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The presence of *Microlobius foetidus* cause changes in the antioxidant defense of *Urochloa decumbens*?

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(With 2 figures)

Abstract

Urochloa decumbens (Stapf) R. D. Webster (Poaceae) is an exotic species with has spread rapidly through the Cerrado area of Pantanal, Mato Grosso do Sul, Brazil. It has covered the soil aggressively turning it into cultivated pastures. Thus, it has become a challenge to protect native areas due its capacity of exclusion of native species. It has been observed that *Microlobius foetidus* (Jacq.) M.Sousa & G.Andrade species (Fabaceae) shows a dominant pattern over the development of *U. decumbens*. This work shows that *M. foetidus* interfere on the natural growth of *U. decumbens* within 10 m ratio. Between 15 and 20 m, it was observed an increase of Importance Value index (IVI) and Relative cover (RC) values. It was also observed a variation on the antioxidant defense system of *U. decumbens* within 10m ratio from *M. foetidus*. The enzymes superoxide dismutase, catalase and peroxidase present higher levels of activity then those found for glutathione reductase. This data indicates that *M. foetidus* may have an effect on *U. decumbens*, increase the activity of antioxidant enzymes. This effect probably happens as means to neutralize the toxic effects of the oxygen generated due to the presence of allelochemicals, which increases oxidative stress.

*Keywords***:** antioxidant enzymes, signal grass, garlic stick, competition.

A presença de *Microlobius foetidus* causa alteraçõess na defesa antioxidante de *Urochloa decumbens*?

Resumo

Urochloa decumbens (Stapf) R. D. Webster (Poaceae) é uma espécie exótica que se expandiu rapidamente no Cerrado do Pantanal de Mato Grosso do sul, e cobre o solo de maneira agressiva convertendo a vegetação natural em pastagens cultivadas e se tornando um desafio para o controle em áreas protegidas devido a sua capacidade de excluir espécies nativas. Observações demonstraram que a espécie *Microlobius foetidus* (Jacq.) M.Sousa & G.Andrade (Fabaceae) apresenta padrões de dominância, interferindo no desenvolvimento de *U. decumbens* e alterações na fitofisionomia e sistema de defesa antioxidante desta espécie foram investigados. Nossos estudos demonstraram que *M. foetidus* interfere no crescimento de *U. decumbens* nos primeiros 10 metros de distância, sendo que aos 15 e 20 metros, é verificado um aumento nos valores de IVI e CR. Alterações no sistema de defesa antioxidante de *U. decumbens* também foram verificados nos indivíduos amostrados até 10 metros de distância. Superóxido dismutase, catalase e peroxidase foram provavelmente as enzimas cruciais envolvidas na neutralização de espécies reativas de oxigênio, uma vez que apresentaram maiores níveis de atividade em comparação com outras enzimas, tais como glutationa redutase. Os dados indicam que a proximidade de *U. decumbens* as áreas onde se encontram populações de *M. foetidus*, aumentam a atividade das enzimas antioxidantes. Este efeito provavelmente ocorre como meio para neutralizar os efeitos tóxicos do oxigênio gerado, devido à presença de aleloquímicos, o que aumenta o estresse oxidativo.

Palavras-chave: enzimas antioxidantes, capim brachiaria, pau- alho, competição.

1. Introduction

Human occupation in Pantanal environment introduced a new fauna and flora, breaking the natural balance among the different native species, and the biodiversity has been threatened as the natural structure of the community has been changed. About 176 exotic species are known in Brazil within terrestrial environments (Brasil, 2006).

Exotics species have grown rapidly at the *Cerrado* of Pantanal. Among them, two species have predominated: *Urochloa decumbens* and *Urochloa humidicola* (Rendle) Morrone & Zuloaga. These two species are aggressive and they have been used to change the native vegetation into pastures. It has been reported to be a challenge to avoid that these herbs invade the Pantanal protected areas (Matos and Pivello, 2009).

Microlobius foetidus subsp. *paraguensis* (Jacq.) M. Sousa & G. Andrade (Fabaceae), which is popularly known as garlic stick, is a tree that can reach up to 18 m (Pott and Pott, 1994). It appears quite naturally in modifies areas in the Pantanal, Mato Grosso do Sul and the *Chaco* of Porto Murtinho. It grows again very easily after cutting and burning. Leaves also exude strong garlicky odor by the production of volatile oils and local population use the leaves such a natural repellent (Silva et al., 2014).

