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BIOMASS YIELD AND FLAVONOID AND PHENOL CONTENT OF *Schinus terebinthifolius* CULTIVATED IN SINGLE OR DOUBLE ROW WITH POULTRY LITTER

PRODUÇÃO DE BIOMASSA E CONTEÚDO DE FENÓIS E FLAVONOIDES DE *Schinus terebinthifolius* CULTIVADA EM FILEIRA SIMPLES E DUPLA COM CAMA DE FRANGO

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ABSTRACT

The aim of this study was to evaluate the influence of the addition of poultry litter on growth, biomass yield, flavonoid and phenol content, and antioxidant activity in leaves of pink pepper. The experiment was carried out at the Federal University of Grande Dourados, in Dourados, Mato Grosso do Sul state, from October 2009 to May 2010. Pink pepper was grown in single or double row in soil with incorporated poultry litter at doses of 0, 5, 10, 15 and 20 t ha^{-1}. Treatments were arranged as 2 x 5 factorial in a randomized block design with four replications. There was a significant interaction between the doses of poultry litter and evaluation times for plant height and chlorophyll content. A linear increase in leaf area, fresh and dry weight of leaves and diameter of main stems with increasing doses of poultry litter was observed. Plants grown in single row showed higher fresh weight of stem with increasing doses of poultry litter. Poultry litter at 15 and 20 t ha^{-1} promoted an increase in flavonoid and phenolic contents in the leaves. No significant effect on the antioxidant activity was observed by the chemical method using DPPH. Therefore, it is recommended the cultivation of pink pepper plants with double row and 20 t ha^{-1} of poultry litter to higher growth, biomass yield, and flavonoid and phenol content.
Keywords: organic waste; pink pepper; plant density.

RESUMO

O objetivo deste estudo foi avaliar a influência da adição da cama de frango ao solo sobre o crescimento,

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produção de biomassa, conteúdo de fenóis e flavonoides e atividade antioxidante em folhas de pimenta-rosa. O experimento foi realizado na Universidade Federal da Grande Dourados, em Dourados-MS, de outubro de 2009 a maio de 2010. A pimenta-rosa foi cultivada em fileira simples e dupla com cama de frango incorporada nas doses de 0, 5, 10, 15 e 20 t ha^{-1}. Os tratamentos foram arranjados em fatorial 2 x 5 em blocos casualizados com quatro repetições. Houve uma interação significativa entre doses de cama de frango e épocas de avaliação para altura de plantas e conteúdo de clorofila. Foi observado um aumento linear na área foliar, massa fresca e seca de folhas e diâmetro dos ramos principais com o aumento das doses de cama de frango. As plantas cultivadas em fileira simples apresentaram maior massa fresca de caules com o aumento das doses de cama de frango. A cama de frango nas doses de 15 e 20 t ha^{-1} promoveu um incremento no conteúdo de fenóis e flavonoides nas folhas. Nenhum efeito significativo na atividade antioxidante foi observado com o método químico utilizando DPPH. Portanto, recomenda-se o cultivo de plantas de pimenta-rosa em fileira dupla e 20 t ha^{-1} de cama de frango para maior crescimento, produção de biomassa, e conteúdo de fenóis e flavonoides.
Palavras-chave: resíduo orgânico; pimenta-rosa, densidade de plantas.

INTRODUCTION

Schinus terebinthifolius Raddi (Anacardiaceae) is a perennial tree or shrub native from South America, occurring in Brazil, from Bahia state down to Rio Grande do Sul state (CORRÊA, 1974). It is popularly called pink pepper or Brazilian pepper, and "pimenta-rosa" in Brazil (PIRES et al., 2004). In traditional medicine it has been used for the treatment of inflammations (GAZZANEO et al., 2005), respiratory problems, wounds, rheumatism, and diarrhea, as well as antiseptic and haemostatic (MEDEIROS et al., 2007).

