Article

ATRAZINE AND MESOTRIONE-INDUCED OXIDATIVE STRESS AND IMPACT ON ANTIOXIDANT ENZYMES AND CHLOROPHYLL CONTENTS IN BERMUDAGRASS

Estresse Oxidativo Induzido por Atrazina e Mesotrione e Impacto em Enzimas Antioxidantes e Teores de Clorofila em Grama-Seda

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ABSTRACT - The effect of atrazine, mesotrione, and joint activity of atrazine plus mesotrione on pigment, lipid peroxidation, and antioxidant enzyme activity was studied. Atrazine and mesotrione treatments significantly reduced chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), carotenoid concentrations, and protein content in bermudagrass (*Cynodon dactylon* L.) plants, whereas they significantly enhanced lipid peroxidation. The treatment of atrazine plus mesotrione caused greater phototoxic effect on bermudagrass than either herbicide alone, which was evident from the significantly decreased membrane stability noted as a function of the enhanced singlet oxygen and malondialdehyde (MDA) contents, as well as from the greater reduction in Chl *a*, Chl *b*, and carotenoid contents. Although bermudagrass activated the antioxidant enzymes catalase (CAT), peroxidase (POD), and glutathione S-transferase (GST), it was significantly injured after the herbicide treatments. Thus, results suggested that the enzymatic and non-enzymatic antioxidants of bermudagrass was overloaded after the treatment of atrazine plus mesotrione, and the reactive oxygen species (ROS) subsequently caused lipid peroxidation, pigment and protein degradation, as well as other cellular constituent damage.

Keywords: antioxidant, carotenoids, herbicide, reactive oxygen species.

RESUMO - O efeito de atrazina e mesotrione e da atividade conjunta de atrazina mais mesotrione no pigmento, na peroxidação lipídica e na atividade da enzima antioxidante foi estudado. Os tratamentos com atrazina e mesotrione reduziram significativamente as concentrações de clorofila a (Clf a), de clorofila b (Clf b), de carotenoides e o teor de proteína em plantas de grama-seda (Cynodon dactylon L.), mas aumentaram significativamente a peroxidação lipídica. O tratamento com atrazina mais mesotrione causou maior efeito fitotóxico na grama-seda do que o herbicida isolado, o que foi evidenciado pela diminuição significativa da estabilidade da membrana em função dos teores de oxigênio singlete e malondialdeído (MDA), bem como pela maior redução dos teores de Clf a, Clf b e carotenoides. Embora a grama-seda ativasse as enzimas antioxidantes catalase (CAT), peroxidase (POD) e glutationa S-transferase (GST), ela foi significativamente danificada após os tratamentos com herbicidas. Assim, os resultados sugerem que os antioxidantes enzimáticos e não enzimáticos da grama-seda foram sobrecarregados após o tratamento com atrazina mais mesotrione, e as espécies reativas de oxigênio (ERO) causaram peroxidação lipídica, degradação de pigmentos e proteínas, além de outros danos a constituintes celulares.

Palavras-chave: antioxidante, carotenoides, herbicida, espécies reativas de oxigênio.

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INTRODUCTION

Under abiotic and biotic stresses, plants produce excessive amount of reactive oxygen species (ROS), resulting in oxidative damage (Jung et al., 2000; Ramel et al., 2009; Gill and Tuteja, 2010; Del Buono et al., 2011; Yang et al., 2015; Zhang et al., 2015). The excessive formation of ROS including hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), single oxygen (O^{2-}), and superoxide radicals ($\cdot O_2^-$) can react with cellular constituents, causing a cascade of oxidative reactions including chlorophyll, protein, pigment, DNA degradation, and lipid peroxidation (Hess, 2000; Sena and Chandel, 2012; Dixon and Stockwell, 2014).

To cope with the adverse effects of ROS, plants have developed a complex antioxidant system for scavenging ROS and alleviating the oxidative damage. The capability of a plant to control and remove the ROS is closely associated with its stress tolerance, including herbicidal stress (Jung et al., 2000; Kim et al., 2004; Del Buono et al., 2011; Sharma et al., 2012). The primary free radical scavengers in antioxidant system include ascorbates, carotenoids, tocopherols, and several enzymes such as ascorbate peroxidase (APX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6), glutathione reductase (GR; EC 1.6.4.2), peroxidase (POD; EC 1.11.1.7), and glutathione S-transferase (GST; EC 2.5.1.18) (Allen, 1995; Zhang and Kirkham, 1996; Edge et al., 1997; Lin et al., 2004; Sharma et al., 2012; Zhang et al., 2015). These free radical scavengers can enzymatically or chemically react with ROS to reduce the toxic effects. For example, thylakoid-bound SOD quickly dismutes $\cdot O_2^-$ into H_2O_2. Thylakoid-bound APX transforms H_2O_2 to water mediated by ascorbic acid as the reducing agent (Allen, 1995). Moreover, CAT, an enzyme often involved in the metabolism of long chain fatty acids in peroxisomes, can transform H_2O_2 to water and oxygen (Peixoto et al., 2008).

