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Departamento de Biologia

CAROLINA MUSSO

FATORES AMBIENTAIS, CORTE E QUEIMA: EFEITOS SOBRE GRAMÍNEAS NATIVAS E INVASORAS DO CERRADO (SAVANA BRASILEIRA)

ENVIRONMENTAL FACTORS, CLIPPING AND FIRE: EFFECTS ON CERRADO (BRAZILIAN SAVANNA) NATIVE AND INVASIVE GRASSES

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Susana Loureiro investigadora auxiliar do Departamento de Biologia e CESAM da Universidade de Aveiro e sob coorientação do Prof. Amadeu Mortágua Velho da Maia Soares, professor catedrático da Universidade de Aveiro.

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Aos meus pais

“ There is a theory which states that if ever
anyone discovers exacly what the Universe
is for and why it is here, it will instantly
disappear and be replaced by something
even more bizarre and inexplicable.
There is another theory which states that
this has already happened”

Douglas Adams

o júri

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orientadora	Doutora Susana Patrícia Mendes Loureiro Investigadora Auxiliar Departamento de Biologia da Universidade de Aveiro

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palavras-chave Estação, Solo, Fogo, Ecologia Terrestre, Manejo de espécies invasoras

resumo

O Cerrado brasileiro abriga uma enorme biodiversidade e é considerado um bioma prioritário para conservação. Uma das maiores ameaças à integridade desse ecossistema é a introdução de gramíneas africanas, que têm a capacidade de excluir competitivamente as gramíneas nativas, causando alterações no microclima e nos regimes de perturbação do ecossistema. O Cerrado é um mosaico de vegetação que oferece combinações diferentes em termos espaciais e temporais, de fatores de stress natural para vegetação herbácea (disponibilidade de água, nutrientes, luz etc.). Os diferentes cenários no ambiente físico refletem diferenças nas relações biológicas entre espécies nativas (*Aristida recurvata*, *Aristida setifolia*, *Axonopus barbigerus*, *Echinolaena inflexa*, *Gymnopogon spicatus*, *Paspalum gardnerianum*, *Paspalum stellatum*, *Schizachyrium microstachyum*, *Schizachyrium sanguineum*) e invasoras (*Melinis minutiflora, Andropogon gayanus*), afetando a competição entre elas e criando situações (local / estação) que são mais ou menos suscetíveis à invasão. Este estudo tem como objetivo identificar as diferentes respostas biológicas de gramíneas nativas (e invasoras às variações de stress ambiental natural e à perturbação (fogo e corte), a fim de compreender as mudanças no funcionamento do ecossistema e os processos de competição entre essas espécies, ajudando assim a compreender a dinâmica de invasão neste ecossistema. No campo, a presença de espécies invasoras mostrou afetar o funcionamento dos ecossistemas, aumentando a atividade dos detritívoros do solo. Estas diferenças não foram, porém, observadas na estação seca nem quando o fogo é frequente, mostrando que a disponibilidade de água e de fogo são mais determinantes para a atividade dos detritívoros do solo do que para a vegetação. Os experimentos de laboratório mostraram que tanto a seca quando o excesso de água causa danos em ambas as espécies, porém as espécies invasoras apresentam um melhor desempenho em qualquer condição de rega e respondem melhor à adição de fertilizantes. Os mecanismos subjacentes, como a eficiência da fotossíntese e mecanismos antioxidantes, ajudam a explicar esse comportamento. Com estes mecanismos ativos, a espécie invasora tem capacidade de crescer mais rapidamente, apresentando menos danos celulares e um fotossistema saudável, apresentando taxas de assimilação mais elevadas. No entanto, estas discrepâncias entre espécies nativas e invasora são reduzidos com a aplicação do corte, especialmente nos solos secos e sem fertilização, onde as espécies nativas recuperaram melhor, em relação aos valores em pré-corte. O excesso de água representa um stress semelhante ao induzido pela seca, mas em solos com melhor drenagem, as espécies invasoras são capazes de contornar esse problema de forma mais eficiente que a nativa. O fogo provou ser mais prejudicial que o corte, provocando uma recuperação mais lenta. Além disso, as temperaturas pós- fogo são capazes de afetar a germinação de sementes tanto de espécies nativas quanto da invasora, podendo ser um fator importante e que influencia a persistência da biodiversidade no campo. Os resultados desse trabalho contribuirão para a escolha da técnica de manejo mais adequada para a conservação da biodiversidade do Cerrado.

keywords Season, soil, fire, terrestrial ecology, management of invasive grasses, seeds

abstract

The Brazilian Cerrado houses a hugely diverse biota and is considered a conservation hotspot. One of the greatest threats to the integrity of this ecosystem is introduced African grasses, which can competitively exclude native grasses and cause changes in the microclimate and other disturbances. The Cerrado is a mosaic vegetation that provides different combinations, both spatially and temporally, of conditions that can become natural stressors to the herbaceous vegetation (water, nutrient and light availability). These mosaics are reflected in differences in relationships among native and invasive species, affecting competition and creating situations (place/season) that are more, or less, susceptible to invasion. The present study aimed to identify the different biological responses of native (*Aristida recurvata*, *Aristida setifolia*, *Axonopus barbigerus*, *Echinolaena inflexa*, *Gymnopogon spicatus*, *Paspalum gardnerianum*, *Paspalum stellatum*, *Schizachyrium microstachyum*, *Schizachyrium sanguineum*) and invasive (*Melinis minutiflora* and *Andropogon gayanus*) grasses to variations in natural stressors and to disturbance (fire and clipping), in order to understand changes in ecosystem functioning and competition processes between the grasses, and to understand invasion dynamics in this ecosystem. The presence of invasive species proved to affect the ecosystem functioning by increasing soil feeding activity. These differences were no longer observed in the dry season or when fires were frequent, showing that water availability and fire are more detrimental to soil feeding activity than is the vegetation. Laboratory experiments showed that both drought and flood simulated scenarios damaged both species, although the invasive species performed better under all watering conditions and responded better to fertilization. Underlying mechanisms such as the efficiency of photosynthesis and antioxidant mechanisms helped to explain this behavior. The invasive species grew faster and showed less cellular damage and a healthier photosystem, reflected in higher assimilation rates under stress. These differences between the native and invasive species were reduced with clipping, especially in dry soil with no fertilization, where the native species recovered better in relation to the pre-clipping levels. Flooding was as stressful as drought, but the invasive species can bypass this issue by growing an extensive root system, especially in the better-drained soils. Fire is more detrimental than clipping, with a slower recovery, while post-fire temperatures affect the germination of both invasive and native seeds and may be an important factor influencing the persistence of a diverse biota. This approach will finally contribute to the choice of the appropriate management techniques to preserve the Cerrado's biodiversity.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE:

GENERAL INDRODUCTION

THE CERRADO

The Cerrado is a tropical savanna that covers more than 2 million km^2 or about 25% of the area of Brazil, from 5 °N to 34° S, including all or parts of several states (Figure 1). It is the second-largest biome in the country, smaller only than the Amazon Forest. Changes in land use, mainly farming and ranching, have reduced the natural Cerrado area by half, i.e., over three times the deforested area in the Amazon basin.

Figure 1. Cerrado distribution along Brazilian territory. Source http://coffee4missions.com/brazillian-cerrado-daterra-farms/. Accessed on august 2014.

Cerrado is the home of a hugely diverse biota including 12,000 plant species and >160 mammals > 800 bird, >120 amphibian, >150 reptile, and >1200 fish species, many of them endemic and endangered, which makes it a conservation hotspot (Myers *et al.* 2000, Klink & Machado 2005, Mendonça *et al.* 2008). However, less than 5% of the remaining natural Cerrado is presently protected.

In addition to deforestation, the Cerrado is threatened by biological invasions, mainly by African grasses. These grasses were introduced into Brazil either by accident during the colonial period or intentionally for improving pastures. Exotic grasses presently cover over 500,000 km^2 and can also spread to conservation units and deplete the local biota (Klink & Machado 2005).

CLIMATE AND SOIL

The Cerrado climate is seasonal and tropical (Köppen's CWA), with two well-marked seasons, a dry winter and a wet summer. Mean annual temperatures are around 22-23 °C and oscillate little during the year. Mean annual precipitation is around 1600 mm, and 90% of the rain falls from October to April. During the dry season, monthly precipitation may reach 0%, and air relative humidity can be below 10% (Eiten 1972) (Figure 2).

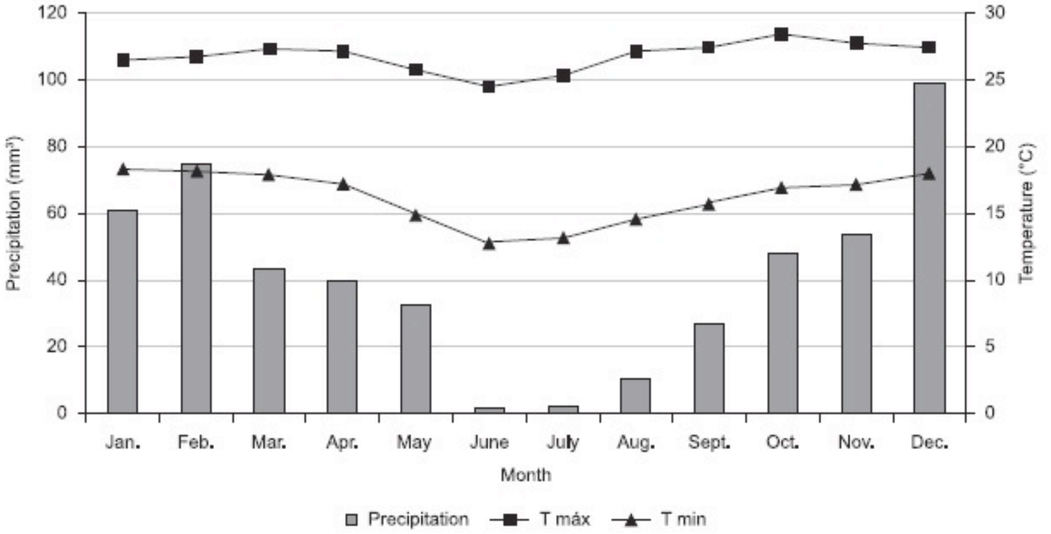

Figure 2. Seasonality expressed as monthly averages of precipitation and temperature in the Cerrado region, Brazil. (França & Braz 2013)

The most frequent soil type is red-latosols (oxisols or acrustox soil according to the American classification), i.e., old, well drained, deep, highly weathered and strongly acid, dystrophic (poor in nutrients), and with high concentrations of aluminum (Haridasan 1994). For farming and pastures, the pH is corrected with limestone and fertilizers are added. Worldwide, human activities and population growth account for over twice as much as the natural production of nitrogen on the land surface, affecting the world nitrogen cycle (Kauffman *et al.* 1994, Galloway *et al.* 2004). In the Cerrado, anthropogenic inputs originate mainly from pastures and soybean plantations (Filoso *et al.* 2006). Soil fertility significantly influences the species composition in Cerrado ecosystems (Ratter & Dargie 1992), and changes in soil chemical properties may alter soil-plant interactions and competition among plants.

VEGETATION

Cerrado is often referred to as the Brazilian Savanna, and it has the richest flora among world savannas (over 10,000 plant species, of which 44% are endemic (Klink & Machado 2005). The wide variety of structural forms or physiognomies range from open grasslands (campo-limpo/campo-sujo) to closed-canopy forests (cerradão) (Ribeiro & Walter 1998) (Figure 3). The vegetation gradient is related to edaphic and topographic factors such as depth, distance from the water table, fertility, mother rock composition, and is also related to fire (Coutinho 1978) (Figure 3). Aboveground biomass, root-shoot ratio, and floristic composition differ along the gradient (Castro & Kauffman 1993).

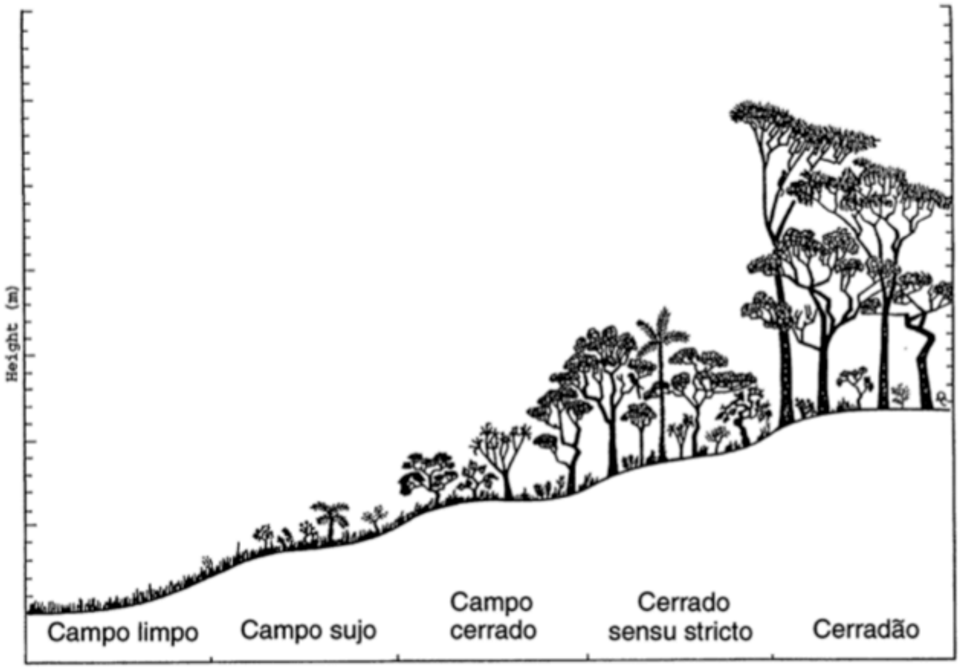

Figure 3. Cerrado vegetation forms along a gradient of soil depth and distance to the water table. The canopy gradient goes from open grassland physiognomies ("campo limpo) to closed canopy forests ("cerradão). (Castro & Kauffman 1993).

Aboveground biomass in the Cerrado is very low in comparison to other tropical ecosystems, because of the low productivity resulting from water stress, low nutrient content and aluminum toxicity (Eiten 1972, Haridasan 1992, Castro & Kauffman 1993). On the other hand, root biomass is high, especially in grasslands where the root biomass can reach over seven times the amount of aboveground biomass (Castro & Kauffman 1993).

In open grasslands and savanna formations, the herbaceous layer is prominent and composed mainly of grasses, which account for over 70% of the primary production of this layer (Batmanian & Haridasan 1985). Grasses (Poaceae) are one of the largest plant families, covering over 30% of the dry land surface of the earth (Watson 1990). They are of great economic and ecological importance, being used since ancient times in agriculture, and support the natural fauna and stabilize the soil (Silva 2000). The grasses have morphological traits and reproductive strategies that enable them to grow and spread rapidly and to tolerate herbivory, fire, drought and flood, recovering quickly after stress events (Watson 1990). In the whole Cerrado biome, there are over 500 species of native grasses while in the Federal district about 300 of these species have been reported (Mendonça *et al.* 2008). These grasses are considered of low palatability and nutritional value to cattle (Filgueiras 1992). Very little is known about their phenology, seeds, and response to water stress, fertility and fire (Almeida 1995, Oliveira 1998, Cole *et al.* 2005, Lindsay & Cunningham 2011).

Cerrado shrubs and trees are xenomorphic vegetation, with thick bark and tortuous trunks (Eiten 1972). The plants are adapted to low soil fertility, and are able to grow accumulating low levels of nutrients in their tissues. They also show high reabsorption rates, resulting in the low decomposition rates that are common in Cerrado plant communities (Resende 2001, Nardoto *et al.* 2006).

In order to endure the dry season and conserve water and nutrients, the plants possess adaptive mechanisms such as leaf scleromorphism, deciduous syndromes, and death of the aerial part (grasses), which enable them to maintain transpiration rates comparable to those in the rainy season (Quesada *et al.* 2004).

FIRE

Fire can be considered an important ecological factor worldwide, influencing patterns of distribution of plants, vegetation structure and floristic composition (Whelan 1995). Fire has become more important with the establishment of C4 grasses, common in tropical savannas. Plants with this photosynthetic pathway show high growth rates in locations with high radiation intensity, and are able to photosynthesize more efficiently by reducing photorespiration. Occupation of space by these grasses promotes fire by creating favorable conditions for the fire front to spread, such as fuel continuity, abundant fine fuel that easily dries, and a biomass that can recover quickly during the rainy season. These characteristics create a positive cycle that benefited the advance of grasslands and the retreat of forests around 10 million years ago, favoring the formation of open landscapes such as savannas (Pennington *et al.* 2006). Phylogenetic studies suggest that the origin of the Brazilian savanna followed this same ecological expansion of flammable C4 grasses, and Cerrado flora evolved from fire-free ecosystems (forests) by developing adaptations to resist fire (Simon *et al.* 2009). However, the most ancient record of fire in the Cerrado is from 32,000 years (Salgado-Labouriau & Ferraz-Vicentini 1994). The lack of evidence of human occupation in the area implies that fires were a natural occurrence. Lightning-caused fires may occur in dry spells during the rainy season or in the transition between dry and wet seasons. However, the human settlement begun 12,000 years ago and changed the fire regimes. Fire was used by humans as a hunting technique and still is to clear land for agriculture, mostly in the dry season, and with a higher frequency than natural fires. Also, accidental or arson fires during the dry season tend to consume much larger areas, and may influence the species composition by impairing the reproduction of many species that disperse their seeds during this season (Coutinho 1990, Munhoz & Felfili 2005).

Cerrado fires are rapid surface fires, consuming mostly the herbaceous layer and thin branches (fine fuel) (Miranda *et al*. 2002), although Cerrado flora is known to be resistant to frequent burns (Coutinho 1990). Shrubs and trees have a thick bark that protects the cambium from high temperatures (Guedes 1993). Trees may lose thin branches after fires, and because flames do not usually reach the canopy, the leaves are not consumed by fire. However, leaves usually die and fall after fires because of lethal temperatures and the exposure time (Miranda *et al.* 2002). The highest temperatures during a fire occur at 60 cm above the ground (over 800 °C) (Miranda *et al.* 1993). Wright (1970) showed that the death of plant tissue depends primarily on moisture content, and is an exponential function of temperature and time. Consequently, the heat tolerance of a plant (the ability of the organs to withstand high temperatures) along with its fire resistance may vary with season, as a result of the seasonal changes in plant water content. Many species have underground storage organs such as xylopodia, and are also able to store nutrients and water in their trunks (Rawitscher & Rachid 1946).

Since fire residence time is low, and the soils function as a thermal insulator, the roots and soil seed bank are protected from the high temperatures of fire. After a fire, these plants have different types of sprouting (basal, subterranean and aerial), reproducing vegetatively. Some species' flowering and seed dispersal are also aided by fire (Haddad & Valio 1993), although frequent fires may reduce the cover. In the case of the herbaceous layer, the species flower a few days after a burn (Coutinho 1990). Although they lose their aerial parts, recovery from their root systems is rapid (Andrade 1998). The seed bank, although somewhat reduced by fire, is also an important source for recovery afterward, and it recovers rapidly due to the induced flowering (Andrade & Miranda 2014). Fire and heating are also an important factor in breaking seed dormancy in other fire-prone environments (Portlock *et al.* 1990).

The reduction of the vegetation cover and the deposition of an ash layer on the soil surface result in a post-fire alteration in soil microclimate. This condition increases the amplitude of soil temperature after the fire on the order of 30°C at 1 cm depth and of 10°C at 5 cm depth, with no alteration at 10 cm depth (Dias 1994; Castro Neves & Miranda 1996) (Figure 4). These alterations in soil microclimate may have some effect on plant colonization, seed germination and soil micro-organisms (Frost & Robertson 1987).

Andrade (1998) reported a recovery of over 80% of the fuel load of the herbaceous layer of a *campo* one year after a fire, while Neto *et al.* (1998) observed that 2 years were enough for *campo sujo* to completely recover from fire. Andrade Miranda reported that the seed bank density recovers one year after fire, and although fire doesn't affect immediately the seed bank, it may reduce the contribution of the monocts , if it occurs during the dispersion period, in late dry season. Oliveira (1998) show that most of native grasses present flowering and anemochoric dispersion in the end of the dry season, when wind is faster favoring their spread (Almeida 1995). Therefore, if fire occurs at this time, the reproduction of grasses would be damaged. Frequent fires may cause even more damage and reduce tree cover.

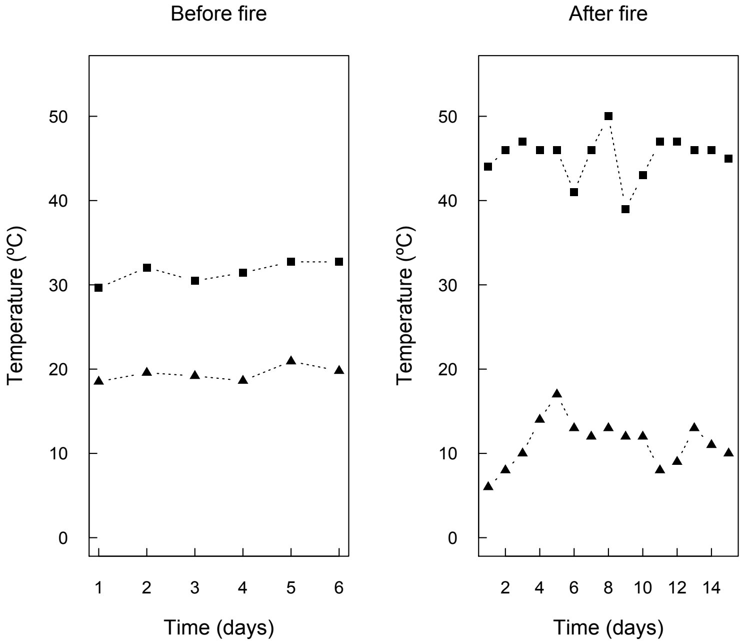

Figure 4. Changes on soil thermal profile with the removal of aboveground biomass after fire. Castro-Neves, unpublished data.

Changes in fire frequency can also favor the establishment of invasive plants, like African grasses (San José & Farinas 1991, D'Antonio & Vitousek 1992, Pivello *et al.* 1999; Williams & Baruch 2000) that are able to accumulate more biomass than Cerrado species, favoring more intense fires.

BIOLOGICAL INVASIONS

Invasive species are exotic species that were introduced intentionally or accidentally and were able not only to establish and adapt to the new environment, but also have the potential to outcompete native species. Only 0.01% of introduced exotic species became invasive, but when succeeded they may cause major problems (Williamson & Fitter 1996, Gurevitch & Padilla 2004). Invasive species usually lack natural enemies, aiding habitat colonization (Randall 1996; Richardson *et al.* 2000). Gurevitch & Padilla (2004) considered invasive species to be one of the greatest causes of biodiversity loss worldwide, after land-use change, and in Brazil this issue is no different (Pivello *et al.* 1999, Richardson *et al.* 2000, Martins 2006). These species may monopolize resources by limiting space, radiation, water and nutrients. Also, they can change ecosystem functions such as nutrient cycling and the fire regime. For instance, African grasses can alter the nutrient cycling due to their ability to uptake and accumulate nutrients more efficiently and in producing litter that decomposes faster than the native plants' litter. They can also alter the fire regime by increasing the frequency and intensity of fire. This is due to a fast recovery rate, tremendous biomass accumulation and higher combustion temperatures. Overall, invasive plants have caused significant changes in vegetation structure and composition in many ecosystems around the world (San José & Fariñas 1991, D'Antonio & Vitouseck 1992, Williams & Baruch 2000, McNeely *et al.* 2001, Brooks *et al*. 2004). The success of an exotic species in becoming an invader is a function of the characteristics of this species but also of the host environment, including natural populations, environmental stress, disturbance frequency, and management practices (MacIntyre *et al.* 1995).

In Brazil, African grasses are the most common and widespread invasive species. These grasses were introduced mainly to improve livestock grazing. Although many Cerrado grasses do not have an equivalent growth rate or palatability, suitable native species for grazing do exist (Filgueiras 1992). Much of the reason that African grasses are used is the lack of knowledge about the value of native species. For the same reason, invasive species are also used in the recovery of degraded areas that have undergone erosion, exploitation and natural disasters, creating another source of invasions (Carneiro *et al.* 2001, Silva *et al.* 2007).

In the Brazilian Federal District, the federative unit where the capital (Brasília) is located, established populations of the African grasses *Melinis minutiflora* (P. Beauv.)*, Andropogon gayanus* Kunth*, Rhynchelytrum repens* (Willd.) C. E. Hubb*, Hyparrhenia rufa* (Nees) Stapf, and *Urochloa decumbens* (Stapf) R.D. Webster, have been reported in conservation units such as the National Park of Brasília (Martins *et al.* 2004, Martins 2006, Zanin 2009) and in the Reserva Ecológica do IBGE (IBGE 2004, Aires 2009). The *M. minutiflora* is reported also all over the country (Martins & Leite 1997, Pivello *et al.* 1999). These grasses produce great numbers of viable seeds that can disperse into natural reserves, where they usually establish in open areas such as *campo limpo* and *campo sujo*[1]. Being C4 species, they also require

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[1] The most common physiognomic forms of Cerrado are grasslands (campo limpo); grasslands with scattered shrubs (campo sujo); open scrub (cerrado ralo); closed scrub (cerrado sensu strictu) and arboreal woodland with a woody underlayer and sparse grasses (cerradão)(Ribeiro and Walter 1998).

intense radiation, which is more available in these open areas (Freitas & Pivello 2005, Pivello *et al.* 1999).

Competition can be defined as an impairment in the performance of an individual when sharing limited resources (space, water, light, nutrients). These invasive grasses outcompete native species by affecting the establishment and development of their seedlings, adding a massive amount of seeds to the soil seed bank and possessing higher growth rates. African grasses have a history of coevolution with large herbivores in Africa, and the absence of equivalent grazers in the Cerrado may explain their higher growth rates in comparison to native species (Baruch *et al*. 1985). Also, in the invaded Cerrado, African grasses limit the development of native species by shading them and growing rapidly below ground. Silva & Castro (1989) found a direct effect of shading by *Andropogon semiberbis* (Nees) Kunth affecting biomass accumulation of native grasses in Venezuela, and Holl (1998) showed the extent of root competition in determining the growth of nearby seedlings in Australia. Aires (2013) studied the competition of native grasses with *M. minutiflora* in the Cerrado, and how both aerial and root biomass impairs native seedling growth. Maintenance of the native biodiversity depends on the recruitment, survival and growth of seeds. Therefore, invasive grasses affect these initial stages of the natives' life cycle.

These grasses are even reported as able to affect water and fire regimes and nutrient cycling of ecosystems (D’Antonio & Vitousek 1992, Williams & Baruch 2000, Hoffmann & Haridasan 2008). Native Cerrado grasses are adapted to these disturbance regimes. Nevertheless, when the invasive grass changes the frequency or intensity of fire, it may affect the response of the native species, delaying its recovery and facilitating invasion and spread of other African grasses, creating a positive feedback favoring the dominance of exotic grass species.

It is believed that a species-rich community is more stable, with overlapping ecological niches, which may impede the establishment of new species (Tilman 1996). More-diverse stable environments usually show healthier functioning, with higher productivity and nutrient cycling (Stachowicz & Tilman 2005). There is evidence that a community may be more susceptible to invasion when disturbed. Even with the natural disturbance of the environment, such as dry seasons and natural fires, native species should be able to better resist invasion, for being naturally adapted to these conditions, having evolved with them (Lockwood *et al.* 2007). Also, African grasses have lower tolerance to water stress than the native species (Baruch & Fernandez 1993). It is also widely accepted that invasive grasses establish more rapidly in soils with high nutrient content (Barger *et al.* 2003).

However, due to the accelerated rate of Cerrado destruction, human impacts, lack of appropriate management, their high propagule pressure and rapid growth rate, African grasses can invade and easily establish in open Cerrado areas (Baruch *et al.* 1989, Rejmánek 1989, Elton 2000, Levine *et al.* 2003, Stachowicz & Tilman 2005). These mixed communities are in a continual dynamic equilibrium, controlled by the competitive interactions between invasive and native species.

***MELINIS MINUTIFLORA* P. Beauv.**

This grass is one of the greatest threats to biodiversity in the Brazilian Cerrado (Martins *et al.* 2004). Also known as molasses grass, *Melinis minutiflora* (P. Beauv., Figure 5) is a perennial grass that can reach up to 180 cm high. Its name comes from the secretion of a volatile oil through the leaf hairs, with a characteristic strong odor (FAO 2011, Partridge 2003). Molasses grass is native to tropical and southern Africa, and was first introduced to South America and India (Duke 1983) in colonial times by accident. More recently, it has been used to improve pastures because of its high growth rate and biomass accumulation.

Figure 5. An area invaded by *M. minutiflora* in Brazil. Source: http://ameninaeasmontanhas.blogspot.com.br. Accessed on august 2014.

Molasses grass yields about 2-20 t/ha depending on moisture and fertilization conditions, far more than the 7 t/ha of native grasslands (Neto *et al.* 1998). Although other species are preferred nowadays, it is still used as the principal component of the diet of grazing cattle in many states. It is a sticky, tufted grass that produces enormous quantities of seeds (Carmona & Martins 2010), most of them viable, although it can also spread vegetatively by stolons. It is considered a weed in Brazil, Colombia, Hawaii, and Venezuela (Duke 1983). However, it is now widely considered as naturalized in many tropical countries (FAO 2011), as it has been introduced so long and is now completely adapted and spread throughout the biome. It is so widespread and is so commonly used by local populations that, by common sense, locals may consider this species as a native species.

Its distribution ranges from 16N and 30.5°S, from sea level to 2500 m altitude at forest edges and on open ground or grassland (Ecocrop 2011, Quattrocchi 2006). It can displace native grasses and form monospecific stands in open areas, and impairs the recruitment of dicot seedlings in forest edges (Hoffman & Haridasan 2008). Molasses grass is a fast-growing grass, both above and below the ground, outcompeting native species for light, space and soil nutrients (Partridge 2003, Freitas & Pivello 2005). It also has impacts on the nitrogen cycle by producing litter with a lower nitrogen content (Silva & Haridasan 2007). Molasses grass thrives in areas with 960-1700 mm annual rainfall and 30°C, and is also relatively tolerant to drought that last up to four to five months. These are precisely the conditions found in the Cerrado biome. During the dry season, cattle prefer other grasses because the palatability and digestibility of this species decreases sharply in the dry season, probably because nutrients are used more efficiently by reabsorbing from dying leaves (Silva & Haridasan 2007). Göhl (1981) found that this species is not resistant to waterlogging, preferring well-drained soils such as latosols. It also tolerates the low-fertility, low-pH Cerrado soils with their high concentration of aluminum. However, it also responds positively to fertilization (Ecocrop 2011). Furthermore, this species has been reported to maintain green biomass longer in the dry season, probably due to its extensive root system (Silva & Haridasan 2007).

The large biomass along with the compounds secreted by the leaf hairs lead to more-intense fires than in native grasslands. Although this species has been reported to alter the fire cycle in Hawaiian ecosystems by increasing fire frequency and intensity, in the Cerrado the native grassland can recover faster from fire than can burned invaded sites (Barros *et al.* 2006), and *M. minutiflora* does not seem to increase the frequency of fire. Marinho & Miranda (2013) also reported that it has a low tolerance to fire, and stated that *M. minutiflora* does not withstand heavy grazing, especially when plants have not reached 15 cm in height (FAO, 2011). Although fire itself may not promote the spread of molasses grass in the Cerrado, it may facilitate invasion by other invasive grasses such as *H. rufa* and *A. gayanus*.

MANAGEMENT

Prevention, control and eradication of invasive species account for significant costs to the economy and may lead to losses in the environment (Zanin 2009). The study and implementation of effective techniques are therefore imperative.

Experiments have been carried out in Brazil in order to test control methods, such as fire, mowing and herbicide application. Although fire does not promote flowering in *M. minutiflora*, and reduces its seed bank, adults can resprout after fire (Marinho & Miranda 2013). Some authors have stated that this species is tolerant to fire (Filgueiras 1990, Pivello & Norton 1996, Martins 2006). On the other hand, Williams & Baruch (2000) claimed that *M. minutiflora* can be controlled by fire. Furthermore, D'Antonio *et al.* (2001) reported high mortality of mature plants after fire, and Marinho & Miranda (2013) showed that annual burns can reduce its cover. Successes in management by fire and clipping are reported in the literature (DiTomaso *et al.* 1999). Nevertheless, no control technique was able to eliminate the individuals established previously during the rainy season (Martins *et al.* 2009).

Furthermore, the different intensities and combinations of the natural environmental variations encountered in the Cerrado (discussed above), or by the frequent human disturbances (such as increases in nitrogen deposition and fire frequency), can influence the susceptibility of a site to invasion. Environmental factors and disturbances can affect the efficiency of management techniques such as clipping. Ferraro & Oesterheld (2002) noted that plants respond differently to herbivory, for example, when growing in different environmental conditions of water and nutrient availability. Environmental factors affect the capability of plants to compete by changing photosynthetic rates, growth, seedling emergence and water relationships (Ballaré *et al.* 1996). Since competition is the main factor determining species dominance in the environment, it is important to understand the dynamics of competition in order to plan management techniques. The methods should be developed taking into account the natural conditions of plant relationships, cost, and applicability to extensive areas. Also, plant invasion dynamics must be analyzed with a view toward the potential effects of climate change.

MAIN OBJECTIVES

The main objective of this study was to measure the effects of different water and nutrient levels and their interactions with fire and clipping, on the performance of native and invasive species of the Cerrado. The treatments simulated the seasons and natural or anthropogenic variations in soil properties in Cerrado areas. Several traits were measured at different levels of organization: morphological, physiological and biochemical. This integrated approach will finally lead to conclusions as to the most appropriate management techniques to be used in order to maintain the sustainability of the Cerrado biome, by implementing higher levels of settlement for the native species.

APPROACH

The objects of study were native and invasive grasses found in the Cerrado and their performance in different environmental conditions. The chosen environmental conditions were: (a) Soil Moisture – assessment of biological activity in the soil in different seasons, assessment of growth in different watering regimes (intermittent and constant) and different levels of soil moisture (from severe drought to flood); (b) Fertilization - assessment of the effects of higher nutrient availability to one invasive and one native species, in combination with different levels of soil moisture. (c) Fire: assessment of the effects of fires on soil feeding activity, effects of post-fire temperatures on seed germination and effects of fire on plant recovery, in combination with differences in soil moisture. (d) Clipping: assessment of the effects of clipping on one native and one invasive species in combination with different watering regimes, different levels of soil moisture, and fertilization.

In order to compare invasive and native plants, plant responses were assessed by measuring several traits at different levels of organization. At a community level, soil feeding activity was assessed. At the population level the germination parameters were assessed, considering for that germination rate, time of germination and viability of different plant species. At the individual level, growth and physiology were the main parameters evaluated. Finally, biochemical parameters were measured to evaluate oxidative stress responses in plant tissue.

MEASURED TRAITS

(A) Bait-Lamina

The bait-lamina test is a simple and inexpensive tool for evaluating the biological community in soils, by assessing soil feeding activity. It consists of visually assessing the consumption of the bait substrate contained within the holes of the lamina strips, by counting the number of empty holes after the lamina strips were inserted in the soil. The test can be performed under different conditions (Kratz 1998). Through this test, it was possible to quantify the effect of the environmental factors: soil moisture (perceived as rainy and dry season), fire (annual fire and protection from fire), and also the invasion on the functioning of the soil community.

(B) Germination

Germination is only one of the important steps in plant competition and establishment; however, it is one of the first bottlenecks affecting the population. An invaded area that is chosen for management to be applied, for instance, will have to be recolonized by the surviving adults and also by the remaining soil seed bank, that will have to germinate. However, the soil seed bank will perceive the managed area in a different way than the unmanaged area. As explained in Figure 4, after fire, due to the removal of vegetation and the reduction of surface albedo, the heat exchange between the soil and the air is performed more easily. Therefore, temperatures in the soil are more extreme during the day and the night. Serveral germination parameters were analysed, simulating the temperatures found in the field after fires, to assess the potencial effect of this modified environment of germination of native and invasive grass seeds.

(C) Gas exchanges and chlorophyll fluorescence: assessing photosynthesis in vivo

In order to better understand the mechanism that lead to the observed morphological characteristics, namely growth impairment, we assessed photosynthetic parameters. Photosynthesis was measured by means of non invasive in vivo techniques: gas exchange parameters with an Infra-red gas analyser (IRGA); leaf chlorophyll fluorescence with a mini-PAM (Pulse modulating fluorometry).

Photosynthesis is the key mechanism in plant physiology. This mechanism is nowadays well elucidated, being dependent on light and water, which interact with the plant's biochemical apparatus. The biochemical pathway is mainly composed by two electron transport chains, which create the conditions for the formation of NADPH, a reducing molecule. This in turn prompts the pH gradient in the thylakoid membranes, creating the force that drives ATP synthase. These molecules (NADPH and ATP) are then used in the Calvin-cycle to reduce atmospheric CO_2 and produce the carbohydrates essentials for plant survival and growth (Figure 6).Therefore, the photosynthesis reactions are the drivers of the morphological parameters and the biochemical responses. To assess photosynthesis in vivo, gas exchange and chlorophyll fluorescence were both assessed.

