compounds (total soluble sugar and total free amino acid). The residue from centrifugation was used to determine starch content by acid hydrolysis method according to Mccready et al. (1950). The total soluble content was determined by using the anthrone method following Graham and Smydzuk (1965). The total free amino acid content was determined by the ninhydrin method, according to Yemm; Cocking and Ricketts (1955).

Data analysis

Data were analyzed *via* one-way ANOVA considering the experiment as a completely randomized design (CRD) with two treatments (two water conditions), all comparisons separated by specie and time of evaluation. The Student's t-test ($p \leq 0.05$) was used to compare the two water conditions for *E. plana* and *P. notatum* by using the software SAS System version 9.0. Data are mean of six replications ± SE (standard error).

2.3 Results

2.3.1 Experiment I – Growth stage effect on drought tolerance

Stomatal conductance (gs) and leaf relative water content (RWC)

We measured stomatal conductance (g_s) and leaf relative water content (RWC) to determine plant water status in *E. plana* and *P. notatum*. In both species water deficit significantly reduced stomatal conductance, regardless of the growth stages (Figure 2A and 2B). RWC presented similar pattern as observed in g_s for both species. At the growth stages of 5-6 leaves, tillering, panicle initiation and grain filling in *E. plana*, the induced water deficit reduced the RWC values by 19%, 32%, 44% and 45%, respectively (Figure 2C). In *P. notatum* the reductions at these stages were about 25%, 29%, 46% and 37%, respectively (Figure 2 D). For *E. plana* and *P. notatum* the reductions on RWC at reproductive stages were likely to be more severe compared to vegetative stages. It could be associated to unfavorable atmosphere conditions (Vapor Pressure Deficit (VPD)= 2.1 kPa (±0.7); Temperature = 29.1°C (±2.8) and RH = 50.1% (±10)) during the evaluation time and/or possibly susceptibility of this stage.

Biometric analysis

The induction of water deficit at 5-6 leaves growth stage in *E. plana* reduced the number of tillers by 34%, while in the other stages has no significative effect (Figure 3A). However, in *P. notatum*, the stress reduced tillers number by 38%, 20%, 32% and 5% at 5-6 leaves, tillering, panicle initiation and grain filling stages, respectively (Figure 3B).

Water deficit negatively affected shoot dry mass accumulation by 40%, 21%, 26% and 14% in *E. plana* and about 82%, 23%, 33% and 23% in *P. notatum* at 5-6 leaves, tillering, panicle initiation and grain filling stages, respectively (Figure 3C and 3D). However, the species increased root dry mass in response to water shortage at tillering (1.30 fold) and grain filling (1.29 fold) for *E. plana* (Figure 3E) and tillering (1.34 fold), panicle initiation (1.62 fold) and grain filling (1.48 fold) for *P. notatum* (Figure 3E). Under water deficit, no changes on root development were observed at 5-6 leaves growth stage in both species.

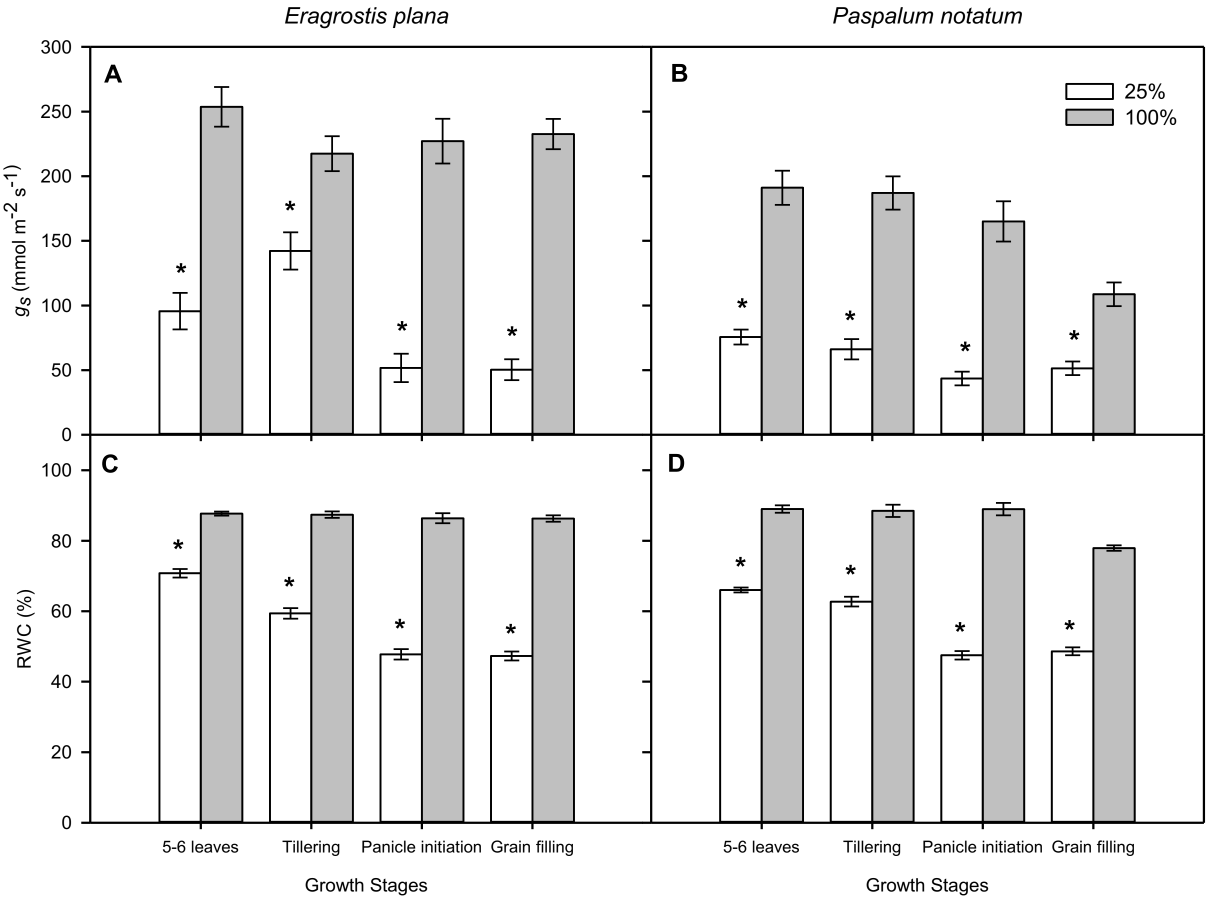


Figure 2 – Stomatal conductance (g_s) and relative water content (RWC) of *Eragrostis plana* and *Paspalum notatum* grown under 100% and 25% water replacement at different growth stages (5-6 leaves, tillering, panicle initiation and grain filling). * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments and error bars represent the standard error (SE).

At tillering stage, the two species were insignificantly affected by water deficit (Figure 4A and 4B) according evaluations of panicles or spikelets. Water stress has a high negative impact on number of panicles at the two late stages for *E. plana*. At panicle stage, the reduction on panicle per plant for *E. plana* was about 75% while for *P. notatum* there was no effect at this stage. However, the reduction on number of spikelets per plant at stage of grain filling reach 73% in *P. notatum* compared to only 45% in number of panicles for *E. plana* (Figure 4A and 4B).

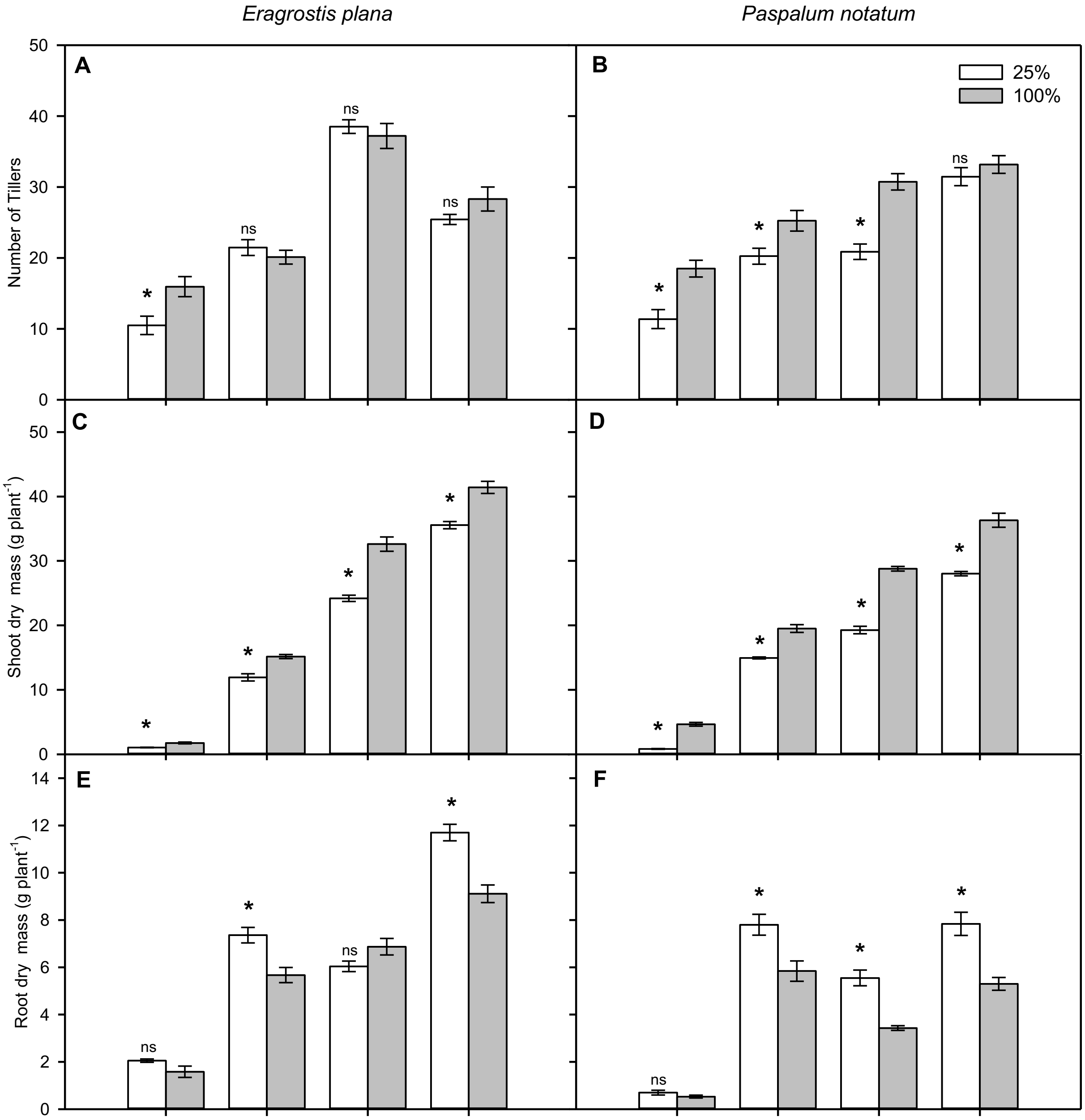


Figure 3 – Number of tillers, shoot dry mass and root dry mass of *Eragrostis plana* and *Paspalum notatum* grown under 100% and 25% water replacement at different growth stages (5-6 leaves, tillering, panicle initiation and grain filling). * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments and error bars represent the standard error (SE).

Principal component analysis

The PCA consider all parameters measured in this experiment resulting for 82.6% and 81.4% of total variation of the original data for *E. plana* and *P. notatum*

(Figure 5), respectively. PC1 explained 51.3% and 48.0% of the variance, whereas PC2 accounted for 31.3% and 33.4% for *E. plana* and *P. notatum*, respectively. The variables that contributed most to discriminate between groups had eigenvector values >0.30. In *E. plana*, the main components for PC1 were shoot dry mass, root dry mass, and panicle number, and were RWC, g_s, and panicle number for PC2. Otherwise, in *P. notatum*, the main components for PC1 were shoot dry mass, tiller number, and spikelet number, and were RWC, g_s, and tiller number for PC2. The spatial ordination of the parameters separated eight groups, allowing for clear distinctions to be made between the four growth stages (left and right quadrants) and the water treatments (upper and lower quadrants) for each species (Figure 5).

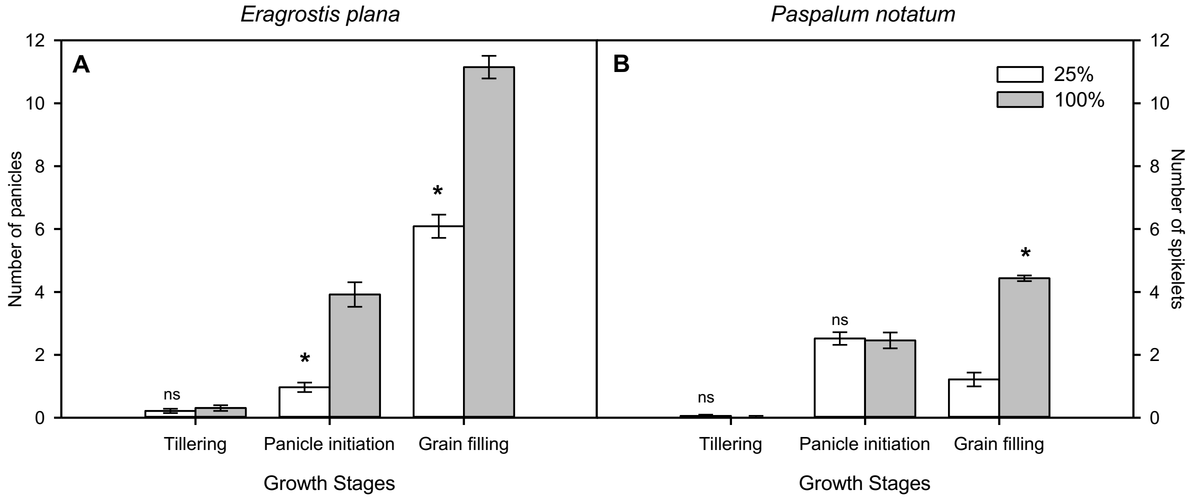


Figure 4 – Number of panicles per plant for *Eragrostis plana* and number of spikelets per plant for *Paspalum notatum* grown under 100% and 25% water replacement at different growth stages (5-6 leaves, tillering, panicle initiation and grain filling). * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments and error bars represent the standard error (SE).

2.3.2 Experiment II – Physiological and biochemical traits of drought tolerance

Stomatal conductance (g_s) and relative water content (RWC)

At the beginning of induction of water deficit, the plants exhibited more than 50% of fully emerged panicle/spikelets. Both species reduced g_s as the time of water deficit increased (Figure 6A and 6B). At 7 and 13 days of water deficit (DWD), both species reached more than 50% and 75% of g_s reduction in relation to well-watered, respectively (Figure 1, 6A and 6B). At 13 DWD, *E. plana* and *P. notatum* reached values of g_s on average of 39.2 and 33.6 mmol m^{-2} s^{-1}, respectively. At the end of 13th DWD, plants were rehydrated by the reestablishment of water irrigation. *E. plana* reach

50% of g_s recovery in the next day after water reestablishment (less than 24 hours) while *P. notatum* took three days of water recovery to attain this percentage. Full recovery (>100%) was reached by *E. plana* and *P. notatum* at 4 and 6 days of rehydration, respectively (Figure 1). Plants kept under well-watered condition (control) showed oscillations in g_s values among the daily evaluations, probably due to a variation on climatic conditions like air temperature, relative humidity (RH) of the air and vapor pressure deficit (VPD) (See Appendix A).

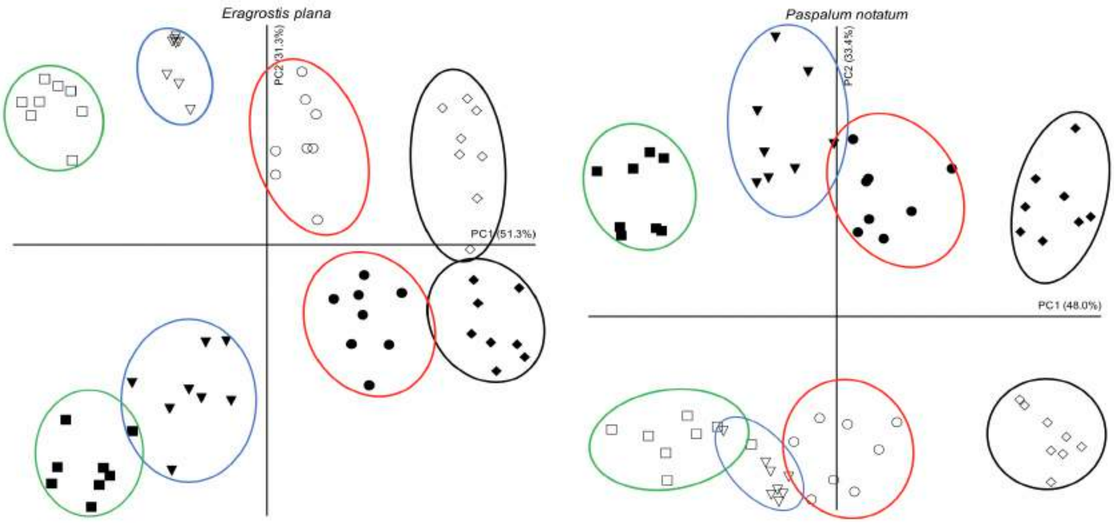


Figure 5 – Ordination generated by principal components analysis based on the entire data set from this experiment. To each species (*E. plana* or *P. notatum*), growth stages are represented by symbols represent the 5-6 leaves (◆ /◇), panicle initiation (▼/▽), tillering (● /○), grain filling (■/□) stage for each species (*E. plana* or *P. notatum*). Open symbols represent 25% water replacement and filled symbols are 100% of water replacement. The percentage of variation explained by each principal component (PC1 and PC2) is shown.

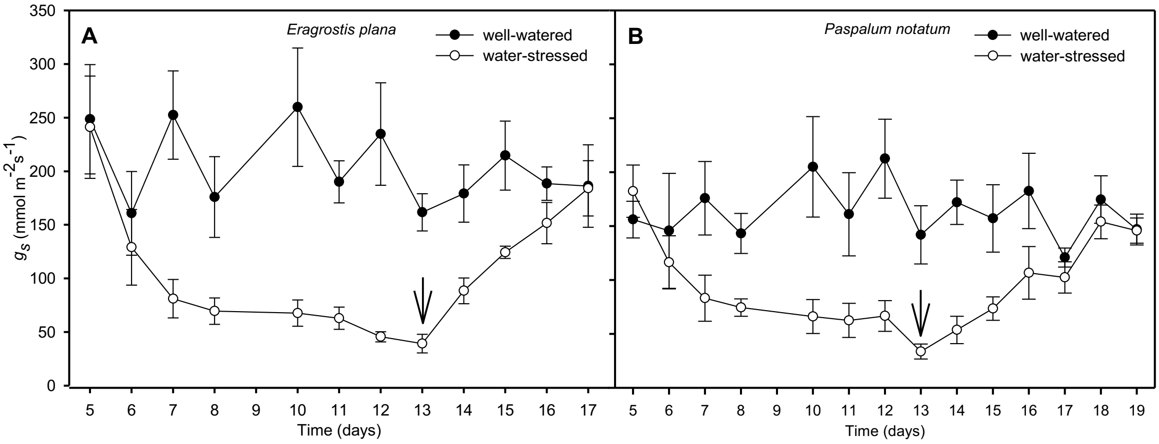
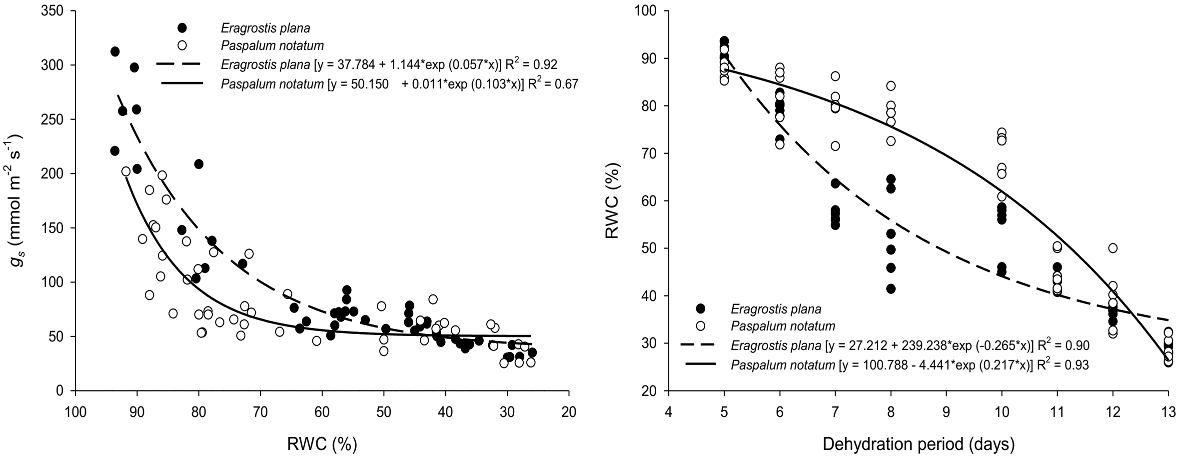


Figure 6 – Stomatal conductance (g_s) during the induction of water deficit (5 to 13 days) and after water recovery (13 to 19 days). The values were measured every day from the 5th day of suspension of irrigation in *Eragrostis plana* and *Paspalum notatum*. Error bars represent the confidence interval. The black arrow indicates the time when the rehydration period began. Means, *n* = 6.