The importance of this plant promoted its inclusion in the Brazilian Pharmacopeia (BRANDÃO et al., 2006) and in the National List of Medicinal Plants of interest to SUS (Unified Health System), which has medicinal plants with potential to generate products of interest to SUS (RENISUS, 2009). Pink pepper has been frequently studied from a chemical viewpoint (DEGASPARI et al., 2005; LIMA et al., 2009; CERUKS et al., 2007; RICHTER et al., 2010). Leaves and fruit are rich in tannins, flavonoids, and essential oil (RICHTER et al., 2010), while saponins are restricted to bark (RIBAS et al., 2006). Oral administration of pink pepper dried extract did not induce any toxic effects in rats, which could stand as an assurance for the medicinal use of this plant in folk medicine (LIMA et al., 2009). For the leaves, fruit, and bark antimicrobial, analgesic, anti-inflammatory, antioxidant, anti-allergic, anti-free radical and insecticidal *in vitro* activities have been described (DI STASI et al., 2002; DEGÁSPARI et al., 2005; CERUKS et al., 2007; CAVALHER-MACHADO et al., 2008). However, there is a lack of detailed agronomic studies in literature, aiming higher biomass yield and secondary metabolites of medicinal interest.

Pink pepper is a pioneer species (SOUTO and BOEGER, 2011) with high growth rates, vigorous re-sprouting and tolerance of fluctuating groundwater levels (EWE and STERNBERG, 2002), being therefore indispensable in the heterogeneous reforestation used for restoration of degraded areas of permanent preservation. Additionally, it is tolerant to shade, fire, saline conditions, and droughts. It also has allelopathic effects on neighboring plants (DONNELLY et al., 2008).

Among the factors that may interfere directly in the growth and biomass yield are plant density, availability of nutrients, and organic matter in soil. Furthermore, the various methods used to cultivate the plants may induce changes in the chemical composition of the plant tissue. Accordingly, poultry litter applications have contributed to increase the soil organic matter (TOMLINSON et al., 2008), C, N, P, K, and other nutrients (MULLINS and BENDFELDT, 2001). It is a valuable organic fertilizer for sustaining soil fertility and supporting plant growth (GUO and SONG, 2009). Moreover, as the Brazilian productive system of broilers generates a very large volume of this waste, land application can be a suitable disposal method avoiding environmental problems. Nutrients provided by poultry litter have been reported to have positive effects on forage, corn and soybean yield (SISTANI et al., 2008; WARREN et al., 2008).

Considering the value of the medicinal plants, not only as a therapeutic but also as a source of economic resources, besides the lack of standardization and the material available in the national and international trade, with low quality and low content of active principles, it is important to establish lines of actions directed toward the development of management techniques or cultivation. Therefore, the aim of this study was to evaluate the growth, biomass yield, flavonoid and phenol content, and the antioxidant activity in leaves of pink pepper plants, cultivated in single or double row and in soil with different doses of semi-decomposed poultry litter.

MATERIAL AND METHOD

The experiment was carried out at the Federal University of Grande Dourados - UFGD, in Dourados-MS state, Brazil, from October 2009 to May 2010. The coordinates of the Medicinal Plant Garden are 22°11'44"S of latitude, 54°56'07W" of longitude, and 452 m of altitude. The climate of Dourados, according to Köppen, is mesothermal humid, Cwa type, with temperature, and annual rainfall averages ranging from 20º to 24ºC and 1250-1500 mm, respectively. Soil in the area of cultivation is classified as a dystrophic red oxisol of clayey texture, with the following chemical characteristics: 4.92 of pH in H_2O; 28.9 g dm^{-3} organic matter; 12.59 mg dm^{-3} P; and 0.0, 4.7, 25.0, 17.0, 53.0, and 25.41 $mmol_c$ dm^{-3} of Al^{+3}, K, Ca, Mg, H+Al, and SB, respectively.

The spread of pink pepper was performed by indirect sowing, in July 2009, with seeds released from the exocarps. Seedlings were produced initially in polystyrene trays with 128 cells, with Plantmax® substrate, kept in the greenhouse with shade 50%, with daily irrigation, and transplanted to the field when the seedlings had about 15 cm height (70 days after sowing).

Pink pepper was grown in single or double row, in soil with incorporated semi-decomposed poultry litter at doses of 0, 5, 10, 15 and 20 t ha^{-1}. Analysis of the semi-decomposed poultry litter showed the following chemical characteristics: 20.74 and 52.66% of total moisture and organic matter, respectively; 7.14 of pH; 6.21, 1.03, 0.85, 3.15, 0.54, and 3.07% of Ca, Mg, K, N, Al, and P, respectively; and 8.75 of C/N. Treatments were arranged as 2 x 5 factorial, in a randomized block design with four replications. Each plot was 2.5 m long x 1.0 m wide, with five plants arranged in single row and 10 plants in double rows, with spacing of 0.5 m between plants. The soil was prepared on the planting day, with one plowing and harrowing, and subsequently elevation of plots with a bedshaper rotary cultivator offset and incorporation of the poultry litter. Irrigations were made using the sprinkler system whenever necessary. Spontaneous vegetation was controlled by weeding with hoe between the plots and manually within the plots.