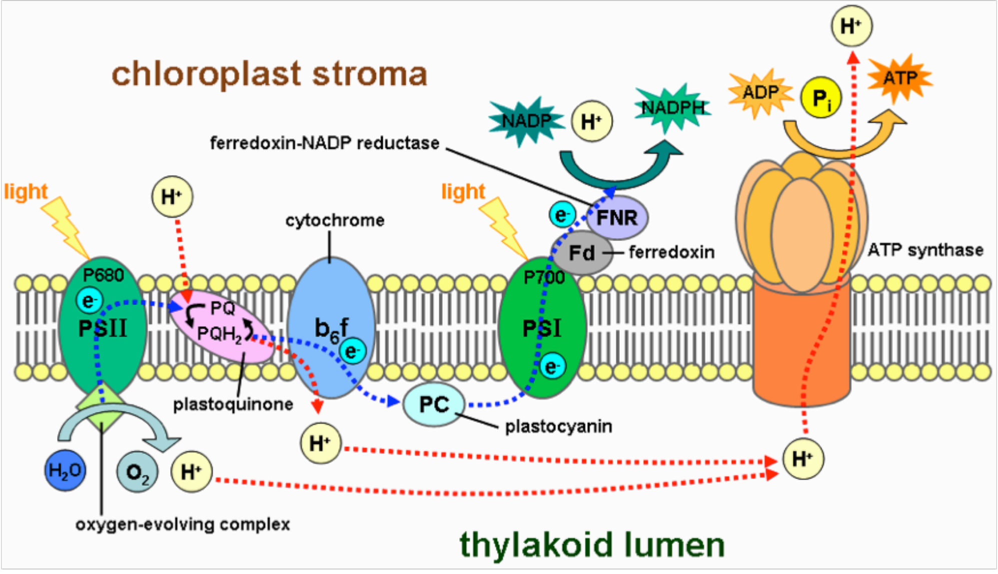

Figure 6. Electron transport chain in thylakoid membrane. Light-phase of photosynthesis where luminous radiation is captured in PSI and PSII and used to create ATP and NAPH. Source: http-//en.wikipedia.org/wiki/Thylakoid assessed on august 2014.

In order to perform photosynthesis, plants need to have access to water, absorbed by the roots, and CO_2 from the atmosphere, which comes in through the stomata. However, for water to reach the photosynthetic tissue, water has to be “pumped” to the leaves. What drives this movement is a gradient of difference in water potential from the root to the leaves. And what is ultimately pumping water is the leaves' transpiration (Larsher 2003). Therefore, stomata play a crucial role not only in absorbing CO_2 but also in water transpiration. The fine control of these cells is what mediates photosynthesis. To assess these gas exchange parameters an infrared gas analyser (IRGA) was used in the present study. This equipment possess a small chamber where leaves are placed, creating a small closed atmosphere. The equipment then measured the absorption of infrared radiation along a period of time, which reflects the change in concentration of the different atmospheric gases that absorb the radiation in different wavelengths. In this chamber, small changes in concentrations of these gases reflect photosynthesis (carbon assimilation), respiration and transpiration. In this work, assimilation rates (amount of CO_2 that is being absorbed from the atmosphere), stomata conductance (which reflects the openness of these structures), transpiration (amount of water released from the leaves though stomata or through the epidermis) C_i/C_a (the ratio between internal and ambient CO_2, also reflects openness of stomata) and Water use efficiency (also known as WUE, is the ratio between Assimilation and transpiration) (Baker 1993) were chosen as study endpoints.

Fluorescence is an inherent property of some substances of changing the wavelength of radiation that was previously absorbed. Usually, emitting fluorescence leads to an increase in the wavelength of this incident radiation. Chlorophyll presents these properties, re-emitting especially the lower energetic radiation (red light), which it absorbs, as fluorescence (Figure 7). The energy that is absorbed in higher energy wavelengths (blue) is partially lost in form of heat, but the energy of red light is lost by fluorescence, that is, a loss of energy by emission of light of longer wavelength (far red). This happens when the excited electrons of the antenna complex emit a photon when returning to its ground state (Taiz & Zeiger 1998).

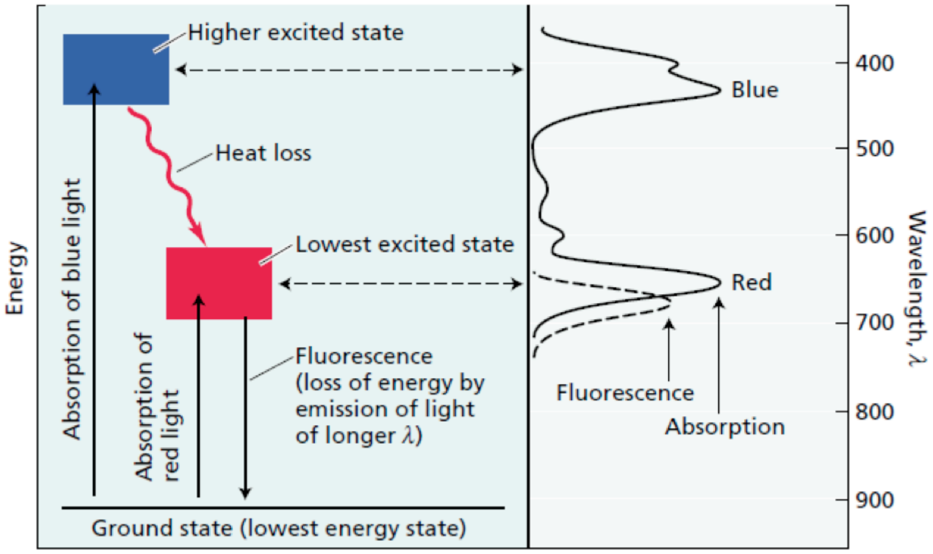

Figure 7. Fluorescence of chlorophyll *a*. Adapted from Taiz & Zeiger (1998).

The antenna complex is an array of protein and pigments (chlorophylls and carotenoids) present in the thylakoid membrane in the plant cells (mainly leaves). This complex is responsible for capturing light energy and transferring it to the reactions centres. There, this energy will drive the photochemical reactions and initiate the photosynthetic energy conversion through the electron transport chains. In optimal conditions, more than 90% of this energy is efficiently converted into fixation of atmospheric carbon. However, depending on the environmental conditions, there is an excess of incident radiation. Therefore, the antenna has to balance this energy input (Figure 8). The plants have several mechanisms to balance energy by dissipating this energy as heat, fluorescence or other quenching mechanisms.

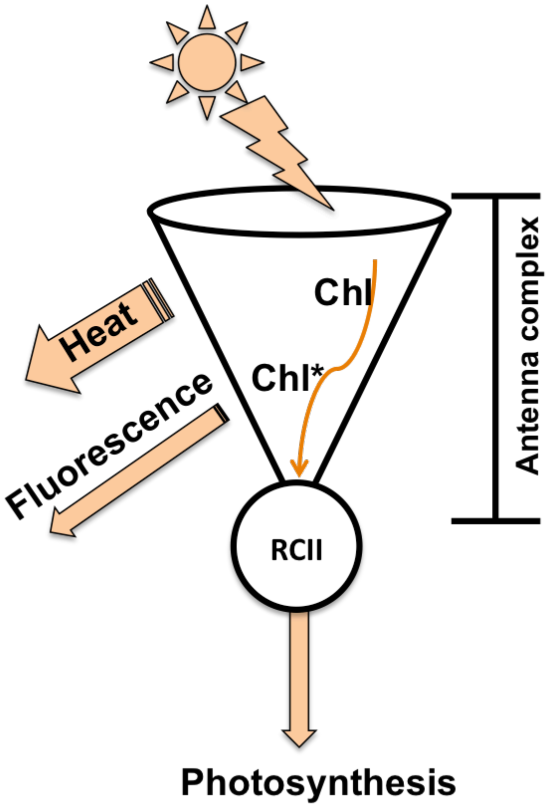

Figure 8. Scheme of the leaf energy balance in the antenna complex. Addapted from Govindjee (2004).

Fluorescence is mostly emitted by chlorophyll a in photosystem II (PS II), because in PS I, the p700 pigment is relatively stable while in PSII the oxidized P680 readily returns to its ground state by electron donation (Figure 7). This energy emitted as fluorescence corresponds to 3% of absorbed light. The intensity of the fluorescence is dependent on the openness of the reaction centres, i.e., it is dependent on the reduction state of the first electron acceptors (quinone_a) of ETC. This acceptor, when being reduced, characterizes a closed centre. If the energy is unable to flow to the reaction centre, it increases fluorescence emission. There are several parameters that one can measure regarding the fluorescence, and it has become increasingly interesting to understand photosystems in vivo; that is, in a non-destructive way (Krause & Weis 1991).

The use of a PAM equipment (pulse amplitude modulated fluorometry) enables the measurement of this emission. Provoking different excitation states of the antennae, with different light intensities, enables the determination of several parameters. These parameters are conservative in the plants and can be used to understand the photochemical reactions:

a) Fluorescence lifetime, for example, is important to study the organization and function of the photosynthetic apparatus and is useful to elucidate energy transfer and kinetics of primary photochemical reactions. Fluorescence measures usually result in the detection of two separate phases: a fast phase, which reflects on the measurement of F_0 and F_m and corresponds to the primary processes of photosynthesis; and a slow phase, in which fluorescence quenching is detected and is related to the induction of Calvin cycle (Figure 9).

b) The F_0 values correspond to the minimal fluorescence, namely, the fluorescence that is emitted when reactions centres are open, when plants are adapted to darkness. In this condition, the first electron acceptors on the reaction centre are ready to receive the energy from the antenna.

c) The F_m values correspond to the maximum fluorescence, which is achieved with a saturating pulse of light that closes all reactions centres. In this case, because the lifetime of the excited state of the pigments is longer, there is a higher probability of the energy being dissipated as fluorescence.

d) The variable fluorescence (F_v) is the difference between these two values, and the F_v/F_m ratio corresponds to the maximum quantum yield of PS II. This ratio is a reflection of the health of the plant's photosynthetic apparatus and is known to be in a narrow range in intact leaves of many species. In theory, environmental stress decreases this ratio, resulting in a lower physiological activity and the presence of photoinhibition.

e) A similar parameter can also be measured in light adapted plants, denoting the effective quantum yield and the photosynthetic activity (ϕ_{PSII}).

f) Fluorescence quenching (q) denotes processes that lower maximum fluorescence. It provides important information on the state of functionality in the plant's photosynthetic apparatus and PS II efficiency. This is related to the regulation of the photosystems according to environmental conditions, as well as the photo-protective capacity of the plant. The quenching occurs due to competition of electrons with different ways of de-excitation. When the energy is captured by oxidized quinones to be used as chemical energy, maximum fluorescence decreases, generating the quenching. However, fluorescence quenching may also occur trough pathways independent of the redox state of quinone; that is, the non-photochemical quenching (NPQ) mechanisms. NPQ operates in the translocation of proteins through the thylakoid membrane (to decrease transfer through PS II), in the xantophylls cycle, or, ultimately, in photoinhibition.

g) ETR values, which correspond to the electron transport rate, are directly related to photosynthetic rate. It can be used to understand acclimation of plants to light intensities. Mutants with reduced ability for fluorescence quenching also presented lower photosynthetic rates and tolerance to stress. For instance, plants acclimated in high light achieve higher ETR values. However the rate of increase is less steep than in plants acclimated to low light, which achieve maximum ETR faster.

F_v/F_m values are faster and more straightforward to achieve. However, light curves or rapid light curves can also be elucidative of the plant's photosynthetic apparatus, pointing to more parameters that can be analyzed. (Krause & Weis 1991, Li *et al.* 2002, Govindjee 2004).

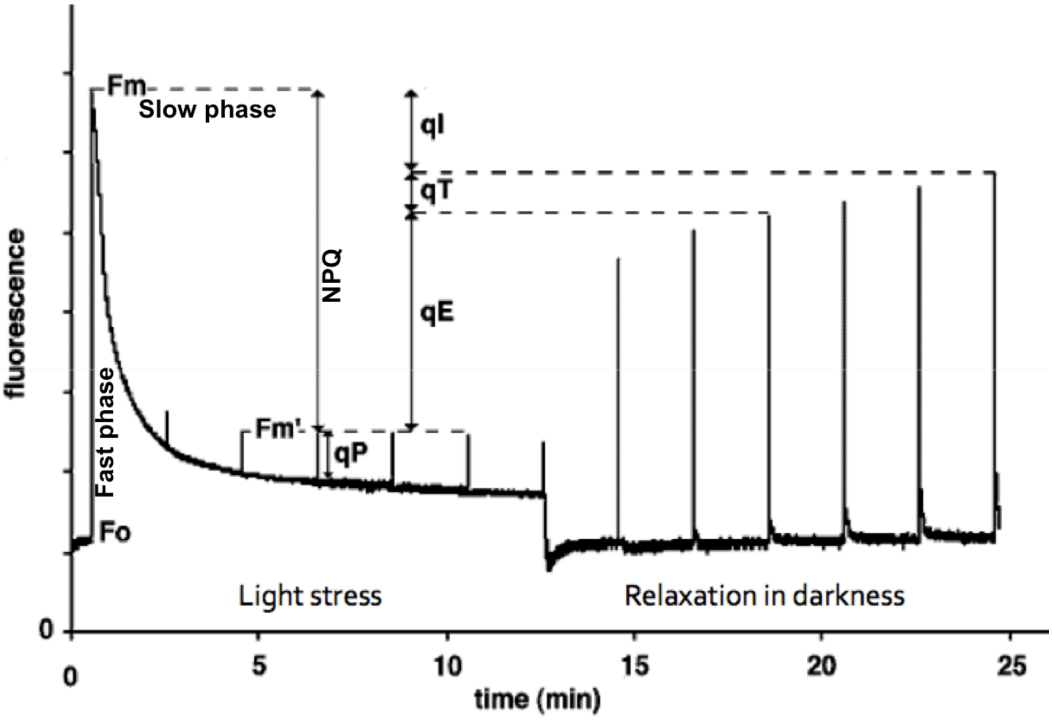

Figure 9. Variation in leaf fluorescence emissions along time following sturating pulses of light (peaks). Adapted from Müller *et al.* (2001).

In this work, a fast and practical approach was chosen were the F_v/F_m ratio (maximum quantum yield) and ϕ_{PSII} (effective quantum yield) were applied as the main means to assess the photosynthetic health of the leaves in relation to the stresses caused by water deficit, fertilization status and damage by clipping and fire. When possible, NPQ was also measured.

(D) Growth: Morphological parameters

Morphological traits are the most usual parameters when studying plant ecophysiology as they represent a visible and macroscopic effect on plants. Plant growth was measured by the endpoints of length and biomass. The partition of biomass was also studied by measuring aerial and root biomass and studied plant architecture by counting number of leaves, tillers and number of senescent leaves.

(E) Oxidative Stress: plant protection

Plants are sessile organisms that have to endure environmental stress by adjusting their internal metabolism. Therefore, although photosynthesis can be highly efficient in optimal conditions, it has to be controlled in stress conditions. One of the tasks that plants have to accomplish is the control of the Reactive Oxygen Species (ROS) equilibrium in their cells.

The production of ROS is a side effect of the evolution of aerobic pathways of energy production and can be used by the plant to monitor and optimise the metabolism according to the environment, regulating transcription factors, hormones, kinases, and post transcriptional modifications in proteins; they are also important in systemic signalling. On the other hand, when ROS is produced in an amount that plants cannot endure, damage begins to appear, with growth impairment and cell death (Foyer & Shigeoka 2011).

Chloroplast organelles, mitochondria and peroxisomes are the main organelles producing ROS, but they can also be produced in the cell membrane and in the apoplast. The functioning of the electron transport chain (ETC) in the membranes naturally produces these radicals (Figure 10). However, overloading the ETC results in greater amounts of O_2 being converted into ROS. Furthermore, radicals like oxygen singlet ($^1O_2$), superoxide ($O_2^{\bullet}$), and peroxide (O_2^{2-}), which are forms of excited O_2 molecules, can form other radicals when interacting with molecules in the cell. They can form radicals like hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$ - when interacting with metals), and carbon radicals ($ROO^{\bullet}$ - when interacting with polyunsaturated fatty acids). These radicals can be very damaging to the cell and can lead to loss in PS II activity, lipid peroxidation, inactivation of enzymes and DNA damage (Arora *et al.* 2002, Gill & Tuteja 2010).

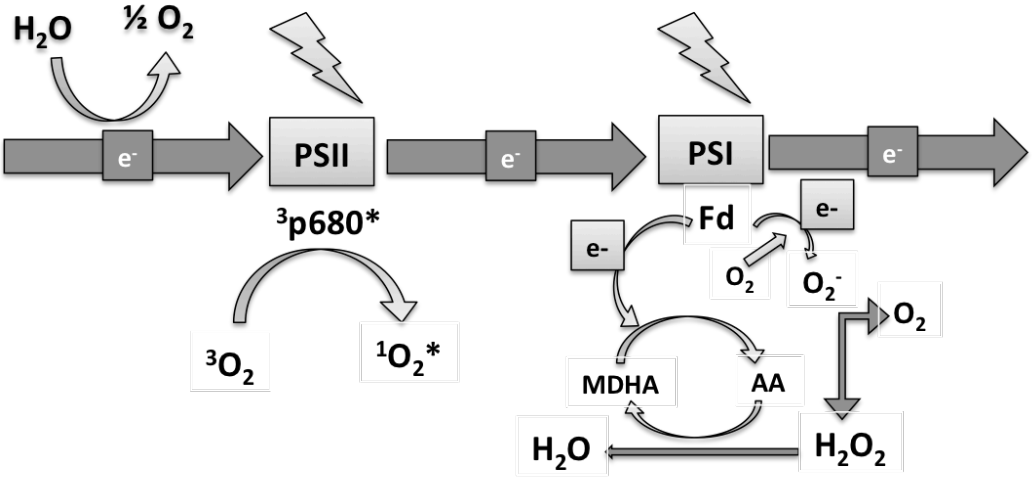

Figure 10. Scheme of ROS production in the ETC of the chloroplast membrane. Adapted from http://www.psc.ac.cn. Accessed on August 2014.

Abiotic stresses such as excess of light or salt, decreases in CO_2 and drought can induce the increase in ROS production. For instance, in drought conditions plants are induced to save water by closing stomata. This closure, however, not only reduces the transpiration, as desired, but also reduces the CO_2 input. However, plants cannot directly control the intensity of sunlight and the antennae continue to be excited by sun's radiation. In this situation, electrons are being excited but are not able to pass normally trough the ETC due to lack of water. In this way, a “leak” of ROS takes place. This ROS production represents the energy that entered the photosystems and was not converted into sugars. Therefore, whenever there is a limiting factor, such as lack of nutrients to produce the enzymes necessary for the Calvin cycle (like RuBisCo), or lack of water, which causes stomata closure, the plant is creating an overload of oxidative radicals.

To balance ROS production, plants have evolved many mechanisms, like leaf movement and chloroplast movement, in a way to reduce the incidence of light in the pigments. However, this avoidance behaviour cannot account for all protection since plants are sessile organisms. Therefore, ROS formation is inevitable and scavenging and repair systems are necessary. Plants make use of antioxidant buffers, antioxidant enzymes, and cyclic electron flow in the transport chains, reducing the pH potential in organelle membranes and repairing damaged molecules.

The main non-enzymatic antioxidants buffers are Ascorbic acid (ascorbate, vitamin C), Glutathione (GSH), Proline, Vitamin E (Tocopherols), Carotenoids and Flavonoids. The main enzymes are Superoxide dismutase (SOD), Catalase (CAT), peroxidases such as Ascorbate Peroxidase (APX), Guaiacol Peroxidase (GPOX) Glutathione Peroxidase (GPX), reductases (GR, MDHAR, DHAR), and Glutathione-S-Transferase (GST) amongst other (Arora *et al.* 2002, Gill & Tuteja 2010, Foyer & Shigeoka 2011).

In the present study we didn't measure the concentration of ROS in the cells, although there are many studies that use hydrogen peroxide concentration, especially, as a biomarker for stress (Silva *et al.* 2013). We chose to measure cell damage caused by ROS using malondialdehyde (MDA) concentration as a marker for lipid peroxidation. We also measured carotenoid concentrations and the activities of APX, G-POX, GST, CAT and SOD.

MDA is formed in the cell when ROS, especially ($OH^{\bullet}$) react with the cell membrane's polyunsaturated fatty acids (PUFA) abstracting one hydrogen and forming alkenals and malondialdehyde. Once lipid peroxidation is initiated, its products can enhance cell damage by reacting with proteins and nucleic acid and propagating lipid peroxidation. Increases in MDA concentration have been reported in plants submitted to drought, and drought tolerant plants have been recognized to present lower concentration of MDA under stress (Arora *et al.* 2002, Gill & Tuteja 2010). Carotenoids are antioxidant pigments responsible for scavenging of singlet oxygen (Knox & Dodge 1985). SOD is an important enzyme that converts superoxide (formed primarily in PS I, but also from quinone$_a$ in PS II) into hydrogen peroxide while being less toxic. It is also the substrate for scavenging other enzymes. Superoxide formation corresponds to 2% of O_2 consumption and its formation is very dangerous and can form the most reactive radical ($OH^\bullet$). Therefore, SOD activity is crucial for the cell's protection and plays a critical role in the survival of the plant under environmental stress. Catalase is one of the enzymes responsible for H_2O_2 removal (along with APX and G-POX). Hydrogen peroxide is a product of SOD, and CAT is responsible for dismutating it. Ascorbate Peroxidase uses ascorbate as an electron donor to reduce H_2O_2 into water. This enzyme also participates in the ASH-GSH cycle, which buffers ROS action (Gill & Tuteja 2010). G-POX is a peroxidase that consumes H_2O_2, but this enzyme prefers the flavonoid Guaiacol (a volatile molecule of strong smell that confers flavour to plants) as an electron donor. This enzyme is known for the ability of catalysing the conjugation of GSH and hydrophobic compounds, by reducing peroxidases. This reaction helps to scavenge cytotoxic and genotoxic compounds. It is related to tolerance to abiotic stress and can increase, for instance, with salinity.

FRAMEWORK

This thesis is divided in seven chapters. The current chapter, Chapter 1, is the General Introduction. Chapters 2 to 6 correspond to the description of the experimental designs, methodologies, statistical analysis and the presentation of the results of this thesis. Finally Chapter 7 constitutes the General Discussion of the obtained results.

Chapter 2 addresses the effect of season, fire, and the presence of the invasive species on soil feeding activity. Using the bait-lamina test, the soil feeding activity was measured in the field and was interpreted as an indicator of soil functioning and organic-matter cycling. Measurements were performed in patches dominated by the invasive grass *Melinis minutiflora* (P. Beauv) , by the invasive grass *Andropogon gayanus* Kunth*,* and by mixed grasslands of native species.

Chapter 3 addressed the effect of post-fire temperatures on seeds of nine native species and one invasive (*Melinis minutiflora* (P. Beauv.). The laboratory treatments simulated the field temperature after fires at the end or in the middle of the dry season. The effects were measured by evaluating the germination rate, mean germination time, and seed viability.

Chapter 4 aimed at quantifying, in laboratory experiments, the plant growth and the underlying physiological and biochemical mechanisms in response to drought and flooding, and to compare these traits between the native species *Schizachyrium microstachyum* (Desv. ex Ham.) Roseng. and the invasive species *Melinis minutiflora* P. Beauv..

Chapter 5 aimed at quantifying, in laboratory experiments, the plant growth and the underlying physiological and biochemical mechanisms in response to drought, flooding, and their combination with fertilization, and to compare these traits between the native species *Schizachyrium microstachyum* (Desv. ex Ham.) Rseng. and the invasive species *Melinis minutiflora* P. Beauv.

Chapter 6 addressed the effect of the disturbances of fire and clipping on the recovery of plants previously grown in the laboratory under different watering regimes, levels of drought, flooding, and the combination of these two factors with fertilization. Plant growth and the underlying physiological and biochemical mechanisms were measured and compared between the native species *Schizachyrium microstachyum* (Desv. ex Ham.) Roseng and the invasive species *Melinis minutiflora* P. Beauv.

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CHAPTER TWO:

BIOLOGICAL ACTIVITY IN CERRADO SOILS: EVALUATION OF VEGETATION, FIRE AND SEASONALITY EFFECTS USING THE "BAIT-LAMINA TEST".

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ABSTRACT

Aims: The Cerrado, a South American savanna, is considered a priority for conservation. In this case study, we assessed soil feeding activity as a way to improve understanding of the ecosystem functioning, in order to support and refine conservation strategies. *Methods:* Soil feeding activity was assessed using the bait-lamina method under different environmental conditions: in the dry and rainy seasons, in burned and unburned areas, and under native and invasive grasses. *Results:* Feeding activity was significantly reduced after fire, but recovered to pre-fire levels with the rains. Activity increased significantly during the rainy season in both areas, being more pronounced in the unburned area. The highest feeding activity was observed under the invasive grass (*Melinis minutiflora* (P. Beauv.). Feeding activity declined with soil depth and was affected by season and fire. *Conclusions:* Seasonality was the most important factor affecting the feeding activity of soil organisms, followed by the fire history and the extant vegetation. Although this method does not allow distinguishing between feeding activity of different organisms, it can provide valuable insights into differences in soil functioning due to changes in environmental conditions.

Keywords: dry season, savanna, burn, soil biota, field experiment, invasive grasses

INTRODUCTION

Ecosystem functioning is directly dependent on soil compartment, which, in turn, is affected by climate, fire and vegetation composition. Microbial biomass and the invertebrate community are regarded as essential for soil functioning. Microbial biomass plays an important role in organic matter decomposition, nutrient cycling, plant–pathogen interactions, and degradation of pollutants. In turn, the invertebrate community maintains the soil physical structure and promotes nutrient turnover by mediating organic-matter decomposition, making it more accessible to microorganisms (Lavelle *et al.* 1997, Wolters 2000, Hattenschwiler *et al.* 2005, Kaschuk *et al.* 2010).

The bait-lamina test is a simple and inexpensive tool for evaluating the biological community in soils, by assessing soil feeding activity. It consists of visually assessing the consumption of the bait substrate contained within the holes of the lamina strips, by counting the number of empty holes after the lamina strips were inserted in the soil. The test can be performed under different conditions (Kratz 1998). Some studies have used this method in risk assessment of contaminated areas and for testing the effects of contaminants (Helling *et al.* 1998, Larink and Sommer 2002, Filzek *et al.* 2004, Niemeyer *et al.* 2010). Hamel *et al.* (2007) and Roembke *et al.* (2006) provided valuable discussions about the effects of vegetation and land use on soil feeding activity in temperate and tropical regions.

The Cerrado, a South American savanna, is considered a priority ecosystem for conservation (Myers *et al.* 2000). Assessing its soil biological activity may contribute to a better understanding of the ecosystem functioning and to support conservation strategies. This biome originally covered up to 25% of the Brazilian territory, mostly on the central plateau. The most frequent soil type is a dystrophic Red Latosol (Acrustox soil according to American classification). The climate has two well-marked seasons: dry,from April to September, and rainy, from October to May, when most of the annual precipitation (1600 mm) occurs (Eiten 1972). The seasonal difference in soil moisture caused by the unevenly distributed rainfall during the year may alter the dynamics of soil chemical properties and biological activity (Bastida *et al.* 2008, Lopes *et al.* 2011).

Fire has been a natural factor in the Cerrado for over 32,000 years (Salgado-Laboriau and Vicentini 1994). Increases in soil temperature during fires are negligible at depths greater than 5 cm (Miranda *et al.* 1993). Nevertheless, the fire regime might be important for soil processes, since it contributes to the circulation and exchange of nutrients between soil and vegetation. Furthermore, human activities have changed the natural fire regime, which was once less frequent and caused by lightning during the rainy season (Ramos-Neto & Pivello 2000).

Although information on the effects of the fire regime on the native Cerrado vegetation and invasive species is abundant (Williams & Baruch 2000, Miranda *et al.* 2009, Marinho & Miranda 2013), very little is known about the effects on the soil compartment (Nardoto & Bustamante 2003, Pivello *et al.* 2010). The native flora is resilient to fire and some common invasive species are capable of benefiting from fire events in some situations (Williams & Baruch 2000, Marinho & Miranda 2013).

The extant vegetation itself is a key factor in ecosystem nutrient cycling. The Cerrado contains a mosaic of vegetation physiognomies with different species compositions and a gradient of tree cover. Changes in plant community are likely to affect soil, and vice versa, since vegetation interacts with the soil in an intricate manner, actively taking up nutrients, interacting and competing for root fixation with other organisms, and producing litter of different varied quality (Hobbie 1996). The open physiognomies of the Cerrado biome are frequently invaded by exotic species. The most common invasive grasses in central Brazil are the African species *Melinis minutiflora* P. Beauv., *Andropogon gayanus* Kunth *and Urochloa decumbens* (Stapf) R. D. Webster. These grasses can markedly change the natural environment by displacing native grasses, forming monospecific stands and increasing the total fuel biomass (Pivello *et al.* 1999, Martins *et al.* 2004).

Considering that data about the biological activity of Cerrado soils are scarce, the aim of this case study was to evaluate the feeding activity of soil organisms under different conditions of rainfall, fire, extant vegetation and soil depth, using the bait-lamina method.

MATERIAL AND METHODS

The experimental areas are located in the National Park of Brasília (PNB - 15º34’ and 15°45’ S and 48º05’ and 48º53’ W). The park has an area of 30,000 ha, which contains the most common physiognomic forms of Cerrado: grasslands (*campo limpo*); grasslands with scattered shrubs (*campo sujo*); open scrub (*cerrado ralo*); closed scrub (*cerrado* sensu strictu) and arboreal woodland with woody underlayer and sparse grasses (*cerradão*) (Ribeiro and Walter 1998). The altitude ranges from 1,070 to 1,200 m and the climate is tropical (Köppen’s CWA), with a marked rainy season (October to April). The mean annual precipitation is 1,600 mm and the mean temperature is 21°C (FUNATURA/IBAMA 1998). This study was included in a research project on prescribed fire settled in the National Park, whose experimental design was originally unreplicated because of the constraints in lighting fires in the Brazilian protected areas. Therefore, this case study was restricted to only one site.

Two different adjacent areas of *cerrado ralo* were used in this study (Figure 11). One area has been protected from fire for over 20 years (unburned), while the other has been subjected to annual prescribed burning since 2005 (burned). Both areas were invaded by *M. minutiflora* P. Beauv. and *A. gayanus* Kunth and contained patches of native vegetation, invasive grasses, and different combinations of native and invasive grasses.

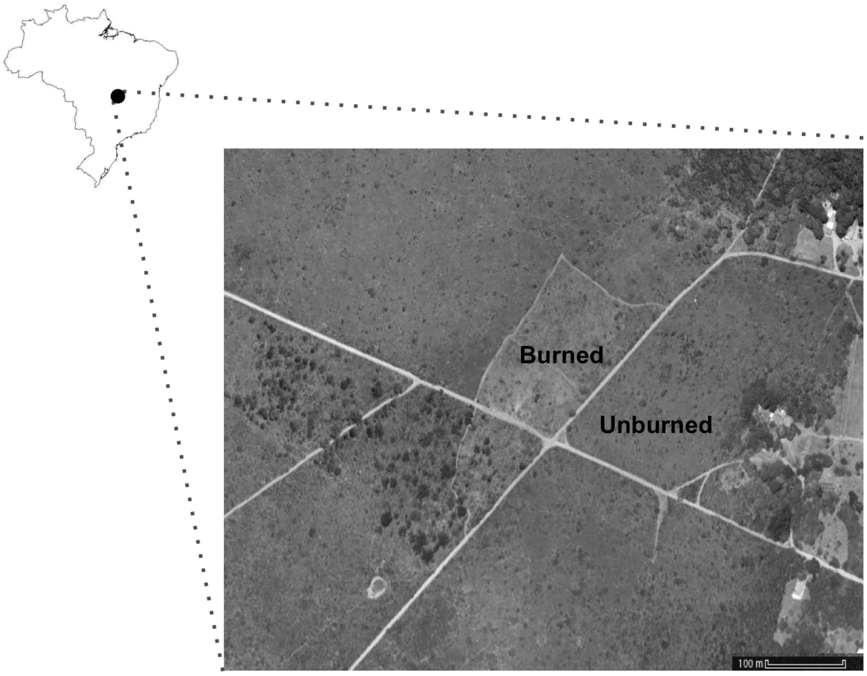

Figure 11. Location of the experimental area in the National Park of Brasília (PNB 15°34' and 15°45' S and 48°05' and 48°53' W), Brasília, Distrito Federal, Brazil (Source https://maps.google.com.br. Accessed in January, 2014).

For each area, five patches of exclusively *M. minutiflora*, exclusively *A. gayanus,* or exclusively native grasses were randomly selected. In each patch type, five sets of 16 bait-lamina strips were used, resulting in 240 lamina strips per area. The laminas consisted of thin strips of about 120 x 6 x 1 mm (length x width x thickness) perforated with 16 holes of 1.5 mm diameter (Kratz 1998). Each set (group of 16 lamina strips) occupied an area of 0.1 m^2 and was placed in the soil according to the design shown in Figure 12. A stainless-steel spatula similar in shape to the lamina was used to perforate the soil so that each lamina could be inserted into the firmly compacted ground. The experimental design is in accordance with other studies using the bait-lamina method (Kratz 1998, Roembke *et al.* 2006). The bait material was adapted from that used by Hamel *et al.* (2007) and consisted of a mixture of finely ground oat grains, cellulose powder (in the proportion of 1 to 3 respectively), agar-agar gel to moisturize the mixture, and traces of activated charcoal.

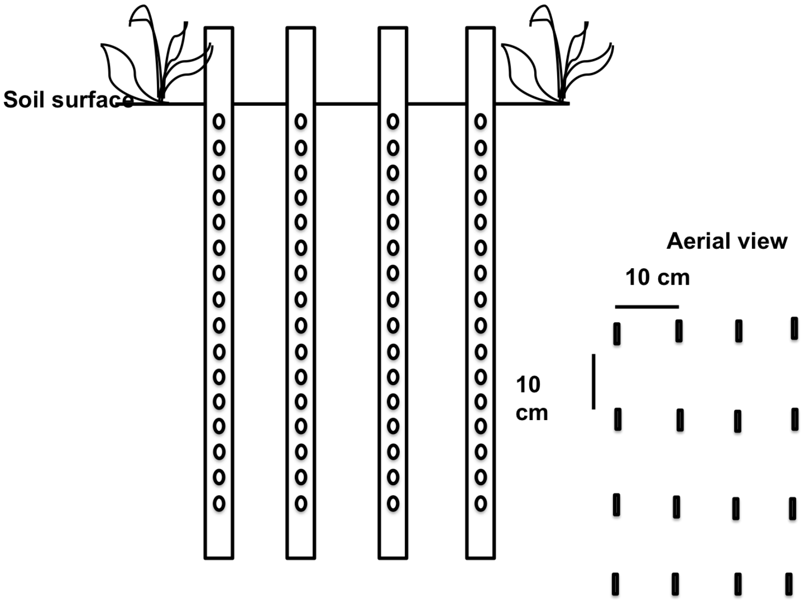

Figure 12. Scheme of a set of bait-lamina strips in the experimental design.

The first sampling was conducted in both areas at the end of the dry season (September), before the scheduled burn. Three days after the prescribed burn, a second sampling was carried out in the burned area. Finally, during the rainy season (November), the same experimental design was repeated in the burned and unburned sites. The bait-lamina strips remained in the soil for 10 days. The time of exposure of the lamina strips and the composition of the bait were previously optimized in preliminary trials. Before the field work, it was necessary to fill the holes with the bait mixture, wait until it dried and fill the holes again, to prevent shrinkage resulting in the loss of bait material (Torne 1990). After the exposure period, the bait-lamina strips were removed, individually wrapped in aluminium foil, and taken to the laboratory. There, they were first carefully cleaned with wet tissue to remove excess soil or biological material adhered to the strips, and then the empty holes were counted against a light source.

The data were analyzed as the mean percentage of empty holes; each value was the mean of 16 bait-lamina strips. The values were standardized by arcsin transformation (√(%/100)). All analyses were carried out with R software (R 3.0.1 binary for Mac OS X 10.6). A factorial ANOVA using season, extant vegetation and fire history as independent variables was performed to compare total feeding activities (total empty holes). A separate analysis was carried out to account for the effect of one specific fire on the biological activity of soil, using a factorial ANOVA with fire and extant vegetation as independent variables, and only data from before and after the fire, both measured during the dry season in the burned area, were used. Tukey's HSD was performed as a *post-hoc* test for multiple comparisons. One-way ANOVA test was applied to compare areas in terms of depth (at each hole level) in order to assess if there was a difference in activities at a specific depth rather than in the total activity. The ANCOVA analysis was used as a complementary approach to the ANOVA, to compare the biological activities among areas, but using depth as a covariant. Also, a Spearman correlation was carried out to account for the significance and importance of a depth effect on soil biological activity according to each vegetation type and season.

RESULTS

In the dry season, feeding activity varied from 16% to 20% in the unburned area and from 15% to 20% in the burned area (Figure 13), with no significant difference between the areas (p=0.8953), independently of the vegetation type. However, during the rainy season the activity in the unburned area was 2.6 to 4.7 times greater (p<0.001) than in the burned area. All three factors (season, fire history and vegetation type) seem to play an important role in explaining the variance in feeding activities, as well as their interactions (Table 1).

Table 1. Three-factor ANOVA testing for effects on season, fire history and extant vegetation on soil feeding activity.

Factors	DF	Sum of squares	F value	P
Vegetation type	2	666	4.482	0.0164
Season	1	5824	78.366	<0.0001
History of fire	1	4817	64.819	<0.0001
Vegetation*Season	2	716	4.819	0.0124
Vegetation*Fire	2	696	4.685	0.0138
Season*Fire	1	3700	49.795	<0.0001
Vegetation*Season*Fire	2	512	3.444	<0.04
Residuals	48	74.40		

In the unburned area, the feeding activity always increased significantly in the rainy season (all p<0.02) and there was a significant interaction between vegetation and season (p=0.007) (Figure 13). The activity in the *M. minutiflora* soil patches was significantly higher than in the other two vegetation types (p<0.05), reaching over 70%, while the feeding activity in soil patches with native grasses and *A. gayanus* was around 40-50% and did not differ between the two (p=0.969). In the burned area, activity was not affected by either vegetation type or season. In the dry and rainy seasons, feeding activity remained around 15%. However, feeding activity increased significantly with the rains (p=0.009) in the burned area where *M. minutiflora* was predominant.