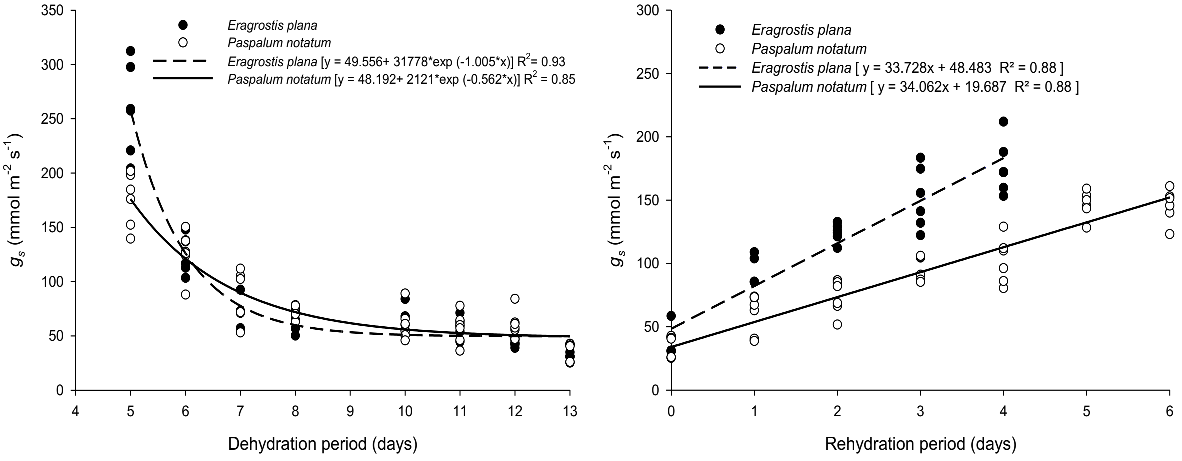
The g_s decreased exponentially when both species reduced the relative water content (Figure 7A). Based on exponential regression, reduction of g_s in *P. notatum* was more sensitive than *E. plana* as the RWC decreased (Figure 7A). Plants of *P. notatum* reached 50% of g_s reduction with approximately 80% RWC, whereas the same response was observed at approximately 55% RWC for *E. plana*. In result of late stomatal regulation, we observed that reduction on RWC in *E. plana* was earlier than *P. notatum* as the days of dehydration increased (Figure 7B). However, at the highest level of water deficit (13th DWD or <75% g_s), the RWC were very similar between the species, approximately 30 and 29 % for *E. plana* and *P. notatum*, respectively.



(A) **(B)**

Figure 7 – The effects of the induction of water deficit on the stomatal conductance (g_s) in relation to the relative water content (RWC) (A) and relative water content (RWC) in relation to dehydration period (B) in *Eragrostis plana* and *Paspalum notatum*. The values were measured every day from the 5th day of suspension of irrigation in *Eragrostis plana* and *Paspalum notatum*. Means, n = 6.

As the days of dehydration increased, the g_s values decrease rapidly for both species. Based on an exponential regression, we observed that g_s showed a more pronounced reduction for *E. plana* plants when compared to *P. notatum*, indicating that the responses of g_s as a result of increasing days of dehydration in *E. plana* were more sensitive than *P. notatum* (Figure 8A). The recovery of g_s increased more rapidly in *E. plana* versus *P. notatum* as the days of rehydration increased (Figure 8 B), illustrated by high values of the slope for *E. plana* (y = 33.728) compared to *P. notatum* (y = 19.687). The rate of g_s recovery was 1.7 faster in *E. plana* compared to *P. notatum*. *E. plana* reached full recovery after 4 days of rehydration while *P. notatum* took 6 days to reach completed rehydration.



(A) **(B)**

Figure 8 – The stomatal conductance (g_s) during the period of dehydration (A) and rehydration (B). The values were measured every day from the 5th day of suspension of irrigation in *Eragrostis plana* and *Paspalum notatum*. Means, $n = 6$.

Leaf chlorophyll index

The reduction on leaf chlorophyll started decreasing at moderated water deficit and still reducing until the phase of medium recovery for *E. plana* (Figure 9A). Similarly occurred in *P. notatum*, except that reduction began only at severe water deficit. At full recovery phase, both species recovered chlorophyll content.

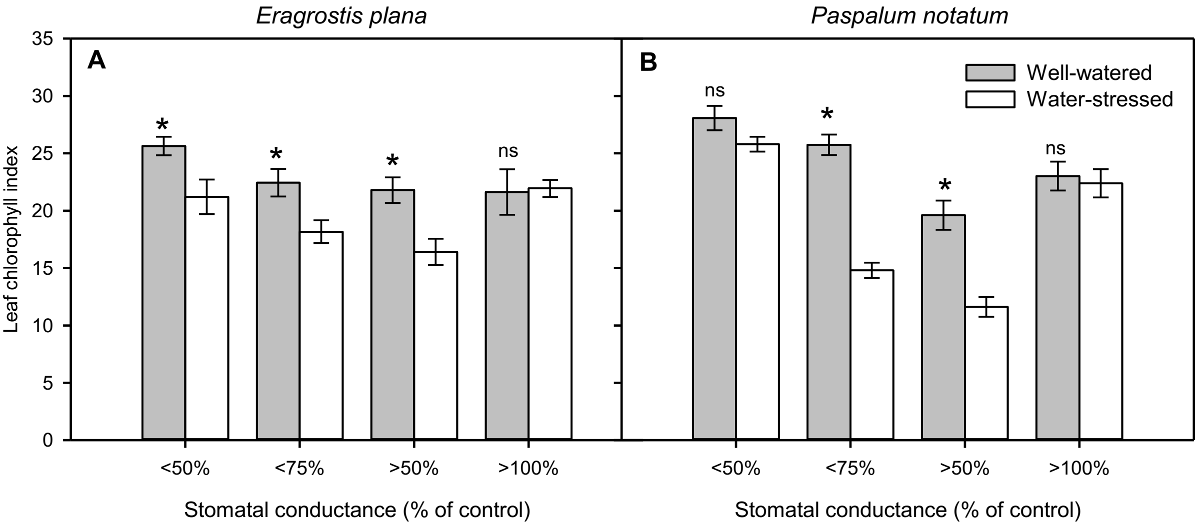


Figure 9 – The effects of the induction of water deficit on the leaf chlorophyll *index* in relation to the stomatal conductance (g_s) during the period of dehydration and rehydration in *Eragrostis plana* and *Paspalum notatum*. * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments and error bars represent the standard error (SE).

Chlorophyll a fluorescence response

The chlorophyll *a* transient fluorescence is the direct measure of the photosynthetic behavior of PSII and provides information about the physiological differences between water stress effect in the chosen species. We evaluated the ET_0/RC (flux of electron transport from QA^- to PQ per RC), DI_0/RC (dissipated energy flux per reaction center) and F_v/F_m (maximum yield of primary photochemistry of PSII) (Figure 10). Plants of *E. plana* only reduced ET_0/RC under severe water deficit (Figure 10A). On the other hand, *P. notatum* only reduced ET_0/RC at recovery time compared to plants kept under well-watered conditions (Figure 10B). There were no changes in DI_0/RC at the first evaluation (moderated water stress) for *E. plana* (Figure 10C) and *P. notatum* (Figure 10D). At severe water deficit, plants subjected to water shortage increased DI_0/RC about 3.4-fold and 2.6-fold for *E. plana* and *P. notatum*, respectively. At medium recovery, both species still dissipating energy about 1.5-fold more than well-watered treatment. In *E. plana*, plants under well-watered conditions showed DI_0/RC higher than plants recovering from water deficit. However, during the full recovery time of *P. notatum*, DI_0/RC was equal between the water treatments. The F_v/F_m increased in plants of *E. plana* subjected to moderated water deficit (Figure 10E), while for *P. notatum* there were no differences between water treatments (Figure 10F). Both species reduced F_v/F_m under severe water deficit and still below to the well-watered treatment during de medium recovery (50%). At full recovery time both species recovery 100% of the maximum yield of primary photochemistry.

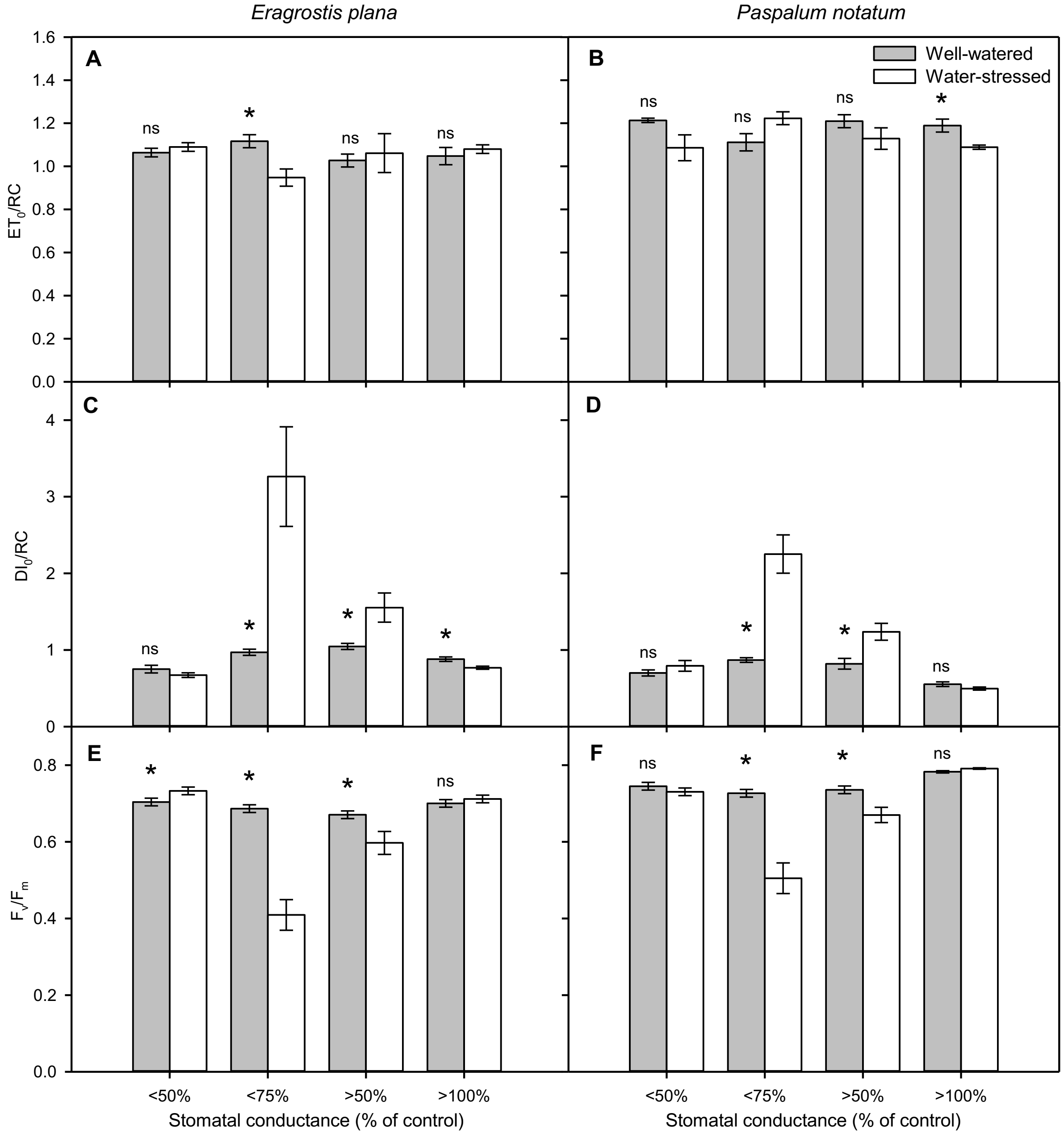


Figure 10 – The effects of the induction of water deficit on the chlorophyll *a* fluorescence parameters in relation to the stomatal conductance (g_s) during the period of dehydration in *Eragrostis plana* and *Paspalum notatum*. ET_0/RC (flux of electron transport from QA^- to PQ per RC), DI_0/RC (dissipated energy flux per reaction center) and F_v/F_m (maximum yield of primary photochemistry of PSII). * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments. *n* = 6 replicates.

Hydrogen peroxide accumulation and lipid peroxidation

Water shortage induced accumulation of H_2O_2 in *E. plana* exclusively at the severe water deficit (<75% g_s) as revealed by an increase of 2-fold compared to well-watered treatment (Figure 11A). Otherwise, in *P. notatum*, the production of H_2O_2 was 1.8-fold (<50% g_s) and 3.7-fold (<75% g_s) higher than well-watered treatment during the dehydration period (Figure 11B). Plants of *E. plana* that was recovering from water deficit reduced the content of H_2O_2, where at full recovery (>100%), this concentration was less than 50% compared to irrigated plants. Similarly, it was observed for *P. notatum*, however significative differences between water-stressed and well-watered were observed only at full recovery (>100%).

The lipid peroxidation is presented here as a result of MDA-TBA accumulation (Figure 11C and 11D). There was no increase on lipid peroxidation at moderated water deficit for both species. In addition, under water deficit, the species showed lower MDA-TBA content in relation to well-watered condition. At severe water deficit (<75%) both species showed accumulation of MDA-TBA, as well as stress induced the MDA-TBA production of approximately 2 and 1.5-fold higher than well-watered for *E. plana* (Figure 11C) and *P. notatum* (Figure 11D), respectively. Plants of *E. plana* recovering from water stress had values of lipid peroxidation below to well-watered treatment at full recovery time, while *P. notatum* there were no differences between the treatments.

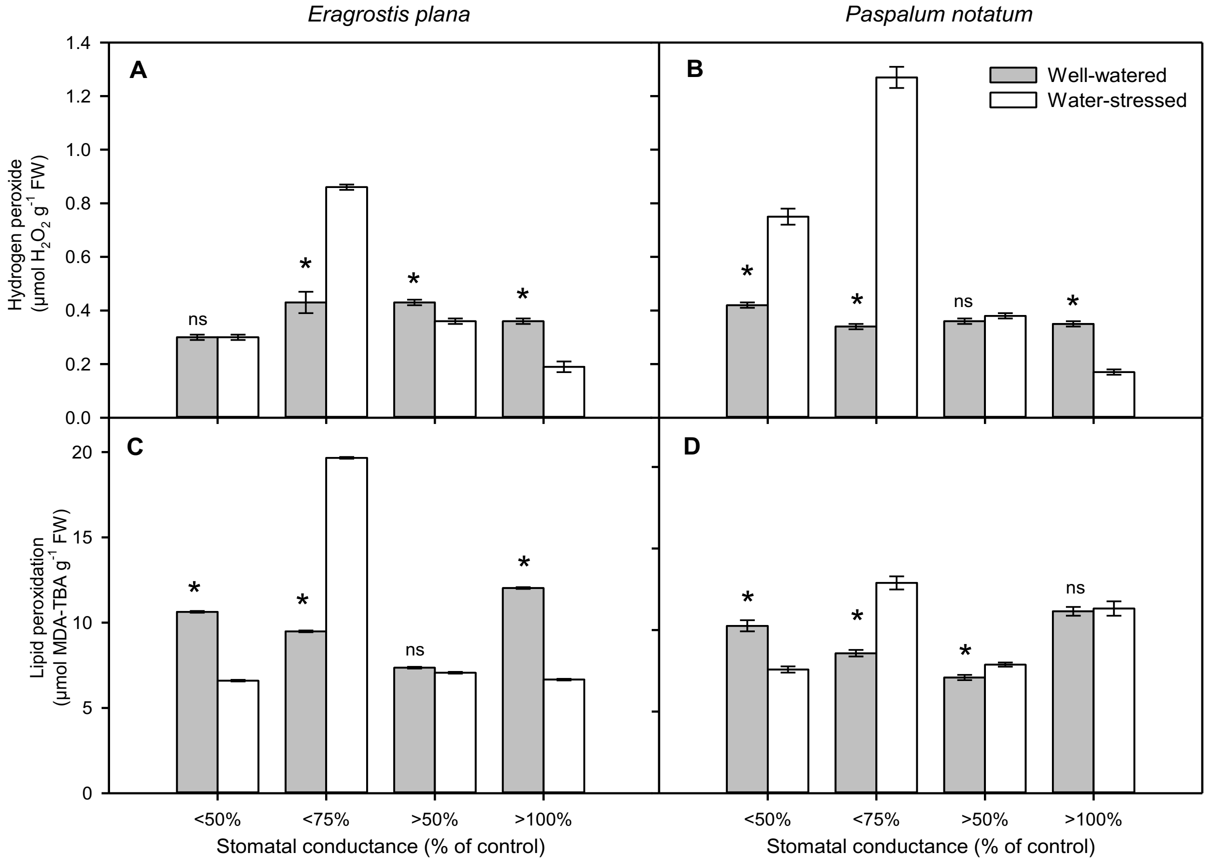


Figure 11 – The effects of the induction of water deficit on the production of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in relation to the stomatal conductance (g_s) in *Eragrostis plana* and *Paspalum notatum*. Means, n = 6. * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments and error bars represent the standard error (SE).

Antioxidant enzymatic activity

The activity of SOD no changed in *E. plana* during the period of water restriction (Figure 12A), while *P. notatum* increased the activity of this enzyme in both periods (Figure 12B). At the recovery phase, plants of *E. plana* recovering from WD reduced SOD activity in relation to control, whereas no changes were observed for *P. notatum* at this time. Plants of *E. plana* subjected to water deficit increased the CAT activity significantly at moderated, severe water deficit and still higher than well-watered treatment at medium recovery (Figure 12C). There were no changes in CAT activity in plants of *P. notatum* during the period of water deficit (Figure 12D). However, during the recovery plants that had experienced water stress showed less activity than well-watered plants. In *E. plana*, activity of APX was no significative at moderated and severe water deficit (Figure 12E), otherwise *P. notatum* raised APX activity only at moderate stress (Figure 12F). During the recovery time, reduced activity in water-stressed plants was observed at medium recovery for *E. plana* and at full recovery for *P. notatum.*

Osmolyte accumulation

The proline content increased significantly in response to water deficit during the phase of dehydration (moderated and severe water deficit) and it was prolongated until medium recovery (>50% g_s) for *E. plana* (Figure 13A). The increasing proline content was delayed in *P. notatum*, being started at severe water deficit and it was kept until full recovery phase (Figure 13B).

The accumulation of amino acids in response to water deficit was significative exclusively at early stages of water deficit (moderated water deficit) and during the full recovery for *E. plana* (Figure 13C). Otherwise, in *P. notatum* the water shortage induced accumulation of amino acid about 2.0, 3.5, 2.7 and 2.8-fold more than well-watered plants at <50%, <75%, >50% and >100% g_s (Figure 13D).

