Spectroscopic and elementary characterization of humic substances in organic substrates

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Abstract

The humic substances, major constituents of the organic matter of the substrates, play an important role in plant development, including: the availability of nutrients, the generation of negative charges (CEC) and the buffering effect. Based on this, this study aimed to use the molecular absorption spectroscopy in the ultraviolet-visible region (UV-VIS), the vibrational spectroscopy Fourier transform infrared (FTIR) and the elementary analysis CHN/O techniques to characterize the humic acid (HA) and fulvic acid (FA) fractions of the humic substances of five organic substrates, named as T1, T2, T3, T4, and T5, which were produced at the Federal University of Tocantins – *Campus* Gurupi. The molecular absorption spectra in UV-VIS and FTIR on the T1, T2, T3, T4 and T5 substrates showed humification, the formation of negative charges, which is related to the cation exchange capacity (CEC) and the complexation of metal ions that are considered nutrients. The biggest humification was shown for the T4 substrate, in function of its higher cabon-acid humic/carbon-acid fulvic ratio and the lower C/N ratio, allied to a negative charge generation, the low E_4/E_6 ratio found in the humic acid fraction. The complexation of metal nutrient ions indicates the greatest potencial of its use for seedlings production and the conservation of the chemical and physical properties of the organic substrate.

Keywords: fulvic acids, humic acid, organic waste

Introduction

The organic substrates used in the production of seedlings are formed by organic materials which contribute to the retention of humidity, nutrient supply, root growth, cation exchange capacity, pH regulation and physical support to ensure the development of the plant with quality (David et al., 2014; Pessoa et al., 2012).

Humic substances (HS), the main constituent of organic matter, present heterogeneous mixture of organic compounds aggregated by weak intermolecular interactions of hydrophobic nature and by hydrogen bonds (Matilainen et al., 2011; Valencia et al., 2013), which are fractionated in humic acids (HA), fulvic acids (FA) and humins (Baldotto et al., 2013); HS have a structural complexity due to the presence of organic functional groups that provide several functions in the nutrition of plants and improvement of the physical and chemical properties of the soil. This heterogeneous composition, derived from physicochemical and biological processes, makes it difficult to characterize SH. However, the composition of SH is always sought in the elaboration of organic substrates used in the production of seedlings.

Many analytical techniques have been used to identify important features of this structural complexity, for example, ultraviolet-visible (UV-

VIS), Fourier transform infrared (FTIR) spectroscopy and elementary analysis (Rodríguez et al., 2014). UV-VIS spectroscopy is useful in determining the absorbance (ABS) of aromatic systems and the ratio E_4/E_6 (ABS at 465 nm/ABS at 665 nm), which are related to the degree of humification of HS and, consequently, to the quality of the soil or the organic substrate (Rodríguez & Núñez, 2011).

Infrared spectroscopy has also been widely used in the characterization of humic substances. This technique allows the identification of functional groups that compose the humic macromolecule, although a specific band may sometimes correspond to the overlap of the absorption of two distinct functional groups (Wang et al., 2014).

Furthermore, the elementary analysis provides data on percentages of carbon, hydrogen, nitrogen, and oxygen elements. The H/C ratio is considered as the aromaticity index and the O/C ratio estimates the abundance of oxygen contained in functional groups. The C/N ratio indicates the degree of incorporation of nitrogen in HS, the degree of humification and the recalcitrant behavior (Silva et al., 2013).

Information regarding the complex chemical structure of HS present in organic substrates is scarce in the literature. This information can predict how these compounds act on the improvements in the chemical, physical and biological properties of the substrate in which the plants develop and, therefore, increase their productivity. Thus, the aim of the present study was to characterize the purified humic and fulvic acids of five organic substrates using the UV-VIS spectroscopy, the FTIR and the CHN/O elementary analysis techniques.

Materials and Methods

The study was conducted at the Federal University of Tocantins - Campus of Gurupi, at the geographical coordinates 11°43' S and 49°04' W, where the five organic substrates were obtained from the composting process of bovine rumen residues enriched with different doses of meat and bone meal. The substrates were designated as T1, T2, T3, T4, and T5, characterized in terms of the contents of N, P, K, Ca, Mg, S, Al, Na, Mo, and Co (Table 1). The substrate characterization analyzes performed according to the Embrapa methodology (2011) were carried out in the soil department of the Federal University of Viçosa in Viçosa, Minas Gerais state, at the geographic coordinates 20°45 'S and 42°55' W.