Macias et al. (2007) also reported that through millions of years of evolution, environmental pressure has ensured that each plant species ends up with its particular solution to the survival problem, thus creating enormous biodiversity. Several morphological and biochemical adaptations have arisen through evolution to solve this problem and ensure plant survival, with a kind of chemical warfare based on toxic secondary metabolite production, storage and, eventually, release into the environment being one important strategy.

In the past, for many years Reactive oxygen species (ROS) were considered as dangerous molecules which must be maintained at low level in cells. However, recently this point of view has been changed. It has been shown that ROS can also play an important role in plant defense against oxidative explosion and can serve as markers of the certain stages of development, such as formation of tracheids and the cross-link in cell walls, lignifications, and programmed cell death and also serve as signaling/alarm molecules in regulation of gene expression process (Schützendübel and Polle, 2002; Sytar et al., 2013).

Changes in growth and antioxidant defense of plants submitted to chemical compounds liberated to the environment by others species have already been reported (Bailly et al., 2008; Pergo and Ishii-Iwamoto, 2011).

Thus, this study was undertaken to investigate the relationship between the oxidative stress in *B. decumbens* with the the predominance of *Microlobius foetidus* in an area of the Pantanal of Mato Grosso do Sul.

2. Material and Methods

2.1. Phytosociological parameters

Studies were performed in an Cerrado area from Pantanal, Mato Grosso do Sul, in June 2011, on the coordinates 19°29'16,20" S and 57°02'35,50" W. A voucher of *M. foetidus* was deposited on herbarium at the Botanic Museum from Curitiba (nº 21739).

The Phytosociological study was conduced in a pasture area with 300 m^2, and 50 individuals of *M. foetidus* (5-15 height meters). Ten plots were distributed in the east-west, with a distance difference of 0, 5, 10, 15 and 20 meters from *M. foetidus* from north to south.

For visual evaluation of species coverage, was observed litter (decomposing plant material), uncovered soil (US), absolute cover (AC), absolute frequency (AF), relative cover (RC), relative frequency (RF), Importance Value index (IVI), decomposing plant material (DPM), Uncover Soil (US) and Total of individuals (TI). The results was calculated according to Brandão et al. (1998) and Lara et al. (2003).

Then five individuals of *U. decumbens* were removed, identified and stored within in a Styrofoam box with ice and taken to the laboratory for the evaluation of antioxidant enzymes.

2.2. Enzymatic assays

To prepare the enzyme extracts solution, 50 mg of each part were crushed with liquid nitrogen. The resultant powder was homogenized with 50 mM sodium phosphate buffer (pH 7.0), 2 mM Ethylenediamine tetraacetic acid (EDTA) and Polyvinylpyrrolidone (PVP) 1,0% (w/v). The liquid was filtered and kept at –18 °C for further use (Marques and Xavier- Filho, 1991). The enzymatic solutions were prepared at 4 °C. The total amount of proteins within the analyzed species was determined using the method described by Bradford (1976). The average protein of the extracts was determined by comparison with a calibration curve of albumin from Bovine serum albumin (BSA) at 594 nm.

Catalase activity (CAT) was measured in a medium containing 67 mM K-phosphate (pH 7.0), 10 mM H_2O_2, and 0.1– 0.4 mg protein of enzyme extract. The consumption of H_2O_2 was monitored at 240 nm (ε, 0.036 $mM^{-1}cm^{-1}$) (Aebi, 1984).

Peroxidase activity (POD) was measured in a medium containing 25 mM K-phosphate (pH 6.8), 10 mM H_2O_2, 2.6 mM guaiacol, and 0.1–0.4 mg protein of enzyme extract. Tetraguaicol formation (ε, 25.5 mM^{-1} cm^{-1}) was measured at 470 nm (Putter, 1974).