The increase of soluble sugar in response to water deficit was significative for both dehydration and rehydration phases in *E. plana* (Figure 13E), while plants of *P. notatum* accumulated soluble sugar only at dehydration period (Figure 13F). Accumulation of starch in response to water deficit was observed at <50%, <75%, and >50% in *E. plana* (Figure 13G) and at <75%, >50% and >100% for *P. notatum* (Figure 13H). It was significative higher the accumulation of starch in plants under water deficit at full recovery (>100%).

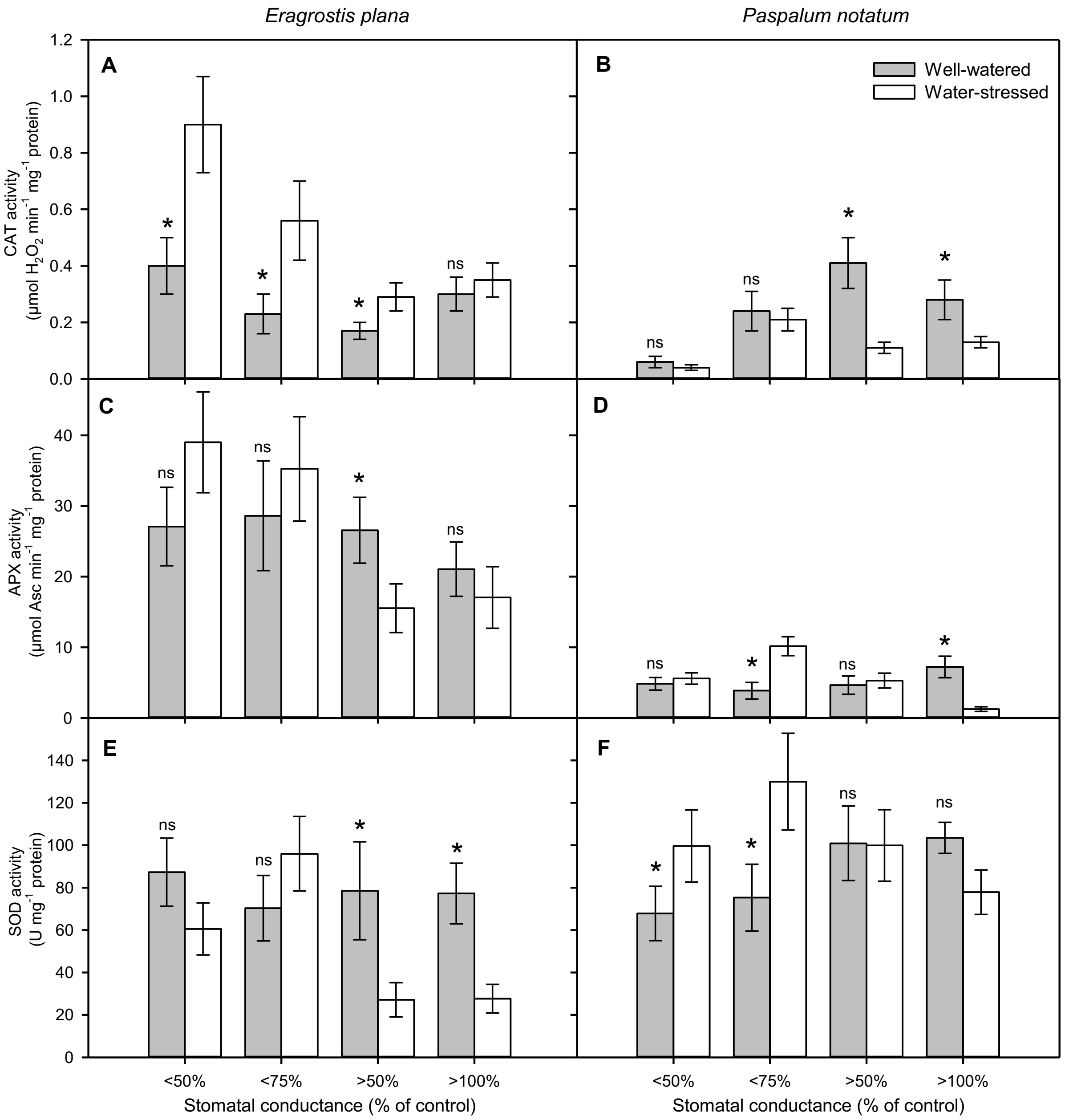


Figure 12 – The effects of the induction of water deficit on the enzymatic activity of catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) in relation to the stomatal conductance (g_s) in *Eragrostis plana* and *Paspalum notatum*. Means, *n* = 6. * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments and error bars represent the standard error (SE).

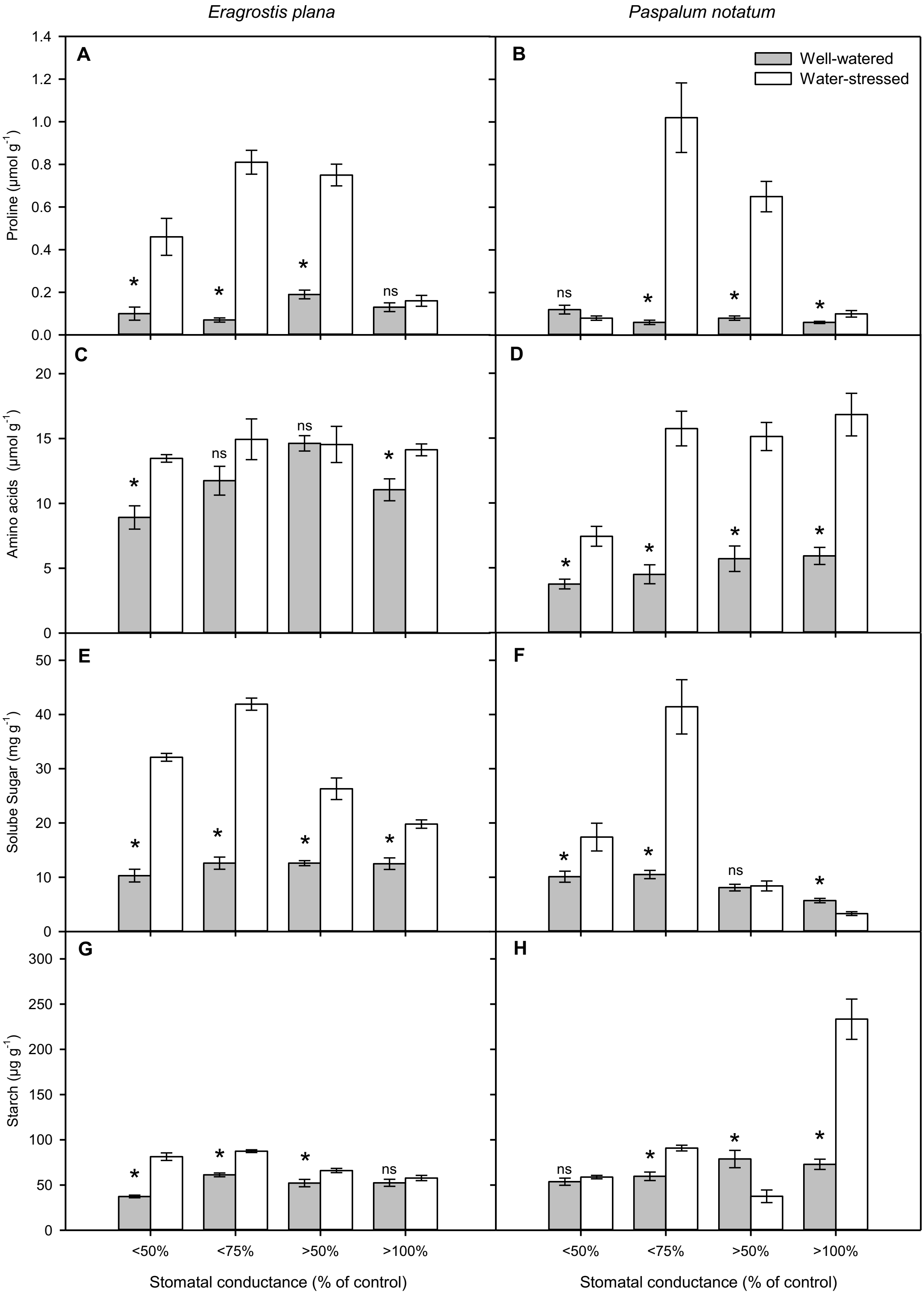


Figure 13 – The effects of water deficit on the content of proline, soluble amino acids, soluble sugar and starch in *Eragrostis plana* and *Paspalum notatum*. * Indicate statistical differences by Student's t-test ($p \leq 0.05$) between water treatments and error bars represent the standard error (SE).

2.4 Discussion

Based on our data obtained from first experiment we could determine the growth stage where *E. plana* and *P. notatum* exhibit the highest and lowest drought tolerance. Then, we choose the most drought-sensitive growth stage of *E. plana* to quantify the changes in physiological and biochemical traits of *E. plana* and *P. notatum* in response to water deficit.

According to Experiment 1, late development stages (panicle initiation and grain filling) for both species had a strong stomatal regulation compared to early stages (5-6 leaves and tillering), such as severe reductions on g_s by 78% and 74% at panicle initiation for *E. plana* and *P. notatum*, respectively. Stomatal control is an important mechanism to reduce water loss and delay tissue dehydration under water shortage (ANJUM et al., 2011). However, it was observed values of RWC below 50% at aforementioned stages, which could be critical for plants sensitive to drought. According to FARRANT et al. (2015), the lowest (critical) plant water status at which mesophytes plants can survive is a RWC of ~40%, therefore it is also dependent of the duration of stress. Thus, our data suggest that both species were able to cope with water deficit due to survival for a period up to 30 days under critical RWC.

Additionally, it is important highlights that the high VPD (2.1 kPa) was probably the main factor inducing stomatal closure in the plants evaluated, whereas it was not enough to avoid water loss by leaves under high evaporative demand. It has been proposed by MERILO et al. (2018) a model where high VPD induces stomatal closure. This possibly occur by three ways: ABA biosynthesis, induction of proteins related to stomatal closure (protein kinase *OST1* for example) or *via* passive hydraulic, when the stomatal closure results from reduced RWC and turgor loses. It happens due to stronger evaporative demand in dry air. Thus, it expected that days with high VPD possibly increased the water loss from leaves. Consequently, plants in advanced growth stages were the most affected by stress due to the soil water content did not supply the demand from evapotranspiration.

The reduction on aerial growth was more evident at 5-6 leaves phase, while in the other growth stages the percentage of reduction on biomass were similarly. Considering the effect of water deficit between species, the reduction on SDM in *P. notatum* at 5-6 leaves was higher than *E. plana*. Similarly, the number of tillers in *P. notatum* was highly affected by drought in all development stages while in *E. plana* it was evident only at 5-6 leaves stage. According to VOLAIRE; BARKAOUI and NORTON (2013) adaptive responses of perennial herbaceous species ranging from resistance to moderate drought with growth maintenance (dehydration avoidance and tolerance of leaf lamina) to growth cessation and survival of plants under severe stress (dehydration avoidance and tolerance of meristems). As an example, the forage grass *Urochloa brizantha* decrease vegetative development, consequently reducing biomass production, as a conservative strategy by lowering metabolism and avoid the dehydration (SANTOS et al., 2013).

Decreasing on aerial biomass was perhaps due to leaf senescence and leaf shedding (as observed visually, in this study). This plant strategy plays an important role in reducing total plant water losses, ensuring cell turgor and therefore in the survival of many species especially under drought, where it has been considered as a mechanism of dehydration avoidance (GEPSTEIN, 2004; MUNNÉ-BOSCH; ALEGRE, 2004; VOLAIRE, 2018). In the same way, reduction on number of tillers is related to lower activity of cell division in the meristematic zone and consequently responsible by the capacity to protect meristematic tissues from dehydration during periods of water restriction (PEZZOPANE et al., 2017). Also, we observed here an increasing on root dry mass allocation under drought stress at tillering and grain filling stage in *E. plana* and at all stages except at 5-6 leaves for *P. notatum*. Biomass partition also is considered a powerful plant strategy to avoid drought by allowing roots to explore water reserves from deep-soil layers (ALOU et al., 2018; OKADA et al., 2002). EZIZ et al. (2017) conducted a meta-analysis by synthesizing 164 published studies found that, on average, drought increased root mass fraction by 9.07% but decreased stem mass fraction, leaf mass fraction, and reproductive mass fraction by 5.55%, 2.29%, and 7.54%, respectively.

Defining “drought tolerance” is a kind of challenging and also misinterpreted, so this definition depends on which scale is being evaluated (tissue organ, whole-plant individual or plant population) as well as what kind of approach is considered, ranging from an analytical (short-time scale) to integrative (long-timescale) studies (VOLAIRE, 2018). From the point of view of yielding biology, a plant resistant to stress is characterized by a high yielding stability, i.e., in water shortage, it yields a little lower than the yield of the plants grown under optimal conditions (STANIAK; KOCOŃ, 2015). Thus, here growth stages presenting less reduction on shoot dry mass and maintenance of tiller production could be a valuable indicator of drought tolerance, mainly for *Paspalum notatum* which represents a high-quality forage in native pastures. This observation is also valid to *E. plana*, where shoot aerial growth has been considered important trait of invasiveness, responsible to overcome native species by shading and reducing light availability to native species (MEDEIROS; FOCHT, 2007). Although, these species are perineal with natural resseding, consequently we should take into account reproductive parameters to properly evaluate the survival in an ecosystem under drought and define which stage would be more tolerant to stress. In *E. plana*, water stress reduced strongly number of panicles at stage of panicle initiation while in *P. notatum* the most sensitive phase was at grain filling. At these stages is expected that water stress induces panicles abortion since at beginning of water induction the panicles were already emitted. Similarly to leaf biomass, plants also tend to reduce the investments in reproductive organs that requires vast amounts of energy and water, because reduction in photosynthesis and changes in phenology (e.g., shortening growth period and early flowering) in relation to drought cause significant reduction in biomass allocation to reproductive parts (EZIZ et al., 2017).

The development stage of panicle initiation was probably the most sensitive stage to drought in *E. plana*. Explained by critical values of stomatal conductance and leaf relative water content (below 50%) that probably caused photosynthesis limitation and consequently affected root growth and the blocking emission of new panicles at this stage. The PCA support this evidence where variables that contributed most to discriminate water stress in *E. plana* were RWC, *gs* and panicle number. In *P. notatum* to define the most drought sensitive stage was challenging, as well as, the PCA separation between water treatments showed similar behavior among the four growth stages. In spite of that we should take into account the number of spikelets as a parameter of survival of this native forage too, thus grain filling is probably the most sensitive stage to water deficit. In the other hand, assuming growth maintenance to produce aerial biomass, the 5-6 leaves stage was the most affected by drought for both species either by reducing shoot dry mass and tiller number. It has been proposed that in perennial grasses, drought survival should be measured after rehydration following the drought period, using values such as the percentage of plant survival and the recovery biomass in the subsequent seasons (MILBAU et al., 2005; POIRIER; DURAND; VOLAIRE, 2012; VOLAIRE et al., 1998). Thus, future studies should take into account the measurements post-stress to better understand the most sensitive stage to drought. Considering water deficit effect on growth and reproductive parameters, we could assume the most tolerant growth stage for both *E. plana* and *P. notatum* must be at tillering stage, explained by the maintenance of RWC above of critical values, percentage of reductions on tiller number and shoot dry mass similar to reproductive phases, increased root mass fraction under water shortage as well as no interference on number of panicles/spikelets.

According to our data from Experiment 1, we choose to perform a deep quantification of changes in physiological and biochemical traits in response to drought at stage of panicle initiation, as the most drought-sensitive growth stage for *E. plana*. All growth stages can be considered tolerant to water stress according our data. However, at panicle initiation in *E. plana,* as the most drought sensitive stage, we expected to observed more changes on plant metabolism in response to water stress if compared to the other growth stages.

In a second experiment, we define the time of our evaluations based on reduction on g_s (% of control) to allow comparations between species considering g_s as the one physiological indicator common for all evaluations. Even, both species reach 50% of g_s reduction together at 7 days of water deficit, the stomatal regulation of *P. notatum* was more sensitive during the early stages of leaf dehydration, where the plants reached 50% of g_s reduction with approximately 80% RWC, whereas the same response was observed at approximately 55% RWC for *E. plana* (Figure 7A). The initial poor stomatal regulation in *E. plana* resulted in more pronounced leaf dehydration as above mentioned, consequently we observed a faster decreasing of RWC compared to *P. notatum* as the water deficit increased (Figure 7B). This level of leaf dehydration is considered critical to maintain cellular homeostasis, so it could have promoted water stress and consequently induced the mechanisms of drought tolerance earlier in *E. plana* compared to *P. notatum* as ours followed discussions through the drought time course (ANJUM et al., 2011). The data of soil water content (see appendix B), showed that *E. plana*, probably due to poor stomatal regulation, consumed higher water percentage compared to *P. notatum*, thus leading a situation of early water stress in *E. plana* plants. According to VOLAIRE (2018), there are two groups of plants based on response to drought; anisohydric plants has low stomatal regulation enabling plants to tolerate rapid and extensive fluctuations in water potential, while isohydric species, the plant water status is maintained at a steady high state by strong stomatal regulation under water deficit. Based on our findings, *E. plana* showed more traits related to anisohydric plants while *P. notatum* had a strong stomatal regulation which is associated to isohydric plants.

Under moderated water deficit (<50% g_s) plants of *E. plana* had low level of leaf water content (55% RWC), however, this condition was not enough to cause any photoinhibition on photosystem II represented here by chlorophyll *a* parameters. Even under moderated water stress, stomatal closure may cause "stomatal" limitations for CO_2 entry, thus in drought-sensitive species the maintenance of the light reactions under reduced internal CO_2 concentrations can result in the over-reduction of the photosynthetic components where photoinhibition is likely to be an essential facet of drought stress (PFANNSCHMIDT et al., 2009).

Our data indicated that chlorophyll index of *E. plana* was reduced by water deficit without any apparent increasing on accumulation of hydrogen peroxide or lipid peroxidation. This indicate cell membrane stability on this level of water stress. The low RWC associated to soil water deficit could have limited the nitrogen availability thus affecting process of chlorophyll biosynthesis/repair. The chlorophyll biosynthesis is substantially down-regulated by water stress, consequently, this could act as a regulatory mechanism in plants to resist drought by minimizing light absorption due to reduced amounts of chlorophyll which would down-regulate the electron transport to reduce the ROS production (DALAL; TRIPATHY, 2012).

The low RWC probably induced the activation of antioxidant system, confirmed by high activity of CAT under water deficit conditions in *E. plana*. CAT and APX in the AsA-GSH cycle of enzymes are responsible for the decomposition of H_2O_2 generated by SOD in different cellular organelles (REN et al., 2016), which could have avoid accumulation of hydrogen peroxide in *E. plana*. Besides that, there was accumulation of compatible osmolytes (proline, soluble sugar and amino acids) and starch content. Proline is an osmoprotector that stabilizes membranes and maintains the conformation of cytosolic enzymes to survive drought stress (PEEVA; CORNIC, 2009), mainly upper-regulated in drought-tolerant plants. Similarly, the accumulation of total amino acids and soluble sugars is related to mechanisms that allow plants to increase their tolerance to low water availability by enabling plants to maintain cellular turgor pressure (BERTOLLI; MAZZAFERA; SOUZA, 2014).

Differently from *E. plana*, plants of *P. notatum* showed a slight reduction of leaf water content (RWC = 80%) at stage of moderated water stress (<50% g_s). The maintenance of leaf water content by early stomatal closure in *P. notatum* as discussed before, probably avoid water loses to atmosphere as well as prevented from damages of water deficit. Thus, there were no changes on chlorophyll *a* parameters and chlorophyll content. Conversely, there was accumulation of hydrogen peroxide which probably came from activity of SOD enzyme at this level of water stress. The percentage of H_2O_2 might was not enough to cause any lipid peroxidation, where the accumulation of osmoprotectants (amino acids and sugar) might have helped membrane stabilization and prevent the damage from H_2O_2 accumulation.

