

Responses in root growth, nitrogen metabolism and nutritional quality in *Brachiaria* with the use of thiamethoxam

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Abstract This study investigated the effects of using increasing doses of thiamethoxam, a neonicotinoid insecticide, applied as seed treatment, on the physiological, biochemical and nutritional composition of *Brachiaria brizantha* cv. Piatã. We conducted two experiments with *B. brizantha* in a greenhouse, in the absence of insects. Biometric parameters and nutritional quality, for feed, were measured in the first experiment, and the root system and the leaf nitrate reductase activity were analyzed in the second experiment. The doses of: 0, 17.5, 35.0, 70.0 and 140.0 g of thiamethoxam per 100 kg of seeds, were applied in both experiments. The results show that the seed treatment with thiamethoxam resulted in a slight decrease in shoot development, but provided root growth, which led to an increase in the nitrate reductase activity that resulted in the higher crude protein content in the shoot. Our data indicate that thiamethoxam alters the metabolism and physiology of *B. brizantha*, and improving the feed quality is thus becoming a technological tool to be applied to crops.

Keywords Pesticide · Bioactivator · Grass · Vigor

Abbreviations

TMX	Thiamethoxam
DAP	Days after planting
NRA	Nitrate reductase (EC 1.6.6.2) activity
DM	Dry matter
CP	Crude protein
NDF	Neutral detergent fiber
IVD	In vitro digestibility

Introduction

In modern agriculture, it is essential to use technologies that promote quality and quantity of plant species for food, feed and fuel, to meet current and future demands (Spiertz and Ewert 2009). The application of agricultural chemicals as seed treatment has been successfully used in agriculture in the world, since they provide the best defense mechanisms and consequently a better initial development of the culture (Castro et al. 2008), however, when an agrochemical activates gene expression that leads to higher water absorption and ion transport, or activates the primary and secondary metabolism resulting in gains in vigor and consequently higher plant growth and yields, it is classified as bioactivator (Castro et al. 2009).

It has been reported in recent literature that the TMX molecule [3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro) amine], a systemic pesticide belonging to the neonicotinoids group of large spectrum of activity at low concentrations (Maienfisch et al. 2001a, b). This molecule is used in the control of sucking insects and some chewing species, because of its

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excellent absorption and translocation in plant tissues (Maienfisch et al. 2001b), leading to a persistent effect in the plant (Maienfisch et al. 2001a) and then adopts its bioactivator action, triggering the physiological and metabolic responses in the plant cycle.

In Brazil, the pesticide TMX is registered under the trademark Cruiser® (Syngenta Crop Protection Ltd., São Paulo, SP, Brazil) and is used for seed treatment of *Brachiaria* sp., at doses ranging from 100 to 300 mL/100 kg of seeds for the control of *Cornitermes cumulans* (MAPA 2011). In the world, TMX is used in 115 cultures, in at least 64 countries, and is the second most commercialized neonicotinoid (Elbert et al. 2008).

This molecule acts to moderate the metabolism of pea, corn and soybean plants (Horii et al. 2007), increasing grain production, viable nodules (Calafiori and Barbieri 2001) and emergence rate (Pynenburg et al. 2011) in bean. Moreover, it stimulates the synthesis of antioxidant enzymes in soybean seedlings subjected to drought conditions (Cataneo et al. 2010), and it boosts growth (Perelló and Dal Bello 2011), metabolism and production (Macedo and Castro 2011) in wheat.

There is a dearth of information concerning the physiological effects of pesticides on tropical grasses, given the high demand of these plants for use in animal feed. In Brazil, pastures occupy circa 172,333,073 ha, accounting for approximately 48.56 % of the agricultural area of the country (IBGE 2006). In this large pasture area, the genus *Brachiaria*, African origin grass, stands out because it shows traits of resistance to acidic and nutrient-deficient soils. Furthermore, it has easy propagation by seeds, great competitive advantage with weeds and presents good animal performance when compared to native pastures (Valle et al. 2010). These facts justify the rapid expansion of this species in tropical savannas in the world (Guenni et al. 2002).