Superoxide dismutase (SOD) activity was measured according to Giannopolitis and Ries (1977). The medium contained 50 mM K-phosphate (pH 7.8) 6.5 mM methionine, 150 µM nitro blue tetrazolium (NBT), 4 µM riboflavin, and 0.02– 0.1 mg protein of enzyme extract. The reaction was started by switching on a light (micro light quantum × m^2 × s^{-1}) and illuminating the medium for 20 min at 30°C. One unit (U) of SOD activity, was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate read at 560 nm, and the results were expressed as U of SOD mg $protein^{-1}$.

The Glutathione reductase activity (GSR) was measured in a medium containing 50 mM potassium phosphate buffer (pH 8.0), 2 mM EDTA, 0.5 mM Glutathione disulfide (GSSG), 0.15 mM Reduced NADP (NADPH), and 0.1-0.4 mg protein of the enzyme extract. The NADPH

oxidation rate was monitored at 340 nm (ε, 6,2 $mM^{-1}cm^{-1}$) according to Foyer and Halliwell (1976).

The lipid peroxidation (LP) was measured in a medium containing (Trichloroacetic acid) TCA 0.1%, thiobarbituric acid 0.5%, and 0.1-0.4 mg protein of the enzyme extract (Gomes-Júnior et al., 2006). The results were measured at 534 nm and the activity was expressed as percentage of lipid peroxidation.

The total concentration of carbohydrates in leaves was determined according to Moraga et al. (2006), using the phenol sulfuric method. The leaves of each sample, had their central nervure taken and were crushed in liquid nitrogen. After that, 300 mg of the mass was extracted with 3 ml of ethanol 80% on an assay tube. The assay tube was agitated and warmed at 60°C during 30 min in a water bath and then centrifuged at 4000 rpm for another 30 min at 4°C.

After the centrifugation, 1 ml of the extract was transferred to assay tubes with 1 ml of chloroform and 1 ml of deionized water. The mixtures were left for 45 min. The pigments migrated to the organic phase and the carbohydrates remaining in the aqueous phase. 200 µl of the aqueous phase were diluted with 1 ml of deionized water. 500 µl of this solution was transferred to assay tubes containing 500 µl of phenol (5%), and 25 µl of concentrated sulfuric acid. The mixture was agitated using a vortex and the measurement was read at 485 nm. To have the total soluble carbohydrates concentration values we have used the calibration curve: y= 0,0081x + 0,0744 (where: y=absorbance; x=carbohydrates concentration; R^2= 0,9988).

To determination of proline, 1 ml of the samples were treated with 5 ml of a 3% sulfo salicylic acid solution. Next, the mixture was centrifuged during 5 min at 3000 rpm at room temperature. 1 ml of the supernatant of each sample was transferred to assay tubes and 1 ml of acid ninhidrin was added followed by 1 ml of glacial acetic acid. The assay tubes were kept at 100°C for hour in water bath. After that the samples were refrigerated in ice and the red complex was extracted with 2 ml of toluene (vortex for 30 seconds). After reaching room temperature, the absorbance of the toluene red-complex solution was measured at 520 nm, according to Bates et al. (1973). The proline concentration measurements were done using the calibration curve: y= 0.0959x – 0.2147 (where: y=absorbance; x= proline concentration; R^2= 0.9926).

To evaluate each sample, three measures was collected and the variation data was analyzed. If any difference was detected by the F analysis, we change to the Dunnet analysis, with 5% probability. If any demanded parameter did not match, non-parametric analysis were performed such as Kruskal-Wallis instead of variance analysis and Mann-Whitney instead of Dunnet analysis.

3. Results and Discussion

The data gathered showed that Microlobius foetidus affects the development of *Urochloa decumbens*. The parameters: uncover soil (18.2%) and decomposing plant material (60.8%) were very high where grows nearby *M. foetidus* (0 m). Such an influence was also detected till 5 to 10 m, and the porcentage of undercover soil and decomposing plant material increases with distance between *U. decumbens* and *M. foetidus* (Table 1).

The average numbers of individuals were also influenced by the distance of *U. decumbens* from *M. foetidus*. The number of the sample individuals of *U. decumbens* decreases as the distance from *M. foetidus* decreases. These data are reinforced when IVI and RC of the 5 distances are compared (Table 1).