The highest level of water stress was reached at less than 75% of g_s reduction and RWC (%) of approximately 30 and 29 % for *E. plana* and *P. notatum*, respectively. This level of g_s reduction is likely to be extremally limiting for CO_2 entry and consequently, the biochemistry of CO_2 fixation would be reduced at lower rates resulting in growth reduction (shoot, shoot and panicle development) as observed on Experiment 1. *E. plana* plants reduced the flux of electron transport from QA^- to PQ per reaction center (ET_0/RC) as a consequence the maximum yield of primary photochemistry of PSII (Fv/Fm) also was reduced. Conversely, our data indicate that ET_0/RC in plants of *P. notatum* were not affect, but Fv/Fm was drastically reduced. Electron transport and formation of ATP and NADPH proceed at much slower rates than light harvesting and energy transfer to reaction center II by antennae. As an immediate consequence, the amount of energy absorbed recurrently overcomes the metabolic energy demands. So, this leads to accumulation of excess energy in the thylakoid membrane that can potentially be harmful to PSII, leading to the permanent closure of the PSII reaction centers and photoinhibition, which can be defined as a sustained reduction of Fv/Fm ratio (ARO; VIRGIN; ANDERSSON, 1993; RUBAN; JOHNSON; DUFFY, 2012). However, the PSII reaction centers are protected by mechanisms that diminish the rate of excitation of PSII by harmlessly dissipating excess energy in the PSII antenna complexes like heat (FOYER, 2018). It was observed here for both species, mainly for *E. plana* which increased DI_0/RC in 3.4-fold while *P. notatum* this value was 2.6-fold higher than well-watered plants. The protection is quantified by processes that can be assessed from nonphotochemical quenching of chlorophyll fluorescence (NPQ) (FOYER, 2018; RUBAN; JOHNSON; DUFFY, 2012).

Here the photoinhibition probably induced the production of reactive oxygen species such as hydrogen peroxide which one cause significant lipid peroxidation and might have reduced even more the chlorophyll index in both species. We should highlight that plants of *P. notatum* under water stress showed 3.7-fold (<75% g_s) higher H_2O_2 content while *E. plana* this was only 2-fold higher than well-watered plants. As a response of higher H_2O_2 content plants of *E. plana* increased CAT activity while *P. notatum* increased significantly SOD and APX activity. We expected that in *E. plana* the activity of SOD at the time of evaluation had already reduced due to earlier exposition to water stress illustrated by lower RWC at previous evaluation (moderated water stress). Thus, we believe that early SOD activity lead to a high production of H_2O_2 to be scavenging by CAT enzyme. Alternatively, plants of *P. notatum* were at the same time detoxifying ROS by SOD, dismuting $O_2^{\cdot -}$ to H_2O_2 and by APX converting H_2O_2 to H_2O. At this level of water stress, the accumulation of compatible osmolytes (proline, amino acids and soluble sugars) in plants of *E. plana* were significantly higher compared to *P. notatum*. Proline has long been recognized as a marker of drought stress (BATES; WALDREN; TEARE, 1973; BOWNE et al., 2012; SÁNCHEZ-MARTÍN et al., 2015). Against this, accumulation of proline in rice and sorghum genotypes under salt stress was deemed to be a symptom of damage rather than an indication of tolerance (ASHRAF; FOOLAD, 2007). In our experiment, the accumulation of proline under water stress was 11.5 and 15.8-fold higher than well-watered plants for *E. plana* and *P. notatum*. We should highlighted here that *P. notatum* accumulated content of H_2O_2 higher than *E. plana* as a result of water stress damage which was directed correlated with higher production of proline, thus agreeing with the theory proposed by ASHRAF and FOOLAD (2007). Besides proline accumulation, both species had accumulated soluble sugar and starch, and specifically for *P. notatum* showed higher accumulation of amino acids. Accumulated carbohydrates during a stress are of importance to plants in three ways: to use in the regrowth, to make new structural components and for osmotic adjustment (WHITE, 1973) as discussed previously.

It has been proposed that in perennial grasses, drought survival should be measured after rehydration following the drought period (MILBAU et al., 2005; POIRIER; DURAND; VOLAIRE, 2012; VOLAIRE et al., 1998). Based on that, we evaluated the recovery after reestablishment of irrigation following the 13 days of water deficit. Plants of *E. plana* reach 50% of *gs* recovery (medium recovery) the day after water reestablishment, with less than 24 hours while *P. notatum* had took 3 days of rehydration. Faster recovery of stomatal conductance could be associate as a good predictor of drought tolerance, where it may help plants recovery the photosynthetic process such as PSII machinery as well as starting over the biochemistry of CO_2

fixation by allowing the entry of atmospheric CO_2 by stomata. At the medium recovery, both species had full recovered of electron transport flux, but still have reduced F_v/F_m and high rate of DI_0/RC which indicate that photosynthetic process still impaired at this time. The repair of chlorophyll content was enough for both species at medium recovery, mainly for *P. notatum*. Besides, there were no accumulation of H_2O_2 and lipid peroxidation indicating the efficient process of detoxification by antioxidant enzymes. However, *E. plana* plants recovering from water stress had high activity of CAT at this time while *P. notatum* showed equal values of APX and lower activity for SOD and CAT compared to well-watered plants. It has been expected that plants recovering from water stress show reduced activity of antioxidant enzymes even lower than plants under optimal water conditions.

Total recovery of stomatal conductance (full recovery time) was reached at 4 and 6 days after water reestablishment for *E. plana* and *P. notatum*, respectively. This behavior is highlighted again where the early response of recovery in plants of *E. plana* should be considered as important trait of drought tolerance. The full recovery of g_s also was attended by reestablishing the PSII machinery and chlorophyll content regardless of both species. Here, the normal operation of PSII also allowed plants to reduce activity of antioxidant enzymes. Plants at full recovery from water stress showed high concentration of compatible osmolytes as observed for amino acids and soluble sugars in *E. plana* and amino acids and proline for *P. notatum*. This osmolytes has an important role in water-stressed plants by accelerating the repair of damages in the recovery time (BANDURSKA; GÓRNY; ZIELEZINSKA, 2008). Solely for *P. notatum* there was a high accumulation of starch at this time, which could be associated to a mechanism to store energy as a mechanism to prevent future events of water deficit.

2.5 Conclusions

In the current study, we can conclude that both species exhibit the highest drought tolerance at tillering stages compared to reproductive growth stage. Plants of *E. plana* are more affected by drought when the water stress is imposed at panicle stage, while for *P. notatum* the highest drought sensibility is observed at stage of grain filling.

The stomatal regulation is key factor to avoid water loses in *P. notatum*, while *E. plana* plants has a late stomatal closure, and can tolerate low RWC for a long time. Both species use mechanisms to dissipate energy excess from photosystems under drought, also associated with an efficient antioxidant system. Proline, amino acids and soluble sugars are produced since early stages of water deficit until complete recovery of stomatal conductance during the rehydration period. Even though *E. plana* enter in water stress prior to *P. notatum* due to poor stomatal regulation, the rate of g_s recovery is almost twice compared to *P. notatum* during the rehydration phase.

Finally, our data suggests that both chosen species use similar mechanisms to tolerate water deficit, however the rate of dehydration/rehydration is a quite different.

3. CHAPTER II - Competition between the invasive weed *Eragrostis plana* and native grass *Paspalum notatum* under drought conditions.

3.1 Introduction

The grassland region located in Pampa Biome is a major livestock production area where the exotic grass *Eragrostis plana* Nees has become a dominant weed in recent decades (ZENNI; ZILLER, 2011). In this region, cattle have been produced for over 300 years and is the central maintainer of Pampa's features (OVERBECK et al., 2007). Farmers have been managed Pampa's grasslands with practices that caused overgrazing, low productivity, and low financial income (NABINGER et al., 2009). Usually, grazed grasslands in this region with moderate grazing intensity are formed by mosaics of intensively grazed patches dominated by prostrate grasses (e.g. *Axonopus affinis* Chase, *Paspalum notatum* Flüggé), and less grazed patches dominated by tussock grasses (e.g *E. plana*), small shrubs or other species less attractive for grazing animals (ANDRADE et al., 2015).

The scenario of Pampa Biome grasslands has areas basically dominated by *P. notatum* (Bahiagrass) and *E. plana* (tough lovegrass, also known in Brazil as capim-annoni), two perennial that differ in their palatability for livestock and in water stress tolerance. The former is a high value forage species native of Pampa Biome while the second species, an unwanted grass by animals, which is native from Africa. The scenario of this areas forces grazers to compensate through overgrazing of remaining, more palatable plants like *P. notatum*, thus creating a positive feedback for the invader (MEDEIROS; FOCHT, 2007), in this case *E. plana*. High and selective grazing pressure decreases the competitive ability of native species. Besides that, many species native from semiarid environments possess physical characteristics that confer low palatability and high tolerance to drought (GRIME, 1979). As the case of *E.*

plana, which growth is favored by animal rejection due to high lignin percentage, low level of crude protein (ALFAYA et al., 2002) and by tolerating drought events. A study conducted by Medeiros, Pillar and Reis (2004) indicated that *E. plana* had invaded up to 20% of Rio Grande do Sul's grassland area by the year 2008, causing severe reduction in forage quality and native plant diversity. The area surpassed one million hectares despite policies to contain its expansion (MEDEIROS; PILLAR; REIS, 2004), and the invasion continues steadily towards the neighboring countries, as identified by Barbosa et al (2016), where there are an optimum climatic conditions for the species development across the South America, and also predicted changes on precipitation patterns including drought events is likely to favor the invasion by *E. plana*.

The competition ability between species is related to several factors, including weed specie, populational density, time of emergence and plant traits. In general, weeds have the potential to better adapt to an environment of competition compared to crop species, besides the weed density is commonly higher than cultivated species (BIANCHI; FLECK; LAMEGO, 2006). The capacity of a weed, especially for invasive weeds, to adapt to changing factors that affect growth is positively related to its capacity to compete for these resources with the cultivated species (LIMA et al., 2016). Likewise, the intensity of the soil water competition depends on the species involved and their survival mechanisms under water deficient conditions. A recent work showed that the water deficit reduced the growth of cowpea plants*, Commelina benghalensis* and *Waltheria indica*, where *W. indica* has a greater water competition capacity with cowpea than *C. benghalensis* (DE OLIVEIRA et al., 2018). However, another study conducted in dry conditions, occurred an intense underground competition between *Festuca pallescens* and *Pappostipa speciose* (two native grass species), but there was no evidence of underground competition with the invasive weed *Rumex acetosella*, where the low competitive ability of *R. acetosella* under water stress may indicate that its invasive potential would be limited by the aridity of the environment (FRANZESE; GHERMANDI, 2014). Changes in competitive ability due to water deficit may be explained by the fact of each species has a wide variation of the dry matter partitioning on the vegetative organs. As an example, soil water deficit reduced the leaves biomass and increased root biomass in *Waltheria indica* and *Crotalaria retusa*, while *Commelina benghalensis* and *Cleome affinis* had an increase only on the roots mass (LIMA et al., 2016). Thus, species tolerant to water stress, which is able to maintain the aerial growth and at the same time increasing the deep of root system is likely to be more competitive under water shortage.

Overall, changes in the timing and amount of precipitation are likely to alter several biological aspects of invasive weeds, where at the community level it is probable that precipitation extremes will favor competition between invasive weeds and crops with subsequent negative effects on crop productivity (ZISKA et al., 2011) These aspects may directly impact the invasive potential of tough lovegrass with native species like Bahiagrass. This native species has been reported to have large and a deep fibrous root system (ACUÑA et al., 2010; SANTOS et al., 2018; TEJERA et al., 2016) which could be associated with plant ability to recover water from deep soil layers and thereby expression of drought tolerance (BENERAGAMA et al., 2018). This perennial C_4 species is commonly established via seed, although its seedlings are slow to establish in shaded conditions and it is reported to be weak competitors with other species (BUSEY; MYERS, 1979). On the other hand, once established the *E.* plana is notoriously difficult to control, largely because of its extensive creeping root system (ABICHEQUER et al., 2009), the tolerance to drought and freezing, production of allelopathic compound (FAVARETTO et al., 2019), selective grazing (MEDEIROS; FOCHT, 2007), high seed production and long period of seed viability (MEDEIROS et al., 2014) among other traits resulting in a weed with a high potential to compete with native species, even *P. notatum* possessing aforementioned those features. Based on traits of both species, it is likely to *E. plana* express a higher competitive ability compared to *P. notatum*, once the invasive species would take advantage in several ways, such as light harvesting (as a tussock grass), increasing the deep of root system, tolerate long period of drought and also by producing allelochemicals that could be harmful to *P. notatum*.

E. plana is considered the main invasive plant of pastures of the Pampa biome, with competitive characteristics that stand out over local species where currently is spread over the state of Rio Grande do Sul. Considering the known competitive ability of this species as well as current and predicted global changes in precipitation patterns, we performed a greenhouse experiment to determine whether drought conditions would increase *Eragrostis plana* competitiveness with the forage grass, *Paspalum notatum*. We hypothesized that *E. plana* outcompetes *P. notatum* under water stress conditions while that *P. notatum* take advantage on competitiveness over *E. plana* under high water availability.

3.2 Material e Methods

Plant material and growth conditions

Seeds from the two species were collected randomly from several individuals and stored in paper bags at room temperature until the experiments were performed. In November 2016, we sowed seeds from each species in plastic crates (18 x 60x 40cm) containing 40kg of sandy loam soil in November 10th, 2016. At the growth stage of 3-4 leaves, we let 32 seedlings to growth, equally spaced in each crate as showed in Figure 14, regardless of competition treatment. The plant density (32 plants) were defined in a previous experiment conducted from November 20th, 2015 to January 20th, 2016 (data not shown). In this experiment we grown monoculture of both *E. plana* e *P. notatum* aiming to determine the population density per m^2 where the shoot dry mass (g m^{-2}) become steady and independent of population according to the “law of constant final yield” (RADOSEVICH; HOLT; GHERSA, 2007).

Plants were watered to field capacity until February 10th, 2017, when we performed the competition treatments. After all evaluations performed in May 1st, 2017, we let the plants to growth during the winter and spring season and use the same plants to perform a second run of this experiment. In November 2nd, 2017, we thinned plants by leaving only 32 plants per crate and also we uniformized plant size by cutting aerial shoot of species. In the same way as first run, plants were kept under field capacity until February 16th, 2018, when we imposed the water stress again and kept plants for 50 days of cycles of drought/water-recovery.

Competition establishment

Plant competition was performed by keeping two plants proportions: without interspecific competition (100% plants of only one species – 100:0) and interspecific competition (50% *E. plana* and 50% *P. notatum*) with a total of 32 plants regardless of competition treatment. Species were allowed to compete all the time of experiment, from emergence to grain filling stage in 50:50% treatment.

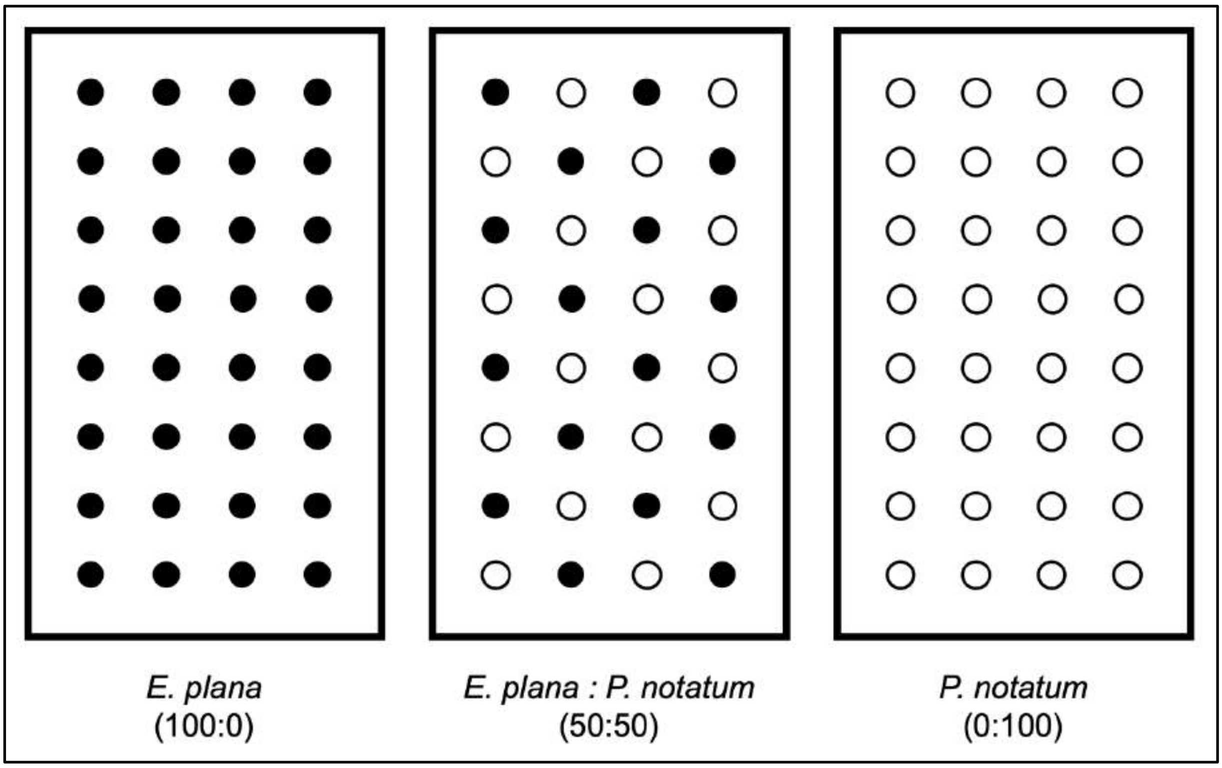


Figure 14 – Distribution scheme of plants across the treatments. *Eragrostis plana* growing without competition (100:0), interspecific competition between *E. plana versus Paspalum notatum* (50:50) and *P. notatum* growing without competition.

Water-Stress Treatment

The plants were maintained at field capacity until the beginning of the water-deficit treatments when the plants reached panicle initiation growth stage (February 2017 or February 2018). Here, we performed a slow water-deficit experiment where the water stress was induced by four cycles of water-deficit (suspension of water replacement) followed by recovery period. In all drought cycles, the water replacement was suspended until the soil reached 5% of volumetric water which was measured by a soil moisture sensor (ECHO EC-5, Decagon Devices América Latina Ltda). The recovery period was reached after each drought period by watering the soil until reach the maximum soil water holding capacity. The plants were kept under these conditions (cycles of drought followed by water recovery) for approximately 50 days. At the end of the last drought cycle (before recovery) at grain filling stage we measured stomatal conductance (g_s), chlorophyll a fluorescence parameters, relative water content (RWC), plant height, tiller and panicle number and above-ground biomass as followed described.

Chl a fluorescence analysis

The chlorophyll *a* fluorescence transient parameters were measured in the last fully expanded leaf between 9-11 h am using a portable fluorometer (Handy PEA, Hansatech Instruments, King's Lynn, Norfolk, UK). Initially, the leaves were adapted for 30 minutes in dark to allow the complete oxidation of the photosynthetic electron transport system. Then, fluorescence emission was induced in a 4mm diameter area by exposing the leaves to a saturating red actinic light at the intensity of 3.000μmol m^{-2} s^{-1}.

Relative water content (RWC)

The RWC was determined as described by Barrs and Weatherley (1962) following the equation: RWC (%) = [(fresh mass - dry mass)/(water saturated mass - dry mass)] X 100. Leaf sections (±10cm length) were taken from the last fully expanded leaf in one plant for each replicate. Then, these segments were weighted (fresh mass) and immediately placed on a plastic box (11x11x3.5cm) fully with deionized water for 24h to obtain the water saturated mass. To determine the dry mass, saturated leaf segments were dried at 60^{o}C until the constant weight was reached.

Stomatal conductance

The stomatal conductance (g_s) was performed by using a steady state porometer (Decagon SC1) (Decagon Devices, Inc., Pullman, Washington) between 9 – 11h am at the last totally expanded leaf.

Plant growth analysis

The biometric analysis was performed at the end of the experiment by measuring tiller number, panicle number and shoot dry mass. The shoot dry mass was obtained by weighting the biomass after drying at 65°C until the constant weight was reached.

