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Spectrophotometric method for quantification of soil microbial biomass carbon

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The search for more suitable alternatives in analytical processes is a strategy to reduce environmental damages. The present study aimed to quantify the soil microbial biomass carbon (SMB-C), establishing a comparison between the methods involving quantification of SMB-C by titration of the samples with ferrous sulfate ammonia and molecular absorption spectrophotometry methods. This comparison was performed by two soil sample tests: (i) areas of grain crops with conventional management versus no-till farming; and (ii) areas with distinct phytophysiognomies in the region southern Mato Grosso do Sul state (Cerrado and Semideciduous Forest). It was found that molecular absorption spectrophotometry was an efficient tool for the determination of soil microbial biomass carbon, allowing replacement of the titrimetric method. There were significant differences in the levels of SMB-C determined spectrophotometrically in relation to those determined by titration. However, for the levels of SMB-C determined by spectrophotometry to be compared with those determined by titration, the values must be corrected by the linear regression equation $Y_{spectrophotometry} = - 151.38 + 0.92532 * X_{titration}$.

Key words: Potassium dichromate, soil quality, titration.

INTRODUCTION

The microbial biomass is the living and most active soil organic matter, being formed primarily of fungi, bacteria and actinomycetes (Jenkinson and Ladd, 1981; Roscoe et al., 2006). The determination of soil microbial biomass carbon (SMB-C) has been used to assess the size of the most active and dynamic reservoir of soil organic matter (Oliveira et al., 2001). Adequate levels of soil microbial biomass (BMS) are essential to the maintenance and productivity of agroecosystems, which depend in large part on processes mediated by microorganisms (Tótola and Chaer, 2002; Mendes et al., 2011).

In this context, the importance of quantifying BMS has been highlighted in gauging the quality of soil, being considered the most sensitive indicator to detect changes in agroecosystems (Mercante et al., 2008; Hungary et al., 2009). Several indicators of soil quality, including

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chemical, physical and biological attributes, have been evaluated for this purpose (Kaschuk et al., 2010). The search for the standardization of an efficient method for determination of BMS is aimed at greater practicality, minimization of the time needed analysis and adequate repeatability, thereby allowing the construction of a database for subsequent identification of soil quality indexes (Roscoe et al., 2006; Mendes et al., 2011). According to Faleiro et al. (2011), finding new standard and reproducible methods to analyze soil microbial biomass, able to avoid or reduce the use of toxic chemicals, is a challenge.

Currently, different methods can be employed to determine BMS. Among the most used are chloroform fumigation-incubation (CFI) and chloroform fumigation-extraction (CFE), described by Jenkinson and Powlson (1976) and Vance et al. (1987), respectively. Both are based on the partial sterilization of soil samples through fumigation with chloroform. The efficiency of these two methods was compared in studies conducted in acidic soils in Australia and New Zealand. According to Brandão-Junior et al. (2008), the CFI was the pioneer and is most widely used as the standard for the calibration of other methods to quantify SMB-C. However, certain analytical methods employed in the quantification of SMB-C, although efficient, require a long time for implementation, in addition to need to handle acids and carcinogenic substances, generating toxic compounds that cannot be discarded without treatment into the environment, such as potassium dichromate. This substance is soluble in water and highly toxic, because it freely diffuses through cell membranes and is a strong oxidizing agent (Knupp e Ferreira, 2011). According to Kotás and Stasicka (2000), chromium (Cr VI), in high concentrations, can cause serious cell damage, due to its high ability to interact with various organic compounds such as important functional enzymes, inhibiting them.

To reduce the pollution of soil and water, development of new methods and/or validation of methods are fundamental to obtain the most appropriate alternatives to reduce the toxic waste generated by the analytical processes. In this sense, spectrophotometry is gaining prominence for the determination of soil microbial biomass carbon (SMB-C) due to its speed, low cost and wide availability in laboratories (Marques et al., 2012). The aim of this study was to compare the methods of titration and molecular absorption spectrophotometry for measurement of soil microbial biomass.