At the initial point (0 m) the IVI (15.02) and RC (3.08) are lower than at 5 m (IVI– 31.49; CR– 14.46). This effect was also observed as the distance from *M. foetidus* increases, 10 m (IVI– 45.,11; RC– 25.23), 15 m (IVI– 53.04; RC– 27.38), and 20 m 10 (IVI– 55.35; RC– 29.85) (Table 1). There was no significant difference between 15 - 20 m distances within the studied parameters, thus establishing a 15 m perimeter of influence of *M. foetidus* over *U. decumbens*.

B. decumbens showed significant difference of total proteins concentration for the distances observed. Individuals from nearby *M. foetidus* (0 meters) displayed a higher total proteins concentration (25 $\mu g.g^{-1}$) when compared to the others. Comparing individuals from all distances studied: 5 m (23.8 $\mu g.g^{-1}$), 10 m (17.9 $\mu g.g^{-1}$), 15 m (12.7 $\mu g.g^{-1}$) and 20 m (12.5 $\mu g.g^{-1}$), it can be observed the decreasing of the total protein concentration in opposition to the increasing distance of *U. decumbens* from *M. foetidus* (Figure 1).

The evaluation of the CAT, POD, SOD (Figures 1b-d) GSH (Figure 2a) enzyme activities showed an effect similar to that one observed with the total protein concentration. The *M. foetidus* nearest individuals displayed higher

Table 1. Phytosociological Parameters of *U. decumbens* at nearby *M. foetidus* in the c*errado* area.

Distance from *M. foetidus* (meters)	DOM* (%)	US* (%)	*TI	AC*	AF*	RC*	RF*	IVI*
0	60.8	18.2	155	10.00	0.17	3.08	11.94	15.02
5	43.5	15.7	221	47.00	0.21	14.46	17.03	31.49
10	32.2	12.4	258	82.00	0.33	25.23	19.88	45.11
15	15.4	10.7	331	89.00	0.73	27.38	25.50	53.04
20	10.1	8.1	333	97.00	1.00	29.85	25.65	55.35

US: Uncover Soil, TI: Total of individuals, AC: Absolute Cover, AF: Absolute Frequency, RC: Relative cover, RF: Relative Frequency, IVI: Importance Value Index. *DOM: Dead Organic Matter.

antioxidant activity then the farther ones as displayed at Figures 1 and 2.

A major percentage of per oxidation of lipids was observed for the individuals closest to *M. foetidus* (0 meters). This effect decreases with the distance from *M. foetidus* as shown in Figure 2b.

The soluble carbohydrate concentration in *B. decumbens* also showed dependence of the distance from *M. foetidus*. The soluble carbohydrate concentration in *B. decumbens* increases with the proximity of *M. foetidus* (Figure 2c). Comparing these results with the ones found for uncovered soil and the amount of dead material in each distance