There was a growing demand for quality forages in the early twenty-first century. This necessity has led companies in the area seek to incorporate new technologies into their products (Teodoro et al. 2011), this gives the TMX become an excellent tool technology being developed for forage seeds.

The objective of this study was to investigate the effects of increasing doses of the TMX on biometric, physiological and metabolic parameters of *Brachiaria brizantha* cv. Piatã under greenhouse conditions.

Materials and methods

Greenhouse experiments

Two experiments were conducted in a greenhouse (20–35 °C), in the absence of insects, using plants of

B. brizantha (A Hochst. Ex A. Rich.) Stapf. cv. Piatã, in the municipality of Piracicaba, São Paulo state, Brazil (22°42'S and 47°38'W). The grass seeds were treated at 0; 17.5; 35; 70 and 140 g of TMX, diluted in 2,000 mL of water for 100 kg⁻¹ of seeds. In both experiments, the pots were filled with a substrate composed of clay, silt and sand, respectively, at the ratio: 267, 113, 620 g kg⁻¹, with the following chemical characteristics: pHCaCl₂ 5.0; O.M. 14.0 g dm⁻³; P 2.9 mmol dm⁻³; K 1.4 mmol dm⁻³; Ca 42 mmol dm⁻³ and Mg 7 mmol dm⁻³. The soil had base saturation of 66 %, a suitable value for the crop in question. The fertilization followed the guidelines for the culture formation, corresponding to 40 kg ha⁻¹ of N; 30 kg ha⁻¹ of K₂O and 20 kg ha⁻¹ of S (Raij et al. 1996).

First experiment

Plants were grown in pots with a capacity of 20 dm³ where we analyzed the growth and development of shoots as well as grass quality using an average of two plants per pot. The planting was carried out on April 30, 2011 and the material was collected on June 9, 2011 (40 days cycle). The experimental design was of randomized blocks, consisting of the application of five TMX doses, with eight samplings for the biometric analyses and four samplings for the analyses of nutritional composition and digestibility.

Biometric analysis

The emergence rate (%) of the plants was measured at 14 DAP. We sowed 10 seeds per pot to estimate the emergence rate; thereafter, we selected the plants to maintain only two plants per pot to study the other variables. Measurements of height (cm) occurred between 21 and 35 DAP, which were taken from the soil surface until the end of the longest leaf of the main stem. At the end of the cycle, 40 DAP, we counted the number of tillers and cut the shoots, separating leaves and stems. Immediately after, the leaves were subjected to the analysis of leaf area (cm²) using the equipment LI-3100 (LI-COR Biosciences, Lincoln, USA). The leaves and stems were weighed on a precision scale to measure their fresh biomass (g), then all the materials were dried in an oven for 72 h at 60 °C to obtain the dry biomass of leaves (g) and stems (g).

Analysis of nutritional composition and digestibility

The DM and CP contents were determined according to the AOAC (1995). The NDF content was established using the method proposed by Van Soest (1994). These variables were expressed in percentage (%) of the total DM at 105 °C.

The IVD evaluation was performed according to the method proposed by Tilley and Terry (1963). The method was conducted using 10 mL of rumen fluid mixed with 40 mL McDougall's buffer to 500 mg of sample and incubated for 48 h in a heating cabinet at 38 °C. The second step of digestion was performed by incubating samples for 46 h with 20 % of hydrochloric acid and 5 % of pepsin. The insoluble residue was then weighed and filtered in distilled water, under slight vacuum, for 2 h and brought to 105 °C for 24 h. They were then weighed again and the difference between the weights determined the value of IVD.