MATERIALS AND METHODS

Characterizations of the experimental area and management systems

Considering that the microbial biomass controls the decomposition and accumulation of organic matter in the environment and is affected by environmental conditions, soil samples were collected in two experimental areas, as follows.

Table 1. Geographical location, altitude and soil type of the experimental areas with native vegetation (Semideciduous Forest and Cerrado) where soil samples were collected in the southern region of Mato Grosso do Sul [1].

[1]Soil	Latitude	Longitude	Altitude (m)
Semideciduous forest			
LVdf	20° 17 ''05 'S	54° 48 ' 37 ''W	385
LV	22° 04 45 'S ''	55° 22 33 ''W '	428
Cerrado			
LVdf	22° 17 '''32 S	54° 48 '''26 W	381
LV	22° '45 'S '05	55° 15 '''22 W	451

[1]LVdf and LV: typical Hapludox and Hapludox, respectively.

Test 1 – Comparison of conventional handling systems and tillage

The soil samples were collected as part of a long-term experiment (established in 1995) in an native forest fragment area (semideciduous forest), used as a reference for the original condition of the soil, and in two comparative management systems established in the experimental field of the Embrapa Western Agricultural Research Unit (Embrapa Agropecuária Oeste), in Dourados-MS (22° 16' S and 54° 49' W), in a typical Dystrophic Red Latosol with highly clayey texture. There were two soil management systems. The first was conventional (CS), consisting of planting soybeans (*Glycine max* (l.) Merrill) in summer and of oats (*Avena strigosa* Schreb) in autumn/winter, where the soil was prepared before each cultivation with disk harrows to 0.20 m depth, with pre-emergence herbicide application, in a 2.0 ha area. The second was a no-till system (NT), planted with soybeans and corn in the summer, rotated with wheat, oats and forage turnip in winter and millet in the spring. The soil sampling was carried out in July, 2012, at a depth of 0 to 0.10 m, with a Dutch auger, to obtain five composite samples from each system.

Test 2 – Evaluation of two phytophysiognomies (Cerrado and Semideciduous Forest) in the south of Mato Grosso do Sul

The soil sampling was carried out in April, 2013, in two distinct natural phytophysiognomies, in the southern region of Mato Grosso do Sul state, in a Red Latosol, with flat relief. The systems of native vegetation were selected on the basis of preliminary floristic surveys (Arruda and Daniel, 2007; Gomes et al., 2007), taking into consideration the two phytophysiognomies. The collections were made in four separate fragments (Table 1), at a depth of 0 to 0.10 m, to collect five samples in each system.

Determination of soil microbial biomass

Two methods were used to quantify the soil microbial biomass carbon (SMB-C): titration and molecular absorption spectrophotometry. The process of preparation and extraction of carbon was identical in both methods. Initially, soil samples were collected, homogenized and packed in plastic bags duly identified and stored

Table 2. Soil microbial biomass carbon (SMB-C), evaluated by spectrophotometry and titration methods, in soil samples collected in an experimental area under conventional system (CS), no-till system (NT) and native vegetation (NV), in typical Hapludox soil.

Systems	Spectrophotometry (µ g C g $^{-1}$ dry soil)	Titration (µ g C g $^{-1}$ dry soil)
NT	252^{abB}	426^{bA}
CS	158^{bB}	361^{bA}
NV	370^{aB}	637^{aA}

Values followed by lowercase letters in columns indicate average contrast between soil management systems and capital letters in rows indicate comparison between the spectrophotometry and titration, by the Tukey test at 5% probability.

in a cold chamber (±4°C), until analysis. The soil samples were ground, homogenized and sieved (< 2 mm), sprayed with distilled and deionized water to maximum capacity, and kept in closed containers for 12 h. After this step, the samples were weighed in cylindrical glass bottles with snap-on lids, using six aliquots of 20 g, repeated three times, for the determination of SMB-C, of which three subsamples were fumigated (packed in the desiccator with 10 mL of chloroform ($CHCl_3$), for 12 h), and three were not fumigated. For extraction of SMB-C, the sub-samples received 50 ml of potassium sulfate (K_2SO_4) 0.5 mol L^{-1}, being agitated horizontally at 30 rpm for 220 min.