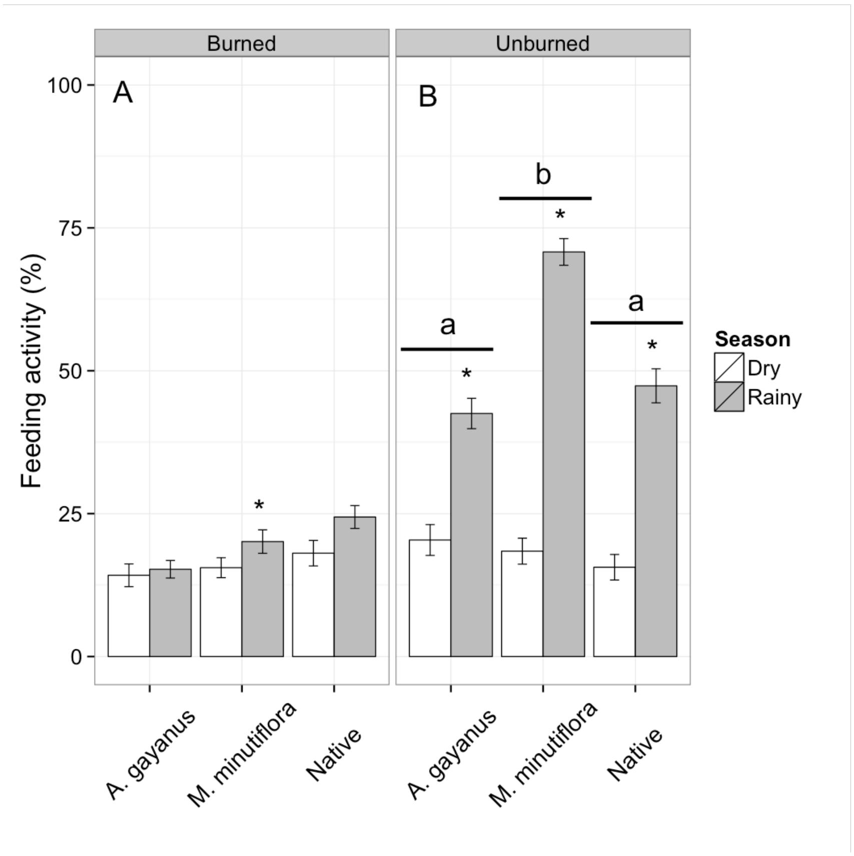

Figure 13. Total soil feeding activity in a Cerrado area in the National Park of Brasília, Distrito Federal, Brazil. Capital letters indicate significant differences between areas, small letters indicate differences among vegetation types, and asterisks indicate differences between seasons. Bars correspond to standard errors (p<0.05, Three-factor ANOVA, Tukey's HSD).

The effect of fire (besides fire history, as observed previously) on soil feeding activity was immediate and significant (p<0.0001). The levels decreased from 20% to 10-15% after fire (Figure 14). The feeding activity immediately after fire decreased significantly in the patches dominated by *M. minutiflora* (p=0.02), while p values were marginally significant for the other two vegetation types (p=0.064, p=0.060, respectively).

Soil depth (p<0.001), fire history (p<0.001), vegetation type (p=0.01647) and season (p<0.001) all significantly affected the feeding activity along the depth gradient. Differences in activity between seasons for each area were observed at each depth level (1 to 16) (Figure 15). The correlation coefficients and the significance of the correlation are listed in Table 2, and were useful to understand the magnitude of the correlation between activity and depth. A negative correlation was significant in all cases, except in the burned area during the rainy season following fire, when correlations tended to be positive, although not significant.

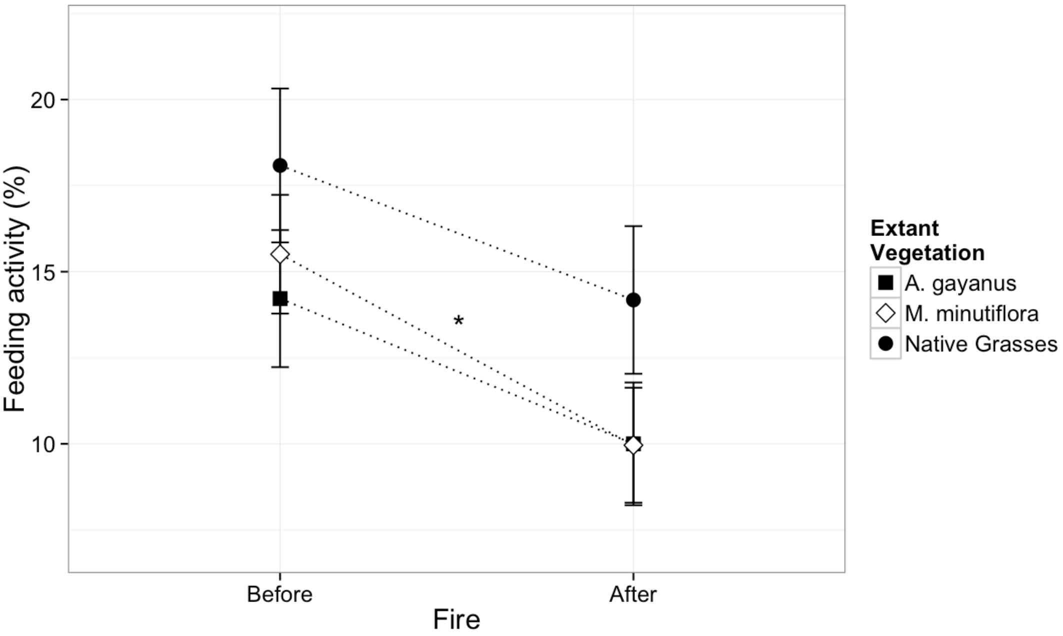

Figure 14. Effect of a dry-season fire on soil feeding activity in a Cerrado area in the National Park of Brasília, Distrito Federal, Brazil. Bars indicate standard errors. Asterisks indicate a significant difference between levels from before and after fire. (p<0.05, T-test).

Table 2. Coefficients for Spearman correlation between soil feeding activity and depth.

	Area	Season	Coefficient	P-value (α=0.05)
***A. gayanus* Kunth**	Unburned	Dry	-0.568	<0.0001
		Rainy	-0.682	<0.0001
	Burned	Dry	-0.505	<0.0001
		Dry – After fire	-0.407	<0.001
		Rainy	0.170	0.1311
***M. minutiflora* P. Beauv.**	Unburned	Dry	-0.363	<0.001
		Rainy	-0.827	<0.0001
	Burned	Dry	-0.391	<0.001
		Dry – After fire	-0.373	<0.001
		Rainy	0.014	0.9023
Native grasses	Unburned	Dry	-0.600	<0.0001
		Rainy	-0.522	<0.0001
	Burned	Dry	-0.526	<0.0001
		Dry – After fire	-0.523	<0.0001
		Rainy	0.117	0.3282

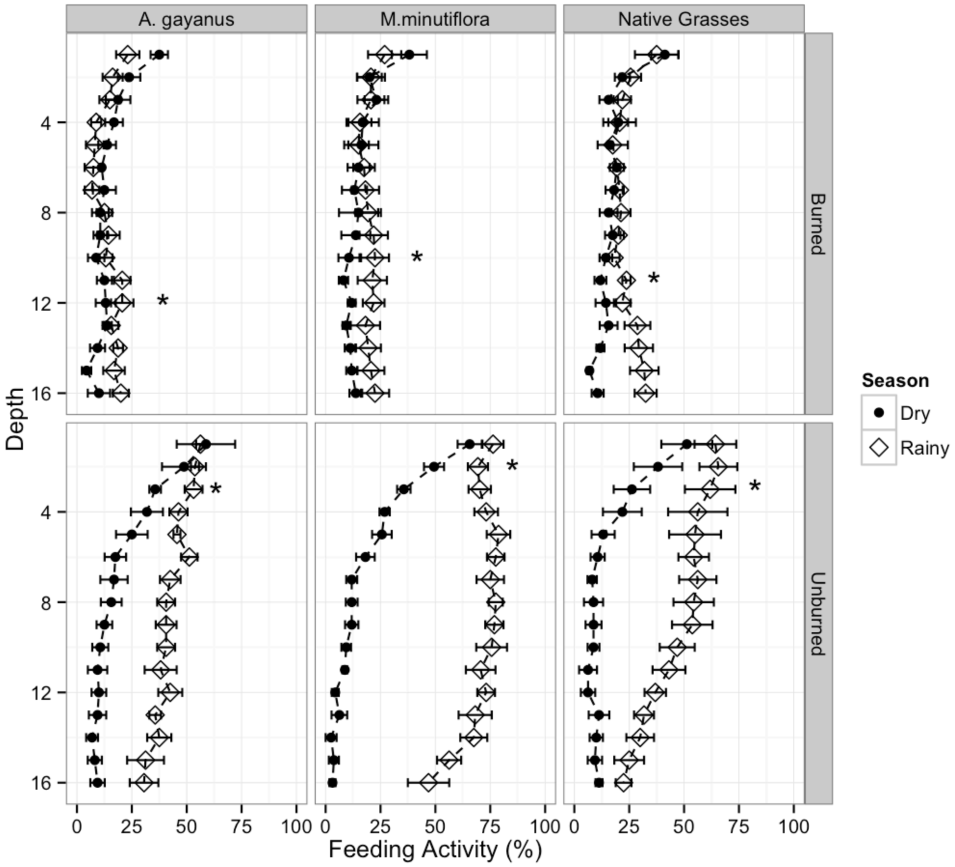

Figure 15. Soil feeding activity along the depth gradient in a Cerrado area in the National Park of Brasília, Distrito Federal, Brazil. Bars indicate standard errors. Asterisks indicate the level at which differences between seasons began to be significant (p<0.05, T-test).

DISCUSSION

With minor adaptations, the bait-lamina test proved to be suitable to evaluate the soil biological function in Cerrado, by measuring the feeding activity of the soil fauna. Although the bait-lamina test is not able to differentiate between feeding activity of different group organisms (Helling *et al.* 1998) and may yield different results compared to other methods for assessing biological activity (Kula & Römbke 1998), it is a relatively easy and sensitive way to evaluate and explain differences among sites in Cerrado. The feeding activity recorded in this study was within the same order of magnitude as those found in other studies with bait-lamina strips in tropical areas (Geissen *et al.* 2001 in Roembke *et al.* 2006; Roembke *et al.* 2006). However, the exposure time adopted in this study (10 days) was shorter than the time reported by Hamel *et al.* (2007) in a dry temperate zone in Canada (65 days), by Helling *et al.* (1998) in laboratory experiments (15-45-days) and by Forster *et al.* (2004) in a grassland field in Germany or in an arable field in Portugal (14 days). On the other hand, it was longer than the time reported by Roembke *et al.* (2006) for the Amazon Forest (4 days) and by Filzek *et al.* (2004) for grasslands in the UK (6 days). In the present study, feeding activity was very low during the dry season; therefore, a 10-day exposure was chosen as the optimal period to measure activity during the dry season without underestimating the activity in the rainy season. In the present study consumption may be attributed to microorganisms (bacteria and fungi) and invertebrates such as nematodes, insect larvae (Coleptera, Diptera, Lepdopterda) and adults (Isoptera, Formicidae, Collembola) and Oligochaete (specially Enchytraeids) abundant in Cerrado soil (Aquino *et al.* 2008). Furthermore, collembolan and enchytraeids have been reported effective feeders of bait-lamina material (Helling *et al.* 1998).

Feeding activity was highly affected by season. Forster *et al.* (2004) also reported that consumption of bait material was significantly related to soil moisture, in several soil types and conditions. The consumption may be attributed to microorganisms and/or to the invertebrate community. Microbial biomass and enzymatic activity are strongly influenced by season and present sharp increases with water availability (Goransson *et al.* 2013). The invertebrate abundance and distribution is also related to soil moisture (Lindberg *et al.* 2002). Besides water content, temperature differences between seasons must also be taken into account, since mean temperatures in the Cerrado during the dry season are 5 to 10ºC lower than in the rainy season and consequently, lower biological activity should be expected. Other soil processes such as CO_2 efflux are also positively related to an increase in temperature (Pinto-Junior *et al.* 2009). This information is consistent with the lower activities found during the dry season in the Cerrado, in both the burned and unburned areas.

Although the feeding activity tended to increase in the rainy season, it was significantly higher in the unburned, compared to the annually burned area, highlighting the importance of the fire history for this parameter. Fire as a management technique is widely used in the Cerrado and has been reported to affect soil parameters and biological activity (Silva *et al.* 2006). For Cerrado, Nardoto and Bustamante (2003) reported lower amounts of inorganic-N cycled annually through mineralization processes in a burned area. On the other hand, Viana *et al.* (2011) found no changes in the microbial density related to fire but did find a significant correlation with plant cover. For other savannas, Decaëns *et al.* (1994) reported a decrease in invertebrate density due to fire in the Colombian Llanos, and Black and Okwakol (1997) found reductions in termite abundance and species richness in burned savannas. Therefore, the bait-lamina measurements may be representing an indirect negative effect of fire on organisms involved in organic-matter decomposition and nutrient cycling. All sample sets were located within the same continuous area (Figure 11), with no topographic differences. Although soil properties were not measured, the burned and unburned areas are 3 m apart and the discrepancies between the areas are unlikely to result from differences in soil type.

The direct effect of fire was significant only for *M. minutiflora* patches. Similarly, Decaëns *et al.* (1994) found a significant change in the invertebrate community and its distribution with depth, immediately after a fire in the Colombian Llanos. The consumption of surface litter by fire directly damages the aboveground biota; for the Amazon Forest, Roembke *et al.* (2006) reported a reduction in feeding activity due to removal of aboveground litter. Nevertheless, it is known that soil temperatures during Cerrado fires are negligible below 5 cm (Miranda *et al.* 2009) and, therefore, the soil biota is protected from high temperatures. However, the consumption of vegetation changes the soil-temperature profiles and increases transpiration (Castro-Neves & Miranda 1996). Together, post-fire soil temperatures and transpiration may be responsible for the reduction in soil activity observed in this study, by negatively affecting microorganisms or making the top soil layers an unfavourable environment for soil fauna. Similarly to the observations of Decaëns *et al.* (1994), the recovery of the soil community to pre-fire levels is relatively rapid (6 months). Loureiro *et al.* (2007) also reported increase in soil enzymatic activity in soils under recovery. This was evident in the present study from the higher feeding activity levels measured in the rainy season following fire.

The vegetation also affected the feeding activity levels in soil, with higher activity found under patches of *M. minutiflora*. This species has been reported to produce litter with a lower concentration of nitrogen, compared to native Cerrado grasses (Silva & Haridasan 2007); and higher nitrification rates have been reported in the soil under this species (Asner and Beatty 1996). The invasion might be affecting the microbilogical community and the nitrogen cycle, however the mechanism is unclear and further studies are needed. Furthermore, an increase in invertebrate abundance in soils under an African invasive species has being reported by Benito *et al.* (2004). Therefore, *M. minutiflora* might affect both soil microbial and invertebrate community. On the other hand, *A. gayanus* does not seem to have the same effect, but this species was present in the initial stages of invasion in the area (visual estimation), possibly resulting in the lower soil feeding activity. In general, the importance of vegetation in feeding activity was no longer evident during the dry season, nor in the annually burned area. Marginal influence of vegetation type on feeding activity was also revealed by a bait-lamina test in dry grassland in Canada (Hamel *et al.* 2007). Thus, season and fire seem to be the most important factors affecting soil feeding activity.

Finally, depth is also an important factor to consider when using bait-lamina strips to evaluate feeding activity in soils (Filzek *et al.* 2004, Roembke *et al.* 2006, Hamel *et al.* 2007). The significant interaction between depth, season and history of fire can be observed in Figure 15. The pattern is similar for all situations but the activity decreases less sharply in the rainy season. While the feeding activity in the unburned area differs between seasons at the soil surface, in the burned area in the rainy season the activity is higher at lower depths (below 5 cm). This pattern is confirmed by the correlation analysis, which showed that the only situation where the activity does not decrease with depth was during the rainy season in the burned area. The absence of live vegetation may facilitate percolation of water to the deeper layer of soils, and enhance evaporation from the first centimetres of soil during the frequent dry spells, which may last up to five to ten days (Assad *et al.* 1993).

CONCLUSION

The bait-lamina method was sensitive enough to detect effects of fire events, fire history, extant vegetation type and season on the feeding activity of Cerrado soil organisms. In addition, similar studies would benefit from information on soil properties and soil fauna to better understand the feeding activity mechanism in the study areas. The effects of season were the most important. As expected, feeding activity was very low during the dry season, regardless of other factors. In the following rainy season, the fire history tended to decrease soil feeding activity, probably through reduction of vegetation cover; while a single burn had showed little immediate effect on soil feeding activity. Also, feeding activity was generally negatively correlated with depth, and showed to be higher in areas invaded by *M. minutiflora*, especially if protected from fire. The bait-lamina test is an inexpensive and easy-to-use technique and may provide a good indication of soil biological function.

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CHAPTER THREE:

SIMULATED POST-FIRE TEMPERATURE AFFECTS GERMINATION OF NATIVE AND INVASIVE GRASSES IN CERRADO (BRAZILIAN SAVANNA)

Carolina Musso, Heloísa Sinátora Miranda, Stefano Salvo Aires, Ana Catarina Bastos, Amadeu M. V. M. Soares and Susana Loureiro

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ABSTRACT

Background: Although fire is an important factor in determining cerrado vegetation, information on its effects on seed banks is sparse. Cerrado fires are rapidly moving surface fires with low residence time, producing only short-term heating of the uppermost centimetres of soil. However, the reduction of vegetation cover and deposition of ashes increases the daily amplitude of soil temperature up to 35°C.

Aims: Assess the effects post-fire daily soil temperatures on the germination of one alien and nine native grasses.

Methods: Seeds were stored at alternating temperatures of 45 ºC/10 ºC (10 h /14 h) for 7 d or 30 d, simulating two different storage times in the soil seed bank before the onset of the rainy season. Germination was monitored for 30 d.

Results: The temperature treatment had a significant effect on seed germination in some species, either enhancing (*Aristida setifolia*) or reducing germination rate (*Schizachyrium sanguineum*). Increased storage time reduced the viability of *S. sanguineum* and *Echinolaena inflexa*. The invasive *Melinis minutiflora* had the highest germination rate and best tolerated post-fire conditions (45 ºC/10 ºC) after 7 d, with a significant reduction in the germination time after 30 d.

Conclusions: Fire seems to have a significant effect in early life stages of cerrado grasses. Some native species responded positively to the temperature oscillation, suggesting that they would be better prepared to compete with the invasive species after a fire, more of their seeds germinating and/or at a more rapid rate.

Keywords: alien grass, burn, mean time of germination, seed germination, seed viability, soil seed bank, soil temperature.

INTRODUCTION

Fire is an important ecological factor in many ecosystems, especially in savannas and grasslands with C_4 grasses (Keeley & Rundel 2005, Bowman *et al.* 2009). The cerrado, or Brazilian savanna, has been affected by natural fires for millennia (Simon *et al.* 2009). The climate in the cerrado region is seasonal, with a well-defined wet season (90% of the precipitation occurs between October and April), and a pronounced dry season, when fires may occur. Although the natural fire frequency is unknown, nowadays fires may recur every 1 to 4 years on average (Coutinho 1990), and may alter the composition and structure of the vegetation (Hoffmann 1998; Munhoz & Felfilli 2006, Miranda *et al.* 2009), changing denser cerrado types to open ones (Moreira 2000).

Cerrado fires are surface fires, and consume mostly the fine fuel of the herbaceous layer. During fires, flame temperature may rise to 800 ºC (Miranda *et al.* 1993) and soil surface temperature may reach a maximum of 280 ºC (Castro-Neves & Miranda 1996). Due to the low fire residence time, significant increases in temperature have been recorded only in the first centimetre of soil: 100 to 111 ºC at 0.5 cm depth, during fires in Venezuelan savannas (Silva *et al.* 1990) and 29 to 55 ºC at 1 cm depth during cerrado fires (Miranda *et al.* 1993, Miranda *et al.* 2009). Therefore, seeds in the first centimetre of soil, which comprise over 90% of the soil seed bank (Andrade *et al.* 2002), are exposed to non-lethal temperatures during fires.

Went *et al.* (1952), Gashaw and Michelsen (2002) and Scott *et al.* (2010) have hypothesised that the effect of fire on germination was not necessarily related to heating during a fire, but rather to the subsequent indirect effects, such as an increase in radiation intensity on the soil surface, reduction of competition, and smoke (Read and Bellairs 1999; Dayamba *et al.* 2008; Figueroa *et al.* 2009). Although some plant families (e.g. Leguminosae and Cistaceae) respond positively to short pulses of heat (60 to 150 ºC), probably due to scarification of the hard seed coat, grass seeds rarely require heat shock to germinate, and the speed of germination is not increased by heating (Portlock *et al.* 1990; Tarrega *et al.* 1992; González-Rabanal & Casal 1995, Herranz *et al.* 1998, Hanley & Lamont 2000). On the other hand, alternation of temperature seems to be important to promote germination in many plant families (Morinaga 1926, Thompson & Grime 1983, Carmona *et al.* 1998, Baskin & Bakin 2001). Pierce & Moll (1994) and Auld & Bradstock (1996) concluded that daily soil temperature increases significantly and is likely to improve seed germination.

Depending on tree cover, the cerrado soil seed bank density varies from 70 to 144 seeds m^{-2} (Andrade *et al.* 2002, Salazar *et al.* 2011); and, as for other savannas, seeds of species that constitute the herbaceous layer comprise most of the soil bank (Tybirk *et al.* 1994, Williams *et al.* 2005, Scott *et al.* 2010). Few studies examined the effects of fire on the seed bank (Andrade et al. 2002; Ikeda et al. 2008), and the effects of high temperatures on germination of native species (Overbeck *et al.* 2006, Schmidt *et al.* 2005, Zaidan and Carreira 2008, Fichino *et al.* 2012, Ribeiro *et al.* 2013).

For some native cerrado grasses and dicots heat shock alone (50 to 110 ºC for 2 min) or long exposition time at constant temperatures (10 to 40 min at 60 ºC, 5 to 20 min at 80 ºC or 2.5 to 10 min at 100 ºC) did not stimulate or reduce seed germination (Overbeck *et al.* 2006, Schmidt *et al.* 2005, Ribeiro *et al.* 2013), suggesting that fire did not have an immediate effect on germination of the seeds stored in the seed bank. However, it is well known that storage and oscillating temperatures may have a positive effect on seed germination (Labourau 1983, Baskin & Baskin 2001).

Considering that seeds stored in the soil bank may not have their germination stimulated by the heat pulse during cerrado fires, and that the expressive increase in post-fire daily amplitude of soil temperature may last to the onset of the rainy season or until the herbaceous vegetation is recovered (Catro-Neves & Miranda 1996), our study examined the effect of increased post-fire soil temperature oscillation on the germination of one exotic and nine native grasses common in the cerrado. The following questions were addressed: (1) Does the increase in daily amplitude of soil temperature after a fire have a significant effect on germination of soil-stored seed? (2) Would different storage periods, i.e. time between fire and the onset of rainy season, affect seed germination under the increased post-fore soil temperature oscillation?

MATERIALS AND METHODS

Seed collection

The seeds of 10 common cerrado grass species (Clayton *et al.* 2006) were used: nine native (*Aristida recurvata* Kunth, *Aristida setifolia* Kunth, *Axonopus barbigerus* (Kunth) Hitch, *Echinolaena inflexa* (Poir.) Chase, *Gymnopogon spicatus* (Spreng.) Kuntze., *Paspalum gardnerianum* Nees, *Paspalum stellatum* Humb. and Bonpl. ex Flüggé, *Schizachyrium microstachyum* (Desv. ex Ham.) Roseng, *Schizachyrium sanguineum* (Retx.) Alston.) and one invasive (*Melinis minutiflora* P. Beauv). All species, except for *E. inflexa* and *M. minutiflora*, form a transient seed bank (Andrade 2000, Martins *et al.* 2009). The seeds were collected in the IBGE

Ecological Reserve (Reserva Ecológica do Instituto Brasileiro de Geografia e Estatística), 35 km south of Brasília, 15° 56' S and 47° 52' W. The climate of the Reserve is tropical (Köppen's CWA), with a distinct rainy season (October to April). The mean annual precipitation is 1453 mm and the mean temperature is 21 °C (IBGE 2004).

The collection of the seeds was made by manual harvesting of the panicles from May through July 2010. To obtain the largest number of mature seeds, harvesting took place after ca. one-third of the seeds had been dispersed (Baréa *et al.* 2007). As most tropical grasses have embryonic dormancy, with the embryos partially mature at dispersal (Adkins *et al.* 2002), the seeds of each species were pooled, and stored in paper bags at room temperature (ca. 25 °C) and humidity (ca. 50%) until use, i.e., the end of August 2010 to ensure that most seeds were completely mature.

Germination experiment

Germination tests were carried out in a controlled-temperature chamber programmed to simulate daily post-fire soil temperatures of 45 ºC / 10 ºC, as reported by Castro-Neves & Miranda (1996); hereafter called the heat, or temperature treatment. The duration of the heat treatment simulated different lengths of periods to the rainy season: 30 d (fire in mid-dry season – H30) and 7 d (fire at the end of the dry season – H7). At the end of the heat treatment, the chamber temperature was changed to 37 ºC / 22 ºC to simulate the soil temperatures at the onset of the rainy season (Andrade *et al.* 2000) and the photoperiod was set to 10 h (2.34 ± 1.37 W m^{-2}).

During the H30 and H7 treatments, for each species, four batches of 100 fertile seeds were left in the chamber in paper bags. After the treatment, the seeds were placed in sterilised Petri dishes containing filter paper moistened with distilled water. To evaluate the effect of each heat treatment on germination and germination time, four batches of non-treated seeds (U30 and U7) were added to the chamber. The number of germinated seeds was recorded during 30 d; the geotropic curvature of the radical was used as the criterion for germination (Labouriau 1983). For *A. barbigerus*, *G. spicatus*, *M. minutiflora*, *P. gardnerianum* and *S. sanguineum* the number of fertile seeds g^{-1} was calculated from five batches of 100 seeds. Each seed was manually tested to verify the presence of an embryo (Brasil 2009); for the remaining species, values from Aires *et al.* (2013) were used.

At the end of the observation period, the seeds were tested for viability with a 1% solution of 2,3,5-triphenyltetrazolium chloride. Seeds that stained were considered viable, but dormant (Lakon 1948).

Data analysis

ANOVA was used for statistical analysis after arcsine transformation of germination rate and viability values. For each measured parameter (germination rate, viability and germination time), means were compared with factorial ANOVAs with species, heat treatment (H or U) and storage duration (30 d or 7 d) as independent variables. For each species, the means of germination rate, viability and germination time were compared with a factorial ANOVA with heat treatment and storage duration as independent variables and adjusted for multiple comparisons using Bonferroni correction. Using a scaled covariance data matrix, a Principal Components Analysis (PCA) was carried out to explore and highlight the relationships and patterns between species and treatments. Analysis of the component loadings allowed identifying which variables contributed the most (or the least) to explaining the discrimination between the different species along PC1 and PC2, as a response to post-fire conditions. The PCA was carried out by using the entire set of the measured variables (germination rate, viability, and mean germination time at H7 and at H30), to avoid loss of variance within the dataset and increase the confidence of the results. The input data were calculated by subtracting the mean values of the control from the mean values for the treatments, for ease in data handling and interpretation. Vectors represent absolute changes, calculated as post-fires mean treatment values (H30 and H7) minus the mean untreated values (U30 and U7). Data were analysed using the R software (R 3.0.1 binary for Mac OS X 10.6).

RESULTS

Germination rate and viability

The germination rate differed among species ($P<0.001$, Table 3) and within species of the same genus. Germination rates were > 80% for *A. barbigerus*, *M. minutiflora*, *P. stellatum*, *S. microstachyum* and *S. sanguineum*; ranged between 30% and 60% for *A. recurvata* and *P. gardnerianum*; and were < 20% for the other native species (Figure 16).

Table 3. Factorial ANOVA scores for three measured dependent variables (germination rate, viability and germination time), using three independent variables as factors (species, storage duration and heat treatment) for a set of nine cerrado grass species and one invasive species subjected to daily post-fire soil temperatures (45 °C / 10 °C) for 7 or 30 days before germination test (37 °C / 22 °C, simulating soil temperature at the onset of the rainy season). Df= Degrees of freedom

Independent Variables	Df	F	Sum of Squares	*P*	F	Sum of Squares	*P*	F	Sum of Squares	*P*
Species (Sp)	9	21.393	205.539	< 0.001	16.297	120.884	< 0.001	10.716	56.448	< 0.001
Storage duration (Sd)	1	0.159	13.768	< 0.001	0.134	8.971	0.003	2.807	133.067	< 0.001
Heat treatment (Ht)	1	0.021	1.842	0.177	0.001	0.055	0.815	0.480	22.741	< 0.001
Sp x Sd	9	0.525	5.045	< 0.001	1.158	8.591	< 0.001	2.725	14.354	< 0.001
Sp x Ht	9	0.396	3.807	< 0.001	1.096	8.127	< 0.001	2.249	11.846	< 0.001
Sd x Ht	1	0.001	0.066	0.798	0.079	5.286	0.023	0.172	8.157	0.005
Sp x Sd x Ht	9	0.335	3.215	0.002	0.542	4.017	< 0.001	0.480	2.527	< 0.001
Residuals	120	1.388	205.539	< 0.001	1.798	120.884	< 0.001	2.531	56.448	< 0.001

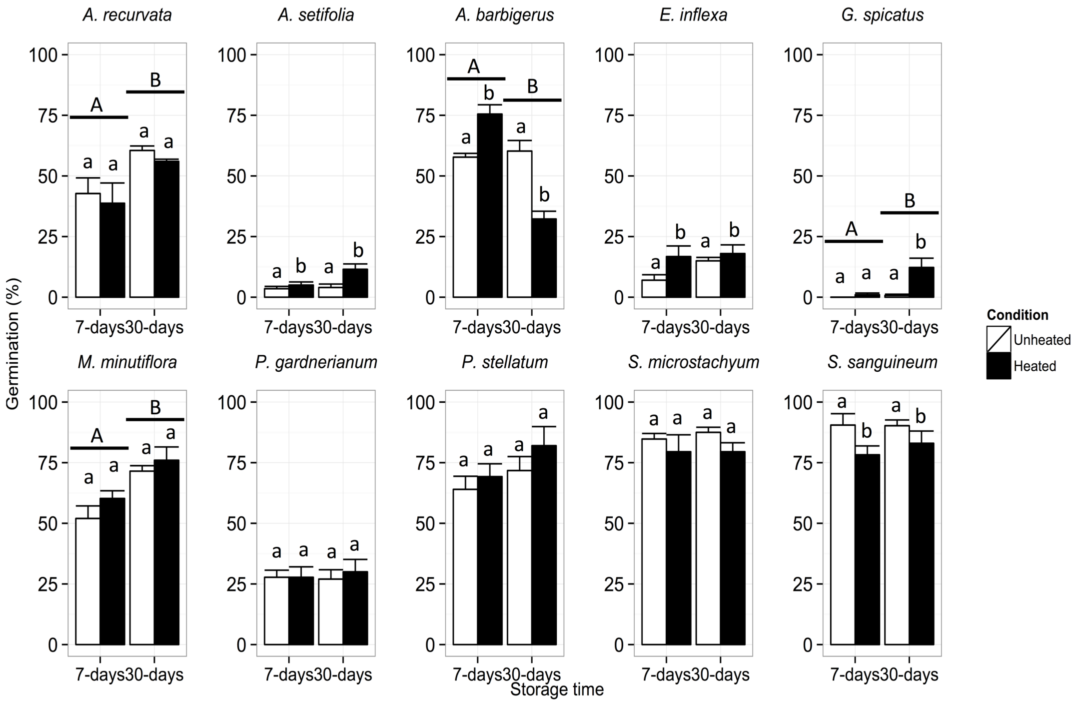

Figure 16. Germination rates of nine native and one exotic grass species (Melinis minutiflora) exposed to simulated daily post-fire soil temperatures (45 °C / 10 °C) for seven (H7) and 30 days (H30) or Untreated (U30, U7), i.e., not subjected to heat treatment. Error bars correspond to standard errors. Capital letters above bars correspond to significant differences between storage duration (30-d or 7-d) and small letters to differences in treatment (H or U). White bars represent untreated seeds. (P<0.05, Factorial ANOVA with heating treatment (H or U) and storage duration (30-d or 7-d) as independent variables, multiple comparisons were corrected with Bonferroni test). Germination was tested at 37 °C / 22 °C that simulated soil temperature at the onset of the rainy season.

The duration of heat treatment (7 d, H7 or 30 d, H30) had a significant effect on seed germination ($P < 0.001$, Table 3). Species did not respond all in the same way to H7 or H30, as indicated by the significant interaction between these variables ($P < 0.001$, Table 3). Although no significant effect of heat treatment (simulated post-fire soil temperature oscillation) was detected at this stage, there was a significant interaction between species and heat treatment ($P<0.001$) and species and storage duration and heat treatment ($P<0.01$, Table 3).

The germination rates of *E. inflexa*, *P. gardnerianum, P. stellatum* and *S. microstachyum* were not affected by the simulated daily amplitude of soil temperature after a fire (45° C / 10 °C) or by the storage duration. However, there was a significant effect of storage duration on the germination rate of *A. recurvata* ($P<0.01$) and *M. minutiflora* ($P<0.01$), with an increase in germination rate at H30.

For *A*. *barbigerus*, *G. spicatus* and *S. sanguineum,* the storage duration seemed to play an important role in germination rate (Figure 16). The H30 treatment resulted in a significant increase in the germination of *G. spicatus*, but no significant effect was observed for it in the H7 treatment (Figure 16). For *A. barbigerus*, germination increased at H7 ($P<0.05$) and decreased at H30 ($P<0.001$). Heating reduced the germination of *S. sanguineum* ($P<0.05$). The viability of seeds of *A. recurvata* ($P<0.001$), *A. barbigerus* ($P<0.001$), *E. inflexa* ($P<0.05$) and *S. sanguineum* ($P<0.01$) decreased significantly at H30.

Germination time

The mean germination time differed among species, and was affected by the storage duration, post-fire temperature, and the interaction among these variables (all

$P<0.05$, Table 3). In general, the mean germination time did not change for seeds at H7 (Table 4). However, a reduction was observed for *G. spicatus* ($P<0.005$), *M. minutiflora* ($P<0.05$), *S. microstachyum* ($P<0.05$) and *S. sanguineum* ($P<0.01$) at H30. The germination times of *A. barbigerus, A. recurvata, A. setifolia* and *P. gardnerianum* did not differ significantly between heated and non-treated groups within H7 nor within H30. However, mean germination times were shorter for *A. barbigerus* ($P<0.05$), *G. spicatus*, *M. minutiflora*, *P. stellatum*, *S. microstachyum* and *S. sanguineum* at H30 than at H7 ($P< 0.05$).

Table 4. Mean (±SE) germination times of nine cerrado grass species and one invasive species subjected to daily post-fire soil temperatures of 45 °C / 10 °C for seven (H7), 30 days (H30). Untreated seeds (U7, U30) not subjected to heat treatment. Germination was tested at 37 °C / 22 °C simulating soil temperature at the onset of the rainy season. Capital letters above bars correspond to significant differences between storage duration (30-d or 7-d) and small letters to differences in treatment (H or U).

	Mean time of germination (days)			
Species	**U7**	**H7**	**U30**	**H30**
Axonopus barbigerus (Kunth) Hitch	12.2 (±0.3)^{Aa}	11.4 (±0.5)^{Aa}	9.5 (±0.8)^{Ba}	10.5 (±2.3)^{Ba}
Aristida recurvate Kunth	15.2 (±0.3)^{Aa}	16.2 (±1.1)^{Aa}	20.8 (±2.1)^{Ba}	16.0 (±2.4)^{Bb}
Aristida setifolia Kunth	17.3 (±6.8)^{a}	20.4 (±1.1)^{a}	17.4 (±3.6)^{a}	13.2 (±3.7)^{a}
Echinolaena inflexa (Poir) Chase	10.0 (±2.0)^{Aa}	12.1 (±0.9)^{Aa}	8.7 (±1.7)^{Ba}	9.6 (±1.5)^{Ba}
Gymnopogon spicatus (Spreng) Kuntze	56.0 (±0.1)^{Aa}	23.0 (±3.7)^{Ab}	20.5 (±1.0)^{Ba}	10.9 (±3.7)^{Bb}
Melinis minutiflora P. Beauv.	15.3 (±2.4)^{Aa}	16.2 (±0.7)^{Aa}	12.5 (±0.8)^{Ba}	9.6 (±1.9)^{Bb}
Paspalum gardnerianum Nees	10.3 (±0.6)^{a}	10.3 (±0.6)^{a}	10.6 (±0.9)^{a}	9.8 (±0.5)^{a}
Paspalum stellatum Humb. and Bompl. ex Flüggé	14.4 (±1.4)^{Aa}	14.0 (±1.9)^{Aa}	9.8 (±0.7)^{Ba}	9.1 (±0.1)^{Ba}
Schizachyrium microstachyum (Desv. ex Ham.) Roseng.	12.3 (±0.4)^{Aa}	11.7 (±0.8)^{Ab}	10.1 (±0.1)^{Ba}	9.0 (±0.8)^{Ba}
Schizachyrium sanguineum (Retx.) Alston	16.8 (±0.5)^{Aa}	18.1 (±1.1)^{Aa}	13.7 (±1-0)^{Ba}	11.7 (±1.3)^{Bb}

Principal Components Analysis

The species separated along the axes and were related to germination rate, viability and mean germination time at H7 and H30 (Figure 17). PCA axes 1 and 2 explained 83% of the data variance, and were therefore considered representative. *A. barbigerus*, *G. spicatus* and *S. sanguineum* clearly differed from the remaining species.

Seed viability, germination rate, and mean germination time at H30 were the most important variables represented by PC1, which explained 57% of the overall variance. Nevertheless, while the correlation with PC1 was positive for seed viability and germination rate, it was negative for mean germination time, for both H7 and H30. PC2 accounted for 26% of the variance, explained mostly by germination rate and viability at H7.