From 30 to 180 days after transplant (DAT) (every 30 days), we evaluated plant height (with ruler in cm) and chlorophyll content (SPAD-502) in all plants in the plot. At 180 DAT, all plants of each plot were harvested, cut close to the soil, and evaluated fresh and dry weight of leaves and stem, leaf area (with electronic integrator LI 3000), and diameter of main branches (results are expressed as g $plant^{-1}$). Furthermore, we determined the total phenolic and flavonoid content and antioxidant activity of the dry material of leaves of each treatment. For this, air-dried leaves of pink pepper (200 g) were exhaustively extracted by maceration with methanol at room temperature. Evaporation of the solvent achieved the methanolic extract (13 g).

Total phenolic content in the methanolic extract of leaves of pink pepper was determined by Folin-Ciocalteu method (MEDA et al., 2005). Specifically, each 100 μL of methanolic extract (1.0 g L^{-1}) was mixed with 1.0 mL of distilled water and 0.5 mL of Folin-Ciocalteu's (1:10 v/v) reagent. After 3 min, 1.5 mL saturated solution of Na_2CO_3 (2%) was added. After 30 min, the absorbance was measured at 765 nm using spectrophotometer. Quantification was done on the basis of the standard curve of gallic acid prepared in 80% methanol and the results expressed in mg gallic acid equivalent per gram of extract. Methanol was used as blank. Assays were carried out in triplicate.

To determine the total flavonoid content, to each 500 μL of methanolic extract was added 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride ($AlCl_3.6H_2O$), 0.1 mL of acetate sodium ($NaC_2H_3O_2.3H_2O$) (1 mol L^{-1}), and 2.8 mL of distilled water. The tubes were kept at room temperature for 40 min. The optical density was measured at 415 nm using spectrophotometer (LIN and TANG, 2007). To calculate the concentration of flavonoids, a calibration curve was prepared using quercetin as standard. Along with these data, a liner regression was made, and the line equation was obtained. And then, the data was used in the calculation of real samples. Results were expressed in mg quercetin equivalents per gram of extract. Assays were carried out in triplicate.

For qualitative analysis of the antioxidant activity, methanolic extracts were analyzed by thin layer chromatography (TLC) using quercetin as a positive control. The plates were eluted in $CHCl_3$/MeOH 10% and after drying they were nebulized with a solution of 0.4 mmol L^{-1} DPPH in MeOH. The plates were observed until the appearance of yellow spots on a background of purple coloration, indicating a possible antioxidant activity (SOLER-RIVAS et al., 2000).

The determination of the antioxidant activity was carried out by *in vitro* photocolorimetric method of stable free radical 2,2-diphenyl-1-picryl-hydrazyl (DPPH), using quercetin as positive control. The method consists in monitoring the consumption of DPPH free radical by the methanolic samples by measuring the decrease in absorbance. In the aliquots of 1 mL of the methanolic samples were added 2 mL of DPPH solution 0.004% and incubated at room temperature for 30 min. The reading of the absorbance of each sample was performed in the spectrophotometer at 517 nm (BLOIS, 1958). All tests were carried out in triplicate. Inhibition percentage (%I) was calculated as follows: $\%I = (A_0 - A) / A_0) \times 100$, where A_0 is the absorbance of DPPH (control) and A is the absorbance of each sample more DPPH.

For all variables of the experiment, the normal distribution of errors was checked by the Anderson-Darling test and homogeneity of error variances by Bartlett test. After attended these assumptions, all data were subjected to the analysis of variance and when the significance was found by F test, data were subjected to regression analysis and Tukey test, all the 5% probability, with the SOC statistic package (Software Científico: NTIA/EMBRAPA).

RESULTS AND DISCUSSION

There was no significant interaction between the doses of poultry litter and rows of plants in the plots for plant height in any of the periods evaluated, indicating that there was no competition for light, water, or nutrients between the plants at higher density. However, we observed a significant interaction between the doses of poultry litter and evaluation times (Figure 1a).