Then, they were filtered through quantitative filter paper (125 mm) for separation of the extract. Soil moisture was determined by placing 50 g of moist samples in an oven at 105°C for 12 h and then weighed again.

For titration, an aliquot of 8.0 ml of each extract was placed in an Erlenmeyer flask and 2.0 ml of potassium dichromate ($K_2Cr_2O_7$), 0.066 mol L^{-1}, 5 ml of concentrated phosphoric acid (H_3PO_4) and 10 ml of concentrated sulfuric acid (H_2SO_4) were added. Then, the extract was heated over a hot plate (± 300°C) for 5 min and then cooled, after which 80 ml of distilled and deionized water was added. The aliquots were titrated with ferrous ammonium sulfate [(NH_4Fe $(SO_4)_{2.2}$ 6:00 0.033 mol L]$^{-1}$, using 1% diphenylamine (($(C_6H_5)_2NH$) as indicator (turning point of violet to green).

In the spectrophotometric method, a 2 ml aliquot of the same extract used for titration was placed in a test tube. Then, 3.0 ml of distilled and deionized water, 2.5 ml of working solution (300 ml of sodium pyrophosphate ($Na_2P_2O_7$) 0.1 mol L^{-1}, 46 ml of sulfuric acid (H_2SO_4) 0.5 mol L^{-1}, 20 ml of potassium permanganate ($Na_2P_2O_7$) 0.1 mol L^{-1}, 80 ml of manganese sulfate monohydrate ($MnSO_4H_2O$) 0.1 mol L^{-1}) and 2.5 ml of sulfuric acid (H_2SO_4) were added to each aliquot. The samples were then vortexed and left at rest for 2 h, and then submitted to molecular absorption spectrophotometry at a wavelength of 495 nm (Bartlett and Ross, 1988).

The results were submitted to analysis of variance and means were compared by Tukey test, with 5% probability. The statistical analyses were carried out with the Assistat program (Silva and Azevedo, 2009). The average values of SMB-C obtained in the two experiments were subjected to regression analysis, through the use of SAEG software (v. 9.1-2007), to establish the model best fitted to the levels determined by spectrophotometry versus those obtained by titration.

Among the regression models tested (linear, quadratic, and exponential square root), the linear model had the highest coefficient of determination, with significance of at least 5%.

RESULTS AND DISCUSSION

In the first test, we compared the levels of SMB-C obtained in native forest in relation to the two management systems (no-till and conventional system), in a typical Hapludox with highly clayey texture (Table 2). The values ranged between 159 (CS) and 637 µg C g^{-1} dry soil (native forest). In both the analytical methods evaluated, the no-till system provided higher SMB-C values compared to management under conventional tillage, which was expected, since in the no-till system the soil micro-aggregates are maintained, preserving the main niche for activity of microorganisms (Mendes et al., 2003). A similar result was observed by Balota et al. (1998), when evaluating SMB-C in soils submitted to successions of wheat/wheat/corn and soybean, under conventional and zero tillage.

The values of SMB-C determined in soil samples collected in the two locations, with two distinct natural physiognomies (Semideciduous Forest and Cerrado) (Table 3), ranged from 218 to 761 µg C g^{-1} dry soil. The samples collected in the forest area showed significantly higher values ($p < 0.05$) than those found in the Cerrado area. According to a study conducted in different native biomes in Brazil (Roscoe et al., 2006), the soil from the forest area had the highest figures. According to Borges et al. (2009), these values can be explained by the denser formation and the presence of a higher stratum in native vegetation found in forest systems. The absence of tillage, greater floristic diversity, maintenance of fungal hyphae and accumulation of litter on the soil surface contribute to more favorable conditions in forest areas than in cropped areas (Mercante et al., 2008). According to Roscoe et al. (2006), the main cause of low SMB-C values in soils with natural vegetation in Cerrado areas is due to the low concentrations of total organic carbon in this ecosystem. This result shows a significant correlation for Brazilian soils, since the measurements were performed in soils with varied textures, vegetation and management systems. When comparing the analytical methods evaluated (Tables 2 and 3), the levels of SMB-C determined by titration were significantly higher ($p < 0.05$) than the values obtained by spectrophotometry, regardless of the type of soil management adopted.