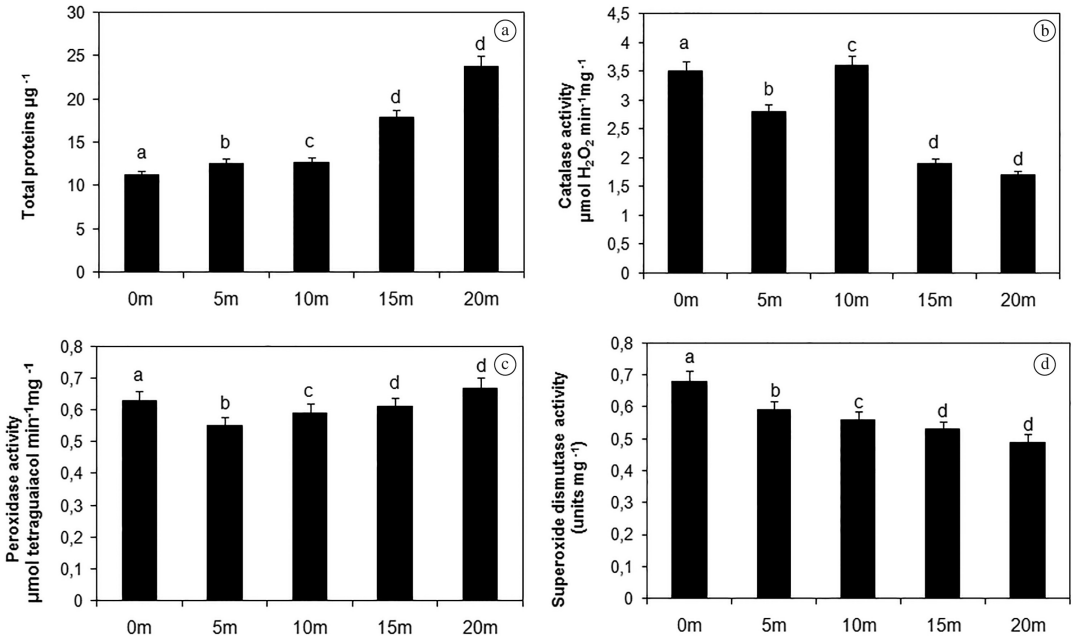

Figure 1. Total amount of Proteins (a), Catalase (b), Peroxidase (c) and superoxide dismutase activities (d) of *U. decumbens*, collected in different distances from *M. foetidus*. The means followed by the same letter from the control have no significant difference among them, according to Dunnet test ($p < 0.05$).

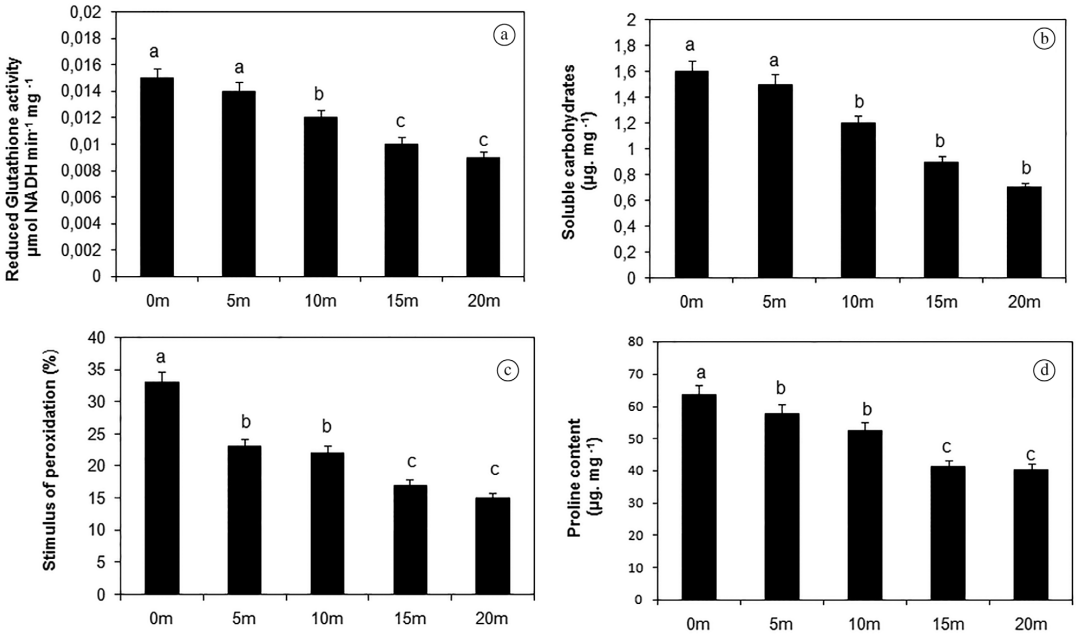

Figure 2. Glutathione reductase activity (a), stimulation of lipid per oxidation (b), soluble carbohydrates (c) and proline concentration (d); of *U. decumbens* collected in different distances from *M. foetidus*. The means followed by the same letter from the control have no significant difference among them according to Dunnet test ($p < 0.05$).

studied, it can be inferred that individuals that grow nearby *M. foetidus* can suffer interference from the compounds freed to the environment by the material originated from dead leaves of this species.

The proline concentration within leaves of *B. decumbens* also displays variation as a function of the distance from *M. foetidus*. It was observed that individuals from 0 m (43.4 $\mu g.g^{-1}$), 5 m (57.8 $\mu g.g^{-1}$) and 10 m (52.4 $\mu g.g^{-1}$) displayed a higher proline concentration then that observed for the distances of 15 m (41.2 $\mu g.g^{-1}$) and 20 m (40.3 $\mu g.g^{-1}$) (Figure 2d). This effect happens probably due the need for protection against lipid peroxidation, activating or even removing directly the oxygen reactive species.