Table 1. Chemical characterization of the five organic substrates

Substrate	N	P	K	Ca	Mg	S	Na	Mo	Co
				g kg				%	
T1	20.18	10.19	1.40	20.20	10.28	3.70	1.80	0.0016	0.0006
T2	20.30	20.04	1.60	20.86	10.34	3.60	2.00	0.0016	0.0003
T3	20.25	30.98	1.80	30.82	10.53	4.40	2.60	0.0015	0.0
T4	20.38	50.43	1.80	40.90	10.67	4.40	3.30	0.0015	0.0005
T5	10.92	1.40	1.30	7.60	8.40	3.20	0.7	0.0015	0.0004

The experiment was carried out in two parts using the five substrates. In the first part, the humic and fulvic acid fractions were extracted, purified, lyophilized, and submitted to the spectroscopic and elemental analyzes. In the second part, a completely randomized design with four replications was followed. Each replicate was obtained by successive quartering, yielding four replicates of the substrates with unpurified total mass, each replicate being then subjected only to the CHN/O elementary analysis.

Humic acids (HA) and fulvic acids (FA) were extracted, purified and lyophilized according to IHSS (Swift, 1996). The extraction was carried out using alkaline solution (NaOH 0.1 mol L^{-1}, at the rate of 10 mL per gram of substrate) for solubilization of the humic and fulvic acid fractions and separation by centrifugation. After separation by centrifugation, the humic extract was acidified to pH 2.0 for the separation of the two fractions by centrifugation. The FAs were purified using two columns with XAD-8 resins and Amberlite IR 120+ for the removal of impurities and salts, and then frozen and lyophilized, obtaining the dry material for analysis. The HAs were purified by means of 15 attacks with 10% HF solution + 0.5% HCl for the removal of the HA-bound to the mineral fraction, after which they

were dialysed in deionized water for the removal of salts, frozen and lyophilized, obtaining the dry material for analysis. The dried material was then subjected to UV-VIS spectroscopy analysis and infrared spectroscopy and elemental analysis.

The measurements in the UV-VIS range were made using a GBC brand UV / VIS 911A scanning spectrophotometer, using quartz cells with a 1-cm optical path. The readings in the visible range, both the scanning (230 to 700 nm) and the determination of the absorbances at 465 and 665 nm were made in solutions of purified fulvic and humic acids, diluted appropriately in 0.05 mol L^{-1} sodium bicarbonate. The measurements in the ultraviolet range were made in solutions of purified fulvic acids, diluted in pH-adjusted solutions to 2.0 with HCl and 12.0 with NaOH, in addition to the neutral buffered bicarbonate solution. Differential spectra were calculated by the difference between the spectra obtained at pH 12.0 and 2.0, expressing the absorbance data (Coelho et al., 2013).

The Fourier transform infrared spectra of humic and fulvic acids were read in the range of 4000-400 cm^{-1} in KBr pellets, using a Fourier transform infrared spectrometer FTIR Spectrum 1000. Each spectrum was obtained by the average of 128 measurements with resolution of 4 cm^{-1}.

Elementary analysis was determined via combustion at 925 °C using an Perkin Elmer CHN Analyzer 2400 Series II elemental analyzer, directly quantifying the C, H and N elements and obtaining the O content by difference (%O = 100% - %C - %H - %N). With the percentages of the elements C, H, N, and O, the elementary ratios C/N, H/C e O/C for all HAs and AFs, as well as the ratio between the C contents of the AH and the AF (CHA/CFA) were calculated.

The data were submitted to analysis of variance, and the means were compared by the Tukey test at 1 and 5% probability, using the Assistat 7.7 software (Silva & Azevedo, 2016).