Atrazine, a triazine herbicide, has its site of action in photosystem II (PS II) in the photosynthesis light reaction (Hess, 2000). In PS II, single chlorophyll cannot transfer to the PS II reaction center when electron flow is blocked by PS II-inhibitors in the Q_B pocket of the D1 protein. Consequently, the short-lived singlet chlorophyll accumulates in the chloroplasts and some of them transform to more reactive and longer lived triplet chlorophylls. The excess triplet chlorophylls react with oxygen molecular and produce singlet oxygen. Both triplet chlorophyll and singlet oxygen extract hydrogen from unsaturated lipids, causing a chain reaction of lipid peroxidation (Kasai, 1997; Krieger-Liszkay, 2005; Krieger-Liszkay et al., 2008; Sharma et al., 2012).

Mesotrione, a herbicide of the triketone group, inhibits carotenoid biosynthesis by inhibiting 4-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme. Following mesotrione treatment, carotenoid biosynthesis is inhibited, leading to foliar bleaching, necrosis, and eventually plant death (Hess, 2000; Yu and McCullough, 2016a,b). HPPD enzyme converts hydroxyphenylpyruvate to homogentisate, producing α-tocopherols and plastoquinones (PQ) (Hess, 2000). In the pathway for carotenoid biosynthesis, PQ is an electron acceptor for the phytoene desaturase (Hess, 2000). Researchers have documented a synergistic interaction between atrazine and mesotrione. The synergism is achieved through the following combined biochemical effects: atrazine blocks the electron transport at the Q_B binding site in PS II, resulting in the accumulation of ROS and free radicals (Armel et al., 2005; Woodyard et al., 2009; Abendroth et al., 2009; Akbulut and Yigit, 2010), while mesotrione depletes carotenoids, tocopherols, and PQ (Triantaphylidès and Havaux, 2009).

The link between antioxidant enzyme activities under herbicidal stress and its relationship with oxidative protection is seldom studied but appears to be complex and warrants further research. Moreover, relevant studies so far have focused on the response of antioxidant enzymes in agronomic crops such as rice (*Oryza sativa* L.), soybean (*Glycine max* L. Merr.), maize (*Zea mays* L.), and wheat (*Triticum aestivum* L.) (Zhu et al., 2009; Jiang and Yang, 2009; Akbulut and Yigit, 2010; Del Buono et al., 2011; Zhang et al., 2014, whereas they ignored the antioxidant enzyme activity in atrazine- and mesotrione- susceptible turfgrass species, such as bermudagrass (*Cynodon dactylon* L.), when exposed to atrazine and mesotrione. In addition, the joint effect of atrazine plus mesotrione on antioxidant enzyme activity has not been reported. Therefore, in this research, the effect of atrazine, mesotrione, and the joint activity of atrazine and mesotrione on pigment, lipid peroxidation, and antioxidant activity were investigated in bermudagrass.

MATERIAL AND METHODS

Greenhouse experiments

Greenhouse experiments were conducted in spring of 2016 at Chinese Academy of Tropical Agricultural Sciences, in Danzhou, Hainan, China (19.5 ºN, 109.6 ºE). Experiments were conducted to examine injury and shoot mass reduction following atrazine and mesotrione applications. Common bermudagrass (*Cynodon dactylon*) seeds were purchased from a commercial seed vendor (Yuesannong Co., Ltd. Shenzhen, China). Ten seeds were seeded in containers (5 cm diam x 20 cm depth) filled with sand : peat moss (80:20, v/v). Containers were placed in a greenhouse set at 32/25 ºC day/night with an average light intensity of 300 μmol m^{-1} s^{-1}, and 12 h photoperiod. Plants were thinned to three per pot after emergence. Plants were irrigated daily as needed to prevent wilting and fertigated weekly with a water-soluble fertilizer (N-P-K, 28:10:7).