In the transplant, no significant difference was observed among the treatments for plant height, indicating that the plants were with uniform size. Similarly, 30, 60 and 150 days after transplant (DAT), there was no significant effect of poultry litter doses on plant height. No statistical difference was observed between the doses of poultry litter 30 and 60 DAT, since probably there was no decomposition and nutrient release from the poultry litter. On the other hand, 150 DAT the early formation of flowers might have become potentially strong sinks for photossimilates in detriment to vegetative growth, since the partitioning of dry matter among various organs depends on their sink strength, i.e., their competitive ability to attract assimilates (MARCELIS, 1996). According to Jackson (2003), in fruit trees, the early stage of fruit development is particularly important, since strong competition exists between the vegetative and reproductive organs both before and after anthesis.

Pink pepper plants showed a decrease in the growth in height 90 DAT with increasing doses of poultry litter (Figure 1a). On the other hand, at 120 and 180 DAT was observed a linear increase in plant height with increasing doses of poultry litter. This increase is probably due to the release of nutrients and organic matter from the poultry litter. Taller plants require more biomass for vertical growth; however, they expose the leaves to higher light levels, which may lead to increased photosynthetic rates (SCHMITT et al., 2003).

For the relative chlorophyll index, there was a significant interaction only for doses of poultry litter and evaluation times from 90 DAT (Figure 1b); a linear increase in chlorophyll content with increasing doses of poultry litter was observed at 90, 120, 150 and 180 DAT. These results are consistent with those for leaf area (Figure 1c), which showed significant effect only of poultry litter. A linear increase was observed in the leaf area of pink pepper plants with increased doses of poultry litter, with maximum leaf area, at 20 t ha^{-1}, of 26,700.06 cm^2 $plant^{-1}$. Leaf area and relative chlorophyll index are good indicators of growth and soil conditions for plant yield, being positively correlated with biomass yield. This increase in the leaf area and chlorophyll content in high doses of poultry litter can maximize the photosynthetic efficiency of plants, mainly by improving the interception of active photosynthetically radiation for more efficient conversion of intercepted radiation into dry weight and photoassimilate partition in the reproductive organs, resulting in higher yield.

Similarly to leaf area, there was a significant effect only of poultry litter doses for fresh (Figure 2a) and dry weight (Figure 2b) of leaves, and diameter of main stems (Figure 3a), having a linear