Second experiment

The second experiment aimed to study the root growth and enzyme activity in leaves. The seeds were sown in long pots with the capacity of 3 dm³, allowing for better root development and is more practical for roots extraction, for evaluation. We sowed five seeds per pot and at 7 DAP, we selected the plants to maintain one plant per pot. The planting was carried out on May 5, 2011 and the material was collected on June 14, 2011, completing a cycle of 40 days. The experimental design was completely randomized and consisted of the application of five TMX doses, with five replicates for the analyses of root growth and three replicates for the analyses of NRA.

Root image analysis

At 40 DAP, the roots were washed in running water to remove excess soil and dried on paper towels. For the scanning process, the roots were spread on a glass plate disposed directly on the desktop scanner HP Scanjet 2400, 200 dpi resolution, and covered with a thin water layer. Then, the scanned images were submitted to the SAFIRA[®] software to measure area and root volume (Jorge and Silva 2010).

Nitrate reductase (E.C. 1.6.6.2) activity assay

The NRA analysis was performed at 40 DAP using a methodology adapted from Radin (1974). The collection of fresh leaves occurred between 8:00 and 9:00 'o'clock, then, the material was placed in coolers filled with ice and transported to the laboratory where it was cleaned on the surface with deionized water and dried on paper towels. For the NRA assay, 100 mg of fresh tissue cut in discs was transferred to assay tubes containing 3 mL of phosphate buffer solution pH 7.4 and 200 mM of KNO₃. Thereafter, the assay tubes were incubated in a water bath at 37 °C for 30 min wrapped in aluminum foil against the light. The reaction was stopped by adding 1 mL of 1 % sulfanilamide

in 2 mol L⁻¹ HCl solution and after that, 1 mL of 0.05 % naphthalenediamine solution. The nitrite (NO₂⁻) produced was measured in a spectrophotometer at 540 nm, using a nitrite standard calibration curve. The enzyme activity was directly related to the amount of NO₂⁻ and the results were expressed in μmol NO₂⁻ g⁻¹ h⁻¹ FM.

Statistical analysis

The data were analyzed with SAS software 9.2 (SAS 2009) by the analysis of variance (ANOVA) and, in case of significance, we carried out a regression analysis. The mathematical models proved more suitable when the analysis of the *F* test for regression was significant ($P < 0.10$; $P < 0.05$; $P < 0.01$) and the coefficient of determination (R^2) showed greater accuracy, electing the linear model ($\hat{y} = a_0 + a_1x + \varepsilon$) or quadratic polynomial ($\hat{y} = a_0 + a_1x + a_2x^2 + \varepsilon$) when more adjusted. Data on area and root volume were transformed into log¹⁰ following assumptions of guided data analysis (SAS 2009).

Results

Emergence and plant height

We found that at 14 DAP, for the regression analysis, no differences were observed for the emergence rate (%), and height (cm), at 21, 28 and 35 DAP, when seeds were treated with TMX (Supplementary Table 1). It is assumed that under adequate conditions of light, temperature and nutrients, TMX presents a stable behavior, with no interference on germination and growth, when compared to the control. These effects have already been reported in the literature, with the use of bioactivator.

Tillering and leaf area

We did not observe differences between TMX doses for the number of tillers per plant (Supplementary Table 1). However, we found later that for the leaf area, measured in cm² (Fig. 1), there was a weak negative correlation among mean true leaf area at different levels of treatment ($P < 0.10$). We also observed a high value for the coefficient of variation (Supplementary Table 1).