Grasses selected were at three to five tiller stage. Grasses were selected on the basis of size and population uniformity. Atrazine (380 g L^{-1} SC, Drexel Chemical Co., Ltd.) was applied alone at 0, 280, and 560 kg a.i. ha^{-1}. Mesotrione (200 g L^{-1}, Syngenta Co., Ltd.) was applied either alone at 0, 140 and 280 kg a.i. ha^{-1} or in combination with all atrazine rates. A nonionic surfactant at 0.25% v v^{-1} was included in the herbicide treatments. A non-treated control was included in each replication. Herbicide treatments were applied with an electric-powered sprayer calibrated to deliver 374 L ha^{-1} spray volume with a 9504E flat-fan nozzle (Electric-Powered Backpack SprayerÿModel XF-16M9). Grasses were not watered for 24 h after herbicide treatment but received irrigation thereafter to prevent wilting. Plant injury was visually assessed at four days interval for a total of 20 days, where 0 equaled no injury and 100 equaled complete desiccation. Above-ground biomass was harvested at 20 days after treatment (DAT), and then oven-dried at 65 ºC for three days. Shoot mass data were calculated to percent reductions from the non-treated control by replication.

Physiological parameters

Experiments were conducted in spring of 2016 at Chinese Academy of Tropical Agricultural Sciences, in Danzhou, Hainan, China to evaluate the effect of atrazine, mesotrione, and their combination on pigment concentration, protein content, lipid peroxidation, singlet oxygen, and antioxidant enzyme activity in bermudagrass. Bermudagrass was grown and established in greenhouse containers as previously described. Grasses were divided into four groups. Atrazine was applied alone at 0 and 280 g ha^{-1}. Mesotrione was either applied alone at 0 and 140 g ha^{-1} or in combination with all atrazine rates. A non-treated control was also included. Herbicide treatments were applied with the aforementioned sprayer calibrated to deliver 374 L ha^{-1} with a 9504E flat-fan nozzle. A nonionic surfactant was included in the herbicide treatments. Shoots were sampled at 1, 4, and 8 DAT and the following physiological parameters were measured.

Pigment concentrations were determined according to a procedure described by Lichtenthaler (1983), with minor modifications. Plant stems were removed, and leaf samples were homogenized in liquid nitrogen with a mortar and pestle. A subsample (0.1 g) was transferred into a plastic tube filled with 10 mL of acetone. The samples were homogenized. Then, supernatants were measured for chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), and total carotenoids (including *β*-carotene, lutein, and xanthophylls) at absorbance of 470.0, 644.8, and 661.6 nm, respectively, using a spectrophotometer (UV-Vis Spectrophotometer, UV-1700 Series, Nakagyo-ku, Kyoto, Japan). Chl *a*, Chl *b*, and carotenoids concentrations were determined from absorbance results according to the following formulae.

$$Chl\ a = 11.24A_{661}.6 - 2.04A_{644}.8$$

$$Chl\ b = 20.13A_{646}.8 - 5.10A_{663}.2$$

$$Carotenoids = [1000A_{470}.0 - 1.90(Chl\ a) - 63.14(Chl\ b)]/214$$

Malondialdehyde (MDA) content was determined based on the procedures described by Zhang et al. (2015), with only minor modifications. Shoots of bermudagrass (0.1 g) were ground to fine power with liquid nitrogen. The powder was suspended and vortexed in 1 mL 0.1% w/v trichloroacetic acid (TCA) for 3 s, and the resulting suspension was then centrifuged at 10,000 g for 10 min. Thereafter, 0.5 mL supernatant was transferred to the separate tube filled with 2 mL of 20% TCA and 0.5% thiobarbituric acid (TBA), incubating at 95 ℃ for 15 min. Samples were then cooled for 2 min in an ice bath to stop the reaction. After cooling, samples were centrifuged at 10,000 g for 10 min. The optical density was measured at 532 nm with a spectrophotometer. The MDA content was calculated using the established standard curve.

The measurement of O^{2-} content was conducted with a methodology described by Doke (1983). The O^{2-} content was determined according to its capability to reduce nitroblue tetrazolium. Fresh leaf tissue (0.5 g) was sampled and immersed in tubes filled with 10 mM potassium phosphate buffer (pH 7.8) containing 0.05% nitro blue tetrazolium and 10 mM NaN_3. Then, the samples were incubated at room temperature (22 ℃) for 1 h. A 2 mL of the reaction solution was heated at 85 ℃ for 15 min and then cooled in an ice bath. Optical density of the solution was measured at 560 nm for 15 min with a spectrophotometer.

The CAT activity was determined according to the procedure described by Chance (1995), with minor modifications. The CAT activity was measured by adding 300 µL of 30 mM H_2O_2 and 100 µL of enzymatic extract to 3 mL of 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.0). The consumption of H_2O_2 was determined with a spectrophotometer at 240 nm at 30 ℃ for 1 min.

The POD activity was determined by using a method from previous reports, with slight modification (Bradford, 1976; Wang, 2005). A 3 mL reaction solution, containing 50 mM phosphate buffer (pH 5), 20 mM guaiacol, and 40 mM H_2O_2, was used to assay the POD activity. Changes in absorbance at 470 nm were read for every 20 s to determine POD activity. One unit of POD activity was defined as the change of absorbance of 0.01 units per min.