Results and e discussion

The visible ultraviolet (UV-VIS) absorption spectra of the humic acids (HA) and fulvic acids (FA) of the five organic substrates showed decreases in absorbance (ABS) with increasing wavelengths (Figure 1a and 1b). The HA (Figure 1a) showed a small peak of ABS in at 270 nm, which was evident in the five substrates. This absorbance occurs due to $\pi \rightarrow \pi$ * electronic transitions in aromatic systems such as phenolic groups, aniline derivatives of benzoic acids, polyenes and polycyclic aromatic hydrocarbons (Rodríguez et al., 2016). The FA (Figure 1b) did not show a peak at 270 nm, suggesting a decrease in the amount of aromatic structures and, thus, their stability in relation to HA, corroborating with the structure suggested for the FA (Stevenson, 1994).

All HAs and AFs in the T1 and T2
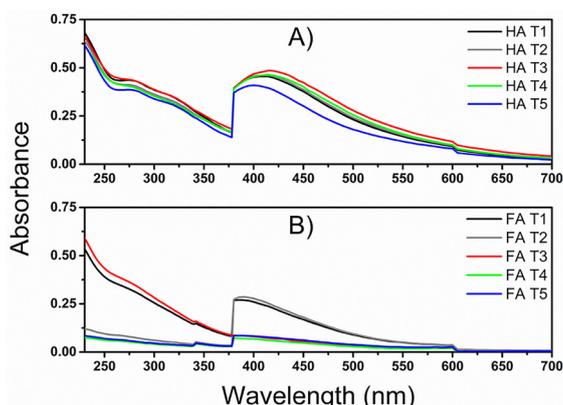
Figure 1. Ultraviolet-visible molecular absorption spectra of humic acids (1A) and fulvic acids (1B) purified from the five organic substrates.

treatments showed ABS band at 390 nm. This is a characteristic behavior of HS substances and occurs due to the overlapping of peaks relative to different chromophoric groups, such as aromatic structures located in several points of the macromolecule and in several forms of conjugation of double bonds with simple bonds, occurring the splinting of the peaks and the

junction of the same in a misshapen spectrum (McCarthy & Rice, 1985).

The E4/E6 ratios of HA were lower than those of AF (Figure 2). High E_4/E_6 is characteristic of FA, since it predicts the existence of less condensed aliphatic structures and a decrease in the conjugations of double and single bonds (Primo et al., 2011). Low E_4/E_6 is related to the increase in molecular weight, condensation of aromatic carbons, increased humification of aromatic structures, and greater conjugation of double and simple bonds characteristics of HA, which caused the studied HA to have greater stability and permanence in the substrate (Zalba et al., 2016; Dobbss et al., 2009).
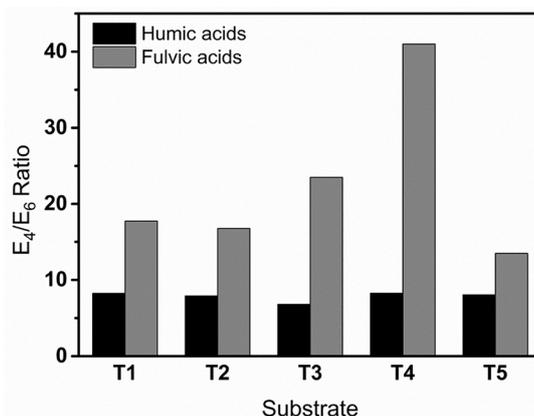
Figure 2. E_4/E_6 ratio of humic acids and fulvic acids purified from the five organic substrates.

The increased humification of the organic matter in the substrates is of great importance since several physiological processes of the plants can be modulated by the humic material, such as the large systems of energy transduction of the plant cell membranes and proton pumps, nutrient absorption, cell permeability and stimulation of H^+-ATPase activity (Trevisan et al., 2010; Canellas et al., 2008)

Together with these physiological effects on plants, HS still play a key role in the root environment in which plants develop. Several positive effects on plant nutrition have been attributed to humic substances, including improvement in seed germination, root growth and initiation, and increases in total biomass (Graber et al., 2015).