U. decumbens is the species most often used for the fast expansion process of cultivated pastures in the Cerrado Pantaneiro, where it aggressively covers the soil turning the natural vegetation into cultivated pastures.

4. Conclusion

There is not much information about *M. foetidus* species, however "*in situ"* observations has shown that this species grows in mono specific population, establishing a strong intra-specific competition, eliminating even others species in areas of dirty field and clean field cerrados. Another important characteristic that favors this species is that after a short period of rain, the plant produces a lot of flowers, increasing the number of seeds. This increase in seed production, makes possible the chance of some germinate and become mature plants, favoring the predominance of this species.

Our results show that the *M. foetidus* species interfere on the development of *B. decumbens*, reducing the number of individuals within 10 meters radium from this specie. Observations in large distances (15 and 20 m) show that the values RC and RF are quite similar, thus *M. foetidus* interferes on the *U. decumbens* only within a 10 meters radium.

The changes in germination and the growth of plants, as well as changes of antioxidant defense activity by the influence of compounds liberated in to the environment have already been reported (Pergo and Ishii-Iwamoto, 2011; Silva et al., 2009). However, few experiments have been done at natural environments that could help to prove these effects in nature. Del Moral et al. (1978) compared the competitive and chemical effects of two *Eucalyptus* species, demonstrating that the growth inhibition of other species was due to the liberation of leachates from the leaves of *Eucalyptus baxteri*.

Reactive Oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and the superoxide radical (O_2^-), may react with several molecules generated by cells causing oxidative damage which can interfere with the regular development of the cells (Marino et al., 2009; Silveira et al., 2003). The plants protect their cells and sub-cell invasive geophyte species. Organs from cytotoxic effects of the ROS with the help of antioxidant enzymes such as super oxide dismutase (SOD), glutathione reductase (GSR), catalase (CAT) among others (Scandalios, 1993; Mittler, 2002).

Comparing the changes of the antioxidant defense activity of *U. decumbens*, it was observed that this activity increases as the distance from *M. foetidus* decreases. These results indicate that *U. decumbens* suffers an oxidative stress, increasing the levels of ROS as a counterpart to the inhospitable environment.

The enzyme activities analyzed show that SOD and CAT display higher activity, thus, having the main role of the ROS neutralization in *U. decumbens*. This specie also has secondary enzymes such as peroxidases. The peroxidases may appear in several forms and are involved in several physiologic processes such as ionic or covalent interactions with polymers from the cellular wall, thus having a role at the biosynthetic pathway of lignin from the cellular wall (Passardi et al., 2005; Quiroga et al., 2000). In the other hand, GSH has no significant role in the elimination of H_2O_2 in *U. decumbens* due its low level of activity when compared to other analyzed enzymes.

The physiologic roles of these enzymes at germination, growth and development of the plants are not completely known. It was suggested that these enzymes play a major role at the hydration of tissues, cells enlarging, nitrogen accumulation (Rodriguez-Concepción and Beltrán, 1995; Tranbarger et al., 1991), and lipid degradation (Feussner et al., 1997).

The increasing lipid per oxidation activity induced by ROS, shown on *U. decumbens* happens as a result of the changes of lipid molecules what also affects the structure of the cell membrane (Moore and Siedow, 1991; Baker and Orlandi, 1995; Thaler, 1999; Porta and Rocha-Sosa, 2002; Blokhina et al., 2003). Peroxidation of the poli-insaturated lipid acids decreases the membranes fluidity, thus loosing cellular content or organelles, together with secondary damage to the membrane proteins (Halliwell, 2006).

When the antioxidant defense system of *U. decumbens* individuals is changed as well as the tillers number, decreases as indicated by the percentage of uncovered soil (Table 1), the soluble carbohydrates can be dislocated from senescent leaves to other parts of the plant, favoring the increase of soluble carbohydrates in individuals that are growing near of M. foetidus.