The direction of the arrow in germination rate and viability suggests a positive influence of the simulated daily amplitude of soil temperature after fire, and of the duration of heat treatment. Similarly, the direction of the arrow in mean germination time suggests a negative effect of the daily amplitude of soil temperature after a fire. These vectors indicated four groups of strategies: (a) indifferent to post-fire soil temperature conditions: *P. stellatum* (Ps) and *P. gardnerianum* (Pg) were the least affected; (b) stimulated by post-fire soil temperature conditions: *M. minutiflora* (Mm), *A. setifolia* (As) and *G. spicatus* (Gs), with the strongest effect; (c) inhibited by post-fire soil temperature conditions, with the strongest effect represented by *S. sanguineum* (Ss); and (d) variable effect: benefited by H7 and impaired by H30, especially *A. barbigerus* (Ab).

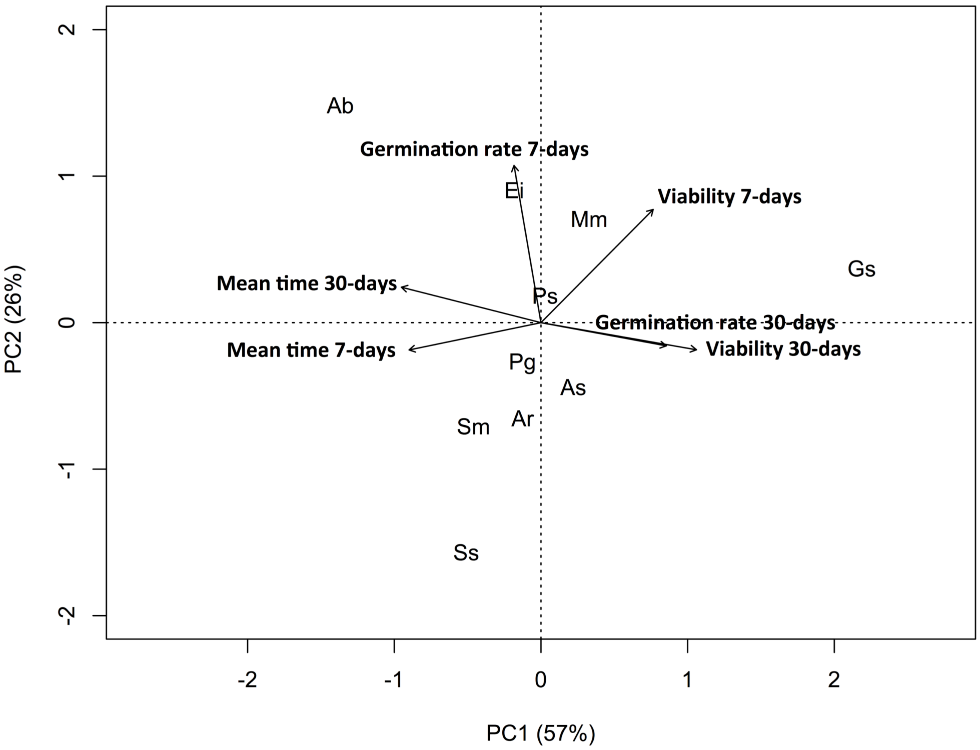

Figure 17. Principal Components Analysis ordination of the species using the of variables germination rate, viability, and mean germination time. Input data were calculated by subtraction of the mean control from the post-fire mean treatment values, here represented as vectors. Letters correspond to initials of species name. Percentage variation accounted for by PC1 and PC2 shown in parentheses on axis labels. Ar, *Aristida recurvata*; As, *Aristida folia*; Ab, *Axonopus barbigerus*; Ei, *Echinolaena inflexa*; Gs, *Gymnopogon spicatus*; Mm, *Melinis minutiflora*; Pg, *Paspalum gardnerianum;* Ps, *P, stellatum*; Sm, *Schizachyrium microstachyum*; Ss*, S. sanguineum.*

DISCUSSION

The germination rate, viability and mean germination time proved to be important parameters for use in assessing the effects of post-fire soil temperature oscillations. Together, they allowed us to differentiate between species-specific responses, which were generally corroborated by the PCA. The viability evaluation complemented the germination pattern by verifying the effects of daily post-fire soil temperatures on the seeds that did not germinate within the 30-d observation period. Assessing the viability of seeds after the 30-d observation period showed that the reduction of germination caused by treatments was due to the killing of the embryo rather than to the induction of dormancy. Mean germination time was important to account for changes in the rate at which seeds might germinate after fires, and can be an indication of early space occupancy that can account for advantages in competition amongst species.

It is important to take into account the variability originating from the method itself when interpreting the results. As it was impossible to select an exact number of viable seeds without damaging the embryos, the 100 ‘viable seeds’ for each experimental batch were selected based on weight at the beginning of the experiment. Although this method is accepted (Carmona *et al.* 1998), it may add variability to the results. Therefore, the results must be interpreted with caution, and potential effects resulting from under- or over-estimating the mass of 100 viable seeds need to be considered. The effect of heat treatment on germination of *G. spicatus* (Figure 16) may have been strongly affected by a failure of the untreated seeds to germinate, resulting in a large discrepancy in comparison to the other species. Although the data may suggest a loss of dormancy for this species, the difference among the groups was less than the internal variability inherent of the estimate of 100 viable seeds (Aires *et al.* 2013).

This study used a more realistic approach than other studies available in the literature, to evaluating the parameters that may affect germination in the field after a fire. The design took into account a more complex scenario of post-fire establishment, based on field data, rather than a single temperature pulse experiment. Although our experiment was a simulation, we can assume that it represented fairly well the field soil temperature conditions of bare soil after a fire in the dry season. Germination is only one of the important steps in plant competition and establishment; however, it is one of the first bottlenecks affecting the population.

A lack of response to fire impacts can be interpreted as tolerance and adaptation to frequent burns (Overbeck *et al.* 2006). Regardless of the treatment, *P. gardnerianum* was not affected with respect to any measured parameter. This species probably has a harder seed coat, as was seen in the difficulty of dissecting it. This may partially explain its lack of response, since thicker seed coats better protect the embryos (Teketay 1996). The congener *P. stellatum* was the second least-affected species by the post-fire conditions. However, both the non-treated and treated groups showed higher germination rate at 30 d, and shorter mean germination times. Both *A. setifolia* and *M. minutiflora* appeared to have followed the same germination timing as that observed for *P. stellatum*, with a higher germination rate and a shorter mean time of germination at 30 d; whereas their viability did not decrease with heat treatment. This can be explained by seed timing, since seeds of some species may show physiological dormancy associated with season (Gramshaw 1972, Justice & Bas 1978, Donohue 2005, Scott *et al.* 2010). This allows them to germinate at higher rates in the late dry season, represented here by the 30-d treatment at daily post-fire soil temperatures of 45 °C / 10° C. This is a strategy to avoid unsuitable conditions for seedling survival. Furthermore, a shorter mean germination time may be more important than germination itself, since seeds that germinate first in a low plant density environment are more likely to grow more rapidly and therefore establish more successfully (Went *et al.* 1952, Ross & Harper 1972).

Taking advantage of post-fire soil temperature conditions results in an increase in germination rate and/or the ability to maintain a higher proportion of germinable seeds, and/or a decrease in mean germination time. These changes could be explained by a loss of dormancy caused by the storage duration itself (30 d or 7 d) or by the conditions of storage, represented by the H or U groups (Gramshaw 1972, Justice & Bas 1978, Melo *et al.* 1979, Baskin & Baskin 2001, Pereira *et al.* 2010). Baskin and Baskin (2001) and Justice & Bas (1978) stated that conditions that simulate the natural environment, including alternating temperatures, may help to overcome dormancy, and McIvor & Howden (2000) observed enhanced germination in annual grass seeds that were stored in alternating temperatures. Although Gramshaw (1972) demonstrated that storage in alternating temperatures did not affect germination of the grass species *Lolium rigidum* Gaudin, Auld & Bradstock (1996) showed that increases in soil temperatures after fires are likely to break the dormancy of buried seeds.

The species that were negatively affected by treatment were *A. barbigerus*, *A. recurvata*, *E. inflexa* and *S. sanguineum* at H30, with lower germination rate or viability, but *A. recurvata* and *S. sanguineum* germinated more rapidly after heat treatment. This confers an advantage in competition when colonising a disturbed site, and may compensate for the disadvantage caused by the reduction of the number of germinated seeds. McIvor & Howden (2000) observed reduced germination and a reduced pool of germinable seeds of perennial grasses stored at oscillating temperatures, and Went *et al.* (1952) observed, for two grass species a negative effect on maintenance of the number of germinable seeds after fire. Negative effects may result from physical damage caused by the oscillating temperatures, or from lipid peroxidation and accumulation of free radicals linked to over-drying or heating of seeds during storage (Hendry *et al.* 1992). Kos & Poschlod (2007) showed that seeds of species that occur under closed canopies perceive soil temperature in a way to favour germination under nurse-plant shade. Thus, these species, negatively affected by the post-fire soil temperatures may be prioritising germination in colonised areas rather than in bare soil, where ruderal species, such as *A. setifolia* and *G. spicatus* are more common.

Our PCA results suggested that *A. barbigerus* was stimulated at H7 and inhibited at H30. The short exposure period probably helps the seed to overcome dormancy, and the longer exposure time kills the embryo, as suggested by the results for viability. The native species *E. inflexa* showed a similar pattern of response (Figure 17); however, the variables related to PC1 seem to have had a weaker effect on this species.

It has been shown that fire may favour obligate seeders by enhancing flowering and promoting seedling recruitment after the removal of competitors (Ferrandis *et al.* 1996, D'Antonio *et al.* 1998, Valbuena & Trabaud 2001, Cirne & Miranda 2008, Scott *et al.* 2010). Furthermore, sexual reproduction and species richness have been suggested to be promoted by fire in savannas (Parron & Hay 1997, Caturla *et al.* 2000, Barros *et al.* 2006), which can also maintain biodiversity and resilience to invasion (Knops *et al.* 1999). The present study emphasises the importance of the transient seed bank in colonisation of burned sites by grasses in the cerrado, in addition to resprouting and vegetative reproduction, which other studies have shown to be prominent factors in post-fire recovery (Hoffmann 1998, Miranda *et al.* 2009).

Species with higher germination rates tend to produce fewer seeds per gram, and vice versa (Zobel *et al.* 2000, Aires *et al.* 2013). Although the production of seeds per gram does not necessarily represent the total production of seeds per plant, it might be related to species composition at the community level. This is true if one considers that plants that produce heavier seeds tend to produce fewer seeds. The data suggest an existence of different strategies among our native species, which may invest in either number (*A. recurvata)* or quality (*S. sanguineum)*.

In the field, *A. barbigerus* may benefit from a fire at the end of the dry season, just before the beginning of the rains (H7), thereby allowing germination to be enhanced. In this situation, this species will be more likely to compete with other species of higher germination rate, such as *S. sanguineum* and *Paspalum* spp. On the other hand, if the fire occurred earlier in the dry season (H30), the seeds of *A. barbigerus* would be exposed for a longer period of time to the new temperature regime and could be harmed. Earlier fire could favour *A. setifolia* that could replace *A. barbigerus* (Almeida 1995).

The invasive species *M. minutiflora* produces a large number of small seeds, with high germination rate (Martins *et al.* 2009, Aires *et al.* 2013). This species tends to displace native species and form monospecific stands (Pivello *et al.* 1999), but it is not tolerant to removal of its aerial part (DiTomaso *et al.* 1999, Valbuena & Trabaud 2001). In Hawaii, studies have shown that fires tended to promote invasion by *M. minutiflora* and other grass species. However, there are no native C_4 grasses in Hawaiian soils. This is not the case in the cerrado, where C_4 grasses comprise the majority of the herbaceous layer. The data for Hawaii support the idea that C_4 grasses are resilient to fire and therefore become invasive, while our data show that in the cerrado, the post-fire condition seems to be a more likely scenario to partially control the invasive species and thereby promote richness (Kucera & Ehrenreich 1962, Parron & Hay 1997). However, our data show that although in the invasive species, *M. minutiflora*, germination does not increase and its viability is not reduced when heated, it does germinate faster than most native species. The reduction in the mean germination time may favour occupation of open space. In addition, it appears that while native cerrado species form a transient soil seed bank (Andrade *et al.* 2002, Salazar *et al.* 2011); *M. minutiflora* possesses a permanent soil bank. In laboratory conditions, 90% of *M. minutiflora* seeds may remain viable for periods as long as 3 years, and 40% for 8 years (Carmona& Martins 2010). Thus, in sites were *M. mintiflora* is the dominant species, with likely concomitant reduction in species richness, fire may reinforce the dominance of this invasive species due to its faster germination and its long maintenance of viability in comparison with native species.

CONCLUSIONS

Our research questions posed were positively answered: (1) The increase in soil daily amplitudes did have a significant effect on seed germination in some species, either enhancing (*A. setifolia*) or reducing (*S. sanguineum*) germination rate. (2) The time of exposure to these conditions could affect species differently, such as for *A. barbigerus*. Although the seeds of some native species were harmed when exposed to the increased soil temperature after fires, the damage was not total suggesting that the species were resilient to this fire effect.

The results also suggest an intricate relationship among species. For instance, in this study *S. microstachyum* and *A. recurvata* could benefit from the pos-fire environment. The first naturally presents a high germination rate and the heat treatment reduced its germination time, while the latter germinated more and faster with heat treatment. On the other hand, the invasive species *M. minutiflora* germination rate was not stimulated by increase in post-fire daily amplitude of soil temperature. However, the significant reduction in its germination time added to its high production of viable seeds and a permanent soil seed bank may reinforce its dominance in the area. Even so, *S. microstachyum* and *A. recurvata* might be the strongest candidates to compete with the invasive grass.

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CHAPTER FOUR:

NATIVE AND INVASIVE CERRADO GRASS SPECIES COPE DIFFERENTLY WITH SOIL WATER AVAILABILITY.

Carolina Musso[1], Gloria Pinto[1], Rhaul Oliveira[1], Carlos Correia[2], José Moutinho-Pereira[2], Amadeu M. V. M. Soares[1], Susana Loureiro[1].

1 - Department of Biology & CESAM (Centre for Environmental and Marine Studies), University of Aveiro, 3810-193 Aveiro (Portugal)
2 - Centre for the Research and Technology of Agro-Environment and Biological Sciences (CITAB), University of Trás-os-Montes e Alto Douro, Apt. 1013, 5001-801 Vila Real, Portugal

ABSTRACT

Invasive grasses are a great threat to the integrity of the Cerrado ecosystem. Considering that the Cerrado region is characterized by its vegetation gradient and climate seasonality, this study aimed to evaluate the responses of an invasive and a native species to water availability, simulating different scenarios present in the field. Individuals of one native (*Schizachyrium microstachyum* (Desv. ex Ham) Roseng) and one invasive (*Melinis minutiflora* P. Beauv.) species were submitted to different soil moisture regimes, from drought to flood simulations (10, 40, 80, 120% of the maximum water holding capacity of the soil), for four weeks at 28ºC in a climatic chamber. Several morphological and physiological parameters recorded in grasses were assessed: height, biomass, number of leaves, tillers, leaf area, fluorescence of chlorophyll a, gas exchange parameters, pigment content, MDA concentration, GST, G-POX, APX and CAT activities. Results showed significant differences among species and treatment. Both species were able to cope with water stress conditions and performed better at 80% of soil moisture where normal Fv/Fm were observed. The invasive species grew more, presented higher net photosynthetic rates in all circumstances and showed less evidence of stress. Flood caused as much stress as drought for the native species, having a stronger effect on its biomass accumulation and triggering a stronger biochemical response.

Key words: Drought, flood, flien plants, *M. minutiflora*, biomarkers

INTRODUCTION

The Brazilian Cerrado is a savanna-like biome that occupies over 25% of Brazilian territory and comprises high levels of biodiversity and endemism of plant species. Many of those species are currently at risk due to high levels of deforestation and changes in land use induced by human activities (Ratter *et al.* 1997) and also to the introduction of exotic species, recently pointed as one of the greatest concerns for local biodiversity. Therefore, the Cerrado is among the world's most critical ecosystems in terms of conservation (Myers *et al.* 2000).

Some exotic species can become invasive and threaten local biodiversity by excluding native species when competing for natural resources. Furthermore, exotic plant species have been reported to cause changes in the microclimate and the natural disturbance regimes, affecting the functioning of the ecosystem (D'Antonio & Vitousek 1992). African grasses are the most threatening invaders in the Cerrado, being considered opportunistic (Barger *et al.* 2003, Martins 2006) and able to displace native grasses and form monospecific stands (Pivello *et al.* 1999).

It is known that competition is the main factor influencing species' dominance. Being aware that plant species respond differently to environmental factors, it is important to consider the potential effects of these factors on competition dynamics and ecosystem vulnerability to invasion. Furthermore, understanding these responses would help planning appropriate control plans to manage invaded areas and sustain biodiversity.

The Cerrado's climate is characterized by two well-marked seasons. Mean annual precipitation is of 1600 mm. However, over 90% of all this rain occurs from October to April (rainy season), while almost no rain is recorded from May to

September (dry season). Along with the high transpiration rates typical of the tropical area and the good drainage of the Cerrado's soils, the dry season imposes strong water stress on plant communities in the Cerrado. The vegetation is represented by different physiognomies, from closed canopy sites (*cerradão*) to savannic formations (*cerrado sensu strictu)* and open sites with scattered trees and shrubs (*campo-sujo* and *campo-limpo*). Distribution of these physiognomies is determined, among other reasons, by distance from the water table (Coutinho 1978). In the campo-sujo and campo-limpo, the herbaceous layer is more representative than in the other physiognomies, and over 70% of the herbaceous species are grasses (family Poaceae) (Ribeiro & Walter 1998, Munhoz & Felfili 2005, Mendonça *et al*. 2008). This ecosystem may, therefore, be more or less susceptible to invasion in different areas and different times of the year depending on how the native and the invasive species cope with the environmental stress factors (Alpert *et al*. 2000).

In this context, the main aim of the present study was to assess the effects of different soil moisture levels on fitness traits of the native grass *Schizachyrium microstachyum* (Desv. ex Ham) Roseng. and the African grass *Melinis minutiflora* P. Beauv.. The experimental trials used in this study tried to simulate seasonal variations that plants are exposed to throughout the year and/or along vegetation gradients, in order to understand the potential competition between them in different soil moisture scenarios.

MATERIAL AND METHODS

Species

Schizachyrium microstachyum (Desv. ex Ham) Roseng. is a common native perennial grass in Cerrado and presents high levels of fertile seeds and germination rates above 70% (Carmona *et al.* 1998). The African perennial grass *Melinis minutiflora* P. Beauv. has been reported to be widely spread in conservation units, being originally introduced in Brazil for use as forage pastures for livestock. It threatens native biodiversity by competitively excluding native species (Pivello *et al.* 1999).

The seed collection took place within the area of the Reserva Ecológica do Instituto Brasileiro de Geografia e Estatística, located 25 km south of Brasília, Brazil (15° 56' 41" S and 47° 53' 07" W GRW). Seeds were collected manually from May to July of 2010, during the dispersion period (Almeida 1995). Seeds were then stored at room temperature until use.

Experimental design

Seeds from both species were primarily germinated in Petri dishes with humidified cotton and filter paper as described by Aires *et al.* (2013), at 37ºC:22ºC and 10h:14h light:dark temperature/photoperiod regime to simulate the soil temperatures at the onset of the rainy season (Andrade *et al.* 2002). Then, seedlings were carefully transplanted to experimental pots containing a 500 mg mixture of organic matter (turf and hummus) and mineral soil (50:50 v:v, Simões & Baruch (1991), with ten individuals per pot. Seedlings were left to grow in constant well-watered soil for four weeks in a climatic chamber set to 28°C and 12h:12h light: dark photoperiod (156 ± 5.37 W m^{-2}). The soil moisture was maintained by capillary action, through a fiberglass wick (between 5 and 10 mm ø) located at the pot's bottom (Loureiro *et al.* 2006). After this period, the smallest seedlings were harvested, and eight individuals were left per pot. Watering was then regulated in order to achieve the desired soil moistures (10%, 40%, 80% and 120% of soil's maximum water holding capacity - WHC). The treatments were maintained in quadruplicate. Soil moisture was kept by weighing the pots and adding the needed amount of water twice daily. The weight of the plant was neglected for this calculation.

After four weeks of growth under the referred conditions, several endpoints were assessed. First, chlorophyll fluorescence and gas exchange measurements were made (see methodology below) and afterwards plants were harvested, measured, weighed, and the number of leaves and tillers were counted. Five leaves of each replicate were separated, scanned with a desk multifunctional printer, dried and weighted. The images were then analyzed with ImageJ software for determination of leaf area. Part of the collected material was dried for determination of water content, nitrate and phosphate concentrations. The remaining material was snapped frozen and stored at -80ºC for biochemical assays.

Chlorophyll fluorescence

Prior to harvesting, chlorophyll fluorescence parameters were measured to calculate the maximum quantum yield of PSII (F_v/F_m) in leaves adapted to the dark, and the effective quantum yield (ϕ_{PSII}) in light-adapted leaves, which are indicators of photoinibitory damage on plants undergoing stress. Chlorophyll a fluorescence parameters were measured on the adaxial side of ten mature leaves, pre-adapted to darkness or adapted to light, located at similar height representing all replicates, by using a Mini-PAM (pulse amplitude modulation system, FMS 2, Hansatech Instruments, Norfolk, England). Minimal fluorescence (F_0) was measured by applying a weak modulated light to leaves which were pre-adapted to darkness for 30 min. Maximal fluorescence (F_m) was measured after applying a 0.7 s long saturating pulse of white light (> 1 500 µmol m^{-2} s^{-1}) to the same leaves. In the light-adapted leaves, steady-state fluorescence (F_s) and maximal fluorescence (F'_m) were also measured following the same methodology. Definitions of fluorescence parameters (F_v/F_m, ϕ_{PSII}) were used as described by Van Kooten & Snel (1990) and non-photochemical quenching (NPQ) was calculated according to Bilger & Björkman (1990), (NPQ = $(F_m - F'_m)/F'_m$).

Gas exchange parameters

One day before sampling of plant material, leaf gas exchange was measured in the oldest intact leaves of ten plants representing all four replicates. Measurements were performed using a portable infrared gas analyser (LCpro+, ADC, Hoddesdon, UK), operating in open mode under ambient conditions. Net photosynthetic rate (A), stomatal conductance (*gs*), transpiration (E), Water use efficiency (WUE – determined by the ratio of A/E) and C_i/C_a ratio (internal and external CO_2 concentration ratio) were determined using the equations developed by Von Caemmerer & Farquhar (1981). The relative humidity of the air, temperature and the airflow rate in the cuvette were set to 60%, 23° C. and 300 mmol s^{-1}, respectively.

Pigments

Pigments were extracted from the leaves in a solution of cold acetone with 50 mM of Tris buffer (pH 7.8; 80:20, v:v) and centrifuged at 5,000 g for 10 min.

Absorbance at 470, 537, 647 and 663 nm was determined in 96-well microplates, each sample in quadruplicate. The contents of chlorophyll a, b, and carotenoids were calculated with the equations (Sims & Gamon 2002):

Chl_a = $0{,}01373\ Abs_{663} - 0{,}000897\ Abs_{537} - 0{,}003046\ Abs_{647}$;

Chl_b = $0{,}02405\ Abs_{647} - 0{,}004305\ Abs_{537} - 0{,}005507\ Abs_{663}$;

Carotenoids = $((Abs_{470} - (17{,}1 \times (Chl_a + Chl_b) - 9{,}479 \times \text{Anthocyanins})) / 119{,}26$;

Anthocyanins = $0{,}08173\ Abs_{537} - 0{,}00697\ Abs_{647} - 0{,}002228\ Abs_{663}$.

Before calculation, absorbance was corrected according to the path length traveled by the light in the samples in the microplates to match the 1-cm cuvette used in the formula (Warren 2008). Values were also used to calculate the Chl_a/ Chl_b ratio to evaluate the balance between these two pigments. Shifts in this ratio can happen due to stress and consequently affect photosynthesis (Kitajima & Hogan, 2003).

Lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) production (Dhindsa *et al.* 1981). For the extraction of MDA, five leaf samples of 0.5g were ground to a powder in a mortar with liquid nitrogen and then homogenized with 2.5 ml of a TCA solution (trichloroacetic acid at 0.1% w/v (g/100ml)) (Santos *et al.* 2001). Then, an aliquot of the supernatant of the centrifuged samples was mixed with an equal volume of the reaction solution (20% TCA 0.5% TBA (m/v)) and left to react for 30 min under heat (95ºC) and then cooled and re-centrifuged. The absorbance was measured in 1ml cuvettes in a Thermo Fisher Scientific spectrophotometer (Waltham, USA) (Genesys 10-uv S). MDA concentration was estimated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm using an absorbance coefficient of extinction (ε), 155 mM^{-1} cm^{-1}.

Enzymatic activity

Leaf samples were pulverized in a mortar with liquid nitrogen and then homogenized in the same mortar with a solution of 100 mM phosphate buffer (pH 7.5) and 0.5 mM EDTA. Samples were then centrifuged at 10,000 g for 20 min to separate the post-mitochondrial supernatant (Howcroft *et al.* 2011, Oliveira *et al.* 2013). All methodologies were adapted to for 96-well microplate reading. GluthationeS-Transferase (GST) activity was measured based on the conjugation product between the 1-chloro-2,4-dinitrobenzene and glutathione at 340 nm for 5 min, according to the method of Habig & Jakoby (1981). Catalase (CAT) activity was determined by measuring the decomposition of the substrate H_2O_2 at 240 nm during 3 minutes, based on the method described by Clairborne (1985). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), and 6 mM H_2O_2, which started the reaction. L-Ascorbate peroxidase (APX) activity was determined considering the ascorbic acid ($\varepsilon = 2.8$ $mM^{-1}.cm^{-1}$) oxidation to dehydroascorbate by H_2O_2, at 25°C, at 290 nm, according to the method of Nakano & Asada (1981). For Guaiacol peroxidase (G-POX) determination, the reaction mixture consisted of 5 µL of plant extract in a 10 mM phosphate buffer (pH 6.1), 12 mM hydrogen peroxide, 96 mM guaiacol solution. Absorbance was recorded at 470 nm for 5 min, and the specific activity was calculated using the 26.6 $mM^{-1}.cm^{-1}$ molar extinction coefficient (Castillo *et al.* 1984). Protein concentration was determined in quadruplicate by the Bradford method (Bradford 1976), at 595 nm, using bovine serum albumin (BSA) as the standard. GST and G-POX activities were corrected by protein content and APX and

CAT by the fresh weight. All measurements were made in a Labsystem Multiskan EX microplate reader (Labsystems Inc., Franklin, MA).

Nitrate and phosphate concentrations

The concentration of nitrate and phosphate in plants was determined with the HACH KIT DR/2000 Spectrophotometer. Dry leaves contents were extracted by homogenization in water with a mortar in the proportion of 1:2 (mass:volume). The extract was filtered with activated coal and filter papers (180 µm of thickness and 11µm pore size for particle retention). The filtered solution was then mixed with Hatch KIT reagents according to the protocols 8151 (Program 363) for nitrate and 8183 (Program 510) for phosphate. Results were presented as the percentage of dry weight.

Data analysis

Under non-normality conditions, data was transformed by either ln(x) or arcsine. For each parameter, means were compared using a factorial ANOVA. Species (*S. michrostachyum* - native, *M minutiflora* - invasive) and water treatments (10%, 40%, 80%, 120%) were the independent variables, and multiple comparisons were carried out using a TukeyHSD test. A Pearson correlation coefficient and significances were calculated for the variables using the whole dataset and for the species separately. All data was analyzed using the R software (R 3.0.1 binary for Mac OS X 10.6).

RESULTS

Growth parameters

The morphological parameters measured in the plants are summarized in Figure 18. The ANOVA results are summarized in Table 5. Native and invasive species differed in the majority of the growth-related parameters regardless of the levels of soil moisture (all $p<0.001$). Out of both species, *M. minutiflora* presented a more vigorous growth. For all treatments, this species presented over twice the length and number of leaves of the native species, three times its fresh weight and root:shoot ratio and at least one more tiller per plant. The native species showed lower water content (~70%) when compared with the invasive one, which presented over 80% of water content. By the end of the experiment, the native species presented a greater percentage of dry leaves in comparison to the invasive one but no difference in specific leaf area was found.

The water stress effects were more pronounced in the native species. The 80% soil moisture treatment was the condition where plants grew better and accumulated more biomass. For both species moisture stress had no effects on the number of leaves, tillers, specific area and water content. The percentage of dry leaves tended to increase with water availability ($p=0.045$) while the flooded treatment (120% WHC) decreased the root:shoot ratio (Figure 18). The results of Pearson's correlation calculated between endpoints pairwise can be observed in Table 6. Growth parameters were significantly ($p<0.05$) and positively ($r>0$) correlated among themselves for the whole dataset and for each species separately, except for the number of dry leaves, which presented a negative ($r<0$) correlation only when analyzed the full dataset.

Table 5. Factorial ANOVA scores for the traits measured for the native *Schizachyrium microstachyum* and invasive grasses *(Melinis minutiflora)* under different soil water availability treatments (10%, 40%, 80% and 120%) and at photoperiod of 12h and temperatures of 22/28°C. Moisture and species were used as independent variables, followed by TukeyHSD (α=0.05).

	Species		Moist treatment		Species X Moist treatment	
	F	p	F	p	F	p
Length (L)	354.64	<0.001	4.30	0.015	3.32	0.037
Fresh weight (FW)	267.04	<0.001	2.34	0.099	0.31	0.822
Number of leaves (NL)	127.55	<0.001	5.04	0.008	1.48	0.245
Number of tillers (NT)	17.95	<0.001	1.81	0.173	1.19	0.333
Dry leaves (DL)	27.33	<0.001	3.11	0.045	1.38	0.272
Water content (WC)	95.21	<0.001	0.46	0.715	0.15	0.929
Specific area (SA)	0.35	0.561	2.10	0.132	0.18	0.909
Root:Shoot (RS)	53.78	<0.001	0.14	0.870	0.11	0.898
Stomatal conductance (g)	47.95	<0.001	4.85	0.014	5.42	0.009
Assimilation rate (A)	14.48	0.001	3.13	0.055	2.57	0.095
Transpiration (E)	2.01	0.167	2.861	0.053	3.24	0.037
Water Use Efficiency (A/E)	30.82	<0.001	5.60	0.005	1.39	0.273
C_i/C_a	19.57	<0.001	3.71	0.022	0.537	0.661
Phosphate	0.05	0.833	9.85	0.001	8.09	0.002
Nitrate	5.59	0.031	1.43	0.268	5.64	0.008
Chlorophyll a	0.08	0.777	0.97	0.419	1.30	0.272
Chlorophyll b	4.69	0.038	0.90	0.453	1.29	0.294
Carotenoids	2.70	0.110	0.52	0.606	1.10	0.363
$\mathrm{Chl}_a/\mathrm{Chl}_b$	14.46	<0.001	3.94	0.012	6.37	0.001
F_v/F_m	14.46	<0.001	3.94	0.012	6.37	0.001
ϕ_{PSII}	37.50	<0.001	2.65	0.058	4.11	0.011
NPQ	0.11	0.7451	1.59	0.203	2.27	0.901
MDA	120.50	<0.001	3.56	0.025	4.00	0.016
GST	211.49	<0.001	3.52	0.026	1.04	0.387
CAT	69.55	<0.001	6.50	0.001	7.45	0.001
G-POX	0.71	0.403	1.34	0.278	3.39	0.099
APX	46.04	<0.001	1.68	0.191	4.25	0.012

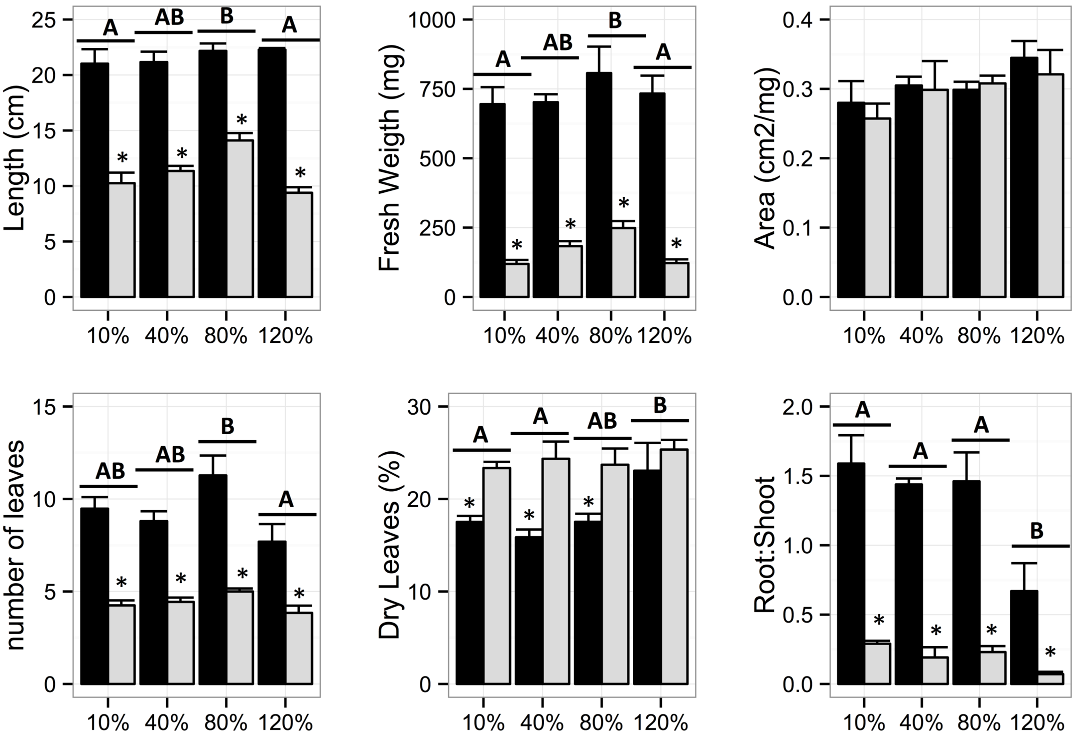

Figure 18. Morphological traits of one invasive (*Melinis minutiflora*) and one Cerrado native grass (*Schizachyrium microstachyum*) grown for 4 weeks on different soil water availability (10, 40, 80 and 120% foil maximum water holding capacity).

Gas exchange parameters

Gas exchange parameters (Figure 19) presented significant changes between species (Table 5). The native species presented higher values of stomatal conductance (p=0.001), while the invasive tended to present greater values of net assimilation rate (p=0.002). Within the flood simulated scenario, stomatal conductance increased significantly for *S. microstachyum* (p=0.002). The greatest assimilation rate was achieved by the invasive species at 80% WHC (Table 5, Figure 19). WUE was also higher in the 80% treatment, and decreased significantly at 120% WHC (p=0.019). WUE didn't differ between species in the 10% and 40% treatments. C_i/C_a was higher for the native species ($p<0.001$) and increased with flood (p=0.013). Stomatal conductance was significantly and negatively correlated with length and fresh weigh while assimilation rate was positively correlated with all growth parameters and negatively correlated with stomatal conductance (Table 6). For each species separately the significant correlation was maintained for the native species).

Pigments and Chlorophyll fluorescence

There was no significant difference in pigment concentration between species (Table 5, Table 7). However, Chl_a/Chl_b ratio was significantly higher for the native species (p=0.034, Figure 20). Both the maximum quantum yield (F_v/F_m) and the effective quantum yield ($\phi_{PSII\ PSII}$) were higher for the invasive species. The 10% soil moisture treatment apparently induced a higher F_v/F_m for both species, but this was not observed for ϕ_{PSII}. The non-photo chemical quenching (NPQ) was not affected by treatment and did not differ among species (data not shown). Pigments content are not correlated with any other parameter but F_v/F_m was negatively correlated with the assimilation rate for both species together and positively correlated to GST activity for the native species (Table 6).

Nitrate and phosphate concentration

Regarding the nutrient accumulation in leaves, a significant interaction between species and soil moisture was observed (p=0.00167, p=0.00783, Table 5, Table 7) for phosphate and nitrate. This interaction may be a reflection of the significantly higher amount of nitrate for the invasive species under drought.

Table 6. Correlation coefficients derived among measured traits (Pearson, α=0.05) for each species individually (Mm = Melinis minutiflora, Sm = Schizachyrium microstachyum) or using the full dataset (Both): Length (L),Fresh Weigh (FW), Number of Tillers (NT), Number of Dry Leaves (DL), Root:Shoot ratio (R:S), stomatal conductance (g), Assimilation rate (A), concentration of MDA (MDA), GST activity (GST), Catalase activity (CAT). Optmum quantum yield (Fv/Fm).