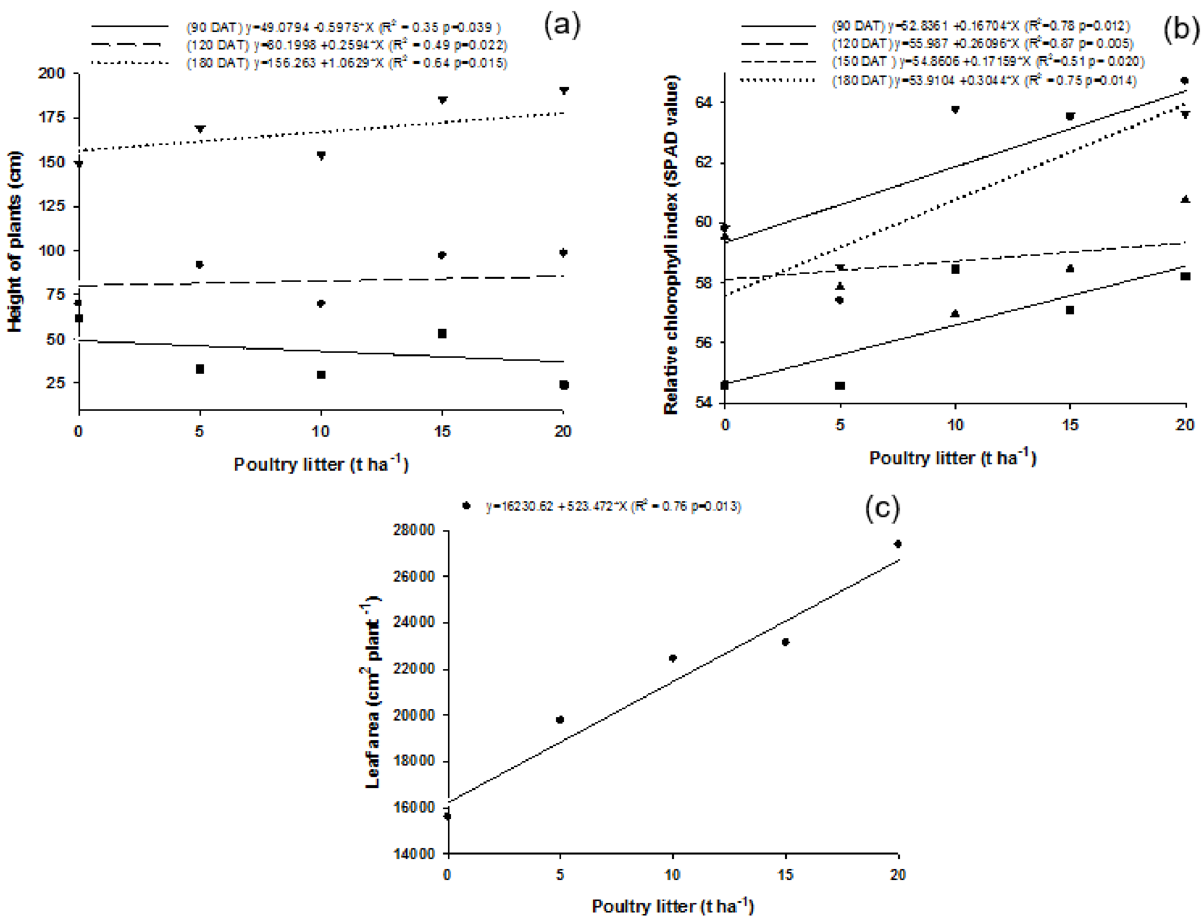

FIGURE 1: Plant height (a) and relative chlorophyll index (b) as a function of poultry litter and evaluation times, and leaf area (c) of pink pepper plants as a function of poultry litter at 180 DAT.

FIGURA 1: Altura de plantas (a) e índice relativo de clorofila (b) em função da cama de frango e épocas de avaliação e área foliar (c) de plantas de pimenta-rosa em função da cama de frango aos 180 DAT.

increase with increasing doses, with maximum yields of 898.09 g plant^{-1}, 69.4 g plant^{-1}, and 19.25 mm, respectively, at 20 t ha^{-1}. Poultry litter has been used as a soil amendment in agriculture to provide nutrients for plant growth (QAFOKU et al., 2001) and increase organic matter in the soil (MELLO and VITTI, 2002). Thus, this increase in biomass yield indicates that the poultry litter provided nutrients and organic matter to soil, improving its chemical and physical attributes (KIEHL, 2008), causing a higher production of photosynthates and, hence, of biomass. Some of these effects are due to the slow release of nutrients from poultry litter, creating greater synchrony with crop nutrient needs (ENDALE et al., 2010). Since the higher investment in height growth (Figure 1a) occurs at the expense of the investment in other plant organs such as the leaves, evidences indicate that the biomass yield capacity of the plant might be reduced (WEINER, 2003). However, in this study the increase in height did not lead to the decrease of total plant biomass.

There was a significant interaction between the doses of poultry litter and rows of plants in the plots to stem fresh weight (Figure 3b). A linear increase in stem fresh weight was observed with increasing doses of poultry litter in both single and double rows; the plants in the single rows showed higher fresh weight of stems. Plants adapt their structural development to the available resources. Probably, the plants growing in single rows had more space for the growth of secondary branches and accumulation of water, since there was no significant effect of factors studied on the dry weight of stems and number of main branches. Moreover,

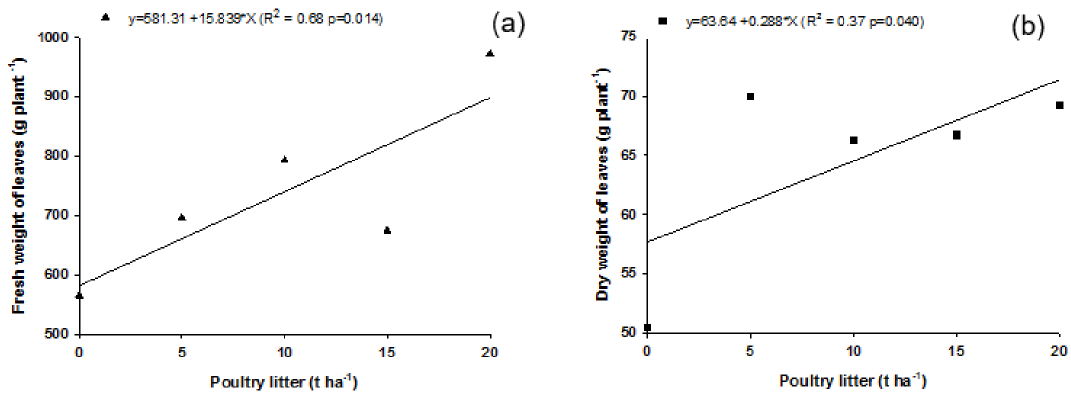

FIGURE 2: Fresh (a) and dry (b) weight of leaves of pink pepper plants as a function of poultry litter at 180 DAT.