Data analysis

Data were analyses *via* two-way ANOVA considering the experiment as a completely randomized design (CRD), arranged in a fully randomized 2 x 2 factorial design (two water treatments and two competitions levels), with four replicates per treatment. The Student's t-test ($p \leq 0.05$) was used to compare the two water conditions and the two competitions levels, by using the software SAS System version 9.0. Data are mean of four replications.

3.3 Results

Even though, the treatments and evaluations were the same for both years of this study, we have decided to analyze each year separated, as long as, in 2016 the plants came from seeds and after the experiment period these plants were kept for the next season to perform the second run (2017/18). We chose this arrangement to simulate a field condition where we have two different scenarios, an one year plant and a perinneal plant which suffered a drought event in the background. Based on that we expected the competition aggressiveness of *E. plana* over *P. notatum* would be enhanced in the second year. There was no significant interactions ($p \geq 0.05$) among plant proportions (competition levels) and water stress factors for both runs. Thus, all data are presented separated by main factors (water treatments or plant proportions).

Chl a fluorescence analysis

There was no effect of *P. notatum* competition or water stress on Chl *a* fluorescence parameters (ET_0/RC), DI_0/RC and F_v/F_m) of *E. plana* in the first run (Table 1). However, in the second run, also plants of *E. plana* increased the flux of electron transport under water stress.

Table 1 – Chlorophyll *a* fluorescence parameters of *Eragrostis plana* under two water treatments and two plant proportions.

Fluorescence parameters	Plant Proportion		Water condition		CV (%)
	100:0	50:50	Well-Watered	Water Stress	
	---------------------------------- 1st Run (2016/17)----------------------------------				
F_v/F_m	0.7016	0.6803	0.6863	0.6955	5.59
DI_0/RC	0.8497	0.9797	0.9573	0.8721	29.49
ET_0/RC	1.0347	1.0234	1.0332	1.0248	7.80
	---------------------------------- 2nd Run (2017/18)----------------------------------				
F_v/F_m	0.6780	0.6793	0.6611	0.6961	4.85
DI_0/RC	0.6975	0.7105	0.7679	0.6409	21.28
ET_0/RC	0.9411	0.8667	0.8256	0.9422 *	7.66

* Indicate statistical differences between plant proportion or water treatments by T test ($p < 0.05$). n = 4 replicates. F_v/F_m (maximum yield of primary photochemistry of PSII), DI_0/RC (dissipated energy flux per reaction center) and ET_0/RC (flux of electron transport from QA^- to PQ per RC).

The effect of *E. plana* competition on Chl *a* fluorescence parameters of *P. notatum* were observed for the two runs of this experiment (Table 2). At first run, plants under competition increased the dissipated energy flux (DI_0/RC) while they decreased the flux of electron transport (ET_0/RC) in the second run. Besides, the main effect of water stress reduced the ET_0/RC, only in the first run.

Table 2 – Chlorophyll *a* fluorescence parameters of *Paspalum notatum* under two water treatments and two plant proportions.

Fluorescence parameters	Plant Proportion		Water condition		CV
	100:0	50:50	Well-Watered	Water Stress	(%)
	---------------------------------- 1st Run (2016/17)----------------------------------				
F_v/F_m	0.7513	0.7367	0.7400	0.7479	3.15
DI_0/RC	0.7006	0.8488 *	0.8179	0.7315	20.12
ET_0/RC	1.2115	1.2129	1.2907 *	1.1337	7.63
	--------------------------------- 2nd Run (2017/18)---------------------------------				
F_v/F_m	0.7716	0.7477	0.7597	0.7596	3.12
DI_0/RC	0.5070	0.4376	0.5042	0.4403	19.58
ET_0/RC	0.9924 *	0.8459	0.9657	0.8726	9.72

* Indicate statistical differences between plant proportion or water treatments by T test ($p < 0.05$). n = 4 replicates. F_v/F_m (maximum yield of primary photochemistry of PSII), DI_0/RC (dissipated energy flux per reaction center) and ET_0/RC (flux of electron transport from QA^- to PQ per RC).

Relative water content (RWC)

The RWC is a suitable predictor of plant water status where in this experiment RWC could inform indirectly how is the competition for water between the chosen species (Table 3). There was no effect of *P. notatum* competition on RWC of *E. plana* regardless of runs. As expected, water stress was enough to reduce RWC of *E. plana* for both runs.

Table 3 – Height, shoot dry matter (SDM), numbers of tillers, numbers of panicles, relative water content (RWC) and stomatal conductance (g_s) of *Eragrostis plana* under two water treatments and two plant proportions.

Main factors	g_s (mmol m^{-2} s^{-1})	RWC (%)	Height (cm)	Tillers (n° plant^{-1})	Panicles (n° plant^{-1})	SDM (g plant^{-1})
	---------------------------------- 1st Run (2016/17)----------------------------------					
100:0	193.0	77.1	106.4*	11.9	2.6	9.5
50:50	185.3	74.2	101.7	10.3	3.7*	12.8*
Well-Watered	228.7 *	88.1*	109.2*	11.0	3.4*	11.8
Water Stress	149.6	63.1	98.8	11.1	2.8	10.5
CV (%)	9.55	11.08	3.23	25.28	11.28	12.18
	-------------------------------- 2nd Run (2017/18)--------------------------------					
100:0	91.1	59.8	134.0	15.0	6.9	14.7
50:50	97.9	70.7	133.4	21.9*	10.4*	21.7*
Well-Watered	130.2*	79.1*	136.1	18.9	9.8*	20.5*
Water Stress	58.7	51.5	131.2	17.9	7.4	15.8
CV (%)	13.36	15.42	5.92	15.17	14.80	10.89

*The lowercase letters indicate statistical differences between plant proportion or water treatments by T test ($p < 0.05$). n = 4 replicates.

The competition with *E. plana* reduced RWC on *P. notatum* plants only at second run while water stress induced a significative reduction on RWC regardless of run (Table4).

Table 4 – Height, shoot dry matter (SDM), numbers of tillers, numbers of spikelets, relative water content (RWC) and stomatal conductance (g_s) of *Paspalum notatum* under two water treatments and two plant proportions.

Main factors	g_s (mmol m^{-2} s^{-1})	RWC (%)	Height (cm)	Tillers (n° plant^{-1})	Spikelets (n° plant^{-1})	SDM (g plant^{-1})
---------------------------------- 1st Run (2016/17)----------------------------------						
100:0	210.9*	73.3	84.0*	7.3*	1.6*	5.3*
50:50	151.7	67.5	72.9	4.5	0.9	3.2
Well-Watered	212.4*	80.7*	81.9	6.2*	1.5*	5.0*
Water Stress	150.3	60.2	75.1	5.6	1.0	3.7
CV (%)	15.28	11.54	8.29	8.44	15.36	8.36
--------------------------------- 2nd Run (2017/18)---------------------------------						
100:0	88.0	69.7*	107.1*	7.6	1.2*	8.3*
50:50	102.3	60.1	93.6	7.1	0.5	4.0
Well-Watered	131.5*	83.2*	102.6*	8.6	0.9	6.6
Water Stress	58.7	46.5	98.0	5.9	0.8	5.7
CV (%)	18.60	11.81	3.75	37.17	33.96	25.18

*The lowercase letters indicate statistical differences between plant proportion or water treatments by T test ($p < 0.05$). n = 4 replicates.

Stomatal conductance

According to our data there was no effect of competition in stomatal conductance of plants of *E. plana* among the two runs (Table 3). However, water stress reduced significatively g_s values in *E. plana* plants, manly in the second run by reducing the values about 2.2-fold compared to 1.5-fold in the first run. It had similar behavior observed on RWC, where in the second run these values were about 10% less compared to first run.

The competition with *E. plana* was significative severe to reduce 1.4-fold the g_s of *P. notatum*, while that second run there was no changes on this parameter (Table 4). The reduction of g_s under water stress followed the same behavior of *E. plana*. As previous observed, reduction due to water shortage were more severe at first run (1.4 fold) compared to second run (2.2-fold).

Plant growth analysis

The only negative effect of *P. notatum* competition on *E. plana* growth was a reduction on plant height particularly at first run (Table 3). The others variables measured like number of panicles and above-ground biomass of *E. plana* increased under competition with similar rate between runs. In the second run, also the number of tillers increased 1.5-fold compared to absence of competition. Main effects of water stress, in the first run, reduced plant height and number of panicles, while that second run besides height, water shortage also reduced shoot dry mass.

Harmful effects of competition with *E. plana* were clear on *P. notatum* growth (Table 4). All variables related to growth reduced under competition, moreover in the second run rates of reduction increased significatively. The only variable not affected by competition was the number of tillers at second run. Similarly, impact of water stress severely reduced *P. notatum* growth, explained by decreasing shoot dry mass, number of tillers, number of spikelets at first run. Alike, plant height, number of tillers and shoot dry mass were reduced at second run.

3.4 Discussion

The induction of water deficit associated to the environment favorable to interspecific competition was enough to change physiological and morphological parameters of both species, thus adding information about invasiveness potential of *E. plana* over the native species *P. notatum* under water stress scenario (Table 3 and 4; Figure 15).

According to our data, it is clearly that competition with *P. notatum* or imposition of water stress does not affect chlorophyll *a* fluorescence parameters, leaf relative water content or stomatal conductance in *E. plana*. However, there was an increasing on dissipated energy flux (first run) and reduction on flux of electron transport (second run) for *P. notatum* when competing with *E. plana*. The coexistence between species could have limited water availability in the medium, where *E. plana* showed to be more efficient to extracted water from soil thus limiting water availability to *P. notatum*. Also, the interference on plant water status was confirmed by reduced stomatal conductance (first run) and low leaf relative water content (second run) in *P. notatum*.

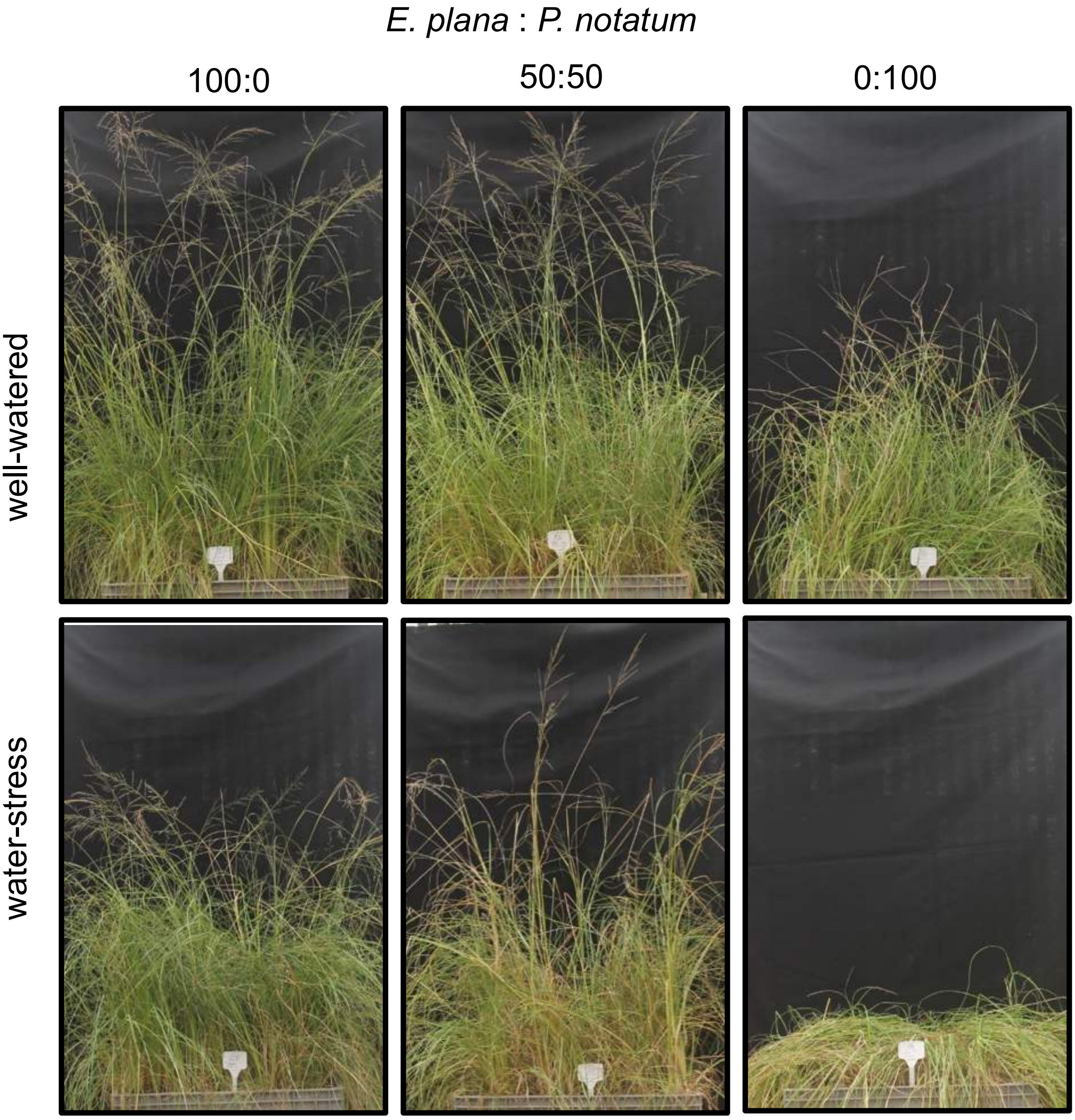


Figure 15 – Plants of *Eragrostis plana* and *Paspalum notatum* under two water treatments (water stress – four cycles of water suspension and well watered – without water suspension) and two plant proportions (100:0 – 100% of one population and 50:50 – 50% of each population).

The water shortage as a result of *E. plana* competition probably result in an impairment on photosynthetic process in *P. notatum*, adding an excess of energy for CO_2 fixation and surplus energy that must be dissipated to prevent serious damages on photosystem machinery. Also, this process to repair photosystem has potential to affect the electron transport fluxes as observed in the second run of the experiment. We should highlight here, that the other effects directed or indirect of competition can interfere on photosynthetic process, among others, competition for light, nutrients, CO_2, etc. The interference of competition on plant water status was enough to interfere on *P. notatum* growth by reducing plant height, number of spikelets, tillers, and above-ground biomass for both runs, except the number of tillers was not changed in the second run (Table 4; Figure 15). The reduced competition performance of *P. notatum* created a favorable environment to enhance the invasiveness potential of *E. plana* confirmed here by increased panicle number, number of tillers (only at second run) and above-ground biomass. Besides that, the absence of significative interaction between plant proportion *versus* water stress, confirmed that *E. plana* had overcome *P. notatum* growth regardless water condition.

The supply of resources may be unlimited in some environments, but limitation is more common and can be caused by unavailability, poor supply, or proximity to neighboring plants, where the presence of neighbors can aggravate an already insufficient resource or create a deficiency where there is ample resource for a single individual (RADOSEVICH; HOLT; GHERSA, 2007). In water-limited environments, it is expected that weeds may have higher leaf water potential than crops, suggesting that a limited amount of water in soil would benefit weeds competitiveness more than crop (CHAUHAN; ABUGHO, 2013; PATTERSON; MAXINE, 1989). Our study observed that, regardless competition environment or water treatments, *E. plana* was taller than *P. notatum* plants (Tablele 3 and 4; Figure 15). Also, these observations are supported by field perceptions of *E. plana* often visible above the *P. notatum* canopy. Thus, *E. plana* as a tussock grass and growing taller than *P. notatum* it is expected to take advantage of the higher light intensity and, at the same time, shading the forage. Besides that, *E. plana* has a deep root system by showing higher root biomass compared to some native species (including *P. notatum*) at 0-40 cm soil depth (ABICHEQUER et al., 2009). The root dry biomass mass of *E. plana* can reach up to 66% higher than roots of native species of Southern grasslands (ABICHEQUER et al., 2006). Also, it is expected that high nitrogen species with rhizomatous and stoloniferous roots like *P. notatum* and *Axonopus afnis* (species predominant in southern grasslands) have a root system less developed and less efficient to explore water and nutrients from soil (CRAINE et al., 2002; MEDEIROS; FOCHT, 2007) than tussock grasses showing poor nitrogen content (LEATHWICK; BOURDÔT, 2012). Thus, if *E. plana* extracted water more efficiently than *P. notatum* then a limited amount of water in soil would benefit its competition potential and the drought tolerance. Another effect that may have influenced the advantage of *E. plana* over *P. notatum* is the allelopathic potential of the weed, where the biosynthesis of allelochemicals may have favored by stress which could be more harmful to the native species under conditions of water shortage (RADOSEVICH; HOLT; GHERSA, 2007).

The isolated effects of water stress over the species were independent of competition treatment. Based on g_s and RWC we can prove that stress was enough to reduce these key variables responsive to drought. Also, data showed that in the second run the level of stress apparently seems to be more severe compared to first run for both species. We can associate data from water status (g_s and RWC) with growth variables to better understand the response to drought. As the reduction of g_s and RWC was more severe at second run, plants of *E. plana* responded reducing above-ground biomass and consequently occurred decreasing on number of panicles, which is considered critical for perpetuation of an invasive plant. We should highlight that in the second run plants of *E. plana* has accumulated more biomass compared to the first run, where in the well-watered condition plants accumulated about 20.5g compared to only 11.8g in the first run, it is 1.7-fold higher biomass. As plant biomass become higher, more water is necessary to supply the plant demand, as a result drought stress was enhanced. Reduction on aerial biomass plays an important role in reducing total plant water losses, ensuring cell turgor and therefore in the survival of many species especially under drought, where it has been considered as a mechanism of dehydration avoidance (GEPSTEIN, 2004; MUNNÉ-BOSCH; ALEGRE, 2004; VOLAIRE, 2018).

Similarly, to leaf biomass, plants also tend to reduce the investments in reproductive organs that requires vast amounts of energy and water, because reduction in photosynthesis and changes in phenology (e.g., shortening growth period and early flowering) in relation to drought cause significant reduction in biomass allocation to reproductive parts (EZIZ et al., 2017).

For *P. notatum*, the increasing on plant biomass was less expressive, being only 1.3-fold more than first run. The less noTablele above-ground growth probably is due to invasive potential of *E. plana* to overcome *P. notatum*, thus less biomass production can be directly correlated to low water requirement. Besides that, both species has the necessity to continuously supply the seed soil bank, thus water stress seems to play an important role affecting this process, mainly for *E. plana*, based on reduction of panicle number observed in the both runs.

3.5 Conclusions

In the current study, we can conclude that, even suffering reduction on plant growth parameters, both *E. plana* or *P. notatum* can survive to continuous cycles of drought. However, *E. plana* can overcome the native grass *P. notatum* either in well-watered or drought conditions, as well as, the invasive potential of *E. plana* is enhanced older than one year.

4. CHAPTER III - Optimizing herbicidal efficacy of glyphosate through ammonium sulphate as adjuvant in *Eragrostis plana* under water stress

4.1 Introduction

Eragrostis plana Nees known as tough lovegrass or South African lovegrass (capim-annoni in Brazil) is a C_4 perennial grass native from South Africa, which has become increasingly problematic in southern Brazilian grasslands (BARBOSA, 2016). The management of this species is extremely complicated after its dispersion across the areas of native pasture. The botanical similarity among *E. plana* and the native species (mostly grasses) have complicated the selective control of weed (GOULART et al., 2012). In recent years, farmers have reported cases of herbicide control failures, even in burndown operations by using glyphosate, where often plants can regrowth after herbicide application. The efficacy of glyphosate in control *E. plana* has been related to be influenced by growth stage. Also, it has been proposed a mechanism called “interdependence among tillers” which is believed that glyphosate do not translocate through whole tillers, especially those considered older, resulting in some levels of tolerance to the herbicide (BASTIANI et al., 2018; CORRÊA et al., 2014).

Rather than applying herbicides as droplets, they can also be wiped directly on to the foliage of weeds from the moistened surface of a “wiper applicator”, where wiping of herbicides can eliminate the risk of spray drift and also allow selective use of non-selective translocated herbicides (HARRINGTON; GHANIZADEH, 2017). Recently, it was developed a wiper applicator exclusively to control *E. plana* in native pasture, where the weed control can reach up to 80% without any injury in the native pasture which is shorter than *E. plana* plants (PEREZ, 2010). Some observations at field of unsatisfactory control of *E. plana* has been related by using wiper applicator or even if applied by conventional spraying. This could be related to several cases, including ununiformed plant size, advanced growth stage, low translocation of glyphosate among tillers, herbicide applications under drought periods, etc. Thus, the knowledge of the processes involved in the tolerance to the herbicide are necessary to perform an efficient weed management.