The GST activity was determined by using a method described by Edwards and Owen (Edwards, 1986). A 25 µL of 40 mM CDNB was added to a solution containing 900 µL of 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 6.5), 25 µL of enzymatic extract, and 50 µL of 0.1 M reduced glutathione (pH 7.0). The conjugate formed by the reaction between CDNB and reduced glutathione was measured spectrophotometrically at 340 nm at 35 ℃ for 60 s.

The protein content was determined using a procedure reported by Bradford (1976).

The design for the growth response and laboratory experiment was a randomized complete block with four replications. Two experimental runs were conducted over time. Data were subjected to analysis of variance in SAS (Version 9.2, 100 SAS Campus Dr., Cary, NC 27513). Treatment means were separated with Fisher's Protected LSD test at α = 0.05. Experiment-by-treatment interaction was not detected, and thus, results were pooled over experimental runs for analysis.

RESULTS AND DISCUSSION

Atrazine applied at 0.28 g ha⁻¹ caused ≤10% bermudagrass injury from 4 to 12 DAT, and 30 and 40% injury at 16 and 20 DAT, respectively (Table 1). Atrazine applied at 0.56 g ha⁻¹ caused ≤10% bermudagrass injury from 4 to 8 DAT, but injury increased to 30% at 12 DAT and >90% from 16 to 20 DAT. Mesotrione applied at 0.14 g ha⁻¹ caused 40% bermudagrass injury at 12 DAT, and increased to >90% from 16 to 20 DAT. Atrazine plus mesotrione combination significantly increased bermudagrass injury compared to atrazine or mesotrione alone. Atrazine at 0.28 g ha⁻¹ plus mesotrione at 0.14 g ha⁻¹ injured bermudagrass 20% at 8 DAT, but injury increased to 80% at 12 DAT, and 100% at 16 and 20 DAT. Atrazine at 0.28 or 0.56 g ha⁻¹ plus mesotrione at 0.14 or 0.28 g ha⁻¹ caused 30 and 85% bermudagrass injury at 8 and 12 DAT, respectively, while injury increased to 100% at 16 DAT.

Atrazine at 0.28 and 0.56 g ha⁻¹ caused comparable bermudagrass shoot mass reduction, averaging 46% of the non-treated control (Table 1). Averaged over mesotrione rates, bermudagrass shoot mass was 30% of the non-treated control. Atrazine at 0.28 g ha⁻¹ plus mesotrione at 0.14 or 0.28 g ha⁻¹, as well as atrazine at 0.56 g ha⁻¹ plus mesotrione at 0.14 g ha⁻¹ caused significantly

Table 1 - Bermudagrass injury and shoot mass reduction following the application of atrazine and mesotrione in two combined greenhouse experiments

Specie	Herbicide		Injury (DAT)					Shoot mass reduction
	Atrazine	Mesotrione	4	8	12	16	20	(% of non-treated)
	(g ai ha⁻¹)		(%)					
Bermudagrass	0.28	0	0 f	3 f	10 f	30 c	40 d	46 a
	0.56	0	0 f	5 e	30 e	98 a	99 ab	45 a
	0	0.14	3 c	10 d	40 d	90 b	98 b	31 b
	0	0.28	5 a	15 c	40 d	88 b	95 c	30 b
	0.28	0.14	1 e	20 b	80 c	100 a	100 a	30 b
	0.28	0.28	2 d	30 a	85 b	100 a	100 a	29 bc
	0.56	0.14	2 d	30 a	85 b	100 a	100 a	28 bc
	0.56	0.28	4 b	30 a	88 a	100 a	100 a	25 c
LSD$_{0.05}$			1	2	2	3	2	4

Different letters in each column indicate significant differences from each other, at the 0.05 significant level according to the Fisher's Protected LSD test. Abbreviation, DAT = days after treatment.

greater shoot mass reduction than atrazine alone at 0.14 or 0.28 g ha⁻¹. Atrazine at 0.56 g ha⁻¹ plus mesotrione at 0.28 g ha⁻¹ caused significantly greater bermudagrass shoot mass reduction than either herbicide alone.

Synergism of PS II-inhibiting herbicides, such as atrazine plus mesotrione, has been previously reported. For example, atrazine plus mesotrione provided greater control of common cocklebur (*Xanthium strumarium*) and yellow nutsedge (*Cyperus esculentus*) than mesotrione alone (Johnson, 2002). McElroy and Walker (2009) reported that atrazine plus mesotrione was generally more injurious to centipedegrass (*Eremochloa ophiuroides*) seedlings for a greater time period and decreased biomass more than mesotrione alone. Willis et al. (2007) noted that mesotrione plus simazine, another PS II-inhibitor, increased white clover (*Trifolium repens*) control than either herbicide alone. Overall, our results suggested that atrazine plus mesotrione was generally more injurious to plants than atrazine or mesotrione alone for bermudagrass.