FIGURA 2: Massa fresca (a) e seca (b) de folhas de plantas de pimenta-rosa em função da cama de frango aos 180 DAT.

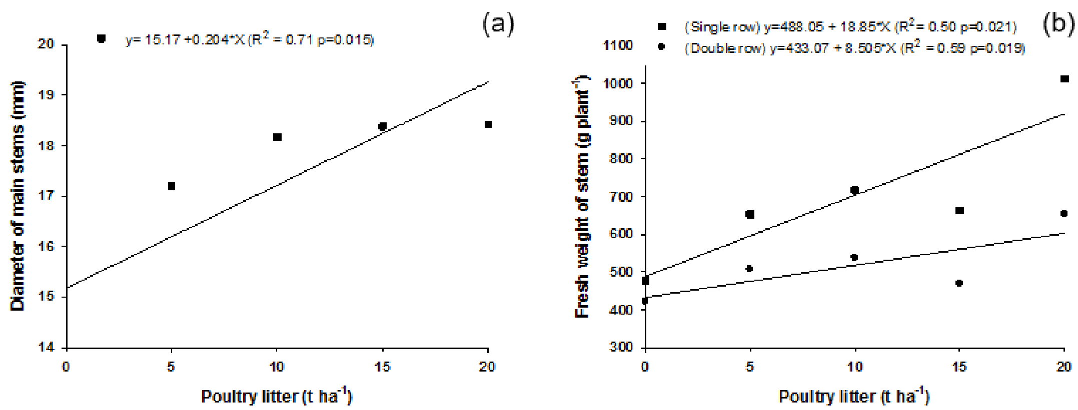

FIGURE 3: Diameter of main stems (a) and fresh weight of stems (b) of pink pepper plants as a function of poultry litter and rows in plot at 180 DAT.

FIGURA 3: Diâmetro dos ramos principais (a) e massa fresca de caules (b) de plantas de pimenta-rosa em função da cama de frango e fileiras de plantas na parcela aos 180 DAT.

plants tend to allocate more biomass to branches when grown in more favorable environment (BONSER and AARSSEN, 2003).

Taking into account the biomass data, it was observed that an increase in the plant density did not influence plant height, chlorophyll content, leaf area, and fresh and dry weight of leaves. Because of this, pink pepper can be grown in double row, seeking the efficient use of land. Moreover, our results indicate that poultry litter can be used as a valuable source of nutrients for pink pepper cultivation.

Concentrations of secondary metabolites change in response to changes in the availability of resources (light, carbon, and minerals) in the habitat (BRYANT et al., 1983). According to pharmacological studies, phenolic compounds lower the risk of coronary heart diseases and lung cancer (KUCHTA, 2001) besides protecting plants against pathogenic agents, herbivores, and competitors (BENNICK, 2002). Phenol contents in the methanolic extract of pink pepper leaves were not influenced by the number of plant rows per plot. However, the phenol contents were significantly influenced by addition of poultry litter in the cultivation of pink pepper. In general, the treatment with poultry litter increased the content of flavonoids and phenolic compounds (Table 1); the highest concentrations were observed at the

TABLE 1: Total phenol and flavonoid content of methanolic extract of pink pepper leaves cultivated with different doses of poultry litter.

TABELA 1: Conteúdo de fenóis totais e flavonoides do extrato metanólico de folhas de pimenta-rosa cultivadas com diferentes doses de cama de frango.