Fresh biomass and dry biomass of shoot

Still reporting on biometric variables, measurements of fresh (g) and dry (g) biomass of leaves and stems did not differ between treatments. However, we found high variability between the observed data, expressed by the high

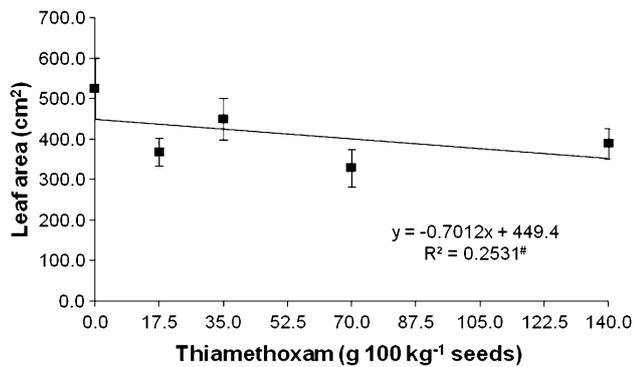


Fig. 1 Leaf area (cm²) of *Brachiaria brizantha* at 40 DAP, subjected to increasing levels of thiamethoxam, via seed treatment. Vertical bars indicate the standard error of mean ($n = 8$), # $P < 0.10$

value for the coefficient of variation of these biometric variables (Supplementary Table 2).

Nutritional quality and in vitro digestibility

The use of increasing doses of TMX affected the CP content, moreover, F test ($P = 0.17$) did not identify differences between the overall averages (Supplementary Table 2); however, applying the regression analysis was found to have a significant effect on the linear regression ($P < 0.05$) (Fig. 2). These data suggest that there is a functional relationship between the variable levels of TMX and CP. This interpretation generates consistent information about the interference of the treatment on this variable (Pimentel-Gomes and Garcia 2002).

For the DM content ($P < 0.01$), we found high statistical significance due to the doses of TMX. Both results expressed in percentage (Supplementary Table 2) showed an increasing linear response to protein in leaves and a decreasing linear response for DM content (Fig. 2). For the qualitative parameters NDF and IVD, the pesticide did not show improvement or degeneration of the plant material (Supplementary Table 2).

Root analysis and enzymatic activity

The assessment of the root system showed increasing linear effect, highly significant ($P < 0.01$) for the regression test (Supplementary Table 3), where the variables root area (cm²) and volume (cm³) increased with the use of increasing doses of TMX (Fig. 3). For the leaf analysis of NRA, we observed a highly significant quadratic regression ($P < 0.01$) (Supplementary Table 3). Due to the increasing doses of the pesticide, we found an increase in NRA, until an optimal point. With the use of a high dose, the activity gradually reduced, possibly due to the toxic effect of the product (Fig. 4).

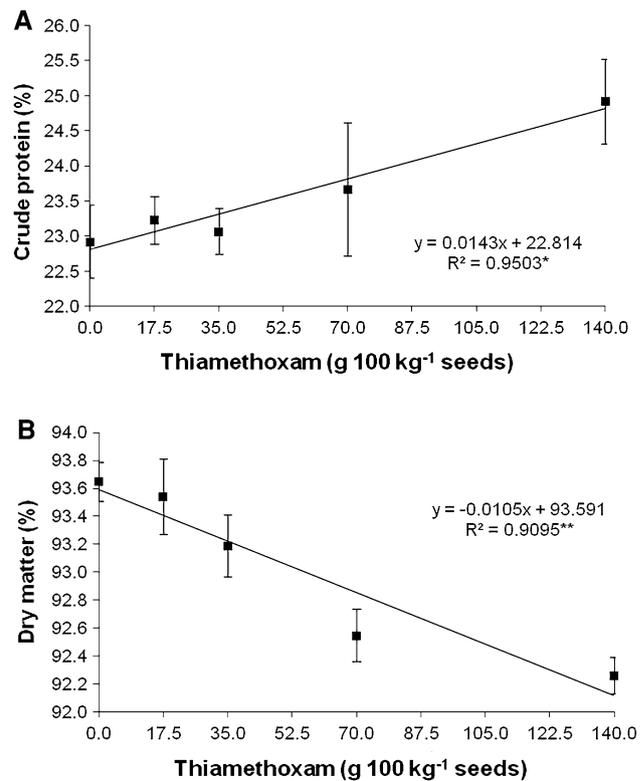


Fig. 2 Crude protein (%) (a) and dry matter (%) (b) of *Brachiaria brizantha* at 40 DAP, subjected to increasing levels of thiamethoxam, via seed treatment. Vertical bars indicate the standard error of mean ($n = 8$), * $P < 0.05$; ** $P < 0.01$