Chl *a* concentration in non-treated bermudagrass plants was 0.909 mg g⁻¹ FW at 1 DAT and declined to 0.726 and 0.788 mg g⁻¹ FW at 5 and 8 DAT, respectively (Table 2). Plant Chl *a* concentrations significantly decreased in plants treated with atrazine, mesotrione, and atrazine plus mesotrione combination. The least Chl *a* concentration was observed when bermudagrass treated with atrazine plus mesotrione. Chl *a* concentrations in plants treated with atrazine plus mesotrione combination were 81, 93, and 90% of the non-treated plants at 1, 5, and 8 DAT, respectively.

Chl *b* concentration in non-treated bermudagrass plants was 0.199 mg g⁻¹ FW at 1 DAT and decreased to 0.194 mg g⁻¹ FW at 5 and 8 DAT, respectively (Table 2). Significant reduction in Chl *b* concentrations in plants treated with atrazine or mesotrione was noted at 1 and 5 DAT, while atrazine or mesotrione did not significantly reduce the Chl *b* concentrations at 8 DAT. Similar to Chl *a*, atrazine plus mesotrione combination caused the greater reduction in Chl *b* concentrations than either herbicide alone. Chl *b* concentrations in plants treated with atrazine plus mesotrione were 82, 92, and 93% of the non-treated plants at 1, 5, and 8 DAT, respectively.

Regarding carotenoid concentrations, non-treated plant carotenoid concentrations were 0.214 mg g⁻¹ FW at 1 DAT, which declined to 0.166 and 0.186 mg g⁻¹ FW at 5 and 8 DAT, respectively (Table 2). Carotenoid concentrations were equally reduced by atrazine and mesotrione at 1 DAT. At 5 and 8 DAT, however, mesotrione was more effective than atrazine in decreasing carotenoid concentrations. Plants treated with atrazine plus mesotrione exhibited the least amount of carotenoid concentrations, averaging 79, 88, and 87% of the non-treated plants at 1, 5, and 8 DAT, respectively.

Determination of singlet oxygen for non-treated samples and samples treated with atrazine and mesotrione showed that the herbicide application significantly increased the singlet oxygen

activity (Table 3). Non-treated plant singlet oxygen was 1.783 nmol s^{-1} µg^{-1} protein at 1 DAT, which increased to 2.206 and 2.979 nmol s^{-1} µg^{-1} protein at 5 and 8 DAT, respectively. For samples treated with atrazine, singlet oxygen activity increased 13 and 21% at 5 and 8 DAT than the non-treated control, respectively. In samples treated with mesotrione, singlet oxygen increased 19 and 53% than the non-treated control at 5 and 8 DAT, respectively. Atrazine plus mesotrione combination significantly increased singlet oxygen activity 23, 26 and 75% at 1, 5 and 8 DAT, respectively. Atrazine plus mesotrione produced significantly more singlet oxygen than either herbicide applied alone.

Table 2 - Bermudagrass pigment concentrations following atrazine and mesotrione treatment, 1, 5, and 8 d after treatment, with respect to non-treated samples

Specie	Herbicide		Chl a			Chl b			Carotenoids		
	Atrazine	Mesotrione	1 DAT	5 DAT	8 DAT	1 DAT	5 DAT	8 DAT	1 DAT	5 DAT	8 DAT
	(g a.i. ha^{-1})		(mg g^{-1} FW)								
Bermudagrass	0	0	0.909 a	0.726 a	0.788 a	0.199 a	0.194 a	0.194 a	0.214 a	0.166 a	0.186 a
	0.28	0	0.733 c	0.718 b	0.726 b	0.162 b	0.182 ab	0.184 ab	0.169 b	0.159 b	0.175 b
	0	0.14	0.743 b	0.685 c	0.724 b	0.167 b	0.185 b	0.187 ab	0.169 b	0.148 c	0.171 c
	0.28	0.14	0.733 c	0.675 d	0.712 c	0.163 b	0.179 b	0.181 b	0.169 b	0.146 c	0.163 d
LSD$_{0.05}$			0.0085	0.0077	0.0064	0.0098	0.0091	0.0111	0.0046	0.0055	0.0033

Different letters in each column indicate significant differences from each other, at the 0.05 significant level according to the Fisher's Protected LSD test. Carotenoids quantified included β-carotene lutein and the oxygenated xanthophylls. Abbreviation, DAT = days after treatment.