		FW	NT	DL	R:S	g	A	MDA	GST	CAT	F_v/F_m
L	***Both***	0.99	0.70	-0.60	0.83	-0.65	0.77	-0.88	-0.89	0.69	ns
	Mm	0.69	0.49	ns	ns	ns	ns	ns	ns	ns	
	Sm	0.88	0.47	ns	ns	-0.67	0.53	-0.54	-0.39	ns	
FW	***Both***		0.71	-0.81	0.56	-0.57	0.77	-0.82	-0.98	0.70	ns
	Mm		0.48	ns	ns	ns	ns	ns	ns	ns	
	Sm		0.48	ns	ns	-0.61	0.58	-0.56	-0.53	ns	
NT	***Both***				0.50	-0.32	0.94	-0.59	-0.60	0.58	ns
	Mm				ns	ns	0.45	ns	ns	ns	
	Sm				ns	ns	ns	-0.38	ns	ns	
DL	***Both***				-0.75	0.47	-0.45	0.48	0.79	-0.54	ns
	Mm				ns	ns	ns	ns	ns	ns	
	Sm				-0.47	ns	-0.49	ns	ns	ns	
R:S	***Both***					-0.58	0.48	-0.69	-0.88	0.73	ns
	Mm					ns	ns	ns	ns	ns	
	Sm					-0.59	0.49	ns	ns	ns	
g	***Both***						-0.33	0.59	0.60	ns	ns
	Mm						0.88	ns	ns		-0.52
	Sm						-0.60	ns	ns		ns
A	***Both***							-0.54	-0.59	ns	-0.32
	Mm							ns	ns		-0.68
	Sm							ns	ns		-0.44
MDA	***Both***								0.80	-0.59	ns
	Mm								ns	ns	
	Sm								ns	ns	
GST	***Both***									-0.56	ns
	Mm									ns	ns
	Sm									ns	0.45

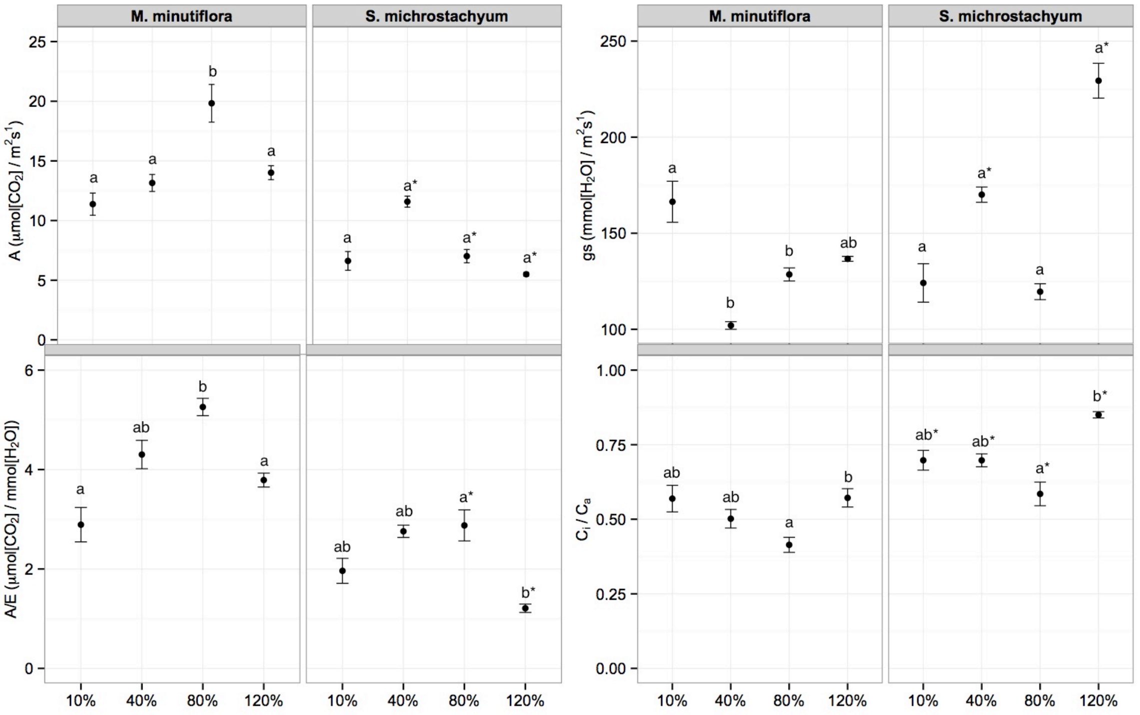

Figure 19. Gas exchange parameters reported for the invasive grass *Melinis minutiflora* and the Cerrado native grass *Schizachyrium michrostachyum* grown for 4 weeks on different soil water availability (10%, 40%, 80% and 120% of soil's maximum water holding capacity). Bars correspond to standard error. Different letters show significant differences among treatments and asterisks show differences between species in that specific treatment.

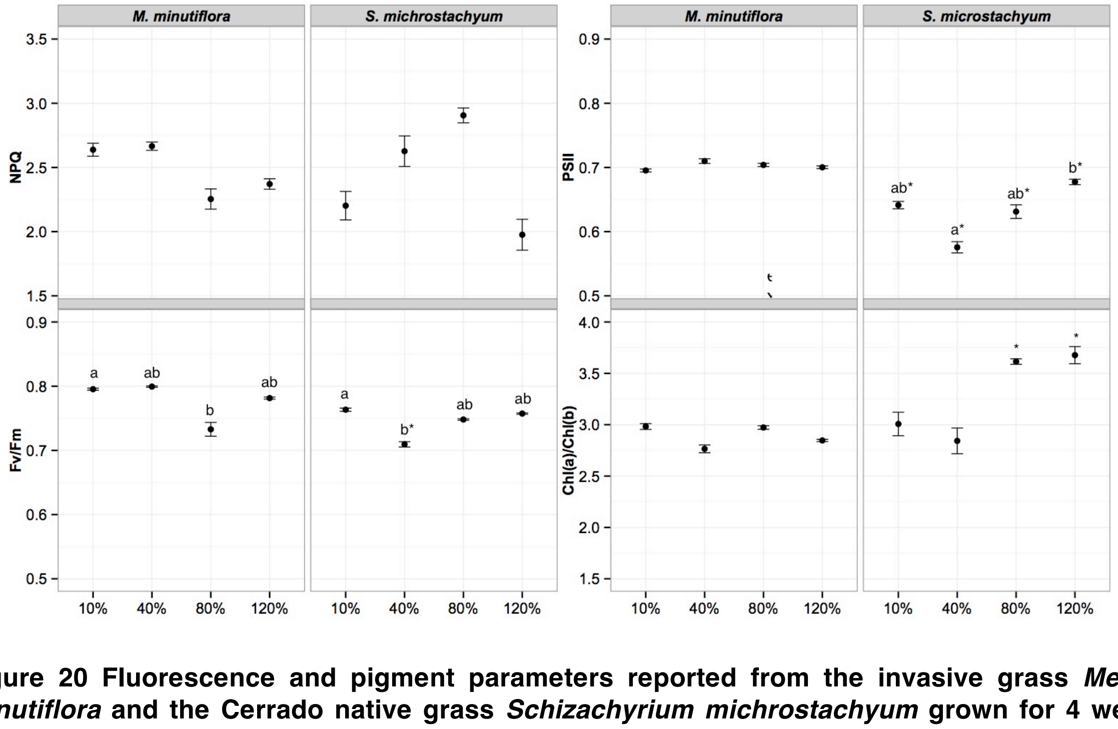

Figure 20 Fluorescence and pigment parameters reported from the invasive grass *Melinis minutiflora* and the Cerrado native grass *Schizachyrium michrostachyum* grown for 4 weeks on different soil water availability (10%, 40%, 80 and 120% of soil's maximum water holding capacity). Bars correspond to standard error. Different letters show significant differences among treatments and asterisks show differences between species in that specific treatment.

Lipid peroxidation and enzymatic activities

The results for lipid peroxidation, expressed as MDA concentration, and enzymatic activities can be visualized in Table 8. The native species presented higher concentrations of MDA ($p<0.0001$) than the invasive species, reaching over twice as much MDA than the measured in *M. minutiflora*, regardless of treatment (Table 5 and Table 8). Only the native species presented a response to drought stress, showing significant higher levels of MDA in the 10% treatment. MDA concentration was significantly and negatively correlated with all growth parameters and is negatively correlated with the assimilation rate for both species together and only for the native species when analyzed separately (Table 6).

APX activity differed among species, where the native species presented the higher activity for all treatments ($p<0.005$). Water stress had no particular effect on this enzyme, but a significant interaction between species and treatment was observed ($p=0.012$, Table 5 and Table 8).

GST activity was affected by the moisture treatment ($p=0.026$, Table 5 and Table 8). Similarly to the APX activity, the native species presented higher values ($p<0.001$) for all treatments. GST activity was also inversely correlated with growth parameters when the full data set was taken into account (Table 6).

CAT activity showed an inverse pattern when compared to GST and APX activities. The invasive species presented the highest activity for this enzyme at any treatment ($p<0.001$) when compared to the native species, but also showed to increase in the driest treatment ($p=0.001$, Table 5 and Table 8). On the other hand, the native species seems to present higher activities in the wetter treatments as suggested by the significant interaction between species and treatment ($p=0.001$).

Regarding both species together, CAT activity was positively correlated with growth (Table 6).

For the G-POX activity, a significant interaction between moisture treatment and species was detected (p=0.029, Table 5 and Table 8), with the activity for the invasive species tending to decrease with water content, while the native species activity showed an increasing pattern. G-POX activity was not clearly correlated to any other parameters (data not shown).

Table 7 Leaf nutrients content and leaf pigments concentration of the native (*Schizachyrium michrostachyum*) and the invasive grasse (*Melinis minutiflora*) under different soil water availability treatments (10, 40, 80 and 120%) and at a photoperiod of 12h and temperatures of 22/28°C. The p column corresponds to significant differences between soil moistures. Asterisks correspond to significant differences between species. Factorial ANOVA with moisture and species as independent variables, followed by TukeyHSD (α=0.05).

Endpoint	**Treatment**	***p***	***M. minutiflora***	***S. microstachyum***
Leaf nitrate (% DW)	10%	a	0.82 (± 0.16)	0.36 (± 0.05)*
	40%	b	0.54 (± 0.03)	0.56 (± 0.11)
	80%	b	0.54 (± 0.04)	0.67 (± 0.03)
	120%	b	0.56 (±0.06)	0.36 (±0.02)
Leaf phosphate (% DW)	10%	a	0.12 (± 0.01)	0.07 (± 0.01)
	40%	bc	0.12 (± 0.01)	0.14 (± 0.02)
	80%	b	0.13 (±0.01)	0.18 (± 0.01)
	120%	ab	0.12 (±0.01)	0.10 (±0.01)
Chlorophyll a (μmol gFW^{-1})	10%	ns	1.0 (±0.09)	1.0 (±0.14)
	40%	ns	0.9 (±0.09)	1.1 (±0.15)
	80%	ns	1.0 (±0.15)	1.2 (±0.22)
	120%	ns	1.1 (±0.09)	0.75 (±0.14)
Chlorophyll b (μmol gFW^{-1})	10%	ns	0.33 (±0.03)	0.21 (±0.06)
	40%	ns	0.33 (±0.03)	0.31 (±0.07)
	80%	ns	0.35 (±0.05)	0.34 (±0.06)
	120%	ns	0.37 (±0.03)	0.21 (±0.04)
Carotenoids (μmol gFW^{-1})	10%	ns	0.66 (±0.06)	0.57 (±0.07)
	40%	ns	0.64 (±0.07)	0.65 (±0.07)
	80%	ns	0.72 (±0.09)	0.70 (±0.11)
	120%	ns	0.77 (±0.08)	0.52 (0.07)

Table 8. MDA and enzymatic activity of the native (*Schizachyrium michrostachyum*) and the invasive grass (*Melinis minutiflora*) under different soil water availability treatments (10%, 40%, 80% and 120%) and at a photoperiod of 12h:12h dark: light and temperatures of 22/28°C. The p column corresponds to significant differences between soil moistures. Asterisks correspond to significant differences between species. Factorial ANOVA using moisture and species as independent variables, followed by TukeyHSD (α=0.05).

Enzyme	Treatment	p	*M. minutiflora*	*S. microstachyum*
MDA (mmol gFW^{-1})	10%	a	4.15 (±0.47)	14.25 (±1.00)*
	40%	ab	3.86 (±0.50)	10.30 (±1.74)*
	80%	b	4.36 (±0.25)	3.86 (±0.50)*
	120%	ab	3.69 (±0.18)	11.52 (±1.29)*
GST (ηmol ml^{-1} prot^{-1} min^{-1})	10%	ns	1.33 (±0.25)	5.41 (±0.58)*
	40%	ns	0.91 (±0.19)	3.98 (±0.31)*
	80%	ns	0.91 (±025)	4.47 (±0.51)*
	120%	ns	1.29 (±0.21)	5.51 (±0.40)
Catalase (mg gFW^{-1})	10%	a	126.00 (±8.92)	21.44 (±3.91)*
	40%	b	52.09 (±4.92)	17.92 (±3.83)*
	80%	b	76.38 (±9.79)	46.72 (±10.60)*
	120%	b	75.50 (±15.27)	31.23 (±8.67)*
APX (µmol gFW^{-1} min^{-1})	10%	ns	2.45 (±0.66)	6.81 (±0.69)*
	40%	ns	4.40 (±0.84)	5.29 (±0.78)*
	80%	ns	0.73 (±0.06)	5.56 (±0.60)*
	120%	ns	3.71 (±1.02)	6.49 (±0.64)*
GPOx (µmol ml^{-1} prot^{-1} min^{-1})	10%	ns	3.48 (±0.65)	1.61 (±0.40)
	40%	ns	1.88 (±0.35)	2.18 (±0.44)
	80%	ns	1.64 (±0.25)	3.03 (±1.02)
	120%	ns	2.40 (±0.72)	4.03 (±0.71)

DISCUSSION

The differences observed in biomass accumulation between species were expected. An invasive species, by definition, is considered a strong competitor to native species and *M. minutiflora* is of great concern in the Brazilian savanna and Hawaii (Pivelo *et al.* 1999, D'Antonio *et al.* 2001). Baruch *et al.* (1989) also reported high levels of biomass accumulation in other invasive African grasses in the llanos, the Venezuelan savanna. African grasses are probably adapted to grow more and faster than native species as an evolutionary response to the large herbivores present in Africa (Baruch *et al.* 1985). Furthermore, the high root:shoot ratio presented by *M. minutiflora* shows that this species also presents a strong investment in underground biomass. Silva & Haridasan (2007) also hypothesized that this species presents high investment in roots, which enables reaching deeper in the soil for water. This hypothesis was raised when they observed that the invasive species was able to maintain above-ground biomass alive during part of the dry season, while the aerial part of the native species was already dead. The same authors also discuss the ability of the invasive species to recycle the nutrients of senescent leaves more efficiently. This ability, along with its prominent root system and higher water content, can help understand why this species showed a smaller percentage of dead leaves by the end of the experiment.

Furthermore, the invasive species showed a higher numbers of tillers, which can confer an advantage in occupying space and displacing native species, for instance, by shading adults, seedlings and seeds (Silva & Castro 1989). However, in the field, other species of the *Schizachyrium* genus have been reported to present intense tillering (Leite *et al.* 1997). Presence of species with such characteristics might help to constrain the advance of *M. minutiflora*. Although the invasive species showed larger leaves, with greater area, there was no difference in specific leaf area, showing that each unit of leaves weighted the same per unit of area.

The 80% treatment seems to simulate the best conditions for both species. In this treatment they presented higher assimilation, biomass and less stress. In the environment, this could be represented by the middle of the rainy season in a well-drained soil, such as the soil of the *campo-sujo* or *cerrado sensu strictu* physiognomies. However, both species were able to grow in all conditions, showing their functional plasticity to cope with environmental stress (Insausti *et al.* 2001, Dalmagro *et al.* 2013). The fluorescence data, for instance, showed that in all situations leaves maintained F_v/F_m values above 0.7, reflecting healthy photosystems. This response was not surprising, since both species have to cope with different environmental conditions in their natural environment. Other studies showed plasticity of grass species to environmental conditions, being able to maintain both growth (Dias-Filho & Carvalho 2000) and active photosynthetic system (Flexas *et al.* 1999, Lima *et al.* 2002).

Water is restricted during the dry season in the Cerrado and during the rainy season soil may become flooded in *campo-limpo* areas or areas adjacent to rivers (Eiten 1972). Although native plants were thus expected to be well adapted to water stress, they were more responsive than the invasive species to these conditions. Both deficit (10%) and excess (120%) of water seemed to be equally harmful to this species, but probably through different mechanisms. Plants under drought stress, in an attempt to save water tend to grow less aboveground and invest more energy, in underground growth (Chaves *et al.* 2003). This situation can be observed in the present data by the root:shoot ratio, which increased with the decrease in water availability. The lack of water can also postpone leaves' senescence, which can be seen in the data through the reduced percentage of dry leaves in the 10% treatment in comparison to the 120% treatment (p=0.045). On the other extreme, a water saturated soil creates an anoxic environment for roots (Kennedy *et al.* 1992). In this way, roots grow less and can show differences in anatomy, with more aerenchyma and less structural tissue, for instance (Insausti *et al.* 2001, Vasellati *et al.* 2001). Accordingly, the results presented showed a reduced root:shoot ratio for both species in the 120% treatment. Furthermore, the native species seems to cope with the flood by further opening their stomata. Grasses have been reported to be intolerant to water in excess, being damaged by flood from early life stages to maturity (Capon & Brock 2006, Baruch 1994), unless they are adapted to wetlands (Kersher & Zedler 2004). It is important to highlight that the method chosen for the experiments provided water daily to the plants, while a suspended watering method (Simões & Baruch 1991, Baruch 1994, Guenni *et al.* 2002) might induce more drastic responses and damages.

The gas exchange parameters showed the invasive species uses water more efficiently, specially at 80% WHC (Figure 19). This species showed a higher assimilation rate despite having lower stomatal conductance, which was negatively correlated with plant length in the whole dataset. Funk & Zachary (2010) showed that an increase in stomatal conductance is related to an increase in the root:shoot ratio, i.e., plants with more roots present permeable stomata (more stomata opened or opened more widely and for a longer time). This relation was not observed in this study, where root:shoot is negatively correlated to stomatal condutance (Table 4). This result was probably a reflection of the damage caused to the roots of the native specie on the flood treatments, where roots were very fragile, together with the attempt of balancing the excess of water opening their stomata. Although stomata must be open for CO_2 assimilation, the native species might not be assimilating it efficiently and losing more water through transpiration in comparison to the invasive species. For instance, at 80% moisture, WUE was higher for the invasive species, although stomatal conductance did not differ between them and the C_i/C_a ratio was higher for the native species. Furthermore, stomatal conductance and C_i/C_a increased within the flood simulated scenario. This data indicates that the limitation in photosynthesis may be a result of a reduced efficiency on the CO_2 fixation for the native species rather than deficiency in CO_2 intake (Hossain *et al*. 2009). Ennahli & Earl (2005) suggested that physiological limitations to photosynthesis with water stress might be a result of chloroplast limitation, such as photoinhibition. Stomatal conductance was expected to reduce with drought, (Flexas *et al.* 1999, Maricle *et al.* 2007), but this response was not observed in this study. This discrepancy might be a result of the methodology. The methods used in the quoted studies consisted of suspended irrigation, that is, they periodically interrupted the water supply, while the method in the present study provided water daily to the plants, even if in low quantities. Therefore, effects on stomata might be less evident. Furthermore, Quesada *et al.* (2004) has already shown that the Cerrado's native flora go through the dry season with no major reduction of the transpiration rate.

The F_v/F_m values represent the maximum quantum yield of the Photosystem II, being used as an indicator of photosynthetic health. This ratio was shown to be lower for the native species, which might partially explain its reduced assimilation rate in comparison to the invasive species. Interestingly, F_v/F_m was slightly lower in plants under a 80% moisture regime than at 10% and was negatively correlated with assimilation rate. Correia *et al.* (2014) also showed increased F_v/F_m values in plants subjected to drought stress. Chaves *et al.* (2003) explained that leaves that survive drought tend to present higher life expectancy and higher photosynthetic capacity, optimizing this rate per area. In further studies, other fluorescence parameters such as rapid light curves (RLC), electron transport rate (ETR) and photochemical quenching provided a more robust explanation on this issue (White & Critchley 1999). No effects of drought on NPQ values were found in this study, although other studies show increases in NPQ with drought stress (Loggini *et al.* 1999, Correia *et al.* 2014).

Studies showed a decrease in grass chlorophyll with drought (Alberte & Thornber 1977, Loggini *et al.* 1999) that was not observed in this study. However, the methodologies used in these two studies (use of hypertonic solution and omitting irrigation, respectively) were very different from those used in the current study. On the other hand, Correia *et al*. (2014) performed a similar methodology to the one used in the present study and found a significant increase in chlorophyll content in clones of *Eucalyptus globulus* Labill. However, this pattern was not observed here. Likewise, Kronfuß *et al*. (1998) used a similar methodology and also showed no effect of drought on leaf chlorophyll content. The higher values of Chl_a/Chl_b found on the native species might reflect adaptation to high light intensity (Ito *et al*. 1993).

Drought seems to have an effect on the leaves' nutritional status. *Melinis minutiflora* presented higher nitrate concentration at 10% soil moisture. Other studies that measured nitrogen concentration showed similar values, as well as the same pattern of higher concentration in dry soils (Pinheiro *et al.* 2001, Baruch 1994). The present study found a negative correlation of phosphate with drought for the native species and no effect of water stress on the invasive species' phosphate content. Accordingly, Baruch (1994) did not find a relation between drought and flood in phosphorus accumulation in leaves from other African grasses. It was not possible to measure the nutritional profile of dead leaves and derive how efficiently they were reutilizing nutrients. Further studies would benefit from these measurements since there has been reported evidence that *M. minutiflora* recycles nitrogen more efficiently than native species from senescent leaves (Silva & Haridasan 2007). Nevertheless, a significantly lower percentage of dry leaves in *M. minutiflora* in comparison to *S. microstachyum*, by the end of the experiment, supports the hypothesis of a more efficient nutrient reabsorption for the invasive species.

As for the oxidative stress responses, the native species presented a pronounced oxidative damage and higher oxidative response in comparison to the invasive species, which can partially explain a lower biomass accumulation. The native species presented higher MDA concentrations, indicating higher lipid peroxidation, which is enhanced by dry or flood-level treatments. Indeed, lipid peroxidation is expected to increase with stress (Moran *et al.* 1994, Zhang & Kirkham 1994, Lima *et al.* 2002). Moreover Gill & Tuteja (2010) and Arora *et al.* (2002) observed lower levels of lipid peroxidation in drought-tolerant plants, presenting lower concentrations of MDA.

APX is a very important antioxidant in plants, responsible for removing hydrogen peroxide. Although oxidative stress is also an important signaling pathway and is a natural byproduct of photosynthesis, environmental and chemical stress might elevate the activity levels of these molecules in the cell and affect internal equilibrium. In the present study, the native species showed higher APX activity and a significant interaction between species and treatment. This observation suggests the activity of APX increases during drought for the native species while, for the invasive species, the activity remains low. Sharma & Dubei (2005) showed that APX activity increased in plants under mild drought and decreased in severe drought.

GST activity was also higher in the native species. GST is related to oxidative response since its substrate is glutathione, an important antioxidant buffer, and it is also related with transportation of flavonoids into the vacuole and signaling in plants (Dixon *et al*. 2002). This study showed significant changes in GST activity depending on treatment (Table 1). Other studies showed effects of drought on other enzymes and antioxidants related to the glutathione cycle, with an increase in glutathione reductase (GR) activity in drought-stressed wheat (Loggini *et al.* 1999), and an increase in reduced glutathione (GSH) (Kronfuß *et al.* 1998) and higher concentration of GSH+GSSH in a variety of wheat less tolerant to drought (Loggini *et al.* 1999). Tobacco clones that over-express GST and glutathione peroxidase (GPX) are also known to be more tolerant to environmental stress (Roxas *et al.* 2000).

G-POX is also related to the oxidative stress signaling (Gill & Tuteja 2010) but it did not seem to play a major role in the way plants dealt with water stress in this study. However, a significant interaction of species and treatment suggested that *M. minutiflora* responds with higher levels of activity during drought while the native species presents high activity during times of flood.

The invasive species presented higher activity of CAT in comparison to the native species, and the activity of this enzyme was enhanced in the 10% treatment. Increases in this defense mechanism might be favoring the maintenance of low cellular damage in invasive species subjected to drought. The removal of reactive oxygen molecules (namely H_2O_2 in the case of CAT) leads to lower levels of MDA and electrolyte leakage under water stress conditions. Drought-tolerant plants do seem to present higher CAT activity (Arora *et al*. 2002) while transgenic plants with low CAT activity tend to respond negatively to environmental stress (Chamnongpol *et al.* 1996). Furthermore, Gamble & Burke (1984) showed an increase in CAT in plants under drought stress.

Although the correlation does not mean a causal relationship, it may provide some insights of the mechanisms underlying the responses. Using the full dataset enabled an overall data visualization to account for the whole variability and possible responses ranges. For instance, it was possible to observe significant correlations between the higher CAT activity, lower MDA concentrations and a higher biomass accumulation. This was observed by the higher growth from the invasive species while the native grew less and presented lower CAT activity and high MDA concentration. On the other hand, the separate analysis for each species showed that the species present different behaviors for the assessed traits. The invasive species showed higher number of non significant correlations, which can reflect better buffering against stress, showing a less steep response.

Although 80% seems to be the best scenario for the native species, it might have more conditions to compete with the invasive species in a situation with lower water availability, where the invasive species also presents levels of stress. This condition probably represents the best situation to apply management. However, in the field it is important to take into account the presence of other species that, together, might be more resilient to the invasion of a strong competitor (Tilman 1996, Sheley & Krueger-Mangold 2003).

In conclusion, the invasive species performed better under all conditions and seems better adapted to lower soil water availability. It grows more both above and below ground, presenting less evidence of biochemical stress, using water more efficiently by controlling stomata and maintaining high assimilation rates and a more active photosystem. Higher nutrient concentration and delayed leaf senescence seemed to indicate more efficiency in nutrient assimilation by the invasive species, partially explaining the higher biomass accumulation.

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CHAPTER FIVE:

NATIVE AND INVASIVE GRASSES IN CERRADO: INTERACTIONS AND EFFECTS OF SOIL MOISTURE AND FERTILIZATION ON MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL TRAITS.

Carolina Musso[1], Gloria Pinto[1], Rhaul Oliveira[1], Carlos Correia[2], José Moutinho-Pereira[2], Amadeu M. V. M. Soares[1], Susana Loureiro[1].

1 - Department of Biology & CESAM (Centre for Environmental and Marine Studies), University of Aveiro, 3810-193 Aveiro (Portugal)

2 - Centre for the Research and Technology of Agro-Environment and Biological Sciences (CITAB), University of Trás-os-Montes e Alto Douro, Apt. 1013, 5001-801 Vila Real, Portugal

ABSTRACT

African grasses are common invaders of tropical ecosystems and are considered a threat to the Cerrado’s integrity, displacing native grasses by outgrowing them and using nutrients more efficiently. The Cerrado is a region characterized by a soil and vegetation mosaic. Plants have to endure low levels of nutrients in the soil and drought during the dry season. However, human activities, especially soy plantations, have increased nitrogen inputs in the Cerrado's soil, changing natural conditions. This study aimed at evaluating the responses of an invasive and a native species to water availability and fertilization, simulating different scenarios which exist in the field. Individuals of one native (*Schizachyrium microstachyum* (Desv. ex Ham.) Roseng.) and one invasive (*Melinis minutiflora* P. Beauv.) species were submitted to different soil irrigation regimes: 5 and 10 day cycles of suspended irrigation (representing drought), watered every day (control) and Over-watering (2 cm flood), with and without the addition of fertilizer (NPK 10-10-10). Several morphological and physiological parameters were assessed after four weeks under 28ºC: plant height, biomass, tillers, leaf area, fluorescence of chlorophyll a, pigment content, MDA concentration, G-POX and SOD activities. Results showed that species reacted differently with the application of the different treatments. Both species were able to cope with the stress conditions but performed better at control conditions (watered every day to field capacity) with fertilization. Under stress, the invasive species grew more and showed less evidence of damage (lower MDA concentration, higher F_v/F_m values) while the native species showed increased SOD and GPOX activity with the combination of fertilization and flood.

Fertilization enhanced morphological differences between species, suggesting a more efficient nutrient assimilation by the invasive species.

Key words: Suspended irrigation, Drought, Flood, Alien plants, *M. minutiflora*, biomarkers, NPK

INTRODUCTION

Alien invasive species are considered a threat to biodiversity worldwide (Gurevitch & Padilla 2004). The introduction of exotic species may occur by accident, but intentional introduction is also frequent. That is the case of many African grass species that have been introduced in South America and Hawaii. They are used as substitutes of native grasses for forage or recovery of eroded areas (Pivello *et al.* 1999, Carneiro *et al.* 2001, Silva *et al.* 2007). African grass species adapt very well to South American savannas and can easily become invaders in the Llanos (Venezuela), Hawaii or in the Cerrado (Central Brazil) since these environments are very similar to their original one (Baruch *et al.* 1989, Barger *et al.* 2003, Freitas & Pivello 2005). They all present a tropical climate and an herbaceous layer that is represented mainly by grasses (Munhoz & Felfili 2005, Mendonça *et al.* 2008).

African grass species are usually able to accumulate greater amounts of biomass and are considered more resilient to defoliation than Cerrado grasses, as an evolutionary response against the large herbivores present in Africa (Simões & Baruch 1991). Therefore, African species tend to displace native grasses and form monospecific stands, diminishing biodiversity (Allison & Vitousek 2004, Baruch & Jackson 2005). The invasive grasses are usually reported as opportunistic, responding more rapidly and efficiently to higher availability of resources (Keeley

1986, Baruch & Bilbao 1999), invading disturbed natural areas and dramatically changing the environment's microclimate and disturbance regimes (D’Antonio & Vitousek 1992).

As to the native grasses, they are considered resilient to the Cerrado's adverse conditions, being resilient to fire, adapted to low bioavailability of nutrients and tolerant to drought (Eiten 1972, Coutinho 1990, Vilela & Haridasan 1994). These adaptations are a result of a coevolution with all these environmental factors: fire-adapted flora has been evolving in the Cerrado for over a millennia (Simon *et al.* 2009), and plants have evolved mechanisms to thrive in the Cerrado's dystrophic, aluminum-rich soil (Haridasan 2008), while also developing strategies to tolerate the severe drought that occurs annually from April to September (Eiten 1972).

Nevertheless, the Cerrado is a mosaic of vegetation and soil types. The local soil's water and nutrient availability vary not only with season but also along the vegetation and soil gradient according to changes in soil type and the distance to the watercourses and to the groundwater (Coutinho 1978). Furthermore, human activities, such as fertilization in agricultural fields and fossil fuel burning in urban areas may become a source of input of nutrients to natural areas by atmospheric deposition or runoff (Jordan & Weller 1996, Vitousek *et al.* 1997).

In order to evaluate which conditions privilege invasive species over native ones, this study aimed at comparing the performance of one native and one invasive grass under different conditions of soil water and nutrient availability. For that, the native species *Schizachyrium microstachyum* (Desv ex Ham.) Roseng. and the invasive species *Melinis minutiflora* P. Beauv. were exposed to different soil irrigation regimes: 5 and 10 day cycles of suspended irrigation (representing drought), watered every day (control) and over-watering (2 cm flood), with and without the addition of fertilizer (NPK 10-10-10). Following this objective, several morphological, physiological and biochemical parameters were assessed in plants grown under different combinations of water and nutrient concentrations under controlled conditions.

MATERIAL AND METHODS

Species

Two species were chosen for the experiments. A native species, *Schizachyrium microstachyum* (Desv. ex Ham.) Roseng. , was chosen for being a common perennial grass in the Cerrado and presenting a high germination rate (Filgueiras 1992, Carmona 1998). An invasive species, the African grass *Melinis minutiflora* P. Beauv., was chosen for being considered one of the biggest threats to biodiversity in Brazil (Martins 2006). Seeds were collected by hand during the dispersion period (from May to July of 2010) in the Reserva Ecológica area of Instituto Brasileiro de Geografia e Estatística (15° 56' 41" S and 47° 53' 07" W GRW), 25 km South of Brasília, Brazil. Seeds were then stored at room temperature until use.

Experimental design and sampling

Seeds from both species were primarily germinated in Petri dishes with humidified cotton and filter paper. Conditions were maintained according to what is described by Andrade *et al.* (2002), considered similar to the environmental conditions at the beginning of the rains and optimum for germination (37ºC/22ºC for 10h of light and 14h of dark, respectively). One-week-old seedlings were then transplanted to experimental clay pots with 20cm height and 20cm top diameter, containing 1kg of soil. Soil was prepared mixing equal parts of organic matter (humus and turf), and mineral soil (adapted from Simões & Baruch 1991). Ten individuals were planted in each pot. Seedlings were left to grow in constant well-watered soil (watered every day to field capacity) for four weeks in a climatic chamber set to 28°C and 12h of light (156 ± 5.37 $W.m^{-2}$). After this period, the smallest seedlings were harvested, equaling a total number of eight individuals per pot, and four different irrigation treatments were then applied: (1) watered every 10 days (10-d); (2) watered every five days (5-d); (3) watered every day (Control); (4) Overwatered every day (Ovw). Treatments representing 10-d, 5-d and Control were always watered until the soil's maximum field capacity was achieved whereas the Ovw group was watered until a 2-cm layer of water was present aboveground (adapted from Baruch 1991). Each treatment had six replicates, from which three were fertilized at the beginning of the irrigation treatment (0.5 g of solid NPK 10-10-10), and the other three lack fertilization. Plants were kept in these conditions for four weeks.

Prior to sampling, fluorescence of chlorophyll a was measured on the adaxial side of ten mature leaves. Firstly, plants were adapted to darkness for 30 min, and then minimal fluorescence (F_0 - fluorescence intensity with all PSII reaction centers open while the photosynthetic membrane is in the non-energized state) was measured. In the same leaves, maximal fluorescence (F_m - fluorescence intensity with all PSII reaction centers closed) was measured after applying a saturating pulse of white light (0.7 s). In light-adapted leaves, steady-state fluorescence (F_s - fluorescence intensity at steady state) was measured and maximal fluorescence (F_m - fluorescence intensity with all PS_{II} reaction centers closed in light-adapted state) was measured after a saturating pulse of white light (0.7 s) (>1 500 mol $m^{-2}s^{-1}$). All measurements were taken using a Mini-PAM (pulse amplitude modulation system, FMS 2, Hansatech Instruments, Norfolk, England). The obtained values were used to calculate the non-photochemical quenching (NPQ) and the optimum quantum yield (F_v/F_m), where F_v is the variable fluorescence in dark-adapted leaves (F_m-F_0) (Van Kooten & Snel 1990). The formula described by Bilger & Björkman (1990), ($NPQ = (F_m - F_m')/F_m'$), was used to calculate NPQ.

After fluorescence measurements, plants were harvested at soil level, immediately measured, weighed and counted for tillers. Five leaves of each replicate were separated for calculation of mean leaf area by the means of a desk multifunctional printer and image analysis with ImageJ software. Roots were carefully washed, and oven dried for root:shoot ratio determination. Approximately half of the aerial biomass was oven dried for determination of nitrate and phosphate concentration while the other part was snap frozen and stored at -80ºC for biochemical assays (Pigments, MDA, SOD and GPOX).

The concentration of nitrate and phosphate was determined according to the HACH KIT method (DR/2000 Spectrophotometer). Dry leaves were ground in a mortar and homogenized in distilled water in a proportion of 1:2 (mass:vol). The plant extract was filtered with activated coal and filter papers (180 µm of thickness and 11µm pore size for particle retention). The filtrate was analyzed according to protocols 8151 (Program 363, 500ηm) for nitrate and 8183 (Program 510, 890 ηm) for phosphate. Results were presented as the percentage of dry weight.

Frozen samples were ground in a mortar with the extraction buffer (a solution of cold acetone and 50 mM Tris buffer, pH 7.8 in a proportion of 80:20, v:v) for pigment extraction. Homogenates were centrifuged at 5,000 g for 10 min.

Absorbance of the supernatant was determined at 470, 537, 647 and 663 nm in microplates. The concentrations were calculated as follows (Sims & Gamon 2002):

$Chl_a = 0.01373\ Abs_{663} – 0.000897\ Abs_{537} – 0.003046\ Abs_{647}$

$Chl_b = 0.02405\ Abs_{647} – 0.004305\ Abs_{537} – 0.005507\ Abs_{663}$

$Carotenoids = ((\ Abs_{470} – (17.1 \times (Chl_a + Chl_b) – 9.479 \times Anthocyanins)) / 119{,}26$

$Anthocyanins = 0.08173\ Abs_{537} – 0.00697\ Abs_{647} – 0.002228\ Abs_{663}$

Lipid peroxidation, an indicative of oxidative damage to the cell membranes, was estimated by measuring malondialdehyde (MDA) production (Dhindsa *et al.* 1981). Five frozen leaf samples of 0.5g were ground to a powder in a mortar with liquid nitrogen and then homogenized with a solution of TCA (0,1% w/v (g/100ml)). Samples were centrifuged and an aliquot was mixed with another solution (TCA 20% (m/v), containing 0.5% (m/v) TBA) and left to react for 30 min at 95ºC. MDA concentration was estimated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm using an absorbance coefficient of extinction (ε), 155 $mM^{-1}\ cm^{-1}$ (Elkahoui *et al.* 2005). Absorbance was measured with a Thermo Fisher Scientific (Waltham, USA) spectrophotometer (Genesys 10-uv S).

For assessment of enzymatic activity of the antioxidant system, frozen leaf samples were ground to a powder in a mortar with liquid nitrogen and then homogenized with an extraction buffer that presented the concentrations of 100 mM of phosphate buffer (pH 7.5) and 0.5 mM of EDTA. After centrifugation (10,000 g, 20 min (Howcroft *et al.* 2011)), the supernatant (heretofore referred to as enzyme extract) was used for determination of Guaiacol peroxidase (G-POX) and Superoxide dismutase (SOD) activity. For GPOX, the reaction mixture presented solute concentrations of 10 mM of phosphate buffer (pH 6.1), 12 mM of hydrogen peroxide, 96 mM of guaiacol, to which 5 µL enzyme extract was added. Absorbance was recorded at 470 nm for 5 min, and the specific activity was calculated using the 26.6 $mM^{-1}.cm^{-1}$ molar extinction coefficient (Castillo *et al.* 1984). SOD activity was estimated by recording the enzyme-induced decrease in absorbance of formazone formed by the nitro-blue tetrazolium with the superoxide radicals (Dhindsa *et al.* 1981). The reaction mixture presented solute concentrations of 13 mM of methionine, 25 mM of nitro-blue tetrazolium chloride (NBT), 0.1 mM of EDTA, 50 of mM phosphate buffer (pH 7.8), 50 of mM calcium carbonate, to which 0.6µl of enzyme extract was added. The reaction was started by adding a solution of 2 mM of riboflavin and placing the microplates under a 15 W fluorescent lamp for 15 min. The absorbance was then recorded at 560 nm and values were calculated based on the curve previously calculated with a standard (Activity = $-2.0789(Abs)^2 + 26.316(Abs) - 1.2766$). Protein concentration was determined in quadruplicate by the Bradford method (Bradford, 1976), at 595 nm, using bovine serum albumin (BSA) as the standard. G-POX and SOD activities were corrected by protein content and fresh weight, respectively. All enzymatic activities protocols were adapted for microplate reader, regarding proportions and a Labsystem Multiskan EX microplate (Labsystems Inc., Franklin, MA) reader was used.