The joint analysis of the means obtained in two runs allowed fitting a linear regression model, with a high

Table 3. Soil microbial biomass carbon (SMB-C), evaluated by spectrophotometry and titration, in samples collected in two experimental areas with two soil types and systems of native vegetation (NV) and Cerrado, in the southern region of Mato Grosso do Sul state.

[1]Soil	System	Spectrophotometry (μ g C g $^{-1}$ dry soil)	Titration (μ g C g $^{-1}$ dry soil)
LVdf	Mata	593^{aA}	761^{aA}
LV	Mata	495^{bA}	660^{aA}
LVdf	Cerrado	314^{cA}	548^{bA}
LV	Cerrado	218^{abA}	347^{bB}

[1]LVdf and LV: typical Hapludox and Hapludox, respectively. Values followed by lowercase letters in columns indicate average contrast between soil management systems and capital letters in rows indicate comparison between the spectrophotometry and titration, by the Tukey test at 5% probability.

correlation coefficient (r = 0.957). This indicates a close relationship between the two analytical methods evaluated. However, according to Oliveira and Leite (2002), the simple observation of a high correlation coefficient between two analytical methods does not mean the results obtained are statistically identical.

Thus, these authors proposed a statistical procedure that combines the F-test with testing the average error and the linear correlation coefficient to check whether the results obtained through an alternate analytical method are equivalent to those obtained by a standard method. From the average SMB-C obtained in the two tests, we estimated the value of F (F_{calc} = 52.23), which was significantly higher ($p < 0.01$) to the F-value in the standard table for 2 degrees of freedom for the treatment and 5 degrees of freedom for the error ($F_{(2.5)}$ = 13.27). According to Leite and Oliveira (2002), in this situation the null hypothesis is rejected and it is assumed that the intercept (b_0) and angular coefficient (b_1) of the adjusted linear regression equation are zero and one, respectively, at 1% significance. These authors point out, however, that in conditions where a high correlation coefficient is obtained, the calculated F-value is greater, increasing the probability of rejecting the null hypothesis mistakenly. For this reason, they recommend applying the t-test in complement to average value $\left\{\bar{e} = \left[\sum \frac{(Y-X)}{X}\right]/n\right\}$ and comparing the correlation coefficients and the average errors [r_{yiyj} in relation to (1-$|\bar{e}|$)].

In this work, the means obtained resulted in error of -0.380, calculated t-value (t_{calc}) of -8.733 and tabled t-value (t_{tab}, in a bilateral test with 6 degrees of freedom for the residuals, at 1% probability) of 3.710. In this way, the value of t_{calc} in absolute value was higher than the value of t_{tab}, so it can be concluded that the differences between the levels determined by two analytical methods evaluated are caused randomly. Finally, since the correlation coefficient is 0.957 (Figure 1) and the expression (1-$|\bar{e}|$) presents a value of 0.620, then r ≥ (1-$|\bar{e}|$). According to the criteria recommended by Leite and Oliveira (2002), the rejection of null hypothesis for $b_0 = 0$ and $b_1 = 1$, coupled with the rejection of the null hypothesis for $b_0 = 0$ and $b_1 = 1$ and the condition that r ≥ (1- $|\bar{e}|$) means that the two methods of determining SMB-C are statistically different.

In other words, there is a systematic discrepancy of 151.38 μg g^{-1} in levels of SMB-C obtained by means of spectrophotometry in relation to those determined by titration. Note also that there is a difference in sensitivity between the analytical methods evaluated, that is the amplitude of variation in levels of SMB-C determined through spectrophotometry is greater than that determined by the standard method. In this context, the results obtained by both analytical method can be compared, so one can correct the SMB-C value obtained by spectrophotometry using the linear regression equation $y_{spectrophotometry} = -151.38 + 0.92532 * x_{titration}$.