Table 3 - Singlet oxygen activity determined in shoots of bermudagrass treated with atrazine and mesotrione, 1, 5, and 8 d after treatment, with respect to non-treated samples

Specie	Herbicide		O^{2-} content		
	Atrazine	Mesotrione	1 DAT	5 DAT	8 DAT
	(g ai ha^{-1})		(nmol s^{-1} µg^{-1} protein)		
Bermudagrass	0	0	1.783 d	2.206 d	2.979 d
	0.28	0	1.909 c	2.502 c	3.620 c
	0	0.14	2.080 b	2.619 b	4.571 b
	0.28	0.14	2.199 a	2.771 a	5.206 a
LSD$_{0.05}$			0.1013	0.1128	0.1166

Different letters in each column indicate significant differences from each other, at the 0.05 significant level according to the Fisher's Protected LSD test. Abbreviation, DAT = days after treatment.

In the present research, carotenoids quantified included β-carotene lutein and the oxygenated xanthophylls (Lichtenthaler and Wellburn, 1983). Results are comparable with those of previous research in which isoxaflutole, an HPPD-inhibiting herbicide, reduced chlorophyll concentrations of 'Prelude' perennial ryegrass (*Lolium perenne*) (Bhowmik and Drohen, 2001). In large crabgrass [*Digitaria sanguinalis*], McCurdy et al. (2009) noted similar pigment reduction, including Chl a, Chl b, and carotenoids, following mesotrione application at 0.28 g ha^{-1}. Reduced Chl a concentrations might be associated with D1 protein destruction in PS II reaction center by ROS. Normally, ROS could be quenched by α-tocopherol, carotenoids, and antioxidant enzymes (Zhang, 1996; Hess, 2000; Sharma, 2012; Zhang et al., 2015). It was noted that α-tocopherol is the most effective antioxidant and react quickly with ROS, including singlet oxygen and lipid peroxide radicals (Hess, 2000). β-carotene lutein is also an important quencher of triplet chlorophyll in addition to singlet oxygen (Edge et al., 1997). However, mesotrione prevents the production of these photoprotecting compounds (Hess 2000; Triantaphylidès and Havaux, 2009; Woodyard et al., 2009), resulting in lipid peroxidation, pigment and protein degradation.

In the present study, atrazine and mesotrione caused similar reduction in Chl a and Chl b concentrations. Chlorophyll degradation following atrazine and mesotrione applications could be attributed to increased formation of ROS. In mesotrione-treated bermudagrass, reduced carotenoid

concentrations could also be attributed to the HPPD inhibition, resulting in increased phytoene concentration owing to the indirect inhibition of phytoene desaturase (Hess, 2000). Atrazine inhibits photosynthesis by competing with plastoquinone (PQ) and binding to the Q_B-binding niche on the D1 protein of the PS II complex in the chloroplast thylakoid membrane. The blockage of electron flow promotes the formation of triplet chlorophyll, which reacts with ground stage oxygen producing single oxygen (Hess, 2000). Both triplet chlorophyll and singlet oxygen attack chlorophylls, carotenoids, lipids, and proteins, as well as other cellular constituents (Krieger-Liszkay, 2005; Krieger-Liszkay et al., 2008; Sharma et al., 2012). PQ is an essential cofactor for phytoene desaturase within the carotenoid desaturation reactions, leading to an indirect inhibition of carotenoid synthesis. Additionally, PQ acts as an intermediate electron carrier between the carotenoid desaturase and the photosynthetic electron transport chain (Norris et al., 1995). For this reason, atrazine plus mesotrione caused greater phototoxic effect on susceptible plant species such as bermudagrass, which was evident from the considerably reduced membrane stability observed as a function of enhanced single oxygen and MDA contents, as well as greater reduction in Chl *a*, Chl *b*, and carotenoid concentrations than either herbicide alone.

Findings concerning MDA content are in agreement with the activity of singlet oxygen. The treatment of bermudagrass plants with atrazine caused significant increases in the MDA content at 1 and 8 DAT that were 13 and 21% more than the non-treated plants (Table 4). The treatment of bermudagrass with mesotrione caused significant increases in MDA content at 1, 5, and 8 DAT that were 17, 19, and 53% more than the non-treated plants. In the case of atrazine plus mesotrione, the greatest accumulation of MDA was noted.