Data analysis

When the data did not present normal distribution, they were transformed by either ln(x) or arcsine. For each parameter, means were compared with factorial ANOVA using species (*S. michrostachyum* - native, *M. minutiflora* - invasive), fertilization (with or without) and water treatment (10-d, 5-d, Control, Ovw) as independent variables and multiple comparisons were carried out using the

TukeyHSD test. Using a scaled covariance data matrix, a Principal Components Analysis (PCA) was carried out to explore and highlight the relationships and patterns between species and treatments, as well as to assess which were the most important parameters in explaining variation among species, as a response to water and nutrient availability.

RESULTS

The summary for the morphological traits can be observed in Figure 21. Overall, the invasive species presented a higher amount of dry biomass, length and leaf area than the native species (all $p<0.0001$, Table 9). These differences were even higher upon fertilization (significant interaction between Species and Fertilization, $p=0.001$, 0.008, 0.023) for all treatments, except the 10-d treatment, suggesting the invasive species is more benefited by fertilization. The number of tillers was not affected by water treatment when not fertilized. However, both species produced more tillers when fertilized, and even more if fertilized and well watered (interaction of Water and Fertilization, $p<0.001$). When fertilized, the invasive species tended to produce more tillers than the native species ($p=0.001$). The root:shoot ratio differed among species ($p=0.001$), being higher for the invasive species. This ratio significantly decreased for the native species with the addition of fertilizer ($p=0.009$).

The means for fluorescence, chlorophyll content and nitrate concentrations can be visualized in Table 10 and the results for ANOVA on Table 1. The F_v/F_m parameter was affected by the Ovw treatment, with a significant reduction in quantum yield. The native species presented lower F_v/F_m than the invasive species when stressed (10-d and Ovw). However, these differences were reduced with fertilization.

NPQ was also higher for the invasive species and was affected by moisture, increasing in 10-d and 5-d treatments, but unaffected by fertilization.

Pigments tended to be more concentrated in the native species (Chl_a $p<0.001$, Chl_b $p=0.008$, Car $p<0.001$), especially in the Control group. Pigment concentrations were not affected by water treatment, and only Chlorophyll a increased with fertilization. Chlorophyll a/b ratio differed among species, with a higher ratio for the native species ($p<0.001$; Table9). Moisture level changed the Chl a/b ratio only in non-fertilized groups with significantly higher levels in the Ovw treatment if compared to Control ($p=0.029$).

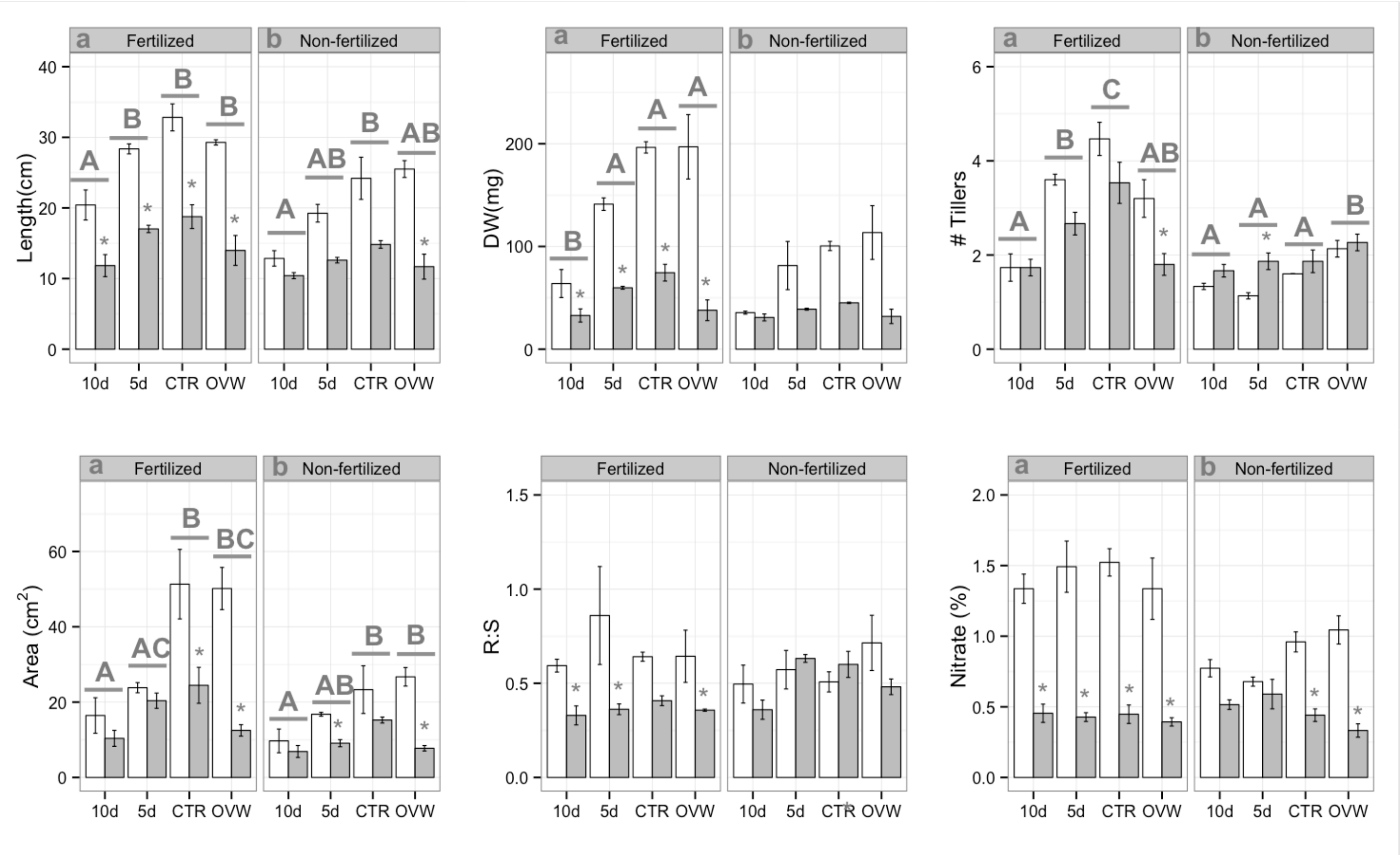

Figure 21. Morphological traits of *Melinis minutiflora* (invasive species, white bars) and *Schizachyrium michrostachyum* (Cerrado native grass, grey bars) grown for 4 weeks under different soil water availability: watered every five (5d) or ten days (10d) (to maximum field capacity), Control (CTR) and Over-watering (OVW) with or without addition of NPK (10-10-10). Error bars correspond to standard deviation. Asterisks refer to significant differences between species, small letters to differences between fertilized and unfertilized treatments and caption letters to differences among watering treatments. (P<0.05, Factorial ANOVA with species, watering treatment and fertilization independent variables, Tukey HSD test for multiple comparisons).

Phosphate concentrations were higher for the invasive species regardless of moisture or fertilization. However, the differences tended to decrease with fertilization (data not shown) and moisture did not affect phosphate concentrations. On the other hand, the invasive species had a significantly higher concentration of nitrate in all treatments ($p<0.001$), especially when fertilized.

As for the biomarkers for oxidative stress, means are summarized in Table 11. The native species showed higher MDA concentrations than the invasive species ($p<0.0001$). When not fertilized, plants from the Control group were the only ones showing equivalent MDA concentration between species. With fertilization, MDA concentration in the native species was reduced. The only exception was the 10-day treatment for the native species, which maintained higher MDA concentration values in relation to other treatments and to the invasive species. SOD activity differed between species ($p=0.002$), showing that their oxidative response is different. When fertilized the SOD activity was higher for the native species than for the invasive species. Furthermore, it increased with water availability while the invasive species presented higher

SOD activity during drought and didn’t increase with fertilization. GPOX activity also differed among species ($p=0.027$). In the invasive species, GPOX activity was not affected by water treatment, while in the native species, values tended to increase with soil water availability. This pattern was intensified when fertilization was added, with significantly higher values of GPOX activity in Ovw treatment in relation to other treatments and to the invasive species (all $p<0.05$).

Table 9. Factorial ANOVA scores for the measured dependent variables using three independent variables as factors: (S) species (*Schizachyrium microtachym* and *Melinis minutiflora)*, (M) watering treatment (5d, 10d, CTR, OWV) and (F) fertilization (With or without). Four-week old plants were grown in a climatic chamber (28 ºC and 12h of light) for 4 weeks.

	Fertilization		Moist		Species		F x M x S	
	F	p	F	p	F	p	F	p
Length	47.452	<0.001	24.438	<0.001	186.289	<0.001	0.436	0.729
Dry weight	38.327	<0.001	18.104	<0.001	120.616	<0.001	0.814	0.496
N of tillers	88.005	<0.001	18.871	<0.001	3.627	0.066	1.610	0.206
Area	37.554	<0.001	16.440	<0.001	52.918	<0.001	2.253	0.101
Root:Shoot	0.192	0.663	1.954	0.140	15.240	<0.001	1.387	0.265
Nitrate	29.362	<0.001	0.472	0.701	209.310	<0.001	2.552	0.073
Phosphate	1.262	0.270	12.601	<0.001	65.787	<0.001	1.840	0.300
Potassium	0.085	0.772	0.543	0.656	0.207	0.652	1.064	0.378
Chlorophyll a	12.054	<0.001	1.641	0.189	53.632	<0.001	0.698	0.556
Chlorophyll b	2.540	0.116	1.976	0.127	7.509	0.008	0.462	0.709
Carotenoids	1.415	0.239	0.952	0.421	48.936	<0.001	0.481	0.693
Chl a/b ratio	4.115	0.010	3.385	0.070	18.404	<0.001	0.315	0.815
F_v/F_m	11.451	0.001	5.100	0.003	59.824	<0.001	1.093	0.359
NPQ	4.265	0.008	22.719	<0.001	204.802	<0.001	2.221	0.094
MDA	23.535	<0.001	35.729	<0.001	120.741	<0.001	6.785	<0.001
G-POX	5.096	0.031	1.996	0.134	5.354	0.027	1.588	0.219
SOD	3.158	0.085	11.158	<0.001	11.845	0.002	4.489	0.617

Table 10. Mean values (±SD) of leaf fluorescence and leaf pigments of the native (*Schizachyrium microtachym)* and the invasive grass (*Melinis minutiflora)* under different soil water and nutrients availability treatments. The p column shows differences between moisture treatments. Caption letters refer to Fertilized treatments and asterisks refer to significant difference between species.

Endpoint	Watering	Fertilized	*p*	*M. minutiflora*	*S. microstachyum*
Chlorphyll a (µmol gFW^{-1})	10 days	No	ns	0.88 (±0.09)	1.42 (±0.14)
	10 days	Yes	ns	1.14 (±0.42)	1.63 (±0.14)
	5 days	No	ns	0.80 (±0.02)	1.63 (±0.07) *
	5 days	Yes	ns	1.52 (±0.13)	1.64 (±0.14)
	Control	No	ns	0.98 (±0.06)	1.73 (±0.18) *
	Control	Yes	ns	1.22 (±0.09)	2.13 (±0.18) *
	OVW	No	ns	1.08 (±0.04)	1.70 (±0.22) *
	OVW	Yes	ns	1.30 (±0.18)	1.74 (±0.15)
Chlorophyll b (µmol gFW^{-1})	10 days	No	b	0.31 (±0.03)	0.41 (±0.03)
	10 days	Yes	ns	0.40 (±0.15)	0.39 (±0.04)
	5 days	No	a	0.29 (±0.03)	0.49 (±0.03)
	5 days	Yes	ns	0.52 (±0.05)	0.46 (±0.03)
	Control	No	a	0.33 (±0.02)	0.60 (±0.13)
	Control	Yes	ns	0.44 (±0.04)	0.54 (±0.05)
	OVW	No	a	0.36 (±0.01)	0.43 (±0.04)
	OVW	Yes	ns	0.44 (±0.06)	0.34 (±0.06)
Carotenoids (µmol gFW^{-1})	10 days	No	ns	0.59 (±0.06)	1.01 (±0.07) *
	10 days	Yes	ns	0.62 (±0.22)	1.01 (±0.05)
	5 days	No	ns	0.58 (±0.04)	1.05 (±0.05) *
	5 days	Yes	ns	0.91 (±0.09)	1.02 (±0.05)
	Control	No	ns	0.63 (±0.03)	1.18 (±0.18) *
	Control	Yes	ns	0.74 (±0.07)	1.13 (±0.07)
	OVW	No	ns	0.70 (±0.03)	0.98 (±0.11)
	OVW	Yes	ns	0.82 (±0.11)	0.92 (±0.06)
Chlorophyll a/b ratio	10 days	No	ab	2.83 (±0.38)	3.41 (±0.15)
	10 days	Yes	ns	2.87 (±0.06)	4.22 (±0.12) *
	5 days	No	a	2.80 (±0.23)	3.34 (±0.18)
	5 days	Yes	ns	2.91(±0.04)	3.97 (±0.12)
	Control	No	a	2.93 (±0.04)	3.14 (±0.35)
	Control	Yes	ns	2.82 (±0.05)	3.95 (±0.07)
	OVW	No	b	3.00 (±0.04)	3.98 (±0.06) *
	OVW	Yes	ns	2.96 (±0.08)	4.04 (±0.56)
Leaf fluorescence F_v/F_m	10 days	No	a	0.796 (±0.005)	0.723 (±0.004) *
	10 days	Yes	A	0.805 (±0.003)	0.765 (±0.013)
	5 days	No	a	0.777 (±0.006)	0.749 (±0.009)
	5 days	Yes	A	0.798 (±0.006)	0.775 (±0.006)
	Control	No	a	0.782 (±0.010)	0.771 (±0.004)
	Control	Yes	A	0.789 (±0.004)	0.779 (±0.003)
	OVW	No	b	0.788 (±0.005)	0.719 (±0.019) *
	OVW	Yes	B	0.774 (±0.004)	0.744 (±0.016)
Non-photo quenching (NPQ)	10 days	No	ns	3.120 (±0.551)	2.243 (±0.207)
	10 days	Yes	A	2.441 (±0.279)	2.441 (±0.287)
	5 days	No	ns	3.550 (±0.598)	2.625 (±0.515)
	5 days	Yes	AB	2.675 (±0.118)	2.386 (±0.301)
	Control	No	ns	4.018 (±0.173)	2.549 (±0.120)
	Control	Yes	AB	3.221 (±0.053)	2.867 (±0.348)
	OVW	No	ns	3.963 (±0.005)	2.602 (±0.396)
	OVW	Yes	B	3.186 (±0.061)	3.023 (±0.455)

Table 11. Mean values (± SD) of MDA and enzymatic activity of the native and invasive grasses under different soil water and nutrient availability treatments. The p column shows differences between moisture treatments. Caption letters refer to Fertilized treatments and asterisks refer to significant difference between species. (FW = Fresh weigh , DW= Dry weigh).

Endpoint	Watering	Fertilized	*p*	*M. minutiflora*	*S. microstachyum*
MDA (mmol gFW^{-1})	10 days	No	a	8.11 (±1.28)	15.67 (±1.26) *
	10 days	Yes	A	5.42 (±0.34)	19.96 (±1.62) *
	5 days	No	a	9.89 (±2.38)	17.00 (±1.62) *
	5 days	Yes	B	4.01 (±0.26)	7.06 (±0.29)
	Control	No	b	4.29 (±0.38)	7.08 (±0.39)
	Control	Yes	B	4.19 (±0.04)	5.73 (±1.64)
	OVW	No	b	3.08 (±0.18)	11.57 (±0.81) *
	OVW	Yes	B	3.26 (±0.31)	5.95 (±0.39)
GPOx (μmol ml^{-1} $prot^{-1}min^{-1}$)	10 days	No	ns	1.89 (±0.33)	-
	10 days	Yes	ab	1.40 (±0.21)	-
	5 days	No	ns	1.56 (±0.43)	0.25 (±0.13)
	5 days	Yes	a	1.63 (±0.21)	1.37 (±0.15)
	Control	No	ns	1.40 (±0.16)	0.90 (±0.78)
	Control	Yes	a	2.17 (±0.19)	2.29 (±0.39)
	OVW	No	ns	1.47 (±0.35)	2.14 (±0.10)
	OVW	Yes	b	0.89 (±0.20)	4.31 (±1.24) *
SOD (mg $gFW^{-1}min^{-1}$)	10 days	No	ab	0.73 (±0.19)	-
	10 days	Yes	AB	0.92 (±0.20)	-
	5 days	No	a	1.59 (±0.51)	2.74 (±0.92) *
	5 days	Yes	A	1.35 (±0.11)	4.57 (±0.51)
	Control	No	b	2.66 (±0.35)	4.16 (±1.39)*
	Control	Yes	A	1.67 (±0.27)	3.09 (±0.53) *
	OVW	No	ab	1.94 (±0.32)	2.57 (±0.02) *
	OVW	Yes	B	1.64 (±0.22)	5.34 (±1.49) *

The PCA showed a clear separation between species (Figure 22). The axis PC1 explained 46% of variation while PC2 explained 24%. In PC1 scores were higher for length, nitrate (positive) and MDA (negative), while in PC2 the variation was due mostly to changes in the leaf pigment concentrations. *M. minutiflora* variables are strongly positively correlated with morphological parameters, F_v/F_m, nitrate and NPQ, while *S. michrostachyum* is correlated with higher values of chlorophyll a/b ratio and MDA content.

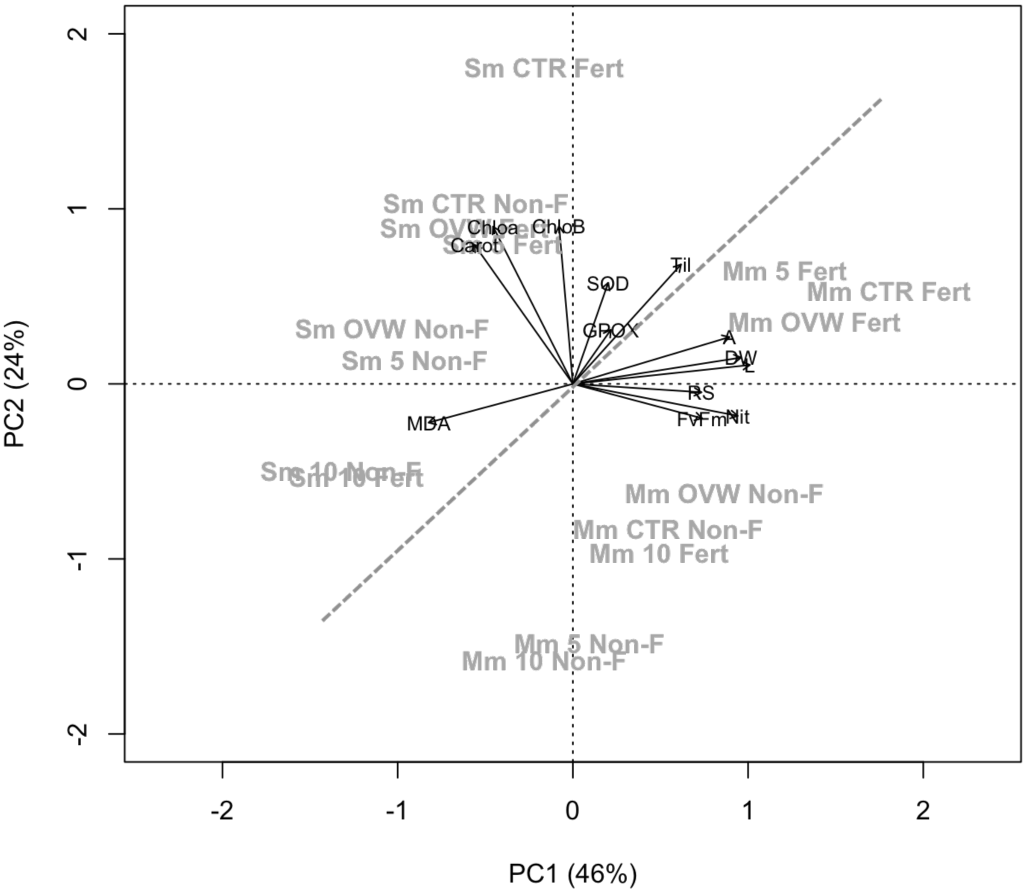

Figure 22. Principal Components Analysis ordination of the species using the variables: Dry weight (DW), leaf area (A), length (L), number of tillers (Til), root:shoot ratio (RS), pigment concentration (ChloA, ChloB, Carot), MDA concentration, GPOX and SOD activities and nitrate concentration (Nit). Percentage of variation accounted for by PC1 and PC2 is shown in parentheses on axis labels. Sm (*Schizachyrium microtachym*) Mm (*Melinis minutiflora*). Fert (Fertilized), Non-F (Non-fertilized), 5 (5-days water suspension), 10 (10-days water suspension), CTR (Control), OVW (Over-watering)

DISCUSSION

The establishment of invasive species in an ecosystem may be related to the availability of resources. Plants are more likely to invade habitats where limitations are removed, and resources are abundant (Galatowitsch *et al*. 1999). As expected, the invasive *M. minutiflora* was able to accumulate more biomass than the native species in well-watered soils, with an intensification of this pattern when soil was richer in nutrients. Another African grass invading the Venezuelan Llanos (*Hyparrhenia rufa* (Nees) Stapf) was also reported to be able to accumulate larger amounts of biomass in wetter and more

fertile habitats (Parsons 1972, San José & Farinas 1991). Rickey & Anderson (2004) reported that the invasive species *Phragmites australis* (Cav.)Trin. ex Steud. also benefited from nitrogen addition when invading marsh areas in the USA.

Drought is the most typical stress savanna plants have to endure, due to the annual dry season. Limitations in the water supply cause changes in plant metabolism, such as arresting photosynthesis, increasing consumption of water and nutrient reserves, altering morphology, shifting plant biomass investment, or causing an overall reduction in growth. Savanna plants are considered resilient to drought either by evading or tolerating such conditions through different mechanisms (Jones *et al*. 1980, Ludlow 1980, Wilson *et al.* 1980, Baruch & Fisher, 1991). In the present study, it was possible to notice that in drought conditions, the difference in growth between the native and the invasive species was not significant, especially in unfertilized sites, corroborating the idea that the Cerrado would be less susceptible to invasion in undisturbed sites in the dry season. A similar pattern was observed in Chapter 4, where well-watered *M. minutiflora* outgrew the native species over twice as much, which the same did not happen in treatments where water was less available. Baruch & Fernández (1993) also reported that native grasses in another neotropical savanna would be better able to compete with African invasive grasses in situations of lower resource availability.

Flooding, on the other hand, occurs during the rainy season on poorly drained soils. Flooding impairs the aerobic respiration of roots, damaging plant metabolism and development (Crawford 1982). Similarly, plants survive flooding by tolerating the anoxic situation, using different strategies such as the development of adventitious rootlets and aerenchyma tissue (Jackson & Drew 1984). In the present study the native species was more negatively affected by flooding than the invasive species, which performed normally when water was abundant. Since the watering method provided water daily, we hypothesized that the greater root biomass of the invasive species was more efficient in using the provided water without creating the anoxious environment that the native species couldn't avoid.

It was noted that the biomass was not only greater in *M. minutiflora,* but also differently partitioned. Invasive plants grew both higher and wider, by producing more tillers and wider leaves and investing more in roots, proportionally, than the native species. Baruch & Jackson (2005) also reported the success of this invasive species in a neotropical savanna, attributing its success to its greater growing rate and efficiency in the synthesis of aerial biomass and leaf area. Wider leaves and a higher number of tillers could confer a better ability to compete for light and space, shading competitors' adults, seedlings and seeds, or even preventing seed showers from reaching the soil (Silva & Castro 1989). Although these characteristics may reduce the success of the invasive species' seedlings as well, it is known that *M. minutiflora* has an enormous production of seeds, with high viability and germination rates (Carmona & Martins 2010), which can compensate for this intraspecific competition. *M. minutiflora* shading is reported to impair even tree regeneration in the Cerrado, significantly reducing tree seedling survival in forest borders when competing for light (Hoffman & Haridasan 2008). Furthermore, Barger *et al*. (2003) and Holl (1988) stress the importance of belowground competition in addition to aerial competition. In the referred study, the experimental reduction of root competition was determinant for increasing height and biomass of seedlings growing nearby adult grasses. Therefore, the greater investments in roots presented by the invasive species, observed in its root:shoot ratio, could confer advantage when competing for nutrients belowground. All attributes together help to explain the displacement of native grasses, in these habitats, by the invasive species.

The pigment results indicate that the native species presents higher concentrations of pigments. When fertilization was added, the difference between native and invasive species reduced. Higher concentration of pigments, however, doesn't seem to confer higher photosynthetic capacity to the native species, which also presented lower F_v/F_m values. Furthermore, the data shows a higher Chlorophyll a/b ratio for the native species, especially in the flood treatment. This response is an evidence of oxidative stress, since, in such conditions, chlorophyll b is degraded before chlorophyll a, increasing the ratio between them (Huang *et al*. 2004). This is in accordance with Ashraf & Habib-ur-Rehman (1999) that also found increased Chla/b ratio in waterlogged plants. Other studies showed stronger effects of water stress on pigment content due to drought and flood on wheat and maize (Alberte & Thornber 1977, Loggini *et al.* 1999).

Optimum quantum yield (F_V/F_M) decreased with flood while NPQ increased. Fernándes (2005) and Rengifo *et al*. (2005) also showed a decrease in F_v/F_m with flood, indicating photodamage. However, just like in the referred studies, the values did not decrease below 0.71, which is considered the threshold for healthy plants (Bolhàr-Nordenkampf & Öquist 1983). Higher NPQ values were also observed for plants under water stress (Correia *et al*. 2014) and the higher NPQ values for the invasive species might be conferring stronger protection against photoinhibition (Li *et al.* 2002)

When fertilization was added, pigment content increased for the invasive species, which also presented higher efficiency in allocating nutrients to leaves, as can be observed by the higher concentration of nitrate. Baruch & Jackson (2005) also reported the better ability of *M. minutiflora* to incorporate nutrients to leaves. Furthermore, Silva & Haridasan (2007) suggested that this species reutilizes nitrogen more efficiently. These characteristics favor *M. minutiflora,* especially in areas with higher nitrogen deposition due to anthropogenic activities (Alpert *et al*. 2000)

As expected water stress does not seem to impair the nutrient assimilation as fertilization does. However, in the unfertilized group with low water availability (10-d and 5-d), nitrate concentration in the native species reached the levels of the invasive species. This finding is in accordance with Baruch (1994), who encountered nitrogen accumulation in the leaves of South American native grasses under drought.

The oxidative stress parameters showed that the native species had a stronger response to water stress and fertilization at a biochemical level. However, this mechanism might not be sufficient to overcome water stress, which was reflected in the low fresh weight values during drought and flood, and in higher oxidative damage during drought, as shown by the MDA content. MDA content suggests elevated lipid peroxidation and damage to the cell membrane for the native species. This damage increased with drought for both species and is in accordance with their lower biomass accumulation. Other studies show an increase of MDA in plants under drought (Zhang & Kirkham 1994, Lima *et al*. 2002, Correia *et al*. 2014). Drought-tolerant plants, however, do not present such high levels in MDA content in these conditions (Gill & Tuteja *et al*. 2010, Arora *et al.* 2002). Therefore, *M. minutiflora* seems to be more tolerant to drought than the native species. This finding was not expected, since the Cerrado's native plants are known to be adapted to drought

(Eiten 1972) and other South American grasses have been reported to tolerate drought better than invasive African species (Baruch & Fernández 1993). Fertilization seemed to diminish lipid peroxidation under drought. When fertilized the native species was affected only in the driest treatment (10-d) while the invasive species was not affected at all. This effect is in accordance to the findings of Qian *et al.* (2012) where the grass *Zoysia matrella* (L.) Merr recovered to normal MDA values with fertilization.

GPOX and SOD are important antioxidant enzymes. SOD is the first line of defense in the scavenging system of Reactive Oxygen Species. It dismutates superoxide ($O_2^{\bullet -}$) into hydrogen peroxide, which can then be reduced by GPOX, which consumes H_2O_2 by oxidizing guaiacol. Although it was not possible to measure the activity of these enzymes in the 10-d treatment for the native species due to lack of material, both enzymes seemed to have their activity enhanced with the combination of watering and fertilization. Water seems to make the nutrients become more available, being diluted in the water, and acting synergistically with fertilization. Other studies showed increases in GPOX and SOD in grass species of rye and wheat exposed to chemical stress (Milone *et al.* 2003, Khan *et al.* 2007, Silva *et al.* 2013). Although fertilization had a positive effect on biomass accumulation, it also seemed to cause oxidative stress in the native species. This response may be a reflection of its adaptation to soils with low nutrient availability.

The PCA shows a clear difference between the two species, with *M. minutiflora* below the diagonal line and *S. microstachyum* above the line (Figure 22). The separation along the horizontal axis (PC1) is mainly due to the scores of dry weight, length and MDA while PC2 discriminates mainly enzymatic activity. Analyzing carefully, the points corresponding to the invasive plants in drought treatments without fertilization (MM 5 C and MM 10 C) are closer to the native species group (SM). This observation suggests that the invasive species' performance was closer to the native species' under low water and nutrient availability, which corroborates the ideas of Baruch & Fernández (1993) that these are better conditions to apply management in.

CONCLUSIONS

The invasive species can outcompete the native species by accumulating more biomass and growing faster, using nutrients and water more efficiently, and investing in wider leaves and a greater underground biomass. Also, invasive species maintain high photon fluxes despite the lower concentrations of pigments and present a weaker oxidative response to drought and fertilization. The native species would perform in a similar pace to the invasive species in conditions of less water and nutrient availability whereas unnatural fertilization inputs and high water availability would benefit the invasive species. In the Cerrado region, sites that present natural levels of nutrients and a good drainage could represent a situation where these native and invasive grasses could compete in a similar pace. On the other hand, disturbed sites with artificial inputs of fertilization, and specially in the rainy season, could prompt the advance of *M. minutiflora*.

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CHAPTER SIX:

CLIPPING AND FIRE TO CONTROL AFRICAN GRASS INVASION IN CERRADO: WHICH , WHEN AND WHERE?

Carolina Musso[1], Gloria Pinto[1], Rhaul Oliveira[1], Carlos Correia[2], José Moutinho-Pereira[2], Amadeu M. V. M. Soares[1], Susana Loureiro[1].

1 - Department of Biology & CESAM (Centre for Environmental and Marine Studies), University of Aveiro, 3810-193 Aveiro (Portugal)
2 - Centre for the Research and Technology of Agro-Environment and Biological Sciences (CITAB), University of Trás-os-Montes e Alto Douro, Apt. 1013, 5001-801 Vila Real, Portugal

ABSTRACT

Biological invasions are believed to be one of the biggest threats to biodiversity conservation. In the Cerrado region, one of the greatest problems is imposed by the African grass *Melinis minutiflora* P. Beauv. and therefore the control of this species is imperative in conservation units. Good management technique should be inexpensive and easily applicable to large areas, and clipping and fire have been applied in the Cerrado region. Considering that management effects might be influenced by other stress factors present in the environment, such as water and nutrient availability, abiotic related effects should also be regarded during management processes. The aim of this study was to assess the effect of clipping and fire in different conditions of soil moisture and fertilization for two grass species, one native and one invasive to the Cerrado. Two-month-old clipped/burned plants were left to recover for four weeks in the same conditions they were in prior to clipping: three different irrigation regimes (Constant moisture, Intermittent Watering, and Intermittent Watering with Fertilization) combined with four levels of water availability (Severe drought, Mild drought, Control and Flood). The invasive species grew more rapidly and presented higher assimilation rates and less lipid peroxidation than the native species. However, clipping reduced the differences in length and dry weight between species when compared to pre-clipping values, especially in the intermittent watering regime with no fertilization. Water availability proved to be more detrimental than fertilization. Pigment and F_v/F_m values were not affected by any factor, and enzymatic activity showed a stronger anti-oxidative response by the invasive species.

Key words: *Melinis minutiflora*, alien plants, management, tropical savannas, stress traits.

INTRODUCION

Biological invasions are one of the biggest threats to conservation of biodiversity, being considered the second most important after land use (Vitousek 1994). This issue has caused a significant loss to both economy and the environment due to the costs of restoration of degraded areas (Gurevitch & Padilla 2004, Richardson *et al*. 2000, Zanin 2009). Invasive species are exotic species that have been accidentally or intentionally introduced to a habitat and that were not only able to successfully adapt to local conditions but also to outcompete native species. Frequently they are able to reduce local and regional biodiversity. In the Cerrado region (the Brazilian savanna and second biggest biome in the country), the greatest problems are imposed by African grasses (Pivello *et al*. 1999, Martins & Leite 1997). The invasive species *Melinis minutiflora* P. Beauv. is considered a serious problem in tropical ecosystems and many studies have reported the effects of its invasion on biodiversity, landscape, soil, fire frequency and fire intensity in natural reserves (San José & Farinas 1991, D'Antonio & Vitousek 1992, Pivello *et al*. 1999, Williams & Baruch 2000). Few studies, however, have focused on techniques to control the spread of such species.

A good management technique should be inexpensive and easily applicable to large areas. Taking these criteria into account, fire, clipping, removal by hand, shading and herbicide use have been proposed as ways to control alien invasive plants (Barros *et al*. 2006, Martins 2006, Marinho & Miranda 2013, Sato *et al*. 2013).

Removing individuals by hand, although feasible in the case of terrestrial plants, it is time consumable and hard work. The application of herbicides is undesirable in natural areas. Therefore, prescribed fire and clipping are the preferred options.

Fire is an important natural factor in many ecosystems, and it has been present in the Cerrado for thousands of years (Whelan 1995, Salgado-Labouriau & Ferraz-Vicentini 1993), and the native flora is considered to be resilient to it. Many species respond positively to fire by being stimulated to flower. Clipping the aerial part of the plant is considered to promote similar effects to fire, but with a less intense response. *M. minutiflora*, on the other hand, is not considered to be tolerant to the removal of its aerial part (William & Baruch 2000).

Nevertheless, the effects of fire or clipping are not that predictable and might be affected by other stress factors present in the environment, such as soil moisture, light, and the availability of nutrients in the soil (Oesterheld & McNaughton 1991, Simões & Baruch 1991, Huston 2004). The Cerrado biome, for instance, is marked by a well-defined rainy and dry seasonality, high presence of dystrophic soils and by a vegetation gradient from grasslands to closed canopy forests (Eiten 1972, Castro & Kauffman 1993). In this scenario, *M. minutiflora* performs better in open areas and in case of soils with more nutrient and water availability (Baruch & Fernandez 1993, Barger *et al.* 2003), and clipping is less harmful to this species when applied in the late rainy season (Klink 1994). Cerrado species, on the other hand, have been reported to respond more positively to fire in the early dry season (Coutinho 1990, Haddad & Valio 1993). In order to plan the best management technique, it is important to understand the responses of the plants to disturbance (clipping or burning) in different conditions encountered in the environment.

The aim of this study was to assess the recovery of two species of grasses after clipping and fire in different soil conditions. The resprouting of the species *Schizachyrium michrostachyum* (Desv. ex Ham.) Roseng (native to the Cerrado) and *Melinis minutiflora* P. Beauv. (commonly-found invasive species) was measured in different soil watering regimes and soil moisture levels. The former was measured in order to simulate differences in soil type, such as drainage and fertilization, and the latter to simulate differences in water availability due to seasonality. The performance of each species was assessed by measuring several traits in different levels of organizations.

MATERIAL AND METHODS

Studied species

Although there are many invasive and native grass species in the Cerrado, only one from each of these groups were chosen for this work. The African grass *M. minutiflora* P. Beauv. is a stoloniferous, perennial, C4 grass also known as "molasses-grass". It is considered a serious problem in American savannas and can displace native grasses population in the Llanos in Venezuela (Baruch & Jackson 2005) and the Cerrado in Brazil (Pivello *et al*. 1999). Although molasses-grass is able not only to affect other grasses but also other herbaceous or woody species (Barros *et al*. 2006, Hoffman & Haridassan 2008), the strongest effects may be considered the effect on grasses. Grasses are of particular importance because the herbaceous layer in the Cerrado exhibits a great richness of grass species, which represents the major part of the biomass of this layer (Munhoz & Felfili 2006). The perennial C4 grass, Schi*zachyrium michrostachyum* (Desv. ex Ham.) Roseng. , is a common

Cerrado grass and presents high levels of seed production and high germination rates (Aires 2013).

These two species were chosen as models for the experimental approach to understand competition among native and invasive species. The panicles were harvested from the field during the seed dispersion period (from May to June of 2010) in order to obtain mature seeds. Harvesting took place in the Reserva Ecológica do Instituto Brasileiro de Geografia e Estatística (15° 56' 41" S and 47° 53' 07" W GRW), 25 km south of Brasília – DF, Brazil. Seeds were taken to the lab, screened and stored in paper bags at room temperature until use.

