LUÍS FERNANDO JANUÁRIO ALMEIDA

TRANSFERÊNCIA DO ^{13}C DE FRAÇÕES BIOQUÍMICAS DE PLANTAS DE EUCALIPTO PARA A MATÉRIA ORGÂNICA DO SOLO

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Solos e Nutrição de Plantas, para obtenção do título de *Magister Scientiae.*

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BIOGRAFIA

LUÍS FERNANDO JANUÁRIO ALMEIDA, filho de Francisco Tibúrcio de Almeida e Maria da Conceição Januário Almeida, nasceu em 24 de março de 1990, em Santo Antônio do Grama/ MG.

Em dezembro de 2007, concluiu o ensino médio na Escola Estadual Effie Rolfs, em Viçosa/ MG.

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Entre agosto de 2010 e março de 2011, foi bolsista do programa CAPES/FIPSE na modalidade "graduação sanduíche", na Florida Agricultural and Mechanical University (FAMU), sob orientação do Professor Dr. Clifford Louime e a Pesquisadora Associada Dr. Amita Jain.

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SUMÁRIO

RESUMO

ALMEIDA, Luís Fernando Januário, M.Sc., Universidade Federal de Viçosa, fevereiro de 2016. T**ransferência do ^{13}C de frações bioquímicas de plantas de eucalipto para a matéria orgânica do solo.** Orientador: Ivo Ribeiro da Silva. Coorientadores: Emanuelle Mercês Barros Soares e Leonardus Vergutz.

A decomposição de resíduos vegetais e a formação da matéria orgânica do solo (MOS) sãos influenciadas pela sua composição bioquímica, a qual está relacionada à concentração relativa de compostos solúveis em água, celulose, lignina e lipídios. Dessa forma, o efeito da qualidade química dos resíduos vegetais na formação da MOS precisa ser estudado mais detalhadamente. Nesse sentido, no presente estudo, avaliou-se a decomposição e a transferência de ^{13}C de frações quimicamente distintas de resíduos de eucalipto para as frações matéria orgânica particulada e aquela associada aos minerais. Conduziu-se um experimento de incubação sob condições controladas, com quatro frações quimicamente distintas extraídas sequencialmente, dentre elas: HWE – Extrativos em água quente (compostos metabólicos); TSE - extrativos totais em solvente (lipídios livres), CF-fração celulósica (principalmente celulose e hemicelulose), e AUR- resíduo não hidrolisável em ácido (a maior parte da lignina e lipídios); proveniente de resíduos vegetais dos componentes de planta (folha, galho, casca e raiz). Ao final do período de incubação, uma sub-amostra do solo foi separada e a MOS fisicamente fracionada usando um método combinado de tamanho e densidade, o que permitiu separar a fração leve não complexada da matéria orgânica pesada por meio da diferença de densidade, e, em seguida, a matéria orgânica particulada daquela fração associada aos minerais (silte + argila) por tamanho. O conteúdo total de C e a abundância relativa de ^{13}C ($\delta\ ^{13}C$) de cada fração da matéria orgânica do solo foram medidos em Espectrômetro de Massa de Razão Isotópica (IRMS). Com o presente estudo foi possível observar que nem todas as frações do material vegetal que são facilmente decompostas são igualmente eficientes em formar associações organo-minerais. Mesmo materiais relativamente mais resistentes à decomposição, tais como lignina e lipídios, foram precursores eficazes da fração mais estável da matéria orgânica, a fração associada ao silte e a argila (MOAM). Nossos resultados levaram a rejeitar nossa hipótese inicial de que materiais vegetais lábeis, metabólicos ou estruturais, são mais eficazes na formação da MOAM Os resultados obtidos também não são compatíveis com as tendências de estudos mais recentes segundo os quais os compostos

mais lábeis contribuem mais para a formação da fração da MOS associada aos minerais ao passo que os compostos mais recalcitrantes são mineralizados ou contribuem mais para a formação da fração particulada da MOS.

ABSTRACT

ALMEIDA, Luís Fernando Januário, M.Sc., Universidade Federal de Viçosa, February, 2016. **Transference of ^{13}C of biochemical fractions from eucalypt plants to soil organic matter.** Adviser: Ivo Ribeiro da Silva. Co-advisers: Emanuelle Mercês Barros Soares and Leonardus Vergutz.

The decomposition of plant litter and the formation of soil organic matter (SOM) are largely affected by its biochemical composition, which is dependent on the relative concentrations of water-soluble compounds, cellulose, lignin and lipids. Thus, the effect of plant litter composition on SOM formation needs to be studied in more detail. In the present study, the decomposition and ^{13}C transfer from chemically distinct labelled litter fractions to the particulate (POM) and mineral associated organic matter (MOAM) fractions were evaluated. An incubation experiment was carried out under controlled conditions with four chemically distinct, sequentially extracted litter fractions, namely: HWE-hot water extractable (metabolic compounds); TSE-total solvent extractable (free lipids), CF-cellulosic fraction (mostly cellulose and hemicellulose), and AUR- acid unhydrolysable residue (mostly lignin and lipids); from four plant litter components (leaves, twigs, bark and roots). At the end of the incubation period, a soil subsample was taken and the SOM was physically fractionated using a size-density combined method, which allowed us to separate the uncomplexed light fraction from the heavy organic matter by density, and then sand (POM) from silt + clay associated organic matter (MOAM) by size. The total content of C and the relative abundance of ^{13}C (δ ^{13}C) of each soil organic matter fraction were measured in a continuous flow Isotope Ratio Mass Spectrometer (IRMS). The results of the current study indicate that not all litter fractions that are easily decomposed are equally effective at promoting the formation of organic-mineral associations. Even materials relatively more resistant to decomposition, such as lignin and lipids, were effective precursors for the more stable silt and clay mineral-bound SOM fraction (MAOM). Our findings led us do refuse our initial hypothesis that microbial labile, whether metabolic or structural, litter fractions are more effective at promoting MAOM formation. They also do not support the more recent propositions that mineral-bond SOM is mainly dependent on labile compounds in metabolic fractions and that recalcitrant materials are mineralized or contribute mostly for the particulate organic matter.

INTRODUÇÃO