Table 4 - Malondialdehyde content determined in shoots of bermudagrass treated with atrazine and mesotrione, 1, 5, and 8 d after treatment, with respect to non-treated samples

Specie	Herbicide		MDA		
	Atrazine	Mesotrione	1 DAT	5 DAT	8 DAT
	(g ai ha^{-1})		(μmol g^{-1} FW)		
Bermudagrass	0	0	0.033 c	0.024 c	0.018 c
	0.28	0	0.034 c	0.025 bc	0.019 c
	0	0.14	0.035 b	0.026 b	0.023 b
	0.28	0.14	0.036 a	0.030 a	0.027 a
LSD$_{0.05}$			0.0005	0.0021	0.0017

Different letters in each column indicate significant differences from each other, at the 0.05 significant level according to the Fisher's Protected LSD test. Abbreviations, DAT = days after treatment; MDA = malondialdehyde.

Data presented in Table 4 showed that the MDA content significantly increased in bermudagrass leaves in response to atrazine, mesotrione, or atrazine plus mesotrione application. It was noted that unsaturated fatty acids of the thylakoids could be completely degraded to ethane and MDA. For this reason, MDA is considered as a good biochemical marker of the structural integrity of plant cell membranes because it indicates the level of lipid damage for reactions of oxidation (Del Buono et al., 2011; Zhang et al., 2015). The higher MDA content in bermudagrass leaves following atrazine plus mesotrione combination is likely due to the joint activity of atrazine plus mesotrione owing to the greater formation of ROS than either herbicide applied alone. These findings were in accordance with the increased singlet oxygen ascertained in herbicide-stressed samples of bermudagrass.

At 1 DAT, atrazine or mesotrione alone did not significantly reduce protein content, while atrazine plus mesotrione combination significantly reduced protein content than either herbicide applied alone (Table 5). Atrazine caused significant decreases of 6 and 18% in protein content at 5 and 8 DAT, respectively. Mesotrione caused significant decreases of 8 and 15% in protein content at 5 and 8 DAT, respectively. Atrazine plus mesotrione significantly decreased protein content more than atrazine or mesotrione alone.

Table 6 reports the effect of the herbicides on the activity of CAT, POD, and GST. Concerning CAT activity, there were significant increases in the enzyme activity in response to the herbicide

Table 5 - Protein content determined in shoots of bermudagrass treated with atrazine and mesotrione, 1, 5, and 8 d after treatment, with respect to non-treated samples

Specie	Herbicide		Protein content		
	Atrazine	Mesotrione	1 DAT	5 DAT	8 DAT
	(g ai ha^{-1})		(µg g^{-1} FW)		
Bermudagrass	0	0	5.542 a	5.188 a	4.364 a
	0.28	0	5.242 ab	4.853 b	3.592 c
	0	0.14	5.364 a	4.791 b	3.724 b
	0.28	0.14	4.942 b	4.736 b	3.506 d
LSD$_{0.05}$			0.3217	0.1549	0.0721

Different letters in each column indicate significant differences from each other, at the 0.05 significant level according to the Fisher's Protected LSD test. Abbreviation, DAT = days after treatment.

Table 6 - Catalase, peroxidase, and glutathione S-transferase activities determined in shoots of bermudagrass treated with atrazine and mesotrione, 1, 5, and 8 d after treatment, with respect to non-treated samples

Specie	Herbicide		CAT			POD			GST		
	Atrazine	Mesotrione	1 DAT	5 DAT	8 DAT	1 DAT	5 DAT	8 DAT	1 DAT	5 DAT	8 DAT
	(g ai ha^{-1})		(Unit µg^{-1} protein)			(Unit µg^{-1} protein)			(µmol s^{-1} µg^{-1} protein)		
Bermudagrass	0	0	7.88 b	7.29 d	5.26 c	7.88 c	5.98 d	4.81 d	2.68 c	2.89 b	2.15 d
	0.28	0	8.04 b	7.67 c	7.43 a	8.50 b	6.93 c	7.52 c	2.59 c	3.03 b	2.70 c
	0	0.14	8.75 a	8.17 b	7.01 b	8.87 a	7.71 b	7.94 b	3.79 a	3.95 a	3.65 a
	0.28	0.14	8.86 a	9.47 a	7.49 a	8.96 a	8.93 a	9.14 a	3.48 b	3.79 a	3.21 b
LSD$_{0.05}$			0.2055	0.3720	0.2043	0.1351	0.1738	0.1167	0.2418	0.2944	0.3660

Different letters in each column indicate significant differences from each other, at the 0.05 significant level according to the Fisher's Protected LSD test. Abbreviations: CAT= catalase; DAT = days after treatment; POD = peroxidase; GST = glutathione reductase.

treatment, particularly at 8 DAT. In particular, the CAT activity in plants treated with atrazine and mesotrione alone increased by 41 and 33%, respectively. Atrazine plus mesotrione combination significantly increased the CAT activity than either herbicide applied alone. Atrazine plus mesotrione combination significantly increased the CAT activity by 13, 30, and 42% at 1, 5, and 8 DAT, respectively.