Seedling and plant preparation

In order to obtain the best seedling success and select appropriate individuals for the experiments, seeds were first germinated and then transplanted to experimental pots. Andrade *et al.* (2002) described the optimum conditions for the germination of Cerrado seeds, simulating natural conditions in the beginning of the rainy season: oscillation temperatures of 37ºC during light (10h) and 22ºC at dark (14h). Seeds were germinated in Petri dishes with sterilized cotton and filter paper, to avoid moss contamination. Seeds were placed in climatic chambers in the described conditions until a desirable amount of seedlings was obtained. The biggest and healthiest-looking ones (no chlorosis, moss or other abnormalities) were transplanted to experimental pots. Then, seedlings were left to grow for four weeks in optimum conditions of water supply and temperature (28ºC, 12h of light 156 ± 5.37 Wm^{-2}). Optimum watering conditions were achieved by capillarity using synthetic fiber (Loureiro *et al*. 2006) or by watering the soil every day to field capacity for the

Constant Watering Regime (CW) and Intermittent Watering regime (IW), respectively (Simões & Baruch 1991).

Treatment – 1st part

In both cases, water stress was only imposed at the end of the fourth week and kept for another four weeks. Pots were either made of plastic (Constant WHC regime, see below) or made of clay (Intermittent Watering regime), containing 500g or 1000g, respectively, of a mixture of organic matter (turf and hummus) and mineral soil (50:50 v:v - adapted from Simões & Baruch (1991). The regimes correspond to distinct methodologies and represent different soil types (distinguished by drainage and/or fertilization status), as described below. Within the watering regime, four levels of soil moisture were created: a severe drought treatment, a mild drought treatment, a control group and a flood treatment. The treatments were achieved for each watering regime as described below.

In the Constant Watering regime, plants were kept at four different constant percentages of the Maximum Water Holding Capacity of the soil (10%, 40%, 80% and 120%, which, in this case, represent the Severe Drought, Mild Drought, Control and Flood treatments, respectively). Moisture was controlled by weighing the pots twice daily and adding the amount of water that was lost (the weight of the plant was ignored). This methodology is commonly used in the laboratory (Kronfuß *et al*. 1998, Lima *et al.* 2011, Correia *et al*. 2014) and enables further precision in controlling the amount of water in the soil. Although it could simulate a soil capable of a high level of water retention, this does not represent most soils found in the Cerrado biome. In the experiment, each treatment presented eight replicates, four of which were burned and another four clipped by the end of the fourth week (see below Treatment- 2nd part).

In the Intermittent Watering regime, one month old plants were watered in the following way: (1) every 10 days (Severe Drought); (2) every five days (Mild Drought); (3) every day (Control); (4) and overwatered every day (Flood) for four weeks. In this regime, the Severe Drought, Mild Drought and Control groups were watered until soil maximum field capacity was achieved, and the OVW group was watered until a 2-cm layer of water was present aboveground. Similar methodologies are also used in ecophysiology studies (Flexas *et al*. 1999, Baruch & Jackson 2005, Maricle *et al*. 2007). Despite the fact that, in this case, the soil moisture is controlled less precisely, it could be a better representation of the Cerrado's well-drained soils. In the experiment, each treatment had six replicates. Three of them were fertilized once (0.5 g of solid NPK 10-10-10, single fertilization at the beginning of the experiment - IWF) and three were not (IW). Because of this experiment's design, there were not enough replicates to sacrifice for burning and therefore the burning experiment was restricted to the CW experiment.

The watering regimes represent soil types (soils with higher or lower water retention or with artificial inputs of nutrients) whereas the moisture levels represent the natural rain seasonality and the Cerrado's topography (in which soils have different levels of access to the water table).

At the end of the fourth week, plants were harvested at soil level (except for the replicates of CW that were burned), and several parameters were measured. Half of the replicates of the CW regime were burned by placing combustible material (dry straw) on top of the pots (see below Treatment- 2nd part), which had previously been wrapped in aluminum foil, by adapting the methodology described by Haddad & Valio

(1993). Data of the first part of the experiment (effects of soil moisture and fertilization) can be visualized in chapters 4 and 5.

Treatment – 2nd part

After clipping or burning, plants were left to regrow for four weeks in the same conditions as described above (Treatment- 1st part). At the end of the fourth week, several parameters were measured again (see below).

Measurements on the day before 2nd harvesting

Prior to harvesting, gas exchange parameters and fluorescence of chlorophyll a were measured. Assimilation rate (A), transpiration (E), stomatal conductance (gs) and C_i/C_a ratio were only measured in leaves from the CW regime. Measurements were performed using a portable infrared gas analyzer (LCpro+, ADC, Hoddesdon, UK) operating in open mode under ambient conditions. In the cuvette, the air temperature and humidity was set at 60%, and 23°C, respectively, and the air flow was of 300 mmol s^{-1}. Calculations of C_i values were estimated using the equations developed by Von Caemmerer and Farquhar (1981): $C_i = [(gs - E/2)\ C_a - A]\ (gs + E/2)^{-1}$, where C_a is the CO_2 concentration in the air under analysis.

Fluorescence of chlorophyll a was measured in five mature leaves for each treatment using a Mini-PAM (pulse amplitude modulation system, FMS 2, Hansatech Instruments, Norfolk, England). Minimal fluorescence (F_0) was measured in 30-min dark-adapted leaves by applying a weak modulated light, and maximal fluorescence (F_m) was measured after applying a 0.7s-long saturating pulse of white light to the same leaves. In light-adapted leaves, steady-state fluorescence (F_s) was measured; then, maximal fluorescence (F_m’) was assessed after a 0.7s-long saturating pulse of white light (>1 500 mol $m^{-2}s^{-1}$). Parameters were used to calculate the optimum quantum yield of Photossystem II (F_v/F_m, where F_v is the variable fluorescence (Van Kooten & Snel 1990)) and the effective quantum yield of PSII (F_v'/F_m' - ϕ_{PSII}). NPQ was calculated according to Bilger & Björkman (1990), (NPQ = $(F_m - F_m')/F_m'$).

Plant tissue sampling

At the end of the fourth week of regrowth, the plants were clipped at soil level. Shoots were immediately measured, weighed, and the number of tillers was recorded. When there was enough plant material, approximately half of the aerial biomass was oven dried for determination of nitrate and phosphate concentrations while the other part was snap frozen and stored at -80ºC for biochemical assays (Pigments, MDA, GPOX, CAT and GST).

Nutrient determination

Using a HACH KIT (DR/2000 Spectrophotometer), the percentage of nitrate and phosphate was measured in the leaves. Plant tissue extract was obtained by mixing the oven-dried leaves with water in a proportion of 1:2 (mass:vol) in a mortar. Leaves were manually ground until forming a visual homogenate. The extract was then filtered with activated coal and filter papers (180 µm of thickness and 11µm pore size for particle retention). For determination of nitrate, protocol 8151 was used (Program 363, measuring at the wavelength of 500ηm) and for phosphate, protocol 8183 was used (Program 510, 890 ηm). Results were presented as the percentage of dry weight.

Pigments

Pigments were extracted from frozen leaves with the extraction buffer composed of acetone and Tris buffer (pH 7.8; 80:20, v:v, Tris at 50mM). Samples were manually homogenized in a mortar and centrifuged at 5 000 g for 10 min. Absorbance was measured in microplates in quadruplicate and were corrected for the path length traveled by the light to match the 1-cm cuvette used in the formula (Warren 2008). Absorbance was measured at 470, 537, 647 and 663 nm wavelengths for the calculation of pigments according to the Sims & Gamon (2002):

$Chl_a = 0.01373\ Abs_{663} – 0.000897\ Abs_{537} – 0.003046\ Abs_{647;}$

$Chl_b = 0.02405\ Abs_{647} – 0.004305\ Abs_{537} – 0.005507\ Abs_{663}$;

Carotenoids = ((Abs_{470} – (17,1 x (Chl_a + Chl_b) – 9.479 x anthocyanins)) / 119.26;

Anthocyanins = 0.08173 Abs_{537} – 0.00697 Abs_{647} – 0,002228 Abs_{663}.

Oxidative stress measurements

In order to measure oxidative damage to cell membranes, lipid peroxidation was assessed by measuring the concentration of malondialdehyde (MDA). Leaf samples (0.5g) were ground to a powder in a mortar with liquid nitrogen and a solution of trichloroacetic acid (TCA 0,1% w/v). After centrifugation, another solution was added to the samples (TCA 20% and thiobarbituric acid 0,5%) that were then heated to 95ºC for 30 min (Santos *et al*. 2001). MDA concentration was estimated by subtracting the nonspecific absorption at 600 nm from the absorption at 532 nm using an absorbance coefficient of extinction (ε), 155 mM^{-1} cm^{-1} (Santos *et al*. 2001). Absorbance was measured with a Thermo Fisher Scientific (Waltham, USA) spectrophotometer (Genesys 10-uv S).

For determination of enzymatic activity, leaf samples were ground to a powder in a mortar with liquid nitrogen. Then, samples were homogenized with an extract solution (Phosphate buffer at 100 mM and pH 7.5, EDTA at 0.5 mM) and centrifuged at 10,000 g for 20min. All enzymatic activity was adapted for microplate proportions and a Labsystem Multiskan EX microplate (Labsystems Inc., Franklin, MA) reader was used. For Guaiacol peroxidase (G-POX) determination, the reaction solution in the microplate wells was made with phosphate buffer (pH 6.1 at contained 10 mM), hydrogen peroxide at 12 mM, Guaiacol at 96 mM and 5 µL of the enzyme extract. Absorbance was recorded at 470 nm (Castillo *et al*., 1984) for 5 min, and the specific activity was corrected by protein content. The GluthationeS-Transferase (GST) activity test was based on the measurement of the product generated by the reaction of 1-chloro-2,4-dinitrobenzene and glutathione. Absorbance was measured at 340 nm for 5 min according to the method of Habig & Jakoby (1981). The reaction solution was composed of phosphate buffer at 100 mM and pH 6.5, reduced glutathione at 10mM and 1-chloro- 2.4-dinitrobenzene at 60mM. Catalase (CAT) activity was determined by measuring the decomposition of the substrate (H_2O_2) at 240 nm during 3 minutes based on the method described by (Clairborne, 1985). The reaction solution was prepared with phosphate buffer (at 0.1 M with pH 7.0) and H_2O_2 (at 6 mM).

Data analysis

For each parameter a three-factor ANOVA was performed using Watering regime (CW, IW, IWF), Species (Invasive, Native) and Moisture Level (Severe Drought-SD, Mild Drought-MD, Control-CTR and Flood-OVW) as independent variables. Then, for each watering regime, means were compared with factorial

ANOVA using species (S. *michrostachyum* - native, *M minutiflora* - invasive) and Moisture Level (SD, MD, CTR and OVW) as independent variables. Multiple comparisons were carried out using the Tukey HSD test. The p values were corrected using the residual error of the whole data set. Data from chapter 4 and 5 was used to calculate the percentage of recovery after clipping/burning in comparison to the previous values. These means were compared with arcsine-transformed data. Comparisons between clipped and burned plants of *M. minutiflora* were made with a factorial ANOVA using management (clipping , burning) and moisture level (SD, MD, CTR and OVW) as independent variables and using the Tukey HSD test for multiple comparisons. All data analysis and graphs were made using the R software (R 3.0.1 binary for Mac OS X 10.6).

RESULTS

The difference between the invasive species and the native species

The results for the morphological traits can be observed in Figure 23 and the results for ANOVA in Table 12. The alien grass, *M. minutiflora,* grew more than the native grass both in height and biomass in any watering regime (Constant Watering, Intermittent Watering and Intermittent Watering with Fertilization) and in any moisture level (Severe Drought, Mild Drought, Control and Flood). It also presented higher assimilation rates and water use efficiency (A/E) in Severe Drought (Figure 24). Regardless of watering regime and moisture level, oxidative damage was lower for the invasive species, reflecting on lower levels MDA (below 4 $mmol.gFW^{-1}$) (Table 13). Molasses-grass also differed from the native species by presenting higher ϕ_{PSII}, lower NPQ and lower Chl_a/Chl_b (Figure 25).

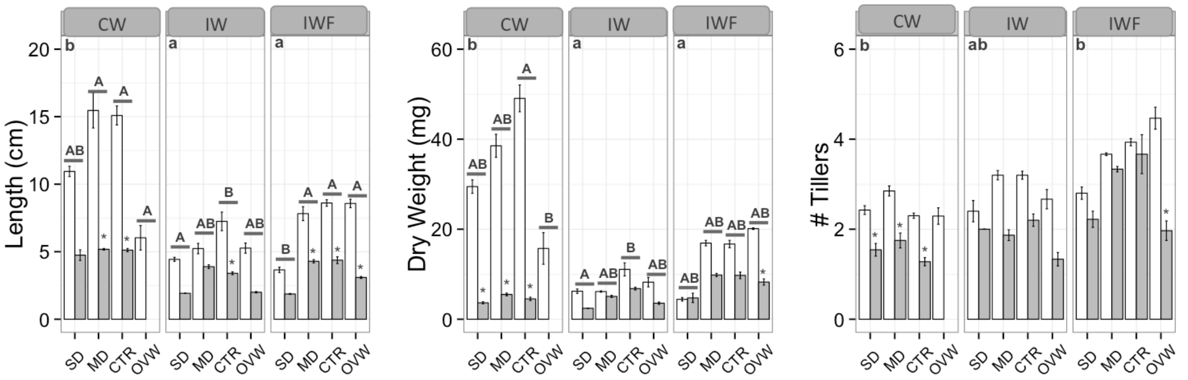

Figure 23. Morphological traits of one invasive (*Melinis minutiflora*, white bars) and one native (*Schizachyrium michrostachyum*, grey bars) grass. Two-month old plants were clipped and left to recover for 4 weeks on three different watering regimes: Constant Watering (CW), Intermittent Watering (IW), Intermittent Watering with NPK (IWF), each with four moisture levels: Severe Drought (SD), Mild drought (MD), Control (CTR) and Flood (OVW). Error bars correspond to standard deviation. Asterisks to significant differences between species, lower-case letters to differences among watering regimes and capital letters to differences among moisture levels. (P<0.05, Factorial ANOVA with watering regimes, species, moisture level as independent variables, Tukey HSD test for multiple comparisons).

Table 12. Factorial ANOVA scores for the measured dependent variables of Two-mont old plants exposed to different watering regimes, clipped and left to recover for 4 weeks, using three independent variables as factors: Species (*Schizachyrium microtachym)* and (*Melinis minutiflora*), Moisture levels (Severe Drought, Mild Drought, Control Flood) and three watering regimes (Constant Watering - CW, Intermittent Watering –IW, and Intermittent Watering with Fertilization – IWF). NS=Not Significant, (-) No comparison made due to lack of material.

	Watering regime (3-way)		CW (2-way)		IW (2-way)		IWF (2-way)	
	Error	**p**	Species	Moisture	Species	Moisture	Species	Moisture
Length	**4.532**	**<0.001**	0.0002	0.0065	0.0464	NS	0.0247	0.0132
Dry weight	**38.433**	**<0.001**	0.0032	0.0001	NS	NS	0.0719	0.0217
N of tillers	**0.749**	**<0.001**	0.0078	NS	NS	NS	NS	NS
Nitrate	**-**	**NS**	NS	NS	-	-	-	-
gs	**-**	**NS**	NS	NS	-	-	-	-
A	**-**	**NS**	0.0008	NS	-	-	-	-
A/E	**-**	**NS**	0.0034	0.0140	-	-	-	-
C_i/C_a	**-**	**NS**	0.0066	0.0105	-	-	-	-
Chlorophyll a	**-**	**NS**	NS	NS	NS	NS	NS	NS
Chlorophyll b	**0.022**	**0.009**	NS	NS	NS	NS	NS	NS
Carotenoids	**-**	**NS**	NS	NS	NS	NS	NS	NS
Chl_a/Chl_b	**0.023**	**NS**	0.0040	NS	NS	NS	NS	NS
F_v/F_m	**0.002**	**<0.001**	NS	NS	NS	NS	NS	NS
ϕ_{PSII}	**0.003**	**0.0079**	NS	0.0451	0.0491	NS	NS	NS
NPQ	**0.517**	**NS**	0.0011	NS	0.0016	NS	0.0011	NS
MDA	**2.413**	**<0.001**	0.0183	NS	NS	-	NS	-
G-POX	**2.297**	**<0.001**	-	NS	NS	NS	0.0246	0.0067
GST	**2.011**	**0.04**	-	NS	11.845	0.002	4.489	0.617
Catalase	**786**	**<0.001**	-	NS	NS	NS	0.1187	0.0449

The differences due to the watering regime (CW vs IW vs IWF)

In general, the CW regime differed from the other two by presenting accentuated growth for both species. The presence of fertilization (IW vs. IWF) didn't cause as high an effect as the change in watering regime (CW vs. IW) did (Figure 24, Table 12). However, fertilization did improve the performance of *M. minutiflora* in mild drought, in comparison to unfertilized groups (IW). Fertilization also increased tillering for both species (all p <0.001).

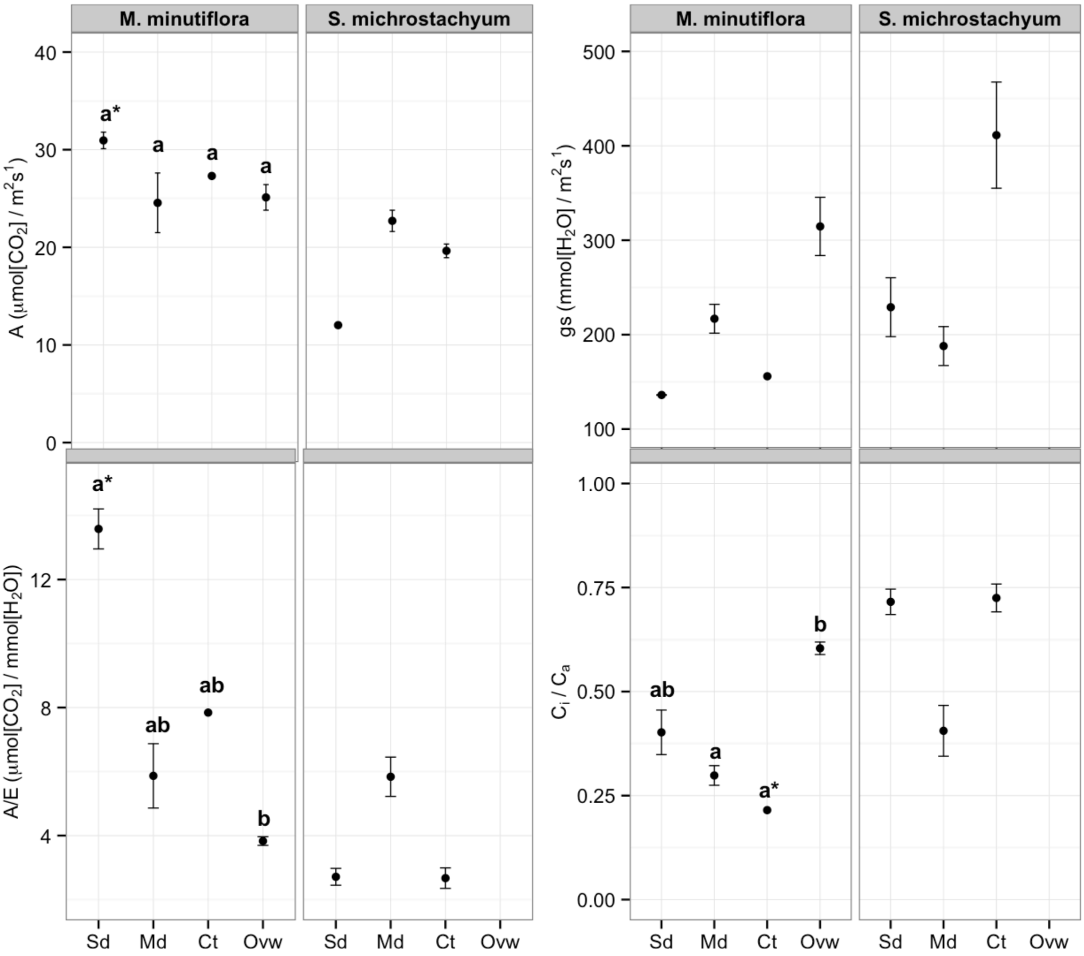

Figure 24 Gas exchange parameters of one invasive (*Melinis minutiflora*) and one native (*Schizachyrium michrostachyum*) grass. Two-month old plants were clipped and left to recover for 4 weeks on different soil moisture levels: Severe Drought (Sd), Mild Drought (Md), Control (Ct), Flood (Ovw). Error bars correspond to standard errors. Big asterisks refer to significant differences between species and small asterisks correspond to significant differences between species in that specific treatment. Lower case letters correspond to differences between treatments. (*P*<0.05, Factorial ANOVA with species and watering level as independent variables, Tukey HSD test for multiple comparisons). Growth was conducted at 28 ºC , with a photoperiod of 12h of ligh.

The differences between species were greater in CW, where invasive plants produced more biomass than in the other regimes (all $p < 0.001$) and more tillers than the native plants ($p=0.008$). Also, the Flooding treatment in the CW regime was more harmful to the native species than the Flooding treatment in other watering regimes. As a result, no native plant was able to re-sprout after clipping when kept in these conditions. Furthermore, the CW regime presented plants with lower concentration of chlorophyll b and slightly lower F_v/F_m values ($p=0.007$, $p<0.001$, Table 14). The moisture level in the CW regime did not affect enzymatic activity.

The difference due to the moisture level (From drought to flood)

Drought affected both the native and invasive species, with significantly less biomass accumulation than control groups (Figure 23, Table 13). The native species' assimilation rate was negatively affected in Severe Drought (all $p<0.001$, Figure 24). The same did not happen to the invasive species: higher assimilation rate reflected in an increased A/E in Severe Drought ($p=0.014$, Figure 24).

Pigment concentration and F_v/F_m values were not affected by moisture level and neither differed among species (Table 13, igure 25). Lipid peroxidation increased significantly in drought only for the native species and was even higher when in Severe Drought ($p=0.018$, Table 13 and Table 14). For *M. minutiflora* samples in Severe Drought in IW, CAT activity increased ($p=0.013$) while GST decreased ($p=0.0053$). GPOX decreased for both species ($p=0.0012$ and $p=0.0044$) with drought. Flood negatively affected both species in CW but didn't affect *M. minutiflora* in IW or IFW (Figure 23).

Table 13. Mean values (± SD) of MDA and enzymatic activity of the native (*Schizachyrium michrostachyum)* and invasive (*Melinis minutiflora*) grasses under different soil watering regimes : Constant Watering (CW), Intermittent Watering (IW) and Intermittent Watering with NPK (IWF). Each one of these regimes has four different soil moisture levels Severe Drought (SD), Mild Drought (MD), Control (CTR) and Flood (OVW). (and moisture levels. The p column shows differences between moisture levels. Letters refer to differences among moisture levels: Severe drought (SD), Mild drought (MD) Control (C) and Flood (F) within each watering regime. Asterisks refer to significant difference between species.

Endpoint	Watering	Fertilized	*p*	*M. minutiflora*	*S. microstachyum*
MDA (mmol gFW^{-1})	CW	SD	a	4.03 (±0.08)	8.32 (±0.38) *
	CW	MD	b	3.50 (±0.20)	6.56 (±0.18) *
	CW	C	c	3.57 (±0.18)	4.99 (±0.03) *
	CW	F	c	2.88 (±0.17)	-
	IW	SD	ns	-	-
	IW	MD	ns	-	-
	IW	C	ns	2.40 (±0.11)	-
	IW	F	ns	2.05 (±0.04)	-
	IWF	SD	ns	-	-
	IWF	MD	ns	2.50 (±0.29)	-
	IWF	C	ns	2.28 (±0.22)	-
	IWF	F	ns	-	-
GPOx (µmol ml^{-1} prot^{-1}	CW	SD	ns	3.10 (±0.41)	-
	CW	MD	ns	2.79 (±0.19)	-
	CW	C	ns	3.01 (±0.40)	-
	CW	F	ns	-	-
	IW	SD	ns	2.20 (±0.59)	-
	IW	MD	ns	33.4 (±0.69)	1.71 (±0.41)
	IW	C	ns	3.01 (±0.51)	2.61 (±0.59)
	IW	F	ns	3.30 (±0.69)	1.8 (±0.01)
	IWF	SD	a	3.70 (±0.49)	-
	IWF	MD	a	4.65 (±0.33)	1.47 (±0.14)
	IWF	C	b	7.42 (±0.44)	3.87 (±0.68) *
	IWF	F	ab	5.99 (±0.80)	4.18 (±0.21)
GST (ηmol ml^{-1} prot^{-1} min^{-}	CW	SD	ns	4.58 (±0.63)	-
	CW	MD	ns	3.65 (±0.34)	-
	CW	C	ns	3.11 (±0.71)	-
	CW	F	ns	-	-
	IW	SD	a	3.19 (±0.68)	-
	IW	MD	b	6.28 (±0.68)	3.76 (±1.17)
	IW	C	a	3.10 (±0.57)	3.73 (±0.24)
	IW	F	a	2.02 (±0.04)	2.18 (±0.01)
	IWF	SD	a	1.07 (±0.08)	-
	IWF	MD	a	1.63 (±0.24)	3.52 (±0.22) *
	IWF	C	b	2.82 (±0.41)	3.86 (±0.29)
	IWF	F	b	3.01 (±0.21)	4.95 (±0.48) *
Catalase (mg gFW^{-1})	CW	SD	ns	64.8 (±8.3)	-
	CW	MD	ns	73.8 (±6.0)	-
	CW	C	ns	93.1 (±12.4)	-
	CW	F	ns	-	-
	IW	SD	ns	51.9 (±4.1)	-
	IW	MD	ns	60.7 (±4.6)	108.0 (±55.3)
	IW	C	ns	58.1 (±2.4)	71.0 (±26.0)
	IW	F	ns	76.9 (±11.8)	23.2 (±1.0)
	IWF	SD	a	71.9 (±6.9)	-
	IWF	MD	b	58.6 (±4.6)	10.2 (±3.5) *
	IWF	C	bc	27.3 (±0.5)	23.4 (±5.7)
	IWF	F	c	18.8 (±7.0)	13.2 (±2.4)

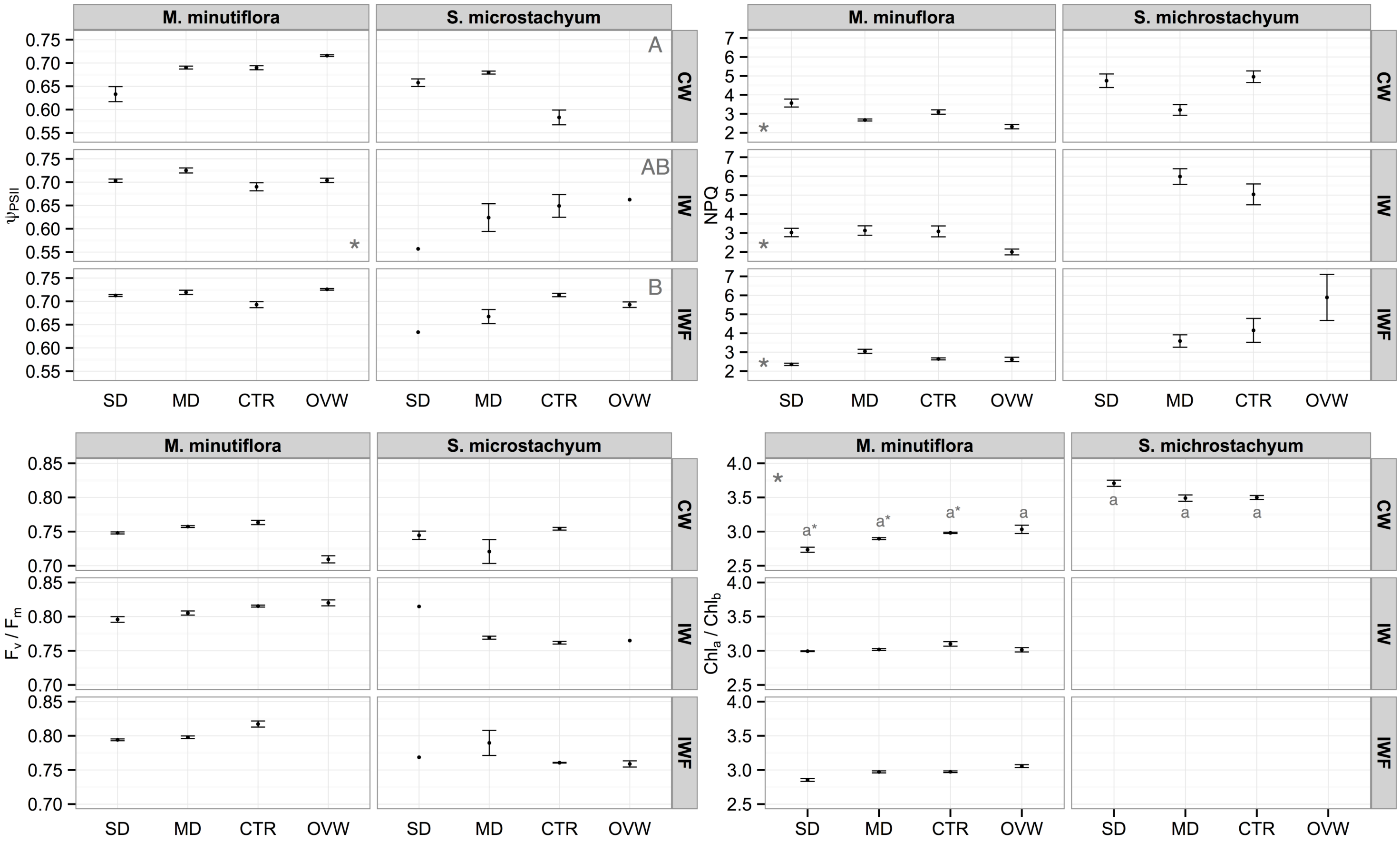

Figure 25. Fluorescence parameters and Chl_a/Chl_b ratio of one invasive (*Melinis minutiflora*) and one cerrado native grass (*Schizachyrium michrostachyum*). Two-month old plants were clipped and left to recover for 4 weeks in three different watering regimes: Constant Watering (CW), Intermittent Watering (IW) and Intermittent Watering with NPK (IWF). Each one of these regimes has four different soil moisture levels Severe Drought(SD), Mild Drought (MD), Control (CTR) and Flood (OVW). Error bars correspond to standard errors. Capital letters refer to significant differences among watering regimes, big asterisks refer to significant differences between species and small asterisks correspond to significant differences between species in that specific treatment. Lower case letters correspond to differences between treatments. (*P*<0.05, Factorial ANOVA with watering regimes, species, moisture level as independent variables, Tukey HSD test for multiple comparisons). Growth was conducted at 28 °C , with photoperiod of 12h of light

The difference between fire and clipping management

Fire was shown to be more harmful to plants than clipping, and only invasive plants were able to sprout after burning. Although tillering and plant length were marginally different between burned and clipped plants, clipped plants were able to accumulate significantly more biomass than burned plants ($p<0.001$) (Figure 26). Also, F_v/F_m values and NPQ were significantly lower for burned plants in Severe Drought and Mild Drought, respectively ($p=0.001$, $p=0.004$).

Table 14. Mean values (±SD) of leaf pigments of the native (*Schizachyrium michrostachyum*). and the invasive grass (*Melinis minutiflora*) under different watering Constant Watering (CW), Intermittent Watering (IW) and Intermittent Watering with NPK (IWF). Each one of these regimes has four different soil moisture levels Severe Drought(SD), Mild Drought (MD), Control (CTR) and Flood (OVW). Letters refer to differences among moisture levels. Asterisks refer to significant difference between species. (-) No comparison made due to lack of material.

Endpoint	Watering	Fertilized	*p*	*M. minutiflora*	*S. microstachyum*
Chlorophyll a (µmol gFW^{-1})	CW	SD	ns	0.92 (±0.08)	1.49 (±0.32)
	CW	MD	ns	0.93 (±0.04)	1.74 (±0.50)
	CW	C	ns	1.19 (±0.08)	0.88 (±0.08)
	CW	F	ns	1.33 (±0.14)	-
	IW	SD	ns	1.34 (±0.04)	-
	IW	MD	ns	1.44 (±0.16)	-
	IW	C	ns	1.13 (±0.11)	-
	IW	F	ns	1.12 (±0.08)	-
	IWF	SD	ns	1.59 (±0.22)	-
	IWF	MD	ns	1.54 (±0.08)	-
	IWF	C	ns	1.18 (±0.13)	-
	IWF	F	ns	1.74 (±0.13)	-
Chlorophyll b (µmol gFW^{-1})	CW	SD	ns	0.34 (±0.03)	0.40 (±0.08)
	CW	MD	ns	0.32 (±0.01)	0.52 (±0.17)
	CW	C	ns	0.40 (±0.03)	0.25 (±0.02)
	CW	F	ns	0.44 (±0.06)	-
	IW	SD	ns	0.48 (±0.01)	-
	IW	MD	ns	0.48 (±0.06)	-
	IW	C	ns	0.36 (±0.04)	-
	IW	F	ns	0.37 (±0.02)	-
	IWF	SD	ns	0.56 (±0.08)	-
	IWF	MD	ns	0.52 (±0.03)	-
	IWF	C	ns	0.40 (±0.04)	-
	IWF	F	ns	0.57 (±0.05)	-
Carotenoids (µmol gFW^{-1})	CW	SD	ns	0.659(±0.041)	0.894 (±0.162)
	CW	MD	ns	0.623 (±0.026)	1.086 (±0.315)
	CW	C	ns	0.787 (±0.052)	0.555 (±0.034)
	CW	F	ns	0.823 (±0.098)	-
	IW	SD	ns	0.780 (±0.015)	-
	IW	MD	ns	0.827 (±0.089)	-
	IW	C	ns	0.695 (±0.037)	-
	IW	F	ns	0.646 (±0.045)	-
	IWF	SD	ns	0.977 (±0.142)	-
	IWF	MD	ns	0.966 (±0.051)	-
	IWF	C	ns	0.680 (±0.067)	-
	IWF	F	ns	1.001 (±0.089)	-

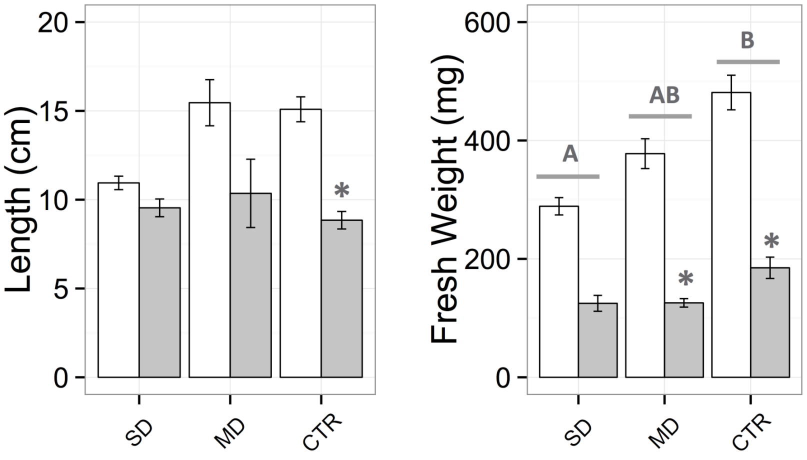

Figure 26. Comparison of growth traits between clipped and burned plants of *Melinis minutiflora*. Two-month old plants were clipped (white bars) or burned (grey bars) and left to recover for 4 weeks on different soil moisture levels: Severe Drought (SD), Mild drought (MD), Control (CTR) and Flood (where no plant recovered). Error bars correspond to standard deviation. Asterisks refer to significant differences between clipped and burned plants, lower-case letters to differences among moisture levels. (*P*<0.05, Factorial ANOVA with watering regimes, species, moisture level as independent variables, Tukey HSD test for multiple comparisons). Growth was conducted at 28 °C , with photoperiod of 12h of light

The recovery

The invasive species recovered to over 75% of the former height and over 50% of previous biomass in CW. The recovery was of 50% and 25% for the same parameters, respectively, in IW and IWF (Figure 27). The native species recovered 50% and 25% of its height and biomass in CW regime and 25% and 15% of the same parameters, respectively, in IW and IWF. Assimilation rate, total chlorophyll and nitrate increased for both species in relation to pre-clipping values (all $p<0.001$). The native species' NPQ and Chl_a/Chl_b values were raised in Severe Drought in relation to pre-clipping values ($p<0.001$, $p=0.049$, data not shown).