In several cases, the differences in the susceptibility of plants to glyphosate, whether in crops or weeds, are related to differences in herbicide absorption and/or translocation. This was observed for *Lolium multiflorum* and *Sorghum halepense* where leaf characteristics were responsible for lower glyphosate absorption (GALVANI et al., 2012; GHANIZADEH et al., 2016). Recently, it was confirmed that absorption and impaired translocation endows glyphosate resistance in *Amaranthus palmeri* populations (PALMA-BAUTISTA et al., 2019). In the same way, plants of *Spermacoce verticillata* may escape control with the herbicide glyphosate due to difficult of absorption and/or translocation (FADIN et al., 2018).

The leaf cuticle and plasma membrane are one of the barriers limiting glyphosate activity (TRAVLOS; CHEIMONA; BILALIS, 2017), by reducing the herbicide absorption. In most cases, neither glyphosate nor its different salts are effective in overcoming these barriers easily without appropriate adjuvants (HESS; CHESTER, 2000; SATCHIVI et al., 2000). Among adjuvants, ammonium fertilizers can prevent the formation of participates in the tank mix, decrease surface tension, increase herbicide spreading and penetration into the leaf (NALEWAJA; MATYSIAK, 2000). Particularly, the addition of ammonium sulphate (AMS), an inorganic salt, to the glyphosate spray solution have been reported for years to improve the efficacy of the herbicide (PLINE; HATZIOS; HAGOOD, 2000; SALISBURY; CHANDLER; MERKLE, 1991; SOLTANI et al., 2016). Additionally, it has been found that salts dissolved in water used as the carrier for glyphosate may reduce its effectiveness, particularly calcium and magnesium salts (WILLS; MCWHORTER, 1985). Also, ammonium sulfate can significantly improve herbicide activity when weeds are grown under water stress conditions (SATCHIVI et al., 2000). Plants that are grown under drought conditions can develop thicker cuticles or leaf pubescence, which inhibit herbicide absorption, and lowered levels of photosynthesis and photoassimilates transport contribute to reduced herbicide translocation (DE RUITER; MEINEN, 1998). Thus, adjuvants, like ammonium sulfate can overcome this problem by enhancing absorption and/or translocation under a scenario of drought, which is likely to happen in areas where *E. plana* has been invaded.

Water shortage can alter a number of biological aspects of plants as weeds including germination, plant size, seed production, and the distribution of waterborne seeds (ZISKA et al., 2011). Also, physiological processes as cell growth, considered one of the most drought-sensitive due to the reduction in turgor pressure (ANJUM et al., 2011), can be affected bu water deficit. Water stress caused impaired mitosis; cell elongation and expansion resulted in reduced growth and yield traits (HUSSAIN et al., 2018).

In order to achieve the main goal of this work, we performed several preliminary studies aiming to (i) evaluate the control of plants in different growth stages with the application of glyphosate salts, (ii) perform a dose-response curve of glyphosate on *E. plana* control and (iii) evaluated the beneficial performance of ammonium sulfate to enhance the glyphosate efficacy on *E. plana* control under water stress. Based on previous findings that *E. plana* exhibit the highest tolerance to drought stress at vegetative stage, we hypothesize that during this stage the plants also become more tolerant to glyphosate regardless the salt formulation, which could be even less efficient under conditions of plant water stress where the addition of adjuvants may reduce the most of harmful effects of stress. Thus, to test theses hypotheses the main objective of this work was understanding the effect of ammonium sulfate tank mixture on absorption and translocation of glyphosate herbicide in *E. plana,* comparing to *Echinochloa colona* as a high glyphosate-sensitive plant.

4.2 Material and Methods

4.2.1 Efficacy of glyphosate salts in *E. plana* control in different growth stages

Plants of *E. plana* were grown under greenhouse conditions at Federal University of Pelotas, Capão do Leão, RS from January 10th, 2017 to May 20th, 2017. The establishment was done by sown the seeds in pots containing 8kg of sandy loam soil in different periods in order to have the following growth stages: 5-6 leaves, tillering and panicle initiation. It was established four plants per pot.

Plants in the aforementioned stages were subjected to glyphosate application at the sub lethal rate of 540g a.e. ha^{-1}. In total, three glyphosate salts treatments plus one control (no herbicide spray) were tested, with four replicates each (Table 5). The herbicide was applied using a backpack sprayer, kept at constant pressure using CO_2 (30 Psi) and equipped with a 2m spray boom and four flat fan nozzles (Tee-Jet AIXR110015) spaced 0.5m apart, which was set to distribute a spray volume of 150L ha^{-1}.

Table 5 – Herbicides, rates and product information for treatments used in *E. plana* control.

Registered brand	Glyphosate salts	Concentration (g a.e. L^{-1})	Commercial rate (L ou Kg ha^{-1})	Rate (g a.e. ha^{-1})
Control	-	-	-	-
Roundup Original	isopropylamine (IPA)	360	1.50	540
Rondup Original DI	diammonium	370	1.46	540
Roundup Transorb R	potassium	480	1.13	540
Roundup WG	ammonium	720	0.75	540

At 60 days after application (DAA), the herbicide control efficacy was evaluated through a visual scale of plant damage, ranging from 0 to 100%, with 0% being the absence of visible symptoms and 100% plant death (SBCPD, 1995). At this time, also above-ground dry weight per pot was determined. The data were subjected to analysis of variance, and when significant, the means were compared by the Tukey test at 5% probability ($P \leq 0.05$).

4.2.2 Glyphosate dose-response curve

The plant material and growth condition were the same as described in the previous experiment. The experiment was performed under greenhouse conditions at Federal University of Pelotas, Capão do Leão, RS from February 5th, 2017 to June 8th, 2017. Glyphosate (potassium salt) was applied to the plants at tillering growth stage, by using a backpack sprayer, at the same calibration form as described before. Glyphosate was applied at rates of 0, 22.5, 45, 90, 180, 360, 720 and 1,440g acid equivalent (a.e.) ha^{-1} to plants of *E. plana*. Above-ground dry weight per pot was determined at 42 days after spraying. Data were plotted and fitted to a nonlinear log–logistic regression model, according to equation [1]:

$$y = c + \left\{(\mathrm{d} - \mathrm{c}) / \left[1 + \left(\frac{x}{e}\right)^{b}\right]\right\} \text{ eq. [1]}$$

where *c* is the lower limit of *y*, *d* the upper limit, *e* is the x that yields response y halfway between *d* and *c*. Finally, *b* is the relative slope. The data from dose-response were analyzed using the drc package in R software, as proposed by Knezevic; Streibig and Ritz (2007).

4.2.2 Ammonium sulfate enhancing the glyphosate efficacy on *E. plana* control

The study was conducted in greenhouse at Altheimer Laboratory Complex of the Dept. of Crop, Soil and Environmental Sciences, University of Arkansas, Fayetteville, USA where the Weed Physiology Laboratory is located, from June 25th, 2017 to December 11st, 2017. The establishment was done by sowing seeds in pots containing 8kg of field soil from experimental field of the Department of Crop, Soil and Environmental Sciences.

All plants were maintained at field capacity until the beginning of the water-deficit treatments when they reached the full tillering stage (four days before the herbicide spraying). The plants were subjected to water deficit by maintaining the water content in the soil at 50% and 100% of the total water storage capacity of the pot (C_w). The C_w was determined using the fresh mass of the soil after water saturation (C_{fm}) and the dry mass (C_{dm}) after soil drying for 24h at 105°C and applied in the equation $C_w = (C_{fm} - C_{dm})/C_{fm} \times 100$ as described by Santos et al. (2015). At the beginning of water treatment all pots were saturated by water, drained and weighted. Then, the pots were weighted every day by replacing the water lost by evapotranspiration assuming the maintenance of C_w at 50 and 100% and considering 1mL = 1g. The plants were kept under these conditions until four days after herbicide application as described by Figure 16.

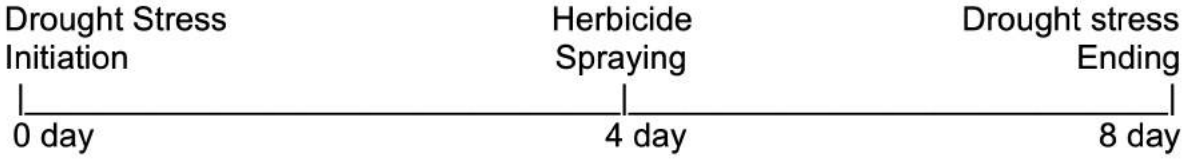


Figure 16 – Scheme of time of water stress induction and herbicide application.

Four days after drought stress initiation, the herbicides treatments were sprayed. The experiment was arranged in a completely randomized design, using a factorial arrangement of treatments of with four replications and conducted twice. For the absorption experiment, factor A was the soil water conditions (50% and 100% of water replacement) and factor B was herbicide treatments [control (no herbicide), glyphosate alone (312g a.e. ha^{-1}), glyphosate (312g a.e. ha^{-1}) + ORO-121 (0,25% v/v) and glyphosate (312g a.e. ha^{-1}) + AMS (1,5% wt/v)]. We defined glyphosate application rate of 312g a.e. ha^{-1} ($LD_{50\%}$ determined in the previous experiment) in order to allow a better performance of adjuvants. The herbicide treatments were applied using spray chamber, kept at constant pressure using CO_2 (40 psi) and equipped with a 1m spray boom and two flat fan nozzles (UNIJET TPU-800067SS) spaced 0.5m apart, which was set to distribute a spray volume of 187L ha^{-1}.

At 60 DAA, the herbicide control efficacy was evaluated through a visual scale of plant damage, ranging from 0 to 100%, with 0% being the absence of visible symptoms and 100% plant death described before, according to SBCPD (1995). At this time, also above-ground dry weight per pot was determined. The data were subjected to analysis of variance, and when significant, the means of the two water conditions were compared by Student's t-test ($p \leq 0.05$) and Tukey test at 5% probability ($P \leq 0.05$) was used to compare herbicide treatments by using the software SAS System version 9.0. Data are mean of eight replications.

4.2.3 Ammonium sulfate effect on absorption and translocation of glyphosate in *E. plana*

The study was conducted in greenhouse conditions at Altheimer Laboratory Complex of the Dept. of Crop, Soil and Environmental Sciences, University of Arkansas, Fayetteville, USA where the Weed Physiology Laboratory is located, in September 14th 2017 to January 22nd, 2018. As the pattern of glyphosate absorption and translocation have not been reported in the literature, we decided to use *Echinochloa colona* as a high glyphosate-sensitive plant to compare with *E. plana*. Seeds of *E. plana* were originate from a native pasture area of Federal University of Pelotas, Capão do Leão, RS, Brazil. For *E. colona*, seeds were collected in rice field in Fayeteville, AR, USA.

The experimental units were pots with soil capacity of 8 kg, filled with field soil from Department of Crop, Soil and Environmental Sciences. The plant material and growth condition were the same as described in the efficacy experiment, except that we let only one plant per pot after the thinning operation.

The experiment was arranged in a completely randomized design, using a factorial arrangement of treatments of with four replications and conducted twice. For the absorption experiment, factor A was the ammonium sulphate addition (glyphosate alone and glyphosate + AMS) and factor B was the sampling times (24, 72 and 144 hours after herbicide treatment - HAT). During each experiment, 4 replications of non-treated plants were grown to remove background radioactivity. We choose a rate of 720g a.e. ha^{-1} that promote 100% of control in *E. plana* (data not shown) and 1.5% wt/v of ammonium sulphate as described on commercial label. Full rate of glyphosate (720 g a.e. ha^{-1}) was based on methodology described by Nandula and Vencill (2015). According to them sublethal rates may be applicable in certain situations, but do not provide the same physiological response as a full rate. Each species was equivalent to one experiment that were evaluated separately.

The herbicide absorption/translocation was performed by using glyphosate (720g a.e. ha^{-1} + radioactive ^{14}C-glyphosate). The radiolabeled herbicide was applied to second and third youngest and fully expanded leaf (Figure 17) using a 50-μL microsyringe in six 2-μL droplets containing 12,500dpm/μL of the aforementioned treatments. Plants were harvested at 24, 72, and 144 HAT. At each harvest time, plants were removed from the pots and fractionated into six parts [above treated leaf (ATL), treated leaf (TL), stem of main tiller (SMT), roots of main tiller (RMT), tillers (TL), roots of tillers (RT)] as described on Figure 17.

The treated leaves were washed just with deionized water to remove the non-absorbed herbicide. The rinsate from the treated leaf of each sample was mixed with 15mL of scintillation cocktail (Ultima Gold™; PerkinElmer Inc., Waltham, MA, USA) and quantified using a liquid scintillation spectrometer (LSS) (Packard Tri-Carb 2100TR liquid scintillation spectrometer; Packard Instrument Co., Downers Grove, IL, USA). Absorption of herbicides were determined as the percentage of total radioactivity recovered (rinsate + radioactivity inside the plant) as described by eq. [2]:

$$Absorption\ (\%) = \frac{total\ of\ radioactivity\ on\ tissues}{total\ of\ radioactivity\ on\ tissues + radioactivity\ from\ rinsate} \times 100 \quad \text{eq. [2]}$$

For the translocation experiment, a factorial 2 X 3 X 6 arrangement of treatments was used. Factor A was the addition or absence of ammonium sulphate; factor B was the harvest time (24, 72 and 144 HAT); and factor C was represented by plant tissue part (Figure 17). After sectioning, the plant portions were dried at 60°C for 48h and then oxidized in a biological oxidizer (OX700™; R. J. Harvey Instrument Corp., Tappan, NY, USA). The $^{14}CO_2$ evolved during sample combustion was trapped in a scintillation vial containing 15mL of scintillation cocktail (Carbon-14 Cocktail; R. J.

Harvey Instrument Corp.) and quantified using LSS, as mentioned above. In the following plants parts: roots of main tiller (RMT), tillers (TL) and roots of tillers (RT), all plant parts were ground in a coffee grinder (Model: BCG211OB, KitchenAid, USA). Then a three subsamples (1g) were taken to oxidize in the same way as described above. Considering an efficiency of 85.2% for the biological oxidizer and 95.6% for the LSC counter, the total amount recovered in the translocation experiment was 83.67 and 84 % for *E. plana* and *E. colona*, respectively.

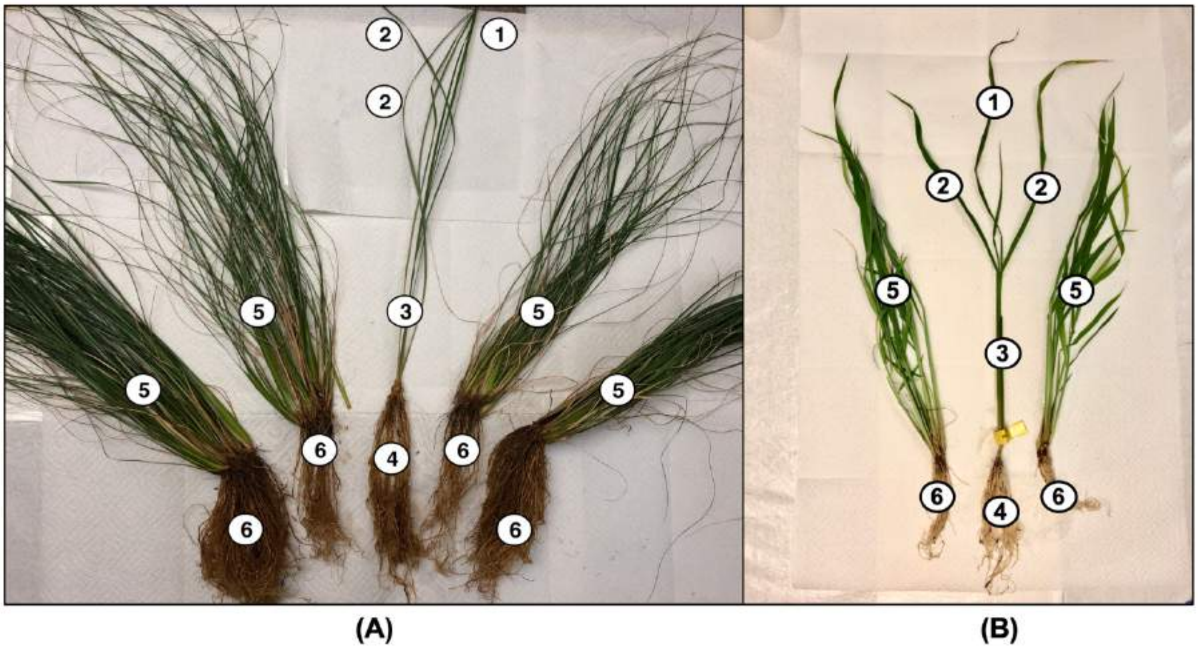


Figure 17 – Plants parts of *E. plana* (A) and *E. colona* (B) used to evaluation of glyphosate translocation. Legend: (1) above treated leaf, (2) treated leaf, (3) stem of main tiller, (4) roots of main tiller, (5) tillers, (6) roots of tillers.

There was no interaction between treatment factors and runs, thus the runs were combined. The interaction among treatment factors of each experiment was investigated. When significant differences were found, Student's t-test ($p \leq 0.05$) was performed to separate means by using R software.

4.3 Results

4.3.1 Efficacy of glyphosate salts in *E. plana* control on different growth stages

As the growth stages of glyphosate application did not match the same time to spray, we decided to analyze them separately. At 7DAT and 5-6 tillers, the potassium salt provided the highest percentage of *E. plana* control followed by diammonium, isopropylamine and ammonium (Table 6). Almost the same behavior was observed for application at panicle initiation stage where potassium and isopropylamine salts at 7 DAT exhibited higher efficacy compared to diammonium and ammonium salt. However, when the glyphosate was sprayed at full tillering stage there were no differences in *E. plana* control at 7, 14 and 21 DAT. As increasing the days after herbicide application, the differences among salts became lower, mainly at 35 DAT, when the control of *E. plana* was equally among salts regardless of growth stage. The data from shoot dry mass showed the same tendency as control at 35 DAT, no differences happened among different salts of glyphosate regardless the stage when glyphosate was sprayed (Table 6).

Table 6 – *E. plana* control (%) at 7, 14, 21, 28 and 35 days after treatment (DAT) and shoot dry matter (SDM) with glyphosate salts (isopropylamine, diammonium, potassium and ammonium) applied in three growth stages of plants.

Glyphosate Salts	*E. plana* control (%)					SDM (g plant^{-1})
	7 DAT	14 DAT	21 DAT	28DAT	35 DAT	
			5-6 tillers			
Check	0 d*	0 c	0d	0 d	0 d	10.5 a
Isopropylamine	15 c	33 b	40 c	45 c	72 a	2.2 b
Diammonium	37 b	72 a	88 a	99 a	100 a	1.5 b
Potassium	55 a	83 a	99 a	100 a	100 a	1.7 b
Ammonium	15 c	32 b	62 b	77 b	86 a	1.3 b
CV (%)	14,04	19,90	7,45	10,67	15,20	16.37
			Full tillering			
Check	0 b*	0 b	0 b	0 c	0 c	17.2 a
Isopropylamine	38 a	45 a	30 a	32 ab	33 b	7.4 b
Diammonium	27 a	33 a	33 a	30 b	35 ab	9.0 b
Potassium	40 a	48 a	47 a	40 ab	55 ab	7.4 b
Ammonium	33 a	42 a	42 a	50 a	57 a	9.8 b
CV (%)	29,20	32,50	35,40	23,70	23,00	20.00
			Panicle initiation			
Check	0 c*	0 d	0 c	0 c	0 b	36.6 a
Isopropylamine	77 a	93 a	97 a	98 a	98 a	21.9 b
Diammonium	43 b	73 b	80 b	83 b	85 a	26.9 ab
Potassium	65 a	93 a	97 a	99 a	99 a	20.0 b
Ammonium	38 b	53 c	75 b	85 b	93 a	19.9 b
CV (%)	12,26	7,02	4,79	3,67	7,25	20.6

*The different lowercase letters indicate statistical differences among glyphosate salts by Tukey test ($p \leq 0.05$). n = 4 replicates.