The main reason for the presence of carbohydrates in plants is the source of energy (Palma et al., 2002). Soluble sugars seem to assume a dual role with respect to ROS. Soluble sugars can be involved in ROS-producing metabolic pathways. In reverse, soluble sugars can also feed NADPH-producing metabolic pathways, such as the oxidative pentose-phosphate (OPP) pathway, which can contribute to ROS scavenging (Russell et al., 2002).

Proline is a protein generator amino acid with exceptional function to the rigid conformation, and it is essential for primary metabolism (Szabados and Savoure, 2010). Proline accumulation has been observed in dry conditions (Choudhary et al., 2005), high salt concentration (Yoshiba et al., 1995), high light intensity and UV radiation (Saradhi et al., 1995), heavy metals (Schat et al., 1997),

oxidative stress (Yang et al., 2009) and biotic stress (Fabro et al., 2004; Haudecoeur et al., 2009).

It is believed that proline accumulation has a role on the adaptation pathways of plants living under stress conditions. It is been proposed that proline acts like an osmolyte and it is a way to the carbon and nitrogen accumulation (Hare and Cress, 1997).

The amount of proline in the study of *U. decumbens* individuals was also influenced by the neighborhood of *M. foetidus* species. The high level of proline is useful to protect against lipid peroxidation (Molinari et al., 2007) removing ROS directly or activating the antioxidant defense system (Carvalho et al., 2013).

It seems that increasing the production of antioxidant enzymes demands more energy from *U. decumbens*, contributing to the reduction of vegetal cover as means to reduce its growth. The grasses are among the invading species with high capability to colonize the forests and denser "cerrado *sensu stricto"* like areas such as those occurring in the Pantanal. The capacity of *U. decumbens* to inhibit, native species has been reported (Matos and Pivello, 2009; Oliveira, 2004). It has been a challenge to control *U. decumbens* in protected areas, especially after a burning, which is a common fact at dry seasons.

Oxidative stress, which frequently or stress, may cause denaturation of functional and structural proteins (Smirnoff, 1998). As a consequence, these diverse environmental stresses often activate similar cell signaling pathways (Knight, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001; Zhu, 2002) and cellular responses, such as the production of stress proteins, up regulation of antioxidants and accumulation of compatible solutes (Vierling and Kimpel, 1992; Zhu et al., 1997; Cushman and Bohnert, 2000; Wang et al., 2003).

Stress-induced ROS accumulation is counteracted by enzymatic antioxidant systems that include a variety of scavengers, such as SOD, POD and CAT and non-enzymatic low molecular metabolites, such as ASH, GSH (Mittler et al., 2004). In addition, proline can now be added to an elite list of non-enzymatic antioxidants that microbes, animals, and plants need to counteract the inhibitory effects of ROS (Chen and Dickman, 2005).

Plant stress tolerance may therefore be improved by the enhancement of in vivo levels of antioxidant enzymes. The above said antioxidants found in almost all cellular compartments, demonstrating the importance of ROS detoxification for cellular survival (Dalton et al., 1999).

In agro-eco systems, the allelochemicals liberated by plants which induce an increasing effect to the oxidative stress may contribute to discover natural compounds that can be used to control invading species (Inderjit and Duke, 2003). In fact, it is already known that some commercial herbicides induce oxidative stress until cell death.

This is a consequence of the interference on the flow of electrons from photo system II (PSII) or, yet, by the inhibition of the biosynthesis of antioxidant compounds such as the carotenoids (Kruse et al., 2006). It is reasonable to suggest that *M. foetidus* have a high potential to inhibit the *U. decumbens* invasion through the induction of the oxidative stress during its growth phase.

The compounds produced in *M. foetidus* leaves can be investigated as a means to establish a control plan for *U. decumbens* at protected areas. Beyond the primary mechanisms of oxidative stress induced by *M. foetidus*, the data we found suggest that, during the growth phase, the investigated antioxidant enzyme activities increases and they neutralize the oxygen reactive species, leaving in situ plants more vulnerable to dysfunction and cell death.

This data indicates that *M. foetidus* may have an effect on *U. decumbens*, increasing antioxidant activity. This effect probably happens as means to neutralize the toxic effects of the oxygen generated due to the presence of allelochemicals, which increases oxidative stress.

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