Poultry litter (t ha^{-1})	% Phenol (mg g^{-1})		% Flavonoid (mg g^{-1})	
	Single row	Double row	Single row	Double row
0	363.32 ± 2.93 b	360.34 ± 1.59 b	84.43 ± 1.36 b	83.24 ± 0.85 b
5	369.02 ± 2.76 b	364.81 ± 2.38 b	85.82 ± 1.11 b	86.38 ± 2.03 b
10	368.88 ± 2.03 b	366.38 ± 1.93 b	88.53 ± 1.93 b	86.74 ± 1.68 b
15	408.18 ± 1.13 a	402.48 ± 3.15 a	106.91 ± 1.21 a	107.56 ± 2.15 a
20	414.41 ± 3.40 a	407.82 ± 3.06 a	111.81 ± 3.05 a	110.35 ± 1.15 a

Em que: Results are expressed as mg gallic acid and mg of quercetin equivalent/g extract, respectively. Means followed by same letter in column do not differ by Tukey Test ($p < 0.05$).

doses of 15 and 20 t ha^{-1}, which differed statistically from the other treatments. On average, there was an increase of 13% of phenol content in the methanolic extracts obtained with the highest doses of poultry litter. Regarding flavonoids, an increase of about 32% occurred on average in extracts conditioned with the doses of 15 and 20 t ha^{-1} both in single or double rows (Table 1).

The results of our study seem to contradict the hypothesis that fertilization with nitrogen should decrease the concentration of carbon-based secondary metabolites such as phenolic compounds (JONES and HARTLEY, 1999). Despite the high concentration of nitrogen present in poultry litter, it may not be available for plants of pink pepper because in acid soils, such as the soil present in the area of cultivation of this study, nitrogen incorporation can be inhibited due to a reduction in the rate of conversion of ammonium to nitrate (WATERMAN and MOLE, 1989). This indicates that, in further studies it is necessary the correction of soil for the cultivation of pink pepper.

The radical scavenging activity was determined by the DPPH test. At first, the extracts were qualitatively assessed by the test on Thin Layer Chromatography and the intensity of formation of halos by the samples were examined visually and compared with the standard (quercetin). It was observed that all the samples showed a strong activity. Subsequently, the quantitative analysis of the antioxidant activity of the samples was determined by the method that also employs the DPPH free radical, with the determination of IC_{50}. As shown in Table 2, regardless the form of cultivation, all samples showed a significant antioxidant activity compared to the positive control quercetin (IC_{50}= 1.6 ± 0.06 µg mL^{-1}); however, there was not any significant difference between treatments. As observed in Table 1, there was an increase in the content of total phenols and flavonoids, therefore an increase in antioxidant activity would be expected. This result may be due to the fact that the DPPH method does not quantify the antioxidant activity in living organisms, but it evaluates only antioxidant activity *in vitro* (ALVES et al., 2010), and thus may have limited this determination. Besides, the type of extract analyzed may have significantly influenced the antioxidant activity, allowing the extraction of higher amounts of bioactive compounds.

The structure of the active component of the extract is an important factor that influences the effectiveness of the natural antioxidant. It was believed that the number of phenolic hydroxyl, the presence of ortho- dihidroxilation system of B ring and a double bond C_2 - C_3 coupled with 4-oxo function in the C ring or of a hydroxyl group at C-3 contribute markedly to the antioxidant ability of flavonoids (BURDA and OLESZEK, 2001). Literature studies have reported the presence of various flavonoids in pink pepper such as myricetin, quercetin, and kaempferol, which have the structural criteria cited, as well as other substances such as gallic acid, ethyl gallate, leucocianindine, triacontane, β-sitosterol, and condensed tannins (LIST and HORHAMMER, 1979). These results suggest ways to cultivate pink pepper to produce large amounts of valuable chemicals in the leaves.

TABLE 2: Antioxidant activity of methanolic extract of leaves of pink pepper plants grown with poultry litter in single or double row.
TABELA 2: Atividade antioxidante do extrato metanólico de folhas de plantas de pimenta-rosa cultivadas com cama de frango em fileira simples ou dupla.

Poultry litter (t ha^{-1})	IC_{50} ($\mu g\ mL^{-1}$)[a]	
	Single row	Double row
0	11.60 ± 1.31 a	11.63 ± 0.69 a
5	12.03 ± 1.44 a	11.75 ± 1.02 a
10	10.76 ± 1.08 a	10.75 ± 1.62 a
15	9.66 ± 1.56 a	9.81 ± 0.63 a
20	9.32 ± 0.77 a	9.30 ± 0.70 a

Em que: [a] IC_{50} = concentration for 50% inhibition of DPPH remaining. Results are expressed as mean ± SD (n = 3).

CONCLUSIONS

In the conditions under which the experiment was developed, the cultivation of pink pepper plants can be performed in double rows and 20 t ha^{-1} of poultry litter to higher growth, biomass yield, and flavonoid and phenol content in the leaves.

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