Despite the fact that statistical comparison among growth stages was not possible, we can highlight here some pattern of glyphosate salts and make some assumptions. If made an average across salts inside of each growth stage the highest efficacy of *E. plana* control was reach when the plants were at panicle stage (94.75%), followed by 5-6 tillers (89.5%) and then by the most tolerant stage represented by full tillering (45%) (Table 6). Although, for SDM, the highest biomass reduction occurred at 5-6 tillers (84%) as expected, followed by full tillering (52%) and panicle initiation (40%).

4.3.2 Glyphosate dose-response curve

Based on data from previous experiment we choose to perform a dose-response curve of glyphosate in the most tolerant growth stage (Figure 18). The main goal of this experiment was to achieve the dose responsible to reduce the growth in 50% (GR_{50}). According to statistical analyzes, the best fit to our data of dry mass accumulation was a log-logistic model with four parameters. The log-logistic model has been extensively used to describe herbicide effects, mainly for determination of GR_{50} in weeds (RITZ; KNISS; STREIBIG, 2015). Based on dose-response curve the GR_{50} for *E. plana* is 312.27g a.e. ha^{-1} of glyphosate.

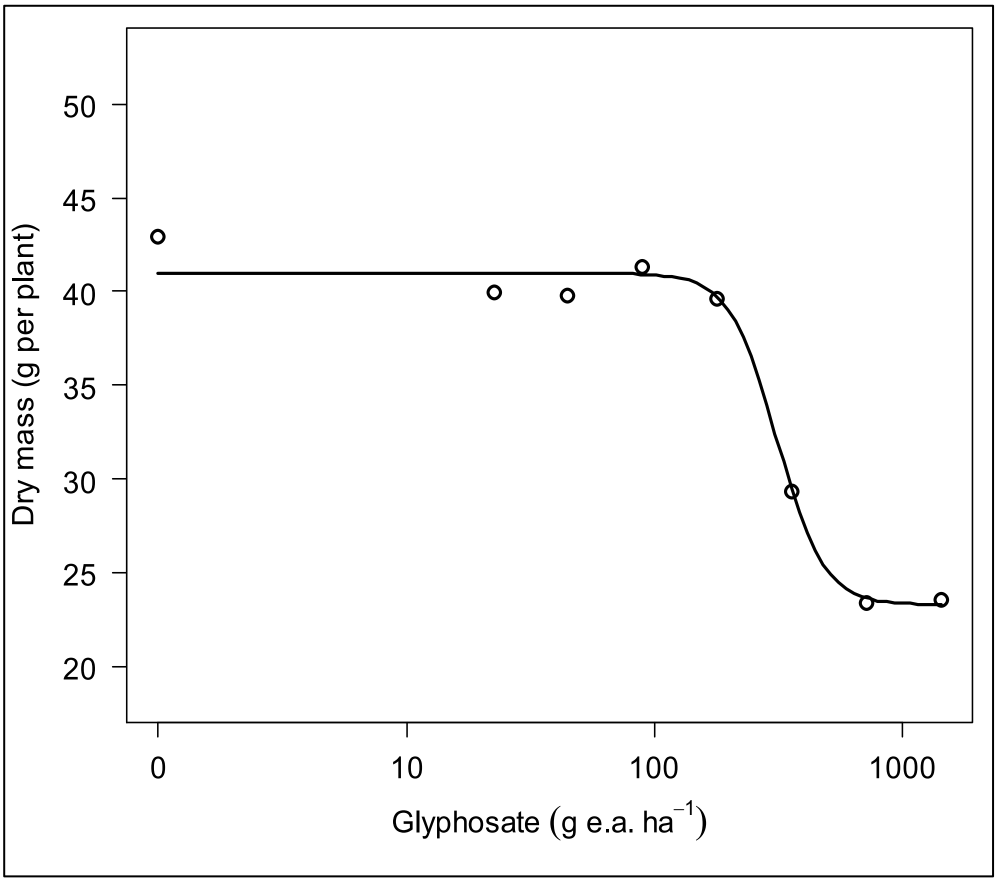


Figure 18 – Dose-response curve to shoot dry mass in *Eragrostis plana* after treatment with glyphosate at 42 days after herbicide application. $y = 23.27 + \left\{(40.97 - 23.27)/\left[1 + \left(\frac{x}{312.27}\right)^{4.63}\right]\right\}$

4.3.3 Ammonium sulfate enhancing the glyphosate efficacy on *E. plana* control

Results indicated that plants drought-stressed showed lower efficacy of control compared to well-watered condition, ranging from 7 to 21 DAT (Table 7) regardless of adjuvant treatment. Except for control using glyphosate + AMS where no differences were observed from 21 DAT. Under drought, there was low efficacy of control using glyphosate plus ORO-121 adjuvant for all visual evaluations performed, including the shoot dry mass. For the treatments with glyphosate alone or glyphosate + AMS, the percentage of *E. plana* control was equally between the two water conditions at 28 and 60 DAT, which resulted in the same accumulation of SDM.

Table 7 – Efficacy of glyphosate sprayed with adjuvants on control (%) and shoot dry mass accumulation of *Eragrostis plana* under drought conditions at 60 days after herbicide treatment.

Water conditions	Herbicide treatments			
	untreated	glyphosate	glyphosate +OR-121	glyphosate +AMS
	Control (%)			
	7 DAT			
Well-watered	0 Ac	20.6 Ab	19.4 Ab	33.8 Aa
Drought	0 Ab	0 Bb	0 Bb	10.6 Ba
	14 DAT			
Well-watered	0 Ac	24.4 Ab	26.9 Ab	51.3 Aa
Drought	0 Ab	20.6 Ba	15.6 Ba	28.8 Ba
	21 DAT			
Well-watered	0 Ac	30.6 Bb	35.0 Ab	68.8 Aa
Drought	0 Ad	40.6 Ab	23.8 Bc	61.9 Aa
	28 DAT			
Well-watered	0 Ac	40.0 Ab	41.3 Ab	77.5 Aa
Drought	0 Ad	36.3 Ab	24.4 Bc	71.3 Aa
	60 DAT			
Well-watered	0 Ac	76.3 Ab	70.0 Ab	99.4 Aa
Drought	0 Ad	63.1 Ab	38.1 Bc	98.1 Aa
	Shoot dry mass			
Well-watered	36.5 Aa	13.9 Abc	17.9 Bb	11.8 Ac
Drought	37.4 Aa	19.3 Ac	29.1 Ab	11.5 Ac

*The different lowercase letters indicate statistical differences among herbicides treatments by Tukey test ($p \leq 0.05$) and uppercases between water conditions by Student's t-test ($p \leq 0.05$). n = 4 replicates.

The comparison among adjuvants allow us to prove that AMS clearly enhanced glyphosate efficacy (Table 7). Even, under water stress at 7 DAT, the first injury symptoms appeared being around 10.6% compared to 0% for glyphosate alone or glyphosate + ORO-121. For all visual evaluations of control, the addition of AMS on glyphosate tank mixture showed higher efficacy compared to the other adjuvants, except at 14 DAT under drought condition. There was no contribution of ORO-121 in enhancing glyphosate efficacy explained by data of visual control, adversely at 28 and

60 DAT, the percentage of control decreased compared to glyphosate alone. Besides, this behavior was confirmed by SDM data, where plants sprayed with glyphosate + ORO-121 showed higher biomass compared to glyphosate alone or glyphosate + AMS. Even though, no differences in SDM at 60 DAT were observed between glyphosate alone and glyphosate + AMS, there was a clear difference in visual control being 99.4 and 98.1% for glyphosate + AMS and 76.3 and 63.1 for glyphosate alone under well-watered and drought, respectively.

4.3.4 Ammonium sulfate effect on absorption and translocation of glyphosate in *E. plana*

Based on the findings from the previous experiment where it was clear the effect of ammonium sulphate on glyphosate efficacy, we decided to perform an experiment of ^{14}C-glyphosate absorption and translocation to better understanding the reason why AMS enhance glyphosate activity in *E. plana*. Thus, as the beneficial effect of AMS was observed for both water treatments, we choose to perform the experiment only under well-watered condition. Besides, as the pattern of glyphosate absorption and translocation have not been reported in the literature, we decided to use *Echinochloa colona* as a high glyphosate-sensitive plant to compare with *E. plana*.

There was no interaction between factors evaluated (harvest times and ammonium sulphate addition) for absorption experiment regardless of species. The ^{14}C-glyphosate absorption in *E. plana* was not enhanced by AMS addition, on the other hand, AMS increased in 5.5% ^{14}C-glyphosate absorption in *E. colona* (Table 8). The absorption of ^{14}C-glyphosate increasing over time for *E. colona* while it was stabilized from 72 to 144 HAT for *E. plana*.

Table 8 – Effect of ammonium sulfate (AMS) addition on total absorption of ^{14}C-glyphosate in *Eragrostis plana* and *Echinochloa colona*.

HAT	*Eragrostis plana*	*Echinochloa colona*
	-- % of applied	--
24	19.55 b	19.05 c
72	27.69 a	26.76 b
144	32.63 a	44.04 a
none	27.66 ^{ns}	27.21*
ams	25.60	32.69
CV (%)	23.87	27.32

The different lowercase letters or * indicate statistical differences among harvest times or addition of AMS by Student's t-test ($p \leq 0.05$) for each species. n = 8 replicates.

As the pattern of absorption, ^{14}C-glyphosate translocation is steady from 72 HAT while that ^{14}C-glyphosate + AMS still increasing until 144 HAT (Table 9). At 24 HAT, the total translocation was higher in ^{14}C-glyphosate alone than ^{14}C-glyphosate + AMS and equal at 72 HAT. However, total translocation of glyphosate plus AMS was 9.2% more than ^{14}C-glyphosate sprayed alone at 144 HAT.

Table 9 – Effect of ammonium sulfate (AMS) addition on total translocation out of treated leaf of ^{14}C-glyphosate in *Eragrostis plana*.

HAT	glyphosate	glyphosate + AMS
	---------------------------------- % of total absorbed	-----------------------------------
24	19.28 Ab	7.21 Bc
72	49.43 Aa	44.95 Ab
144	53.40 Ba	62.68 Aa
CV (%)	11.96	

*The different lowercase letters indicate statistical differences among harvest times and uppercases indicate differences between ammonium sulfate addition by Student's t-test ($p \leq 0.05$). n = 8 replicates.

There was no interaction between factors evaluated for the translocation out of treated leaf for *E. colona*. The analysis of the main factors indicated that highest percentage of ^{14}C-glyphosate was reached at 72 HAT (43.49%), followed by 144 HAT (29.58%) and then by 24 HAT (21.94%) (Table 10). According to our data, addition of AMS increased in 5% the total ^{14}C-glyphosate translocated out of treated leaf.

Table 10 – Effect of ammonium sulfate (AMS) addition on total translocation out of treated leaf of ^{14}C-glyphosate in *Echinochloa colona*.

Main	Translocation out of treated leaf (% of total absorbed)
24	21.94 c
72	43.49 a
144	29.58 b
glyphosate	29.24 b
glyphosate + AMS	34.04 a
CV (%)	27.32

*The different lowercase letters indicate statistical differences among harvest times or ammonium sulfate addition by Student's t-test ($p \leq 0.05$). n = 8 replicates.

The analyses of distribution of ^{14}C-glyphosate in plants parts of *E. plana* (Table 11) at 24 and 72 HAT indicated that the concentration of ^{14}C-glyphosate from highest to lowest percentage was maintained in the treated leaf (TL) > stem of main tiller (SMT) > roots of main tiller (RMT) ≥ tillers (TL) ≥ roots of tillers (RT) ≥ above treated leaf (ATL) regardless of addition or not of AMS. At 144 HAT, when glyphosate was sprayed alone the ^{14}C-glyphosate maintained in the TL was equivalent to percentage in SMT.

However, there was more ^{14}C-glyphosate in STM than TL when performed the application of glyphosate + AMS.

Table 11 – Effect of ammonium sulfate (AMS) addition on distribution of ^{14}C-glyphosate in *Eragrostis plana*.

Plant part	24 HAT				72 HAT				144 HAT			
	glyphosate		glyphosate + AMS		glyphosate		glyphosate + AMS		glyphosate		glyphosate + AMS	
	--------------------------------------- % of total absorbed ---------------------------------------											
Above treated leaf	0	Ad	0	Ac	2	Ad	1	Ad	3	Ac	3	Ad
Treated leaf	81	Aa*	93	Aa	52	Ba*	55	Aa	47	Ca*	37	Ab
Stem of main tiller	15	Cb*	6	Cb	40	Bb	39	Bb	44	Aa*	53	Aa
Roots of main tiller	4	Ac	1	Bc	5	Ac	7	Ac	7	Ab	7	Ac
Tillers	0	Ad	0	Ac	0	Ad	0	Ad	0	Ac	0	Ad
Roots of tilllers	0	Ad	0	Ac	1	Ad	0	Ad	2	Ac	0	Ad

The different lowercase letters indicate statistical differences among plants parts, uppercases indicate differences between harvest times and * indicate statistical differences between ammonium sulfate addition by Student's t-test ($p \leq 0.05$). n = 8 replicates.

Quite interestingly, the addition of AMS increased the quantity of glyphosate translocated out of TL, being maintained mainly in the SMT, but no difference was observed in the quantity of glyphosate translocated to the roots at any time. The ^{14}C-glyphosate translocate to SMT was 9% higher when glyphosate was mixed with AMS in last harvest time. No ^{14}C-glyphosate was quantified in the tillers regardless the addition of AMS. In the RT 1 and 2% of ^{14}C-glyphosate was found in the absence of AMS, while no traces of ^{14}C-glyphosate were quantified for glyphosate + AMS. We hypothesize the traces found in the RT were likely to be a sample contamination during the washing and roots separations process.

Similarly, to *E. plana*, the analyses of distribution of ^{14}C-glyphosate in plants parts of *E. colona* (Table 12) for all harvest times indicated that the concentration of ^{14}C-glyphosate from highest to lowest percentage was maintained in the treated leaf (TL) > stem of main tiller (SMT) > roots of main tiller (RMT) ≥ tillers (TL) ≥ roots of tillers (RT) ≥ above treated leaf (ATL) regardless of addition or not of AMS. At 24 and 72 HAT, when glyphosate was sprayed alone, the ^{14}C-glyphosate maintained in the TL was higher than application of glyphosate + AMS. This induced a higher translocation of ^{14}C-glyphosate out of treated leaf, mainly accumulated in the SMT when AMS was added to glyphosate at 24 and 72 HAT. Although there was no effect of AMS on ^{14}C-glyphosate distribution at 144 HAT. As observed for *E. plana*, AMS did not change the quantity of glyphosate translocated to the roots of *E. colona* at any time. Traces of ^{14}C-glyphosate, ranging from 1 to 2%, were quantified in the tillers and roots of tillers, regardless the addition of AMS.

Table 12 – Effect of ammonium sulfate (AMS) addition on distribution of ^{14}C-glyphosate in *Echinochloa colona*.

Plant part	24 HAT				72 HAT				144 HAT			
	glyphosate		glyphosate + AMS		glyphosate		glyphosate + AMS		glyphosate		glyphosate + AMS	
	------------------------------------ % of total absorbed ------------------------------------											
Above treated leaf	0	Ac	1	Ac	1	Ad	2	Ad	2	Ac	2	Ac
Treated leaf	80	*Aa	74	Aa	61	*Ca	52	Ca	71	Ba	70	Ba
Stem of main tiller	17	*Cb	21	Bb	32	*Ab	37	Ab	22	Bb	22	Bb
Roots of main tiller	2	Ac	3	Ac	5	Ac	6	Ac	3	Ac	4	Ac
Tillers	0	Ac	1	Ac	0	Ad	2	Ad	1	Ac	2	Ac
Roots of tilllers	0	Ac	0	Ac	0	Ad	2	Ad	1	Ac	1	Ac

The different lowercase letters indicate statistical differences among plants parts, uppercases indicate differences between harvest times and * indicate statistical differences between ammonium sulfate addition by Student's t-test ($p \leq 0.05$). n = 8 replicates.

4.4 Discussion

The main concern of this paper was provided information to be used in the management of *Eragrostis plana*. The experiments were based on some related cases of herbicide control failures by using glyphosate, where often plants can regrowth after herbicide application.

Primarily, we perform an experiment to test the performance of different glyphosate salts (isopropylamine, diammonium, potassium and ammonium) applied under three plant growth stages (5-6 tillers, full tillering and panicle initiation). Our findings indicate few differences among salts of glyphosate were observed only by visual control at early evaluations (7, 14 and 21 DAT), which did not change the above-ground biomass. In general, the time reached about 100% of control was shorter when sprayed glyphosate formulated as potassium salt compared to the others (Table 6) mainly observed during the earliest stage (5-6 tillers). The high performance of potassium salt was confirmed when applied at stage of panicle initiation, although the IPA salt also showed high performance at this time.

Some studies have been related few or no differences to control weeds among salts formulations of glyphosate (MUELLER et al., 2006; RICHARDSON et al., 2009; SATCHIVI et al., 2000). A study evaluating three glyphosate formulations (Isopropylamine, Diammonium, and Potassium) found no differences among them to control the weeds *Cyperus esculentus*, *Ipomoea lacunosa*, *Brachiaria platyphylla* and

Amaranthus palmeri, even when some salts affected the pattern of absorption and translocation (MUELLER et al., 2006). It is important highlight that differences could be attributed to different leaf anatomy and surface of broadleaf and grass weeds as previously described by Oliveira et al. (2015). According to their results, the potassium and ammonium salt formulations showed higher wettability when applied to *Bidens pilosa* leaves, while the isopropylamine salt formulation resulted in lower wettability on *Cenchrus echinatus* leaves. Although, evaluating the control of *Amaranthus rudis* L., initial absorption of glyphosate was higher with the isopropylamine formulations compared to the diammonium formulation 2 HAT (LI et al., 2005). However, the initial slight differences in absorption and translocation in the weed species did not affect the overall efficacy of the three glyphosate formulations at 72 HAT. Thus, the interaction among glyphosate formulations and leaf surfaces should be taken into consideration, as they may be crucial to the efficacy of the formulations (TRAVLOS; CHEIMONA; BILALIS, 2017).

These studies aforementioned, help us to understanding the differences found in visual control across the salts and growth stages (See appendix C). As an example, even though without any statistical differences, at 5-6 tillers, the IPA and ammonium salt showed 72 and 86% of control, respectively. This could be explained by interaction among glyphosate formulations and leaf surfaces, as the leaf surfaces changing over time. Besides, the highest efficacy of *E. plana* control (represented by visual control) was reached when the plants were at panicle stage, followed by 5-6 tillers and then by the most tolerant stage represented by full tillering. Overall, younger plants are typically more easily controlled compared with plants at advanced stages, because their tissues are less developed and allow greater absorption of the herbicides (DEGREEFF et al., 2018). In addition, during the life cycle of the plant, the amount of translocated sugar also changes and plants in advanced stages may be less sensitive to herbicides, including glyphosate (FADIN et al., 2018). At reproductive stage plants tend to translocated sugar to roots and reproductive organs direction, thus carrying also the herbicides together and improving the efficacy of control. A previous study indicated that plants of *E. plana* sprayed at panicle initiation translocated more ^{14}C-glyphosate out of treated leaf (mainly to the main stem) compared to tillering stage (BASTIANI et al., 2015).

Assuming full tillering as the most glyphosate-tolerant growth stage, we performed a dose-response curve to define the GR_{50} for *E. plana*. According to log-

logistic model used the GR_{50} for *E. plana* at aforementioned stage is 312g a.e. ha^{-1} of glyphosate. It would have expected a GR_{50} higher than observed here, based on problems of herbicide failures related by farmers. Some species with innate tolerance to glyphosate as *Ipomoea grandifolia* and *Commelina benghalensis* exhibit a GR_{50} around 616 and >1.440g a.e. ha^{-1}, respectively (LACERDA; VICTORIA FILHO, 2004), which is almost 2-fold above of the value found here. Although, velvetbean (*Mucuna pruriens*) a plant also known by having a high tolerance to glyphosate showed GR_{50} of 404g a.e. ha^{-1} (ROJANO-DELGADO et al., 2012). Thus, the GR_{50} does not directly indicated how likely the plants are tolerant to the herbicides. First of all, we should take in to account the mechanisms of tolerance involved. For *E. plana*, the capacity to regrowth after herbicide applications plus the high potential to tillering, seems to be the main factor linked to failures observed at field situations (See appendix C). Visual observations of regrowth were observed even at 5-6 tillers in the previous experiment.