Regarding POD activity, all herbicide treatments significantly increased the enzyme activity. The POD activity exhibited different responses to the treatments. In particular, mesotrione increased POD activity more than atrazine at 5 and 8 DAT. Atrazine plus mesotrione combination significantly increased POD activity; the increases were 14, 49, and 90% at 1, 5, and 8 DAT, respectively.

Concerning GST activity, atrazine did not significantly affect the GST activity at 1 and 5 DAT, while significantly increased the enzyme activity at 8 DAT. In particular, increased GST activity by 26% was determined at 8 DAT. The treatment of bermudagrass with atrazine plus mesotrione significantly increased the GST activity by 30, 31, and 49% more than the non-treated control at 1, 5, and 8 DAT, respectively. In the case of mesotrione alone, significant increases of GST activity by 41, 36, and 70% more than non-treated control were noted at 1, 5, and 8 DAT, respectively.

ROS are naturally generated at the thylakoid and are normally scavenged by a series of antioxidants within the stroma or dissipated as heat (Edge et al., 1997; Sharma et al., 2012; Zhang et al., 2015). In the present study, atrazine and mesotrione treatments caused the production of ROS, which are toxic to the plant cells. To cope with the toxic effect, plants activated antioxidant activities based on non-enzymatic and/or enzymatic systems (Zhang and Kirkham, 1996; Peixoto et al., 2008; Del Buono et al., 2011; Zhang et al., 2015; Jiang et al., 2016). In the present study, the antioxidant enzymes, including CAT, POD, and GST, were assayed. Results showed that atrazine and mesotrione, as well as atrazine plus mesotrine significantly increased CAT, POD, and SOD activities in bermudagrass. CAT is the most important enzyme in regulating

the hydrogen peroxide content (Peixoto et al., 2008). GST is a very important enzyme for the resistance of plants to many classes of herbicides. For example, enhanced GST activity was noted to be responsible for multiple-herbicide resistance in annual ryegrass (*Lolium rigidum*) and blackgrass (*Alopecurus myosuroides*) (Cummins et al., 2013). In a recent investigation, increased antioxidant enzyme activities, including APX, CAT, POD, and SOD, were noted to enable pearl millet (*Pennisetum americanum*) seedlings to cope with the oxidative stress induced by the moderate concentrations of atrazine (Jiang et al., 2016). It was noted that the cooperative functions of the antioxidant enzymes and non-enzymatic scavengers such as α-tocopherol and carotenoids protect plant cells from the oxidative stress and maintain the redox status of the cells (Cho and Seo, 2005; Del Buono et al., 2011; Jiang et al., 2016). In our experiments, although bermudagrass activated the antioxidant enzymes, it was significantly injured by the herbicide treatments. Therefore, we postulated that when ROS are excessively produced and antioxidants become overloaded, the susceptible plants could be significantly injured, even if the antioxidant enzymes are activated.

The roles of antioxidant enzymes in protection against oxidative processes have been shown in transgenic plants overexpressing Mn-SOD, Fe-SOD, POD, and GST (Yun et al., 2000; Yu et al., 2003; Wang et al., 2004). For example, Yun et al. (2000) reported that transgenic tobacco (*Nicotiana tabacum*) plants expressing either a sweet potato (*Ipomoea batatas*) anionic POD or neutral POD had increased capacity in scavenging H_2O_2 and contributed to increased protection against paraquat mediated oxidative damage. Similarly, Yu et al. (2003) reported that the transgenic tobacco plants overexpressing the GST gene were normal in growth and mature compared with control, but had significantly higher levels of GST activities and showed an enhanced resistance to oxidative stress inducted by a low concentration of paraquat. The antioxidant enzyme activities under herbicidal stress and their relationship with oxidative protection have not been previously reported in turfgrasses. Perhaps, increasing enzymatic antioxidant activities is an approach for breeding the herbicide resistant turgrass cultivars.

In summary, this research confirmed that atrazine plus mesotrione could cause greater injury on bermudagrass plants and physiological damage including membrane peroxidation, pigment and protein degradation than each of these herbicides alone. Bermudagrass activated some of its antioxidant enzymes to alleviate the oxidative stress caused by the herbicide treatments. Nevertheless, results from this study showed that bermudagrass was significantly injured by the herbicide treatments, although the plants activated the antioxidant enzymes of CAT, POD, and SOD. These observations are likely due to the excessive formation of ROS in plant cells caused by herbicide treatments. Although the plants activated the antioxidant system, ROS still caused the lipid peroxidation, pigment and protein degradation, as well as other cellular constituent damage.

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