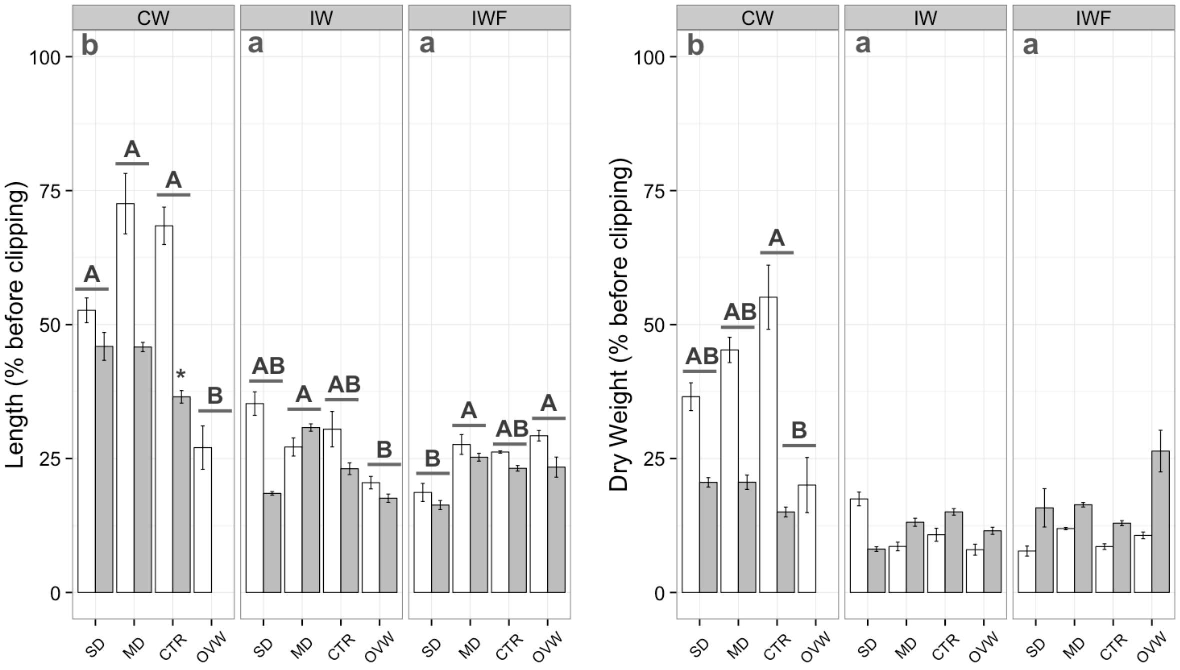

Figure 27. Percentage of recovery in comparison to pre-clipped values (Chapter 4 and 5). Morphological traits of one invasive (*Melinis minutiflora,* white bars) and one native (*Schizachyrium michrostachyum,* grey bars) grass. Two-month old plants were clipped and left to recover for 4 weeks on three different water availability regimes (Constant Watering, Intermittent Watering, Intermittent Watering with Fertilization - CW, IW, IWF) and four moisture levels: Severe Drought (SD), Mild drought (MD), Control (CTR) and Flood (OVW). Error bars correspond to standard deviation. Asterisks refer to significant differences between species, lower-case letters to differences among watering regimes and capital letters to differences among moisture levels. (*P*<0.05, Factorial ANOVA with watering regimes, species, moisture level as independent variables, Tukey HSD test for multiple comparisons. Values were normalized with arcsin transformation). Growth was conducted at 28 ºC, with photoperiod of 12h of light

DISCUSSION

As expected, the invasive species performed better than the native species did, by growing higher, accumulating more biomass and producing more tillers. Similar results were obtained by Baruch & Jackson (2005), Simões & Baruch (1993), Baruch & Bilbao (1999) and Aires (2013). However, it is important to notice that the differences between species were much higher when the watering regime was keeping water continuously available in the soil (CW). This regime could represent poorly drained soils, which even in the dry season are able to retain more water.

Even when in low water availability (SD and MD) molasses-grass could overgrow the native species. In terms of percentage (Figure 27), plants recovered up to 50-75% in CW. This response may represent a compensatory growth since they grew over half as much, in half the time of pre-clipped plants. Many studies reported compensatory growth in plants. Compensation of the removed biomass might appear as an adaptation to herbivory and fire, or as a response to removal of apical dominance (Silva & Raventos 1999, Leite *et al*. 1997, Sarmiento 1992, Hjálten *et al*. 1993). Accordingly, Baruch & Jackson (2005) reported that African grasses recovered faster than South American grasses from simulated herbivory. Tillering could be another efficient way to compete for space. Production of tillers only increased after clipping for the invasive species samples in the IW regime (p=0.0047, Figure 23). Simões & Baruch (1993) also observed an increased tillering in an African species after clipping while the American native species tillered less with clipping. However, Klink (1994) found no effect of clipping on tillering of *M. minutiflora* in the field so this result must be regarded with caution.

On the other hand, plants recovered only 25% in IW and IWF, showing that persistence of the water in the soil was more important than the presence of fertilization. In these regimes, water percolated more easily, and the plants had access to the water for a shorter period of time, what may have influenced their recovery. Interestingly, and IW and IWF presented higher growth rates in comparison to CW plants prior to clipping (Chapter 4 and 5). In this context, Oesterheld & McNaughton (1991) and Hicks & Turkington (2000) explain that plants with lower growing rates and in poorer soils present a more positive response to defoliation than plants growing at higher rates.

Clipping reduced the differences between species in the IW and IWF regimes. In relation to pre clipping values (Chapter 4 and 5), native plants recovered a higher percentage of biomass in comparison to the invasive plants (Figure 27). In CW, the invasive species continued to grow twice as much as the native species did. We hypothesized that when water is available every day, even in low quantities, the invasive species can readily and efficiently use it by taking advantage of its extensive root system (Baruch 1984, Silva & Haridasan 2007). Klink (1994) also showed that clipping in soils where water was more readily available resulted in better recovery for *M. minutilfora*. This observation is in accordance with Zhao (2008), who showed that water availability was also much more important to plant recovery to clipping than the addition of fertilizers. Hicks & Turkington (2000) showed that fertilization reduced the ability of clipped plants to compensate for biomass loss. This information may help to explain the lack of differences between IW and IWF, even though the fertilized group was much heavier than the unfertilized group prior to clipping (Chapter 4 and 5).

On the other hand, when soil is well drained (here represented by the IW and IWF plants that were in clay pots) and water is not always provided every day, both species tend to recover at a closer rate after clipping. Differences between the species in IW were noticed only when they were also fertilized (IWF). Although the invasive plants grew more than the native plants (Figure 23), the native plants recovered more in relation to pre-clipping values (Figure 27). Accordingly, Leriche *et al*. (2003) showed that African grasses needed fertilization to compensate defoliation.

We assume that the IW regime is the best representation of the natural conditions of Cerrado soils: mostly red-latossols, well drained and dystrophic. Irrigation suspension may represent the dry season, when it rarely rains, as well as dry spells that may occur in the rainy season (Eiten 1972). In this scenario both species would have similar recovery after management is applied. However, the Cerrado also presents areas with more water availability, near watercourses, for instance. Also, the Cerrado's soil can have its fertility altered by atmospheric deposition of fertilizers from agricultural areas or from fossil fuel burning in urban areas (Jordan & Weller 1996, Vitousek *et al*. 1997). Our data suggests that in those cases, the invasive species would show better recovery than the native species would.

Fire was only used in the CW regime. No native plant sprouted after fire. Burned invasive plants were slightly shorter (p=0.02) and with lower biomass at Severe Drought (p=0.018) and Control (p=0.0049) than those submitted to clipping. Also, burned plants tended to produce fewer tillers than clipped ones. A similar result was obtained by Leite *et al*. (1997) and Klink (1994), who found a simplification of grass architecture with fire. No burned plant sprouted in the Flood treatment. Although native plants were expected to be able to sprout after fire, it is important to take into account the age and size of the plants. Native plants were much smaller than the invasive plants, which might have interfered in their ability to recover after fire. Also, burning conditions (fire intensity and time of residence) were not controlled. These properties interfere in the effect of fire on plants, so more effort should be applied in controlling these variables in future studies.

However, it was interesting to notice that fire did bring more damage to the invasive species than clipping did. In field conditions, where native species are well established and in greater number and richness, this might be of some advantage to native plants in relation to *M. minutiflora*. Baruch & Bilbao (1999) also found that fire was more harmful than clipping to an African grass in the Venezuelan savanna. For these authors, burning resulted in lighter and shorter resprouted individuals. On the other hand, Haddad & Valio (1993) and Coutinho (1990) showed increased flowering in Cerrado plants after fire and pruning. Furthermore, Sarmiento (1992) describes fire-dependant behaviors and trade-offs in savanna plants. Therefore, in field conditions, colonization by native species might be enhanced by fire. Nevertheless, fire may promote invasion by other invasive species by favoring early successional species (Keeley 2005).

It was not possible to measure some parameters (gas exchange, MDA, pigments and enzymatic activity) in all conditions due to lack of material. However, it was possible to observe that pigment concentration was barely affected and didn't differ among species and moisture levels. Also, F_v/F_m values were always above 0.75 and similar to pre-clipping values (Chapter 4 and 5), showing that the plants' photosystems remained healthy. However, CW plants showed slightly less chlorophyll and smaller F_v/F_m values. However, Chaves *et al.* (2003) explained that leaves with less access to water tend to present higher photosynthetic capacity and chlorophyll content. This optimization might be happening in the IW and IWF regimes. The Chl_a/Chl_b ratio and NPQ were significantly higher while ϕ_{PSII} was significantly lower for the native species. This response might be a result of an increased oxidative stress in the native plants (Huang *et al.* 1997).

Unlike the results from pre-clipping (Chapter 4 and 5), stomatal conductance did not differ among species or among treatments in CW. Assimilation rate continued to be higher for the invasive species, which presented higher water use efficiency in Sever Drought. This result explains the tolerance of the invasive species to drought and its ability to grow faster than the native species after clipping. Many studies showed that African grasses present compensatory growth when defoliated (Sarmiento 1992). Simões & Baruch (1997) found increased assimilation rate with clipping in an African grass; however, in their study the increase was not affected by water stress. Furthermore, Baruch & Jackson (2005) show an increase in growth of *M. minutiflora* after clipping and discuss that the increase in Assimilation rate might be an evolutionary response of African grasses to large herbivores.

Lipid peroxidation and the activity of oxidative stress enzymes displayed the same pattern as pre-clipping values (Chapter 4 and 5). In this case, MDA content was higher in the native species, especially in the driest treatments. High MDA concentration reflects a greater oxidative damage and a weaker tolerance to drought in comparison to the invasive plants (Correia *et al*. 2014, Gill & Tuteja *et al*. 2010). The invasive species showed a higher activity of CAT and GPOX, which may represent a stronger general antioxidant response. CAT activity is important in drought stress with drought-tolerant plants presenting higher CAT activity (Arora e al. 2002), while transgenic plants with low CAT activity tend to respond negatively to environmental stress (Chamnongpol *et al*. 1996). GPOX activity, as the other ezymes, specific to drought stress, but has been shown to increase with chemical stress (Silva *et al*. 2013), which is in accordance with higher activities of this enzyme in the fertilized group. The native species presented higher GST activities than the invasive species, and they increased with water availability. Although this enzyme plays many roles in plants (Dixon *et al*. 2002), its increase may suggest an anti oxidative response. For instance, tobacco clones are more tolerant to environmental stress when they present an overexpression of GST (Roxas *et al.* 2000).

In contrast to pre-clipping values, leaf chlorophyll content and nitrate tended to increase after clipping. Zhao *et al*. (2008) also showed a relation between clipping frequency and increase in chlorophyll concentration in leaves. Furthermore, Oesterheld & McNaughton (1991) reported higher concentrations of nitrogen on plants re-growing after clipping. Also, the increase in NPQ and Chl_a/Chl_b ratio for the native species in drought is an indicative of increased oxidative stress in relation to pre-clipping values. However, a more careful comparison could not be made. Samples from before and after clipping were processed with different dilution factors (due to lack of material) adding more error to the data, and therefore, a direct comparison would be inappropriate.

CONCLUSIONS

The invasive species performed better than the native species even after clipping and fire. However, clipping reduced the differences in growth between native and invasive species. This reduction was evident in mild drought conditions and when watering was intermittent with no fertilization. Such conditions could represent the dry season in the most frequent soil type in the Cerrado (dystrophic red latossol). Fire was more harmful than clipping. Data suggests that clipping should be applied in the early dry season in areas with low nutrient and water availability, and in places with presence of native species/seed bank to colonize the area.

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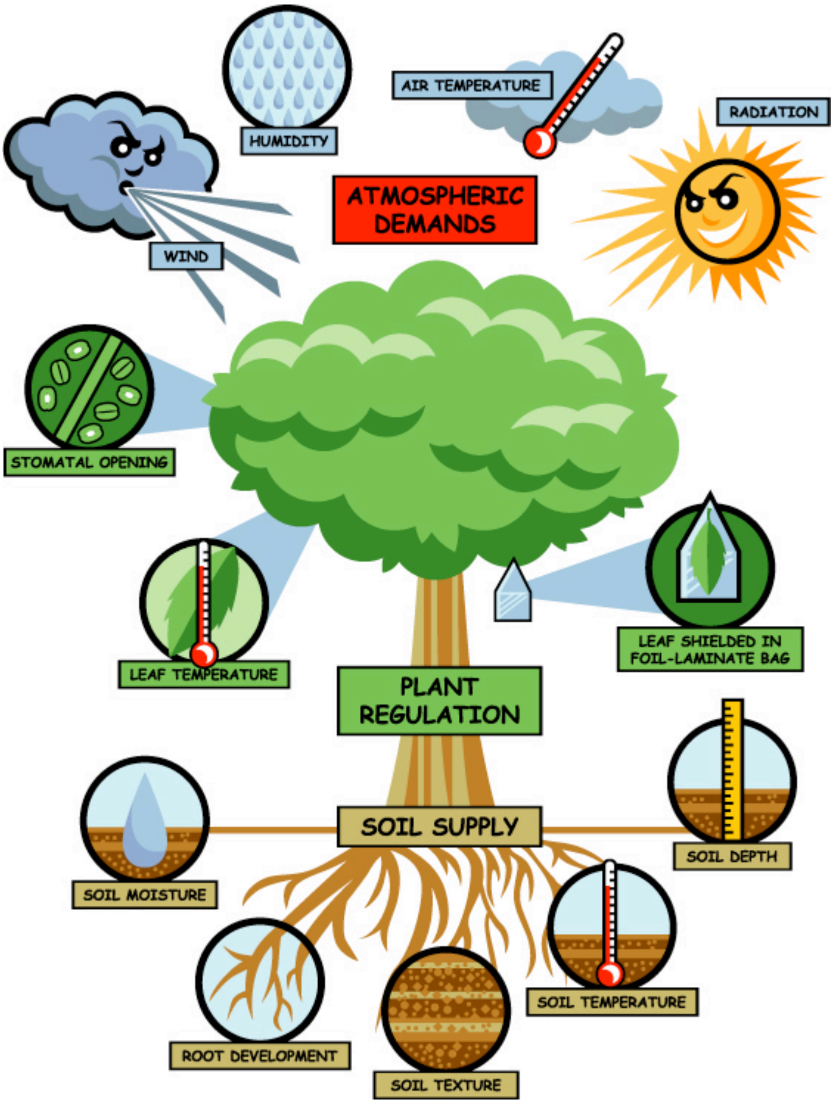
CHAPTER SEVEN:

GENERAL DISCUSSION AND CONCLUSIONS



APPROACH and MEASURED TRAITS

The chosen approach enabled the assessment of the effects of the environmental factors naturally found in the Cerrado and of management techniques on several traits, from the community to the biochemical level. Together these data might help to understand the functioning of the Cerrito ecosystem and shed some light on how susceptible this biome would be to invasion under these different scenarios. Furthermore, the data summarized in this thesis may come in hand when planning effective management techniques to control the invasive grass *M. minutiflora* and serve as a start point for other invasive grass species.

(A) Gas exchanges and chlorophyll fluorescence: assessing photosynthesis in vivo

The invasive species was shown to have higher assimilation rates, especially in CTR conditions. Furthermore, WUE was higher for the native species, which reflected on the strong plant growth. The invasive species was most harmed by flooding conditions, and this reflected on high stomatal conductance, probably in an attempt to manage the excess of water. However, assimilation rates were very low, probably from root damage. Impairment in growth seems to be more related to stomata closure than to damage to PSII (see next section, Zanella *et al.* 2004). Four weeks after clipping, the invasive species still showed higher growth rates. However, the native plants presented a higher relative recovery of biomass when in control conditions.

In general, all measurements showed that leaves had healthy photosynthetic systems, with F_v/F_m values above 0.75, while ϕ_{PSII} (effective quantum yield of PSII) tended to be around 0.6. The effective quantum yield is naturally lower than the optimum quantum yield because of the activation of the reactions centres, i.e., the reduction of maximum fluorescence in light-adapted leaves is a result of the mechanisms of quenching, which initiate with photosynthetic active leaves, thus reducing the variable fluorescence F_V and reducing the yield.

The native species tended to present lower F_v/F_m values. Although this is not a direct measure of the photosynthetic rate and carbon assimilation, it represents an inference of photosynthetic activity, which seems to be less pronounced in this species and may partially explain its lower biomass accumulation. These values are also in conformity with nitrate values, which were lower for this species. Lower nitrate values may reflect less effective absorption of nutrients, which leads to defective enzyme production and lower photosynthetic capacity. However, it is also usual to find inactive photosystems in plants (Chylla & Whitmarsh 1989), so this may be a species specific response. In theory, drought stress could lower the maximum quantum yield, and some studies have confirmed this hypothesis (Bjorkman & Powles 1984, He *et al.* 1995). However, many studies found minor or negligible changes on F_v/F_m values in response to drought and fertilization (Genty *et al.* 1987, Da Matta *et al.* 1997, Sanchez-Rodriguez 1997, Silva *et al.* 2002), confirming the stability of the photosystems to water stress. Other studies show increases in F_v/F_m values during drought (Correia *et al.* 2014), which might be a compensatory response to mild stress. Rather than being due to damage to photosystems, decreases in photosynthesis are mostly attributed to differences in usage of N by photosystems (Heckathorns *et al.* 1997) as well as to hormonal responses that induce stomata closure (Queiroz *et al.* 2002).

Plants under drought activate mechanisms to balance the excessive energy and prevent damage to their photosynthetic apparatuses (Casper *et al.* 1993, Lima *et* *al.* 2002). In this study this could be observed as the higher NPQ values under mild drought. Non-photochemical quenching has been shown to increase with stress and is associated with protective downregulation, reversible once they are watered (Heckathorn *et al*. 1997). In addition, it was observed that mutants of the model plant species *Arabidopsis thaliana* with lower NPQ capacity perform worse (Li *et al*. 2002).

Fluorescence parameters could be particularly interesting in our study to understand the physiological responses of these grasses in different conditions of light, which would simulate the canopy gradient naturally present in the Cerrado (Castro & Kauffman 1993). Invasive grasses are known for being very successful in open areas, while being less effective in invading shaded sites. In fact, shading is also proposed as a control technique for these grasses. Furthermore, all invasive grasses are C4 grasses, which present a differential distribution of the biochemical apparatus for carbon fixation, creating a more effective way of capturing carbon by reducing photorespiration (Taiz & Zeiger 1998). However, this mechanism is sensitive to low light intensities, explaining their success in the tropical savannahs, characterized by high radiation intensities. Although the majority of Cerrado grasses mainly have the C4 photosynthetic pathway, there is also a C3 grass, *Echinolaena inflexa*, which could be an interesting subject to compare to the invasive species in different light regimes. In the present study this grass was only studied regarding its germination in post-fire environments. It is a grass with low seed production and medium germination rate. However, it could, in theory, benefit from fire in closed canopy sites.

Other than using the fluoresce approach in different light regimes, this study could also benefit from the measurement of other parameters as a more detailed research on non photochemical quenching and the use of rapid light curves, which could lead to further insights about the functioning of photosystems in these plants in different light intensities.

(B) Growth: The invasive species performs better

This study assessed the effect of an *M. minutiflora* invasion in an ecosystem's functioning through the bait-lamina test. It also measured seed germination parameters and morphological, physiological and biochemical traits of young plants. The bait lamina test is the approach used in chapter 3 has been reported by Römbke (2014) as a basis for other works within similar regions and case-studies or in order to improve the bait-lamina method.

There is much in evidence in the literature of this invasive species' effects on biodiversity and fire regime. Furthermore, data has shown that it could also affect ecosystem functioning by affecting soil. In places where this plant was well-established soil feeding activity increased tremendously. This study also confirmed the high and fast germination rate of this species, as well as a high seedling growth rate and tolerance to fire and stress.

Germination is the first bottleneck that plants have to go through when colonizing an environment. *M. minutiflora* has high productions of seeds, seed viability and germination rate, as well as a fast germination. This early establishment is very important and Sarukhan & Harper (1973) showed that plants that establish themselves first are less susceptible to competitive effects when mature.

Furthermore, fertilization enhanced the performance of the invasive species in a more pronounced way than in the native species, probably reflecting a better efficiency in nutrient absorption and use. This was confirmed by a higher concentration of nitrate on leaves, especially when fertilized. This study would also benefit from the measurement of nutrients on senescent leaves. This parameter would elucidate the recycling of nutrients. Native species are known to have high rates of nutrient reabsorption from senescent leaves. However, studies have shown even higher rates of nutrient reabsorption for this invasive species when compared to native grasses. Experiments with fertilization in the Cerrado have shown to promote faster nutrient cycling, leading to litter degradation (Jacobson *et al*. 2011). This condition would probably increase the susceptibility of the environment to the opportunist invaders. Baruch *et al*. (1989) has also shown higher efficiency of nutrient allocation to leaves in *M. minutiflora,* which, combined with lower costs for tissue building, helps to explain its high growth rate and rapid response to fertilization.

Constant water availability and/or water in high quantities promote a better performance of the invasive species, making its biomass allocation even greater than the native species'. This is particularly true when we compare data from Chapter 4 and 5, which had different watering regimes. We hypothesized that when water is available every day, even in low quantities, the invasive species is able to readily and efficiently use it with its extensive root system (Silva & Haridasan 2007). Many studies showed the advantages that African grasses take with high availability of resources. Also, Klink (1994) has shown that clipping in soils where water was more available resulted in better recovery for *M. minutilfora*. Baruch & Fernadez (1993) discusses the trade-offs of African grasses that present higher growth rates than American grasses in a non-limiting environment. These same opportunistic grasses presented less plasticity in adverse situations, when the native species present better evasion mechanisms.

This species' fast response to nutrients, tolerance to drought and its effect on the soil's biological activity may also be a reflection of its extensive root biomass. This characteristic may facilitate water uptake from deeper soil levels, also enabling a fast interception of surface water. Cerrado grasses presented a high root:shoot ratio, but *M. minutiflora* presented much greater underground biomass allocation. This root biomass may be the reason of the increase of biological activity in the soil, which is known to be higher near plant roots, due to the favourable microenvironment created for soil biota by root exudates.

Root biomass is also crucial in competition, mainly for nutrient and water acquisition. Many studies showed the importance of underground competition as well as aerial competition (Snaydon & Howe 1986, Silva & Castro 1989). Furthermore, Wilson & Shay (1990) have shown that root competition has a more specific role in competition among grasses. *Paspalum stellatum*, for instance, presents better performance when protected from root competition with *M. minutiflora* (Aires 2013).

All of these evidences confirm the ease this species has to invade and establish itself in the environment, depleting biodiversity by outcompeting native grasses.

This study assessed the effect of an *M. minutiflora* invasion in an ecosystem's functioning through the bait-lamina test. It also measured seed germination parameters and morphological, physiological and biochemical traits of young plants. The bait lamina test is the approach used in chapter 3 has been reported by Römbke (2014) as a basis for other works within similar regions and case-studies or in order to improve the bait-lamina method.

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Germination is the first bottleneck that plants have to go through when colonizing an environment. *M. minutiflora* has high productions of seeds, seed viability and germination rate, as well as a fast germination. This early establishment is very important and Sarukhan & Harper (1973) showed that plants that establish themselves first are less susceptible to competitive effects when mature.

Furthermore, fertilization enhanced the performance of the invasive species in a more pronounced way than in the native species, probably reflecting a better efficiency in nutrient absorption and use. This was confirmed by a higher concentration of nitrate on leaves, especially when fertilized. This study would also benefit from the measurement of nutrients on senescent leaves. This parameter would elucidate the recycling of nutrients. Native species are known to have high rates of nutrient reabsorption from senescent leaves. However, studies have shown even higher rates of nutrient reabsorption for this invasive species when compared to native grasses. Experiments with fertilization in the Cerrado have shown to promote faster nutrient cycling, leading to litter degradation (Jacobson *et al*. 2011). This condition would probably increase the susceptibility of the environment to the opportunist invaders. Baruch *et al.* (1989) has also shown higher efficiency of nutrient allocation to leaves in *M. minutiflora,* which, combined with lower costs for tissue building, helps to explain its high growth rate and rapid response to fertilization.

Constant water availability and/or water in high quantities promote a better performance of the invasive species, making its biomass allocation even greater than the native species'. This is particularly true when we compare data from Chapter 4 and 5, which had different watering regimes. We hypothesized that when water is available every day, even in low quantities, the invasive species is able to readily and efficiently use it with its extensive root system (Silva & Haridasan 2007). Many studies showed the advantages that African grasses take with high availability of resources. Also, Klink (1994) has shown that clipping in soils where water was more available resulted in better recovery for *M. minutilfora*. Baruch & Fernadez (1993) discusses the trade-offs of African grasses that present higher growth rates than American grasses in a non-limiting environment. These same opportunistic grasses presented less plasticity in adverse situations, when the native species present better evasion mechanisms.

This species' fast response to nutrients, tolerance to drought and its effect on the soil's biological activity may also be a reflection of its extensive root biomass. This characteristic may facilitate water uptake from deeper soil levels, also enabling a fast interception of surface water. Cerrado grasses presented a high root:shoot ratio, but *M. minutiflora* presented much greater underground biomass allocation. This root biomass may be the reason of the increase of biological activity in the soil, which is known to be higher near plant roots, due to the favourable microenvironment created for soil biota by root exudates.

Root biomass is also crucial in competition, mainly for nutrient and water acquisition. Many studies showed the importance of underground competition as well as aerial competition (Snaydon & Howe 1986, Silva & Castro 1989). Furthermore, Wilson & Shay (1990) have shown that root competition has a more specific role in competition among grasses. *Paspalum stellatum*, for instance, presents better performance when protected from root competition with *M. minutiflora* (Aires 2013).

All of these evidences confirm the ease this species has to invade and establish itself in the environment, depleting biodiversity by outcompeting native grasses.

Our results showed that the invasive species presented lower concentrations of MDA, a reflection of less damage to its membrane in relation to the native species. Furthermore, the native species showed an increase in MDA concentration with drought, which was not observed for the invasive, regardless of environmental conditions (moisture, fertilization, clipping). Therefore, drought either increases ROS in a more pronounced way in the native species or this is a reflection of a weaker scavenging system. This can be related to the native lower enzymatic activities of G-POX and CAT under drought (see below).

In the present study, there was no significant effect of environmental stress on carotenoid concentration. However, other studies have shown a decrease in carotenoids with environmental stress, which can potentially damage the plant's antioxidant protective system (Sairam & Saxena 2000). Part of the data suggests that the invasive species has a higher production of carotenoids, but the difference between species is reduced with fertilization. Higher carotenoids might confer a stronger antioxidant mechanism that enhanced plant growth and protected membranes from peroxidation in the invasive species. With fertilization, the native species' carotenoid concentration increased, but even then the invasive species still performed better. Therefore, molasses-grass must make use of other mechanisms to deal with environmental stress. Other antioxidants that would be interesting to measure would be the redox state of Glutathione and the concentration of Ascorbate. These molecules are important buffers in controlling the effects of ROS in plants. Ascorbate (ASH) is a soluble antioxidant that participates in detoxification by donating electrons to many reactions. It can directly scavenge superoxide and hydroxyl, and participate in the violaxantine cycle for energy dissipation. Glutathione (GSH) is a substrate for many reactions. It regenerates ASH, scavenges radicals and participates in the detoxification of xenobiotics. It can also conjugate with metabolites with the aid of GST. There are techniques to easily measure the concentration of these antioxidants using microplates (Queval & Noctor 2007). Further studies would benefit from these measurements.

SOD activity was higher for the native species, but only in the well watered treatments that were under fertilization. SOD activity was shown to increase with metal and salt concentrations (Elkahoui *et al.* 2005, Silva *et al*. 2013). Higher nutrient availability might be creating conditions that stimulate the native species to produce more ROS. This species is adapted to low concentrations of nutrients in the soil and doesn't present a positive response to fertilization as strong as *M. minutiflora* does. It was not possible to derive conclusion on this regarding severe drought exposure because the SOD activity was not measured in the native species due to lack of plant material. Higher SOD activity is consistent with MDA values for the native species. Studies showed increases in SOD activity with drought and enhanced drought tolerance in plants overexpressing SOD genes. However, the invasive species showed a decrease in its activity with drought (Zhang & Kirkham 1994, Gill & Tuteja 2010).

The invasive species presented higher levels of this enzyme and a significant increase under drought, even after clipping. This might be the cause of an efficient scavenging system that protected membranes from being damaged, reflecting on the low MDA concentrations under drought for this species. Other studies show increases of CAT with drought and its relations with tolerance to drought (Gamble & Burke 1984, Arora *et al.* 2002, Gill & Tuteja 2010). APX activity was similar between both species but was higher for the native species under drought, which might reflect this species' preferable pathway to deal with ROS, rather than by Catalase and G-

POX activity. Maricle *et al*. (2007) and Lima *et al.* (2002) also showed increase of APX with drought. This enzyme is particularly easy to measure and presents strong activity even at low concentrations, when compared to CAT and APX. However, not so many studies use this enzyme as a biomarker for oxidative stress and comparisons are more easily found in the literature for CAT, APX and SOD (see Gill & Tuteja 2010 for review). G-POX has been reported to increase with toxic compounds such as metals (Gill & Tuteja 2010). In this study, GPOX also increased with fertilization. However, the invasive species tended to present higher GPOX activity, especially under drought, which might be conferring protection against lipid peroxidation. Water availability seems to increase this activity for the native species. It is important to notice that flood was also detrimental to this species, causing damage and reduced growth. This was probably done by damaging the roots with an aerobic environment, thus impairing their growth.

GST activity was higher in native plants. However, GST enzymes are a large and diverse group, and can account for over 1% of a plant's soluble proteins, and its gene family is also highly diverse. GST participates in many reactions in the plant such as the elimination of herbicides, hormone homeostasis, transportation of flavonoids into the vacuole and signalling (Dixon *et al*. 2002). Therefore, GST is not specific to oxidative stress repair and a higher activity in the invasive species might be a reflection of another mechanism present in this species. However, drought and flood seemed to enhance its activity.

THE EFFECT OF MANAGEMENT

(A) Fire

This study has also shown that management could reduce the performance of the invasive species *M. minutiflora* P. Beauv*.* species and partially control its spread, by permitting other grasses to coexist with it.

In places with frequent fires, invaded patches had their soil's biological activity decrease to natural levels. Furthermore, post-fire temperatures, despite accelerating germination, were able to reduce the germination rate and viability of *M. minutiflora* seeds. On the other hand, fire seems to favour the germination of some plants. *Paspalum stelattum* Humb. and Bonpl. Ex Flüggé germination, for example, tended to increase with a longer period of post-fire environment (Chapter 2). Furthermore, Munhoz & Felfilli (2005) reported flowering for this species after burns and Aires (2013) recommends this species for restoration of degraded areas due to its relatively high germination rate, resilience to fire, and especially because of its high recruitment and seedling survival rate. Fire has never been reported to induce flowering in *M. minutiflora*; however, many native species bloom a few days after fire.

Burning has also been shown to be more damaging to this species than clipping, decreasing biomass allocation. Marinho (2013) has shown that frequent burns reduce the cover by this species and Marinho & Miranda (2013) has shown that burns can be lethal to mature plants of this species. D'Antonio *et al.* (2001) found mortality rates of over 70% for molasses-grass after burns. Fire also dramatically reduces its soil seed bank. In contrast, fire doesn't affect the density of native soil seed banks (Andrade & Miranda 2014). The high specific heat coefficient and the architecture of the individuals (with great accumulation of dead biomass near the ground) may create a favourable condition to intense fires, with high residence time in clumps of the invasive species, thus damaging the basal meristems and causing plant death (Baruch & Bilbao 1999, D'Antonio *et al.* 2001). Native species, on the other hand, present a more upright architecture, which might favour a quick fire (and, therefore, less damage). Neto *et al.* (1998) state that with high precipitation, the biomass of a *campo-sujo* (grassland with scattered bushed and small trees) area may completely recover within a year, while Barros *et al.* (2006) stated that this time is not enough to recover the fuel biomass of an invaded site. Also, frequent burns weaken plants by depleting the nutrients stored in roots, due to energy allocated to re-sprouting. In the present study no native species survived the experimental fire. However, further measurement should be done with other native species and in more controlled fire conditions (measuring intensity, time of residence etc.).

Fire, however, may promote invasion by other alien species and also momentarily increase soil fertility due to the input of ash, promoting invasion by opportunist species (Coutinho 1990). In the present study we measured the effects of *A. gayanus* in the functioning of the ecosystem. In the studied area this species didn't present the same effect as *M. minutiflora* in the soil feeding activity. However, other studies showed that this species has higher growth rates and is less damaged by fire (Marinho & Miranda 2013). Even with frequent burns, this species was able to increase its cover. This plant also produces a great amount of seeds, with high germination rate. Therefore, fire has to be regarded with caution and one might not use it in places where this species is present.

Furthermore, in areas with heavy invasions of *M. minutiflora* fuel biomass may reach over twice as much as in natural areas (Silva & Haridasan 2007, Hoffman & Haridasan 2008, Martins *et al.* 2011), promoting fires that are three times more intense (Marinho 2013). This may cause much more damage to native species. If the area does not present a native soil seed bank or nearby sources of seeds, the perpetuation of the invasive species might be enabled by fire.

(B) Clipping

Clipping didn't present a strong negative effect on the invasive species. Also, clipping would be more expensive and would require more labour to apply in extensive areas. However, other characteristics might favour the choice of this method instead of fire. First of all, clipping also promotes flowering of native plants (Haddad & Valio 1993). The effect is not as pronounced as with fire, but it is also present. Furthermore, this effect is not observed in the invasive species *M. minutiflora* or in *A. gayanus*. Klink (1994) has also shown that *M. minutiflora* is not able to recover its prior architecture after clipping. Furthermore, clipping might be an easier approach to convince managers to apply in natural reserves, since it is more controllable than fire. Studies show that clipping applied in the end of rainy season would be more harmful for this invasive species, which is then in the flowering phase. Clipping applied during this period would deplete these plants from energy to resprout, promote flowering of native species and the dead biomass deposed on the soil would shade clipped bushes, preventing resprouting.

Our data shows that clipping reduced the differences among species when watering was intermittent, while in constant moisture the invasive species continued to grow twice as much as the native. However, the invasive species continued to perform better. Many studies have reported compensatory growth in plants as an adaptation to herbivory and fire, being a response to removal of apical dominance (Sarmiento 1992, Hjálten *et al.* 1993, Leite *et al.* 1997, Silva & Raventos 1999).

While in the field and in lab experiments we observe that without disturbance the invasive species will always perform better, and with high availability of nutrients the invasive species will perform better even when disturbed, the lab experiments also showed that in some environmental conditions, disturbance can make competition among the invasive and native grasses more balanced.

For instance, when soil is well drained (here represented by the intermittent watering in clay pots), both species tend to recover in a more similar rate after clipping. Differences between species when watering was intermittent was noticed only when they were also fertilized, and although invasive plants grew more than the native plants, these recovered more in relation to pre-clipping values. Therefore it is assumed that the intermittent watering regime better represents the natural conditions of Cerrado soils, that are mostly red-latossols, well drained and dystrophic.

LIMITATIONS

This study is mostly limited to lab experiments, with the exception of the bait-lamina experiments. For this reason, extrapolation of data to real-life scenarios must be taken with caution. Although in laboratory experiments environmental factors can be controlled to assess the effect of specific variables and conclusions drawn more easily, the conditions are far from real-life situations. Therefore, mesocosm and field experiments would be the next step to complement the present study. It would be interesting to measure plant competition and seedling recruitment in situ, assess plant ecophisyology in the environment and study the effect of management in larger scales. However, management in natural areas in Brazil is difficult due to environmental legislation. Therefore, lab studies are still necessary in parallel.

Additionally, different watering methods led to diverse results (Chapter 4, 5, 6). Methods should be chosen carefully to answer the questions raised, considering realistic needs. In this study it was possible to observe that adding a small amount of water every day for many days is less stressful to plants than irrigating them to soil field capacity and completely suspending irrigation for a few days.

There was also the limitation of physical space and time. For instance, the slow growth rate of the native species constrained the acquisition of biological material sufficient for all analyses. Therefore, there were many parameters that could not be repetitively measured. Since no other study was found using *S. microstchyum* (for the parameters assessed in this study), many preliminary experiments had to be carried out. This optimization of the test conditions became very time-consuming. Furthermore, the native specimens were much harder to measure. Besides lack of material, leaves were also smaller, hindering measurements with Mini-PAM and IRGA chambers for this species. These difficulties led to a greater standard deviation for these parameters in the native species. As for the biomarkers, tissue processing was also problematic. Since tissue was rigid, the homogenization was arduous, which might have led to greater variation in the data.

Legal transportation and collection bureaucracy in Natural Reserves in Brazil also constrained the use of several native species for the lab experiments. Furthermore, multiple seed collections had to take place in the field in Brazil since seed viability and seedling establishment decreased with time of storage. The use of many native species was only possible in one task (Chapter 3), which was carried out in Brazil. From this study it was possible to observe the diverse responses that a plant community could display. Further studies would benefit from using multiple native species, which would reflect a more realistic environment.

Furthermore, further studies would benefit from measurements of other related parameters: leaf elongation rates, leaf and root anatomy, concentration of nutrients in senescent leaves and roots, partition of biomass between leaves and culms, Ascorbate and GSH concentration and oxidation state, Rapid Light Curves, H_2O_2 concentration, Proline, ABA, or DNA damage.

MAIN HIGHLIGHTS AND CONCLUSIONS

(1) The invasive species *M. minutiflora* affects ecosystem functioning by increasing biological activity, except in burned areas. This is probably related to the massive root system of this species.

(2) The other invasive species, *A. gayanus* doesn't present the same effect on soil functioning.

(3) Fire immediately reduces biological activity, which recovers fast with rain, but frequent fires tend to maintain lower soil feeding activity.

(4) The invasive species *M. minutflora* has high germinations rates, viability and fast germination, and it is not harmed by post-fire environments.

(5) The community of species presents diverse germination parameters and multiple responses to post-fire conditions, which may confer resilience to invasion to the ecosystem.

(6) *M. minutiflora's* initial stages (seedlings and 3-month-old plants) perform better than the native in any situation, being more resistant to drought, responding better to fertilization, maintaining healthier

photosynthesis activity and efficient protection against oxidative stress.

(7) Water availability is more detrimental then nutrient availability for the invader's success.

(8) Fire presents stronger negative effects on *M. minutiflora* and *S. michrostachyum* than clipping.

(9) Clipping reduced the difference in performance between *M. minutiflora* and *S. microstachyum*. The latter presented a better relative recovery in comparison to the invasive plants, although these still performed better in absolute values.

(10) Clipping seems a feasible management to be applied in places where the native community is still present and in the absence of other invasive species. Data suggests it would be more efficient when applied in early dry season in places with no fertilization input.

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