The feasibility of assessing SMB-C by spectrophotometry is important since it allows direct determination of carbon in soil extract, which avoids problems related to the variability of results by perception of the turning point in the titration process (Duda et al., 2005). Another advantage of spectrophotometry is the greater agility during the carbon quantification process, due to the possibility of prior preparation of the working solution in significant volumes (2 liters). On average, for a period of eight hours of work, the use of the titration method allows quantification of SMB-C in 10 samples, repeated three times, totaling 30 subsamples. On the other hand, by spectrophotometry in five hours, it is possible to analyze 20 samples with three repetitions, for a total of 60 subsamples.

Finally, spectrophotometry allows the use of potassium permanganate instead of potassium dichromate, which is potentially toxic and carcinogenic (Stout et al., 2008), as well as the reduction of approximately 75% of the need for sulfuric acid, resulting in less toxic waste generation.

In addition to this substantial reduction in environmental impact, the adoption of the spectrophotometric method results in a considerable reduction in the cost of

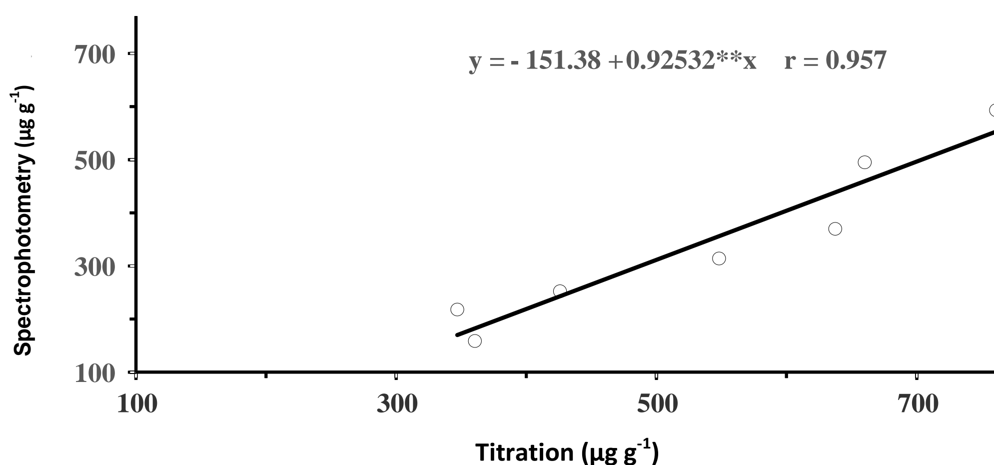

Figure 1. Linear regression between the levels of soil microbial biomass carbon (SMB-C), determined by molecular absorption spectrophotometry and titration in soil samples collected in areas under three management systems (conventional, no-till and native vegetation), two Cerrado systems and two Semidecidual forest in the experimental field of the Embrapa Western Agricultural Research Unit (Embrapa Agropecuária Oeste), Dourados-MS.

laboratory tests, since the phosphoric acid used to digest the solution in the titrimetric method has an average cost of more than five times that of the potassium permanganate used in spectrophotometry. Spectrophotometry is also better for human health, since the technician will not be exposed to potassium dichromate. Therefore, molecular absorption spectrophotometry can be an efficient tool for the evaluation of SMB-C, with the potential to replace the titration method (Ka and Ferreira, 2011).

Conclusions

1. Spectrophotometry is an efficient tool for determination of soil microbial biomass carbon, allowing it to replace the titrimetric method.
2. There are significant differences in the levels of SMB-C determined by spectrophotometry in relation to those determined by titration.
3. The SMB-C values obtained by spectrophotometry can be compared with those determined by titration by applying the linear regression equation $y_{spectrophotometry} = -151.38 + 0.92532 * x_{titration}$.

Conflict of Interests

The authors have not declared any conflict of interests.

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