Discussion

In the literature, there are reports on the growth promotion in cultures of agronomic interest with the use of pesticides (Coosemans and Van Assche 1981; Reddy et al. 1997a, b); there are also studies that report negative biological effects of pesticides on crops (Moore and Kröger 2010; Çavuşoğlu et al. 2011; Dias 2012). However, Khaleeq and Klatt (1986) state that, for wheat crop, the physiological responses of pesticides on plants depend on the product used, as well as the variety studied. This study aims to understand what physiological and biochemical mechanisms of TMX insecticide affect the *B. brizantha* culture and enhance its development.

Regarding the analysis of biometric parameters, no significant effects on emergence rate, height, number of fertile tillers (Supplementary Table 1), fresh and dry shoot biomass (Supplementary Table 2) were observed.

However, we observed a slight linear decrease of leaf area when exposed to the increasing doses of TMX (Fig. 1), which is opposite to results reported by Teodoro et al. (2011) who found no differences between the control treatment and the fipronil insecticide on the leaf area in *B. brizantha*. We therefore believe that the TMX, in the

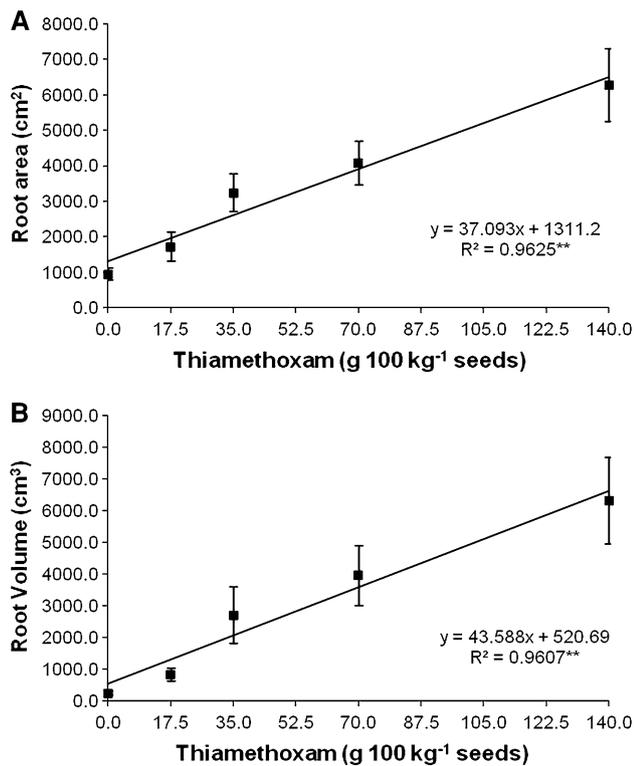


Fig. 3 Root area (cm^2) (a) and root volume (cm^3) (b) of *Brachiaria brizantha* at 40 DAP, subjected to increasing levels of thiamethoxam, via seed treatment. Vertical bars indicate the standard error of mean ($n = 5$), $**P < 0.01$

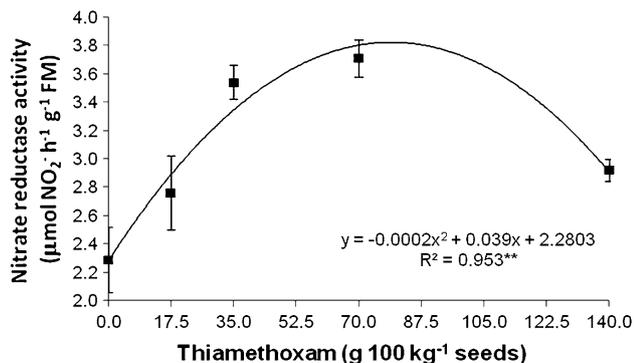


Fig. 4 Nitrate reductase activity ($\mu\text{mol NO}_2^- \text{h}^{-1} \text{g}^{-1} \text{FM}$) in *Brachiaria brizantha* leaf at 40 DAP, subjected to increasing levels of thiamethoxam, via seed treatment. Vertical bars indicate the standard error of mean ($n = 3$), $**P < 0.01$

short term, becomes a cofactor in the negative regulation of the shoot development, which is a negative response, because the leaf area reduction leads to a decrease of plant material used for animal feed.