In relation to the Fourier transform infrared (FTIR) absorption spectra of the HA and FA in the five organic substrates, it is possible to observe the higher structural complexity and relatively higher molecular mass of the HA in relation to the FA. This difference is evidenced by the greater presence of several absorption bands evident in the five substrates (Figures 3 and 4). In the case of humic substances, infrared absorption bands generally cannot be interpreted safely, so the recommendation for humic material is to use the comparative mode, comparing a spectrum against another (Primo et al., 2011). Therefore, the possible attributions of the absorption bands were made based on the studies of Rodríguez et al. (2016), Fernandes et al. (2010) and Stevenson (1994).

There was no major difference in the HA FTIR spectra of the five studied organic substrates (Figure 3). In the region around 3600 to 3000 cm-1 the HA presented a strong and wide absorption band attributed to the symmetrical and/or asymmetric angular stretch of the O–H bond of hydroxyl groups capable of forming hydrogen bonds, mainly carboxylic acids, alcohols and phenols. The presence of oxygenated functional groups favors the interaction of humic substances with organic and inorganic compounds, such as metallic ions (Thorn & Cox, 2009). This interaction evidences the possibility of the HAs to maintain metallic ions present in the substrates, favoring their availability to the plants. Phenolic groups formed during the process of decomposition of organic matter are converted into quinones through oxidation reactions, which are precursors of semiquinone-like free radicals in HA that stabilize with the advancement of humification.
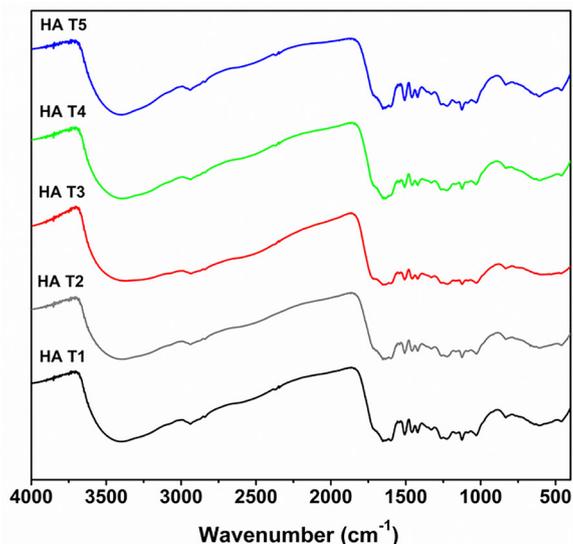
Figure 3. Infrared Spectra with Fourier Transform of purified humic acids from the five organic substrates.
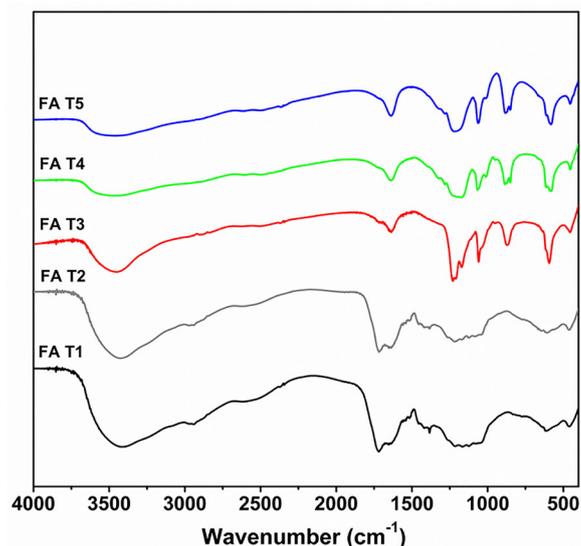
Figure 4. Fourier transform infrared spectra of fulvic acids purified from five organic substrates.

The increased concentration of semiquinone free radicals in HA can estimate the degree of humification of HS (Baldotto et al., 2011).

In the region attributed to the symmetrical stretching of C–H bonds, mainly of methyl (–CH$_3$) groups, the FTIR spectra of the five organic substrates showed a sharp and well-defined absorption band centered at 2940 cm^{-1}. Another acute, well-defined absorption band centered at 2840 cm^{-1} is also evident and is attributed to the symmetrical stretching of –CH$_2$ groups. The existence of a narrow, intense and well-defined absorption band ranging from 1650 to 1590 cm^{-1}, evident in the HA of the five substrates, indicates the presence of a series of symmetrical stretches

of C=O bonds of COO$^-$, secondary amides and quinones. The presence of quinones becomes important since the redox properties of humic substances have been attributed to it, although complexed metals, particularly iron, may also perform redox activities. Thus, humic substances play important roles in the mechanisms of electron transfer during the microbial activity. They act as redox buffers, accepting electrons from microbial respiration under anoxic conditions and, after re-reaction, transfering electrons to oxygen (Aeschbacher et al., 2010).