Situations where drought stress was imposed clearly indicated that plants drought-stressed exhibit lower efficacy of *E. plana* control compared to well-watered plants, regardless of adjuvant treatment. It is known how drought stress has the potential to influence the level of herbicide uptake, translocation, metabolism and efficacy. Plants that are grown under drought conditions can develop thicker cuticles or leaf pubescence, which inhibit herbicide absorption, and lowered levels of photosynthesis and photoassimilates transport contribute to reduced herbicide translocation (PATTERSON, 1995). However, little attention has been done to use adjuvants aiming to improve the efficacy of herbicides under drought scenario. Here, the comparison between adjuvants allow us to prove that AMS clearly enhanced glyphosate efficacy (Table 7, Figure 19).

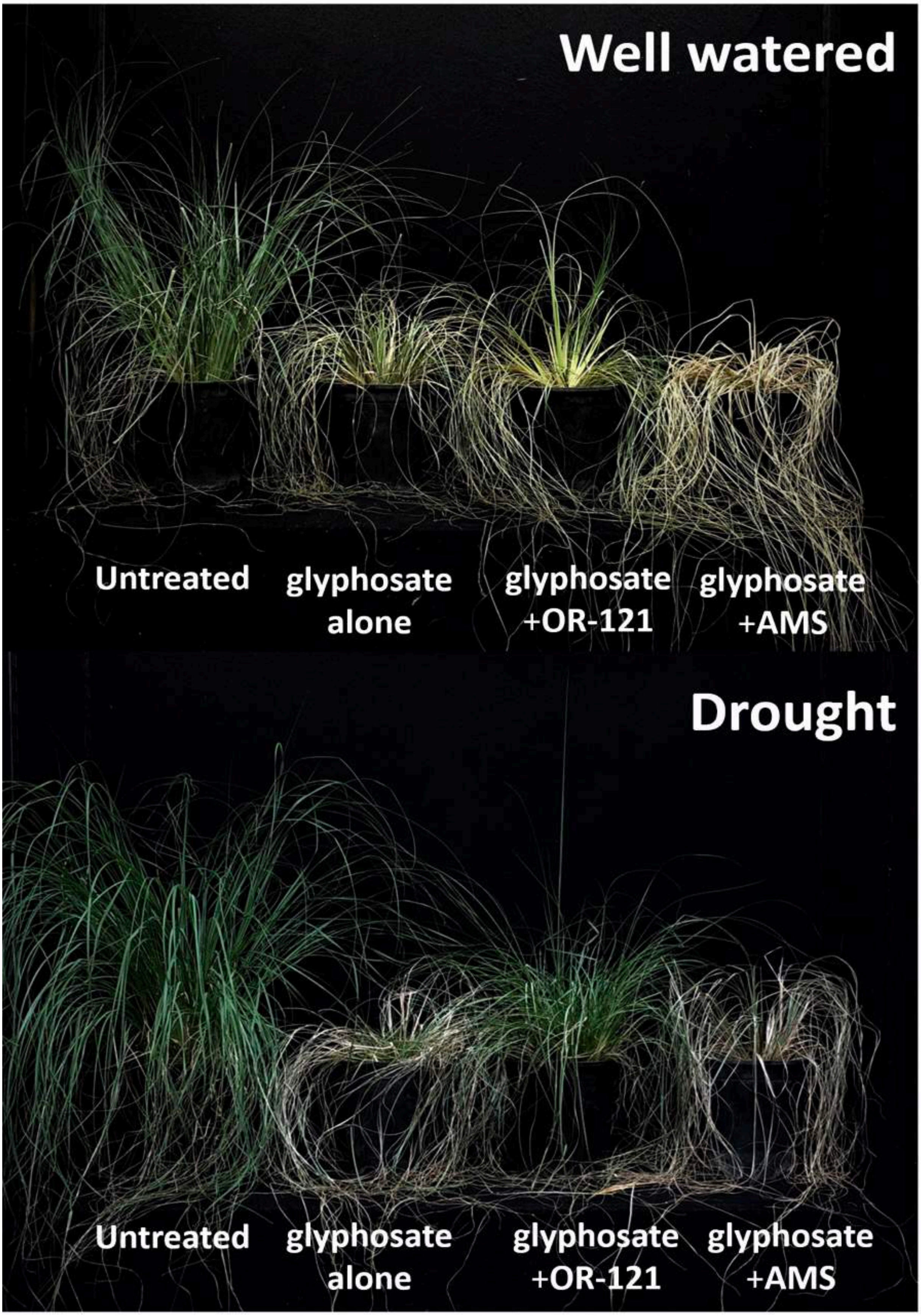


Figure 19 – Efficacy of glyphosate sprayed with adjuvants on control (%) of *Eragrostis plana* under drought conditions at 60 days after herbicide treatment.

The addition of ammonium sulphate (AMS), an inorganic salt, to the glyphosate spray solution have been reported for years to improve the efficacy of the herbicide (PLINE; HATZIOS; HAGOOD, 2000; SALISBURY; CHANDLER; MERKLE, 1991; SOLTANI et al., 2016). Additionally, it has been found that salts dissolved in water used as the carrier for glyphosate may reduce its effectiveness, particularly calcium and magnesium salts (WILLS; MCWHORTER, 1985). According to the analyses of water used in our experiments (data not shown), there were no enough salts dissolved to be considered hard water, so this indicate that beneficial effect of AMS was unlikely to be relative to hard water. Occasionally, AMS may enhance glyphosate efficacy through mechanisms other than overcoming antagonistic salts. Glyphosate plus AMS applied in deionized water reduced *Agropyron repens* fresh weights in the greenhouse significantly more than glyphosate sprayed alone (DE RUITER; MEINEN, 1998). So, the role of AMS in enhance the efficacy of glyphosate still unclear. Some researchers have reported increasing on absorptions and/or translocation, as found by SATCHIVI et al. (2000). They found that glyphosate absorption by *Abutilon theophrasti* and *Setaria faberi* was enhanced by the addition of 1% ammonium sulfate in either isopropylamine or trimethylsulfonium salt formulations.

Also, ammonium sulfate can significantly improve herbicide activity when weeds are grown under water stress conditions (SATCHIVI et al., 2000) which was confirmed by ours experiments. However, the addition of ORO-121 did not overcome the adverse effects of water stress, and this could be attributed to the interaction of adjuvants with leaf surface. The influence of non-ionic (X-77), organosilicone (L-77) adjuvants and methylated seed oil (MSO) was evaluated in *Bidens frondosa* and *Panicum maximum.* In *B. frondosa*, all three adjuvants significantly increased the uptake and translocation of glyphosate, while in *P. maximum*, uptake and translocation of glyphosate increased with both X-77 and MSO, however there was an antagonistic effect on uptake and translocation of glyphosate for L-77. These results are close to found here with ORO-121 (Table 7, Figure 19).

As discussed above, the performance of adjuvants may depend of interaction of adjuvant *versus* leaf surface of weed which probably can be confirmed by the absorption and translocation experiment. According to literature, the addition of AMS is expected to improve glyphosate uptake, however in our experiment this contribution of AMS was only observed for *E. colona*. AMS increased in 5.5% ^{14}C-glyphosate absorption in *E. colona* (Table 8). The same behavior was reported to glyphosate absorption by *Abutilon theophrasti* and *Setaria faberi*, where the addition of 1% (wt/v) ammonium sulfate enhanced the ^{14}C-glyphosate absorption in either isopropylamine or trimethylsulfonium salt formulations. (SATCHIVI et al., 2000). Quite interestingly, the addition of AMS in both glyphosate formulations increased the quantity of glyphosate translocated out of the treated leaf, but no difference was observed in the quantity of glyphosate translocated to the roots at any time. Again, the same response occurred either *E. colona* or *E. plana* in our experiments, where AMS increased in 9.2 and 5% respectively, the total total ^{14}C-glyphosate translocated out of treated leaf. It is important highlighted here, the differential translocation between the species, where the total percentage of ^{14}C-glyphosate translocated out of treated leaf was around 58% for *E. plana* compared to only 30% in *E. colona*. As *E. plana* considered a species with a potential tolerance to glyphosate, it is expected low levels of herbicide translocation compared to a high glyphosate-sensitive plant as *E. colona*. Although, we hypothesize that *E. colona* as specie with a low GR_{50} of 48.45 g a.e. ha^{-1} (data not shown) was likely to be inhibit by glyphosate with a lowest amount of glyphosate translocated to the sinks (e.g. roots) compared to *E. plana* (GR_{50} of 312 g a.e. ha^{-1}). Thus *E. plana* could have translocated more glyphosate than *E. colona*.

Evaluating the distribution of ^{14}C-glyphosate in the whole plant, we noticed that addition of AMS on glyphosate tank mixture promoted a higher translocation out of treated leaf and this ^{14}C-glyphosate translocated is mainly accumulated in the stems of main tiller, which was not observed for *E. colona*. The accumulation of glyphosate in the stems may is the main cause of better performance of glyphosate when adding AMS. Besides, the regrowth in *E. plana* observed at field and also in this experiment (See Appendix D) indicated that new tillers came from the main tiller, thus more concentration of glyphosate in this tissue would be avoid the production of news one.

Based on percentages of absorption and translocation in *E. plana,* compared to *E. colona* (as a high glyphosate-sensitive specie), we could assume that herbicide failures at field is unlikely to be related to mechanism of reduced absorption/translocation. It is explained by the absence of ^{14}C-glyphosate moved to surround tillers when glyphosate was applied at main tiller. Thus, once the glyphosate reaches the leaf surface its able to move in the whole tiller, but cannot move to the surround tillers, as found here. These findings confirm the theory first proposed by Corrêa et al. (2014) and later confirmed by Bastiani et al. (2018), where plants of *E. plana* exhibit a mechanism known as “interdependence among tillers” which is believed that glyphosate do not translocate up to tillers resulting in some levels of tolerance to glyphosate.

4.5 Conclusions

In the current study, we can conclude that there are no relevant differences among salts of glyphosate in the efficacy of *E. plana* control regardless of growth stage, being the full tillering the most glyphosate-tolerant growth stage.

Water shortage cause severe reduction on efficacy of glyphosate in *E. plana* control, where the addition of ammonium sulphate in glyphosate applications can overcome antagonist effect of drought.

The enhanced performance of glyphosate efficacy when adding ammonium sulfate is explained by increasing translocation of the herbicide on plant tissues, mainly to stems.

5. FINAL CONSIDERATIONS

Our results showed that *Eragrostis plana* and *Paspalum notatum* exhibit the highest drought tolerance at tillering growth stage whether compared to reproductive stages. The growth and development of *E. plana* are more affected when the water stress is imposed during the stage of panicle emission; while *P. notatum*, the most sensitive growth stage is during the grain filling. Specifically, during the spikelets emission, the earlier stomatal regulation is the main key to avoid water loses to atmosphere in *P. notatum*. Probably, *E. plana* show late stomatal closure based in decreasing rate of soil water, although the plants tolerate low percentage of leaf water for a long period of water stress. Both species use mechanisms to dissipate excess of photosystems energy under water stress, associated to an efficient antioxidant system and biosynthesis of compatible osmolytes. Even both species show similar mechanisms to tolerate water stress, the rate of stress recovery after the reestablishment of soil water is faster in *E. plana* compared to *P. notatum*.

The occurrence of water stress when *E. plana* e *Paspalum notatum* are in coexistence reduce parameters related to growth for both species. Although *E. plana* show high competitive ability when competing with *P. notatum*. Besides, *E. plana* is able to overcome *P. notatum* growth regardless of water soil condition.

During the tillering stage *E. plana* show the highest tolerance to glyphosate application. Although, there are no difference on efficiency of glyphosate salts to control *E. plana* regardless of growth stage which glyphosate was sprayed. In addition, application of the herbicide under soil water limitatiom decrease the glyphosate efficacy. However, addition of ammonium sulfate in the glyphosate tank mixture has the potential do reduce the antagonic effects of water stress, which is explained by the enhanced herbicide translocation in the whole plant.

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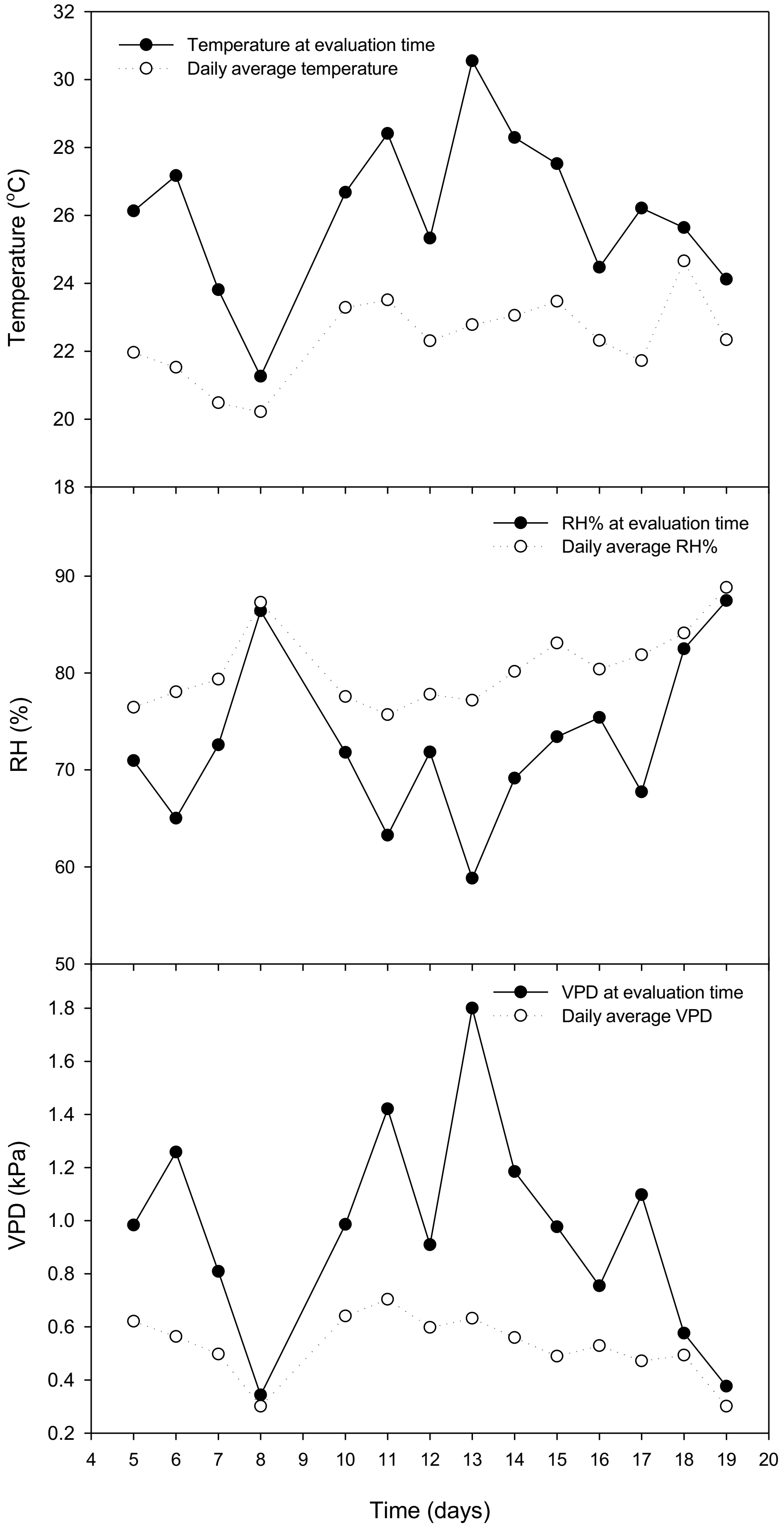
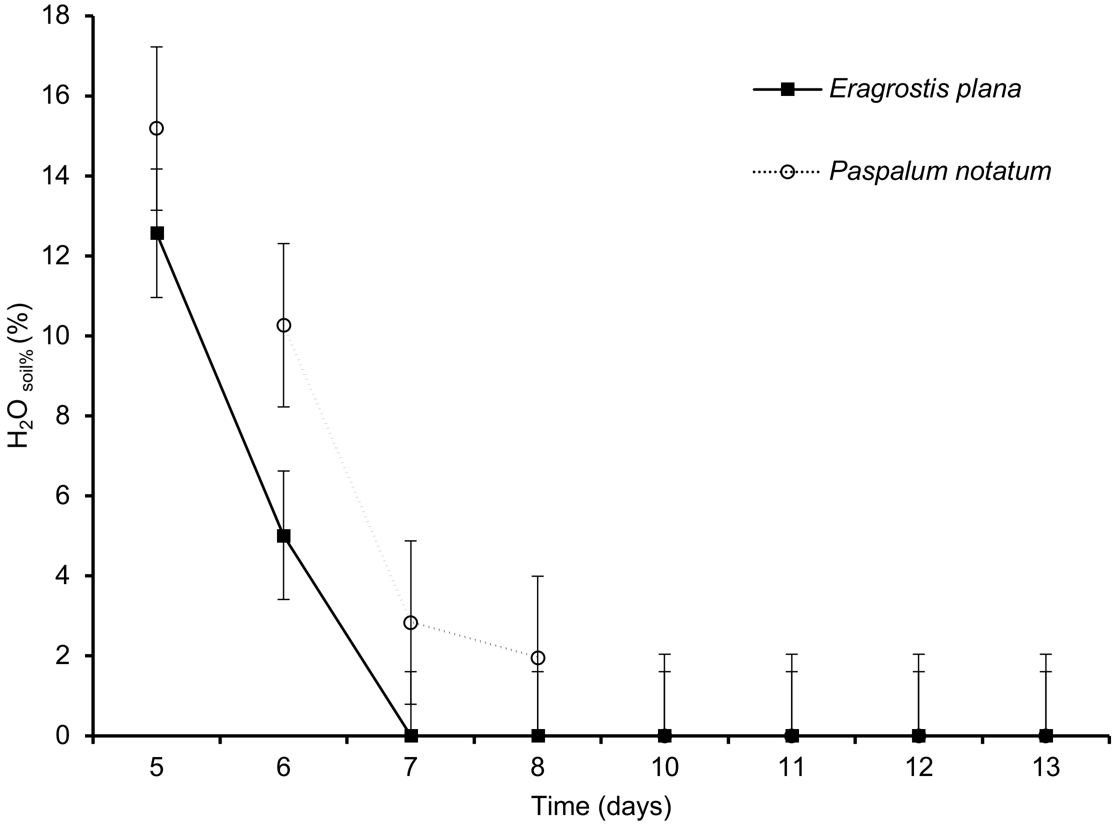
APPENDICES

APPENDIX A – Air temperature (TºC) relative humidity (RH%) and vapor pressure deficit (VPD) during the time of water stress in the experiment II (Chapter I).



APPENDIX B – The soil water percentage (H_2O soil%) during the induction of water deficit in the experiment II (Chapter I). The values were measured every day from the 5th day of suspension of irrigation in pots with *Eragrostis plana* and *Paspalum notatum*. Means, *n* = 6.



APPENDIX C – *E. plana* control (%) at 35 days after treatment (DAT) with glyphosate salts (isopropylamine, diammonium, potassium and ammonium) applied in 5-6 tillers (A), full tillering (B) and panicle initiation (C) growth stages in the experiment I (Chapter III).

(A)

(B)

(C)

APPENDIX D – Observation of regrowth in *E. plana* plants after 60 days of glyphosate (312g a.e. ha^{-1}) treatment at greenhouse (A) and field conditions (B and C). The picture from greenhouse conditions (A) was taken from experiment III of Chapter III and the following pictures (B and C) were from observations of unsatisfactory control of *E. plana* in burndown operations using glyphosate (1,920g a.e. ha^{-1}) in soybean fields (Santana do Livramento, RS).