The leaf area reduction did not result in the losses of the grass nutritional quality (Supplementary Table 2), given that there was an increase in CP (%) in shoots (Fig. 2a). This change in nitrogen compounds content in plant tissues

reflects the good status of nitrogen supply (Miller et al. 2007), providing nutritional quality to the grass that subsequently affects the performance of animals supplemented with this plant material (Waramit et al. 2012). We observed that the high CP, in the control and other treatments, occurred because the cutting of the grass happened near the nitrogen supplementation. The results obtained for the DM content (%) (Fig. 2b) were consistent with those presented for leaf area (Fig. 1), which reduced with the increasing doses of TMX; however, the values were within the expected range for the grass (around 90 %).

Root growth was significantly increased with the increasing TMX doses, resulting in gains of linear area and root volume (Fig. 3a, b, respectively). Similar responses have been reported for roots in sugarcane stalks (Pereira et al. 2010), soybeans (Cataneo et al. 2010) and wheat (Macedo and Castro 2011), when the TMX insecticide was used. This modification of root architecture is an important factor for better use of the soil by plants (Lynch 2007), and therefore it provides better water and nutrient absorption (Castro et al. 2009), which are critical factors for modern agriculture (Lynch 2007).

Castro et al. (2009) have described the enhanced root development at the beginning of seedling growth as the initial process of a chain of metabolic, physiological and anatomical changes that culminate in an increased enzyme activity in the plant during its development. We corroborated this hypothesis by measuring the NRA (Fig. 4), the first enzyme to act in the nitrogen assimilation process by plants, which is controlled by several genetic factors, responsible for converting the nitrate absorbed from soil into nitrite, initiating the synthesis of amino acids in plants (Heldt and Piechulla 2010). Glaab and Kaiser (1999) found a similar result by applying the fungicide kresoxim-methyl on leaf discs of spinach and observed linear gains in the nitrate content and NRA.

We observed an increase in CP (%) in the shoots with the increased availability of nitrogen in the plant (Fig. 2a). This response related to nitrogen metabolism corroborates research by Parween et al. (2011) that used a low dose (0.3 mM) of the insecticide chlorpyrifos in mung bean and found increases in root length, NRA, sugar content and soluble protein content. The low fiber content means greater intake of dry matter, due to the lower physical filling in the rumen, and also a greater digestibility, since this fraction has most of the components that are not digested. While there is no significant response to the variable NDF, it is noticed that in all treatments, the content of this constituent was adequate, showing, with the crude protein content (Mertens 1994), improving quality in animal nutrition.

We emphasize, thus, the potential bioactivator action of some pesticides on the crops of agricultural interest, requiring further studies to prove this action in other plant species.

Conclusions

Thiamethoxam is a molecule with property to alter the metabolism and physiology of *B. brizantha*, when applied to seeds, able to modify root growth and nitrogen uptake, resulting in gains of grass quality.

Author contribution Willian Rodrigues Macedo: design and conducted the experiments, collected and analyzed data, and wrote the manuscript; Gisele Machado Fernandes: participated in the analysis of nutritional quality of grass and assisted in writing the manuscript; Rosana Aparecida Possenti: participated in the analysis of nutritional quality of grass; George Rodrigues Lambais: helped in the biochemical analysis; Paulo Roberto de Camargo e Castro was the research coordinator and assisted in writing the manuscript.

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