The three well-defined and centered fine absorption bands at 1510 cm^{-1} found for all substrates are characteristic of the stretching

of C–C bonds of aromatic rings, indicating the presence of lignins and N=C conjugated systems (Dobbss et al., 2009). Lignin is a compound of difficult decomposition, but it contributes to the formation of humic substances from its degradation and oxidation derivatives (Zhou et al., 2014; Fialho et al., 2010).

In the region between 1460 and 1420 cm^{-1}, it is evident the presence of two absorption peaks attributed to deformations of C–H bonds of methyl and methylene groups, and also possibly to an angular deformation of medium intensity of –CH 2 groups in the HA of the five substrates. The presence of an intense and well-defined band ranging between 1270 and 1225 cm^{-1}, with an absorption center at 1250 cm^{-1}, is attributed to the presence of angular deformations of O–H bonds in carboxylic groups and C–O bonds in esters and the presence of phenols. The presence of carboxylic groups is important since the cation exchange capacity (CEC) has been related to the dissociation of functional groups that are pH-dependent, mainly carboxylic acids (Smith et al., 2015).

The absorption band near 1100 cm^{-1} can be attributed to the presence of carboxylic groups bound to metal cations, both nutrients and pollutants. This suggests the existence of persistent inorganic material even after the HA purification (Miranda et al., 2007). This shows the ability Ahs to maintain the fertility of the substrates by supplying the plants with the necessary minerals to the development, growth, and quality index of the seedlings.

The presence of polysaccharides and alcohols can be attributed to the band centered at 1030 cm^{-1} due to stretches of C–O bond, which is evident in the five organic substrates. However, this band can also be attributed to the presence of aluminosilicates that were not completely removed during the humic acid extraction process (Pernet-Coudrier et al., 2011).

Unlike the FTIR absorption spectra of the HA, the spectra of the FAs (Figure 4) did not present numerous absorption bands, which indicates less complexity of their chemical structure. In addition, there was a difference between the absorption spectra of the substrates, especially those related to the absorption of hydroxyl groups in hydrogen

bonds, and a decrease in the absorption of these bands in FA3, FA4 and FA5, when compared to FA1 and FA2. The fulvic acids FA1 and FA2 showed similar FTIR absorption spectra, with a broad and well-defined absorption band ranging from 3600 to 3000 cm^{-1} that is characteristic of stretches of O–H bonds. On the other hand, FA3, FA4 and FA5 also presented the hydroxyl-related band, however, with lower intensity, being an indication of the decrease in the amount of hydroxyl groups. The presence of methyl groups is evidenced by the absorption band at 2940 cm^{-1}, which is attributed to the presence of aldehyde and ketone carbonyl groups (C=O).

Similarly to HA, FAs also showed absorption peaks related to carboxyl and phenolic hydroxyl functional groups. The presence of these functional groups shows their interaction potential with organic and inorganic molecules. Thus, the structural characteristics of FA and HA influence the behavior of several species, including nutrient metal ions (Havelcová et al., 2009), favoring the improvement of the quality of the organic substrates for the production of seedlings. The decrease in the amount of aromatic structures in FA, suggested by the absence of an ABS peak at 270 nm in the UV-VIS spectrum, is also evidenced by the FTIR spectra of the five FAs, since these did not show a fine absorption and well-defined peak centered at 1510 cm^{-1}. The presence of oxygenated and nitrogenous functional groups, besides the presence of a wide variety of aliphatic and aromatic structures, gives HS a fundamental role in improving the chemical, physical and biological properties of the soil-plant system, which are desirable characteristics in the preparation of organic substrates (Passos et al., 2007).

Observing the values of the F-test, the elemental analysis of the substrates indicated that there was no significant difference at 1 and 5% of probability by the Tukey test in the C, H, N and O contents in the five organic substrates (Table 2). In terms of elemental ratios, there were also no significant differences between H/C and O/C ratios (Table 2). However, a highly significant difference (F = 11.97 and p <0.001) occurred in the C/N elemental ratio between the different organic substrates.

Table 2. Elementary analysis CHN/O and elementary ratios C/N, H/C and O/C of the five organic substrates

Substrate	C	H	N	O	C/N	H/C	O/C
			%				
T1	30.77 a	3.52 a	2.44 a	63.27 a	12.66 b	0.11 a	2.06 a
T2	27.27 a	3.19 a	2.4 ab	67.14 a	11.34 b	0.12 a	2.50 a
T3	28.75 a	3.39 a	2.46 a	65.4 a	11.89 b	0.11a	2.27 a
T4	27.72 a	3.26 a	2.54 a	66.47 a	11.00 b	0.12 a	2.41 a
T5	28.53 a	3.24 a	1.79 b	66.42 a	15.88 a	0.11 a	2.34 a
Mean	28.61	3.32	2.33	65.74	12.56	0.12	2.32
F-value	1.62 ns	0.75 ns	4.55 ns	1.40 ns	11.97 **	1.14 ns	1.40 ns

Mean followed by the same letter in the columns do not differ statistically from each other by the Tukey test at the level of 5 and 1% probability.

The lower N content and, consequently, the higher C/N ratio found in the T5 substrate suggests that in this organic substrate, N forms are mostly not available, as in lignified tissues or in very poorly decomposed or humid material, such as fibers (Ebeling et al., 2011).

The elemental analysis for the purified humic fractions is presented in Table 3. Both fractions showed low H/C values. One consequence of such a reduction is the decrease in the hydration capacity of HS, which leads to the formation of hydrophobic regions. It has been suggested that hydrophobic moieties of humic substances are significantly related to the stimulation of the proton pump, and the preservation of bioactive molecules such as auxins present in organic matter (Dobbss et al.,

2010), and root increments (Loffredo et al., 2010). High H/C values indicate higher alipacity and lower content of aromatic structures in humic substances (Coelho et al., 2013; Goveia et al., 2011; Amir et al., 2010) and consequently, its decrease implies an increase in the degree of humification. Thus, the FA fraction is expected to have higher H/C values in relation to HA. This decrease in H/C was observed in all the studied substrates, especially for the T3, T4 and T5 substrates, which characterizes a greater stability and humification of these substrates during the composting process. The lower the atomic ratio H/C, the higher the resistance of the sample to thermo-degradation, indicating high resistance of aromatic structures to oxidation processes (Cunha et al., 2007).

Table 3. Elementary analysis CHN/O, elementary ratios C/N, H/C and O/C of humic acids and fulvic acids purified from the five organic substrates

Substrate	C	H	N	O	C/N	H/C	O/C
			%				
				Humic acids			
T1	51.52	4.86	3.96	39.66	13.01	0.09	0.77
T2	51.36	5.12	4.49	39.03	11.44	0.10	0.76
T3	50.18	5.16	5.14	39.52	9.76	0.10	0.79
T4	50.15	5.19	6.11	38.55	8.21	0.10	0.77
T5	51.96	5.33	3.56	39.15	14.60	0.10	0.75
Mean	51,03	5,13	4,65	39,18	11,40	0,10	0,77
				Fulvic acid			
	C	H	N	O	C/N	H/C	O/C
T1	41.14	4.55	3.94	50.37	10.44	0.11	1.22
T2	42.13	4.44	4.02	49.41	10.48	0.11	1.17
T3	6.19	2.14	0.82	90.85	7.55	0.35	14.68
T4	2.86	2.37	0.60	94.17	4.77	0.83	32.93
T5	4.35	3.31	0.61	91.73	7.13	0.76	21.09
Mean	19.33	3.36	2.00	75.31	8.07	0.43	14.22

The HAs showed lower O/C values than the FAs. However, for all substrates, the HA fraction presented values higher than 0.5, suggesting the presence of a greater amount of oxygenated functional groups such as

carboxylic and carbohydrates (Fontana et al., 2010). Furthermore, low O/C ratio is another characteristic of the decrease of aliphatic structures and increased humification of SH. For all substrates, the value of the C/N ratio was higher

for the HAs than for the FAs, especially for the T3, T4, and T5 substrates. The lower C/N ratio for FAs, not due to high N contents, but due to the low C content, demonstrates the greater oxidation capacity of these structures (Araújo et al., 2011). These substrates also presented the lowest values in the C/N ratio, except the HA for the T5 substrate. The increase in the value of N may occur as a consequence of the microbial activity and the rapid decomposition of the vegetal tissues that were once part of the organic matter present in the substrates (Campos et al., 2013). The low C/N ratio indicates the incorporation degree of N into the HS structure and the degree of humification. A high C/N ratio indicates low N incorporation and low humification degree (Cunha et al., 2007).

The values of the C/N ratio found in this study were similar to those observed by Baldotto et al. (2013), in which they found average values of 8.3 in deep ocean sediments. However, these values were different from those observed by Campos et al. (2013), where they obtained values ranging from 10.02 to 31.28 for the C/N

ratio in Yellow Latosol. This behavior explains part of the difficulty of predicting the rate of N release for plants by the mineralization of organic nitrogen fractions.

Regarding the analysis of the carbon content of the purified humic fractions, the FA fraction presented the lowest C content, implying humic acid/fulvic acid carbon (CHA/CFA) ratio greater than 1.0 (Table 4). This suggests that the humic substances present in the substrates are in more recalcitrant forms such as AH, due to the presence of aromatic functional groups, the higher degree of polymerization and the lower susceptibility to microbial attack. The CHA/CFA ratio is used as an indicator of the quality of humic substances. This ratio indicates the degree of conversion of the insoluble organic carbon into soluble fractions. Values close to 1 mean a higher equilibrium between the humic fractions and values below 1.0 indicate a higher content of fulvic acids in detriment of the humic acid fraction (Borges et al., 2015).

Table 4. Elementary carbon humic acid/fulvic acid carbon ratio (CAH/CAF) of the five organic substrates

Substrate	CHA/CFA
T1	1.25
T2	1.22
T3	8.11
T4	17.53
T5	11.94
Mean	8.01

The substrates showed CHA/CFA ratio values ranging from 1.25 to 17.53, with an average value of 8.01. Only two substrates, T1 and T2, presented values close to 1.0, which indicates that the organic substrates, in general, have predominance in the humic acid fraction. The contribution of the C content in the AH of the substrates T3, T4, and T5 may favor the better development of the plants. This fraction of SH has already been widely used in agriculture because it can provide crops with an increase in the size and number of roots, increasing nutrient absorption and growth rates. They also act on the microbiological properties of the plants, increasing the endogenous content of diazotrophic bacteria and, consequently, increasing the beneficial effects on the host plant. Finally, they promote plant growth by the

greater rooting and increase in the number of sites of mitosis and emergence of lateral roots (Conceição et al., 2009; Pinheiro et al., 2010).

Conclusions

In the T1, T2, T3, T4, and T5 substrates the molecular absorption spectroscopic techniques in the ultraviolet-visible region, with bands at 270 and 390 nm (aromatic), of the humic acids showed humification. Regarding the infrared technique, bands ranging from 3,600 to 3000 cm^{-1} (carboxylic acids, phenols and alcohols); at 1510 cm^{-1} (aromatic) and at 1100 cm^{-1} (complexes with metals) are present for humic acid and fulvic acids fractions, humification, negative charge formation on substrates (CEC) and metal complexation (nutrients and pollutants).

The infrared absorption spectra for

the fulvic acids in the T3, T4 and T5 substrates showed a decrease in the amount of hydroxyl from alcoholic groups, phenolic and carboxylic groups, showing the lower influence of this fraction on properties such as CEC and metal complexation (nutrients and pollutants).

The T4 substrate shows a higher humification level when compared to T1, T2, T3, and T5 due to the higher carbon-humic acid / fulvic acid-carbon ratio and lower C/N ratio, as well as the low E_4/E_6 and H/C ratios.

The higher humification of the T4 substrate associated with the generation of loads and potential of nutrient complexation indicates its greater potential for use in the production of seedlings with the maintenance of the chemical and physical properties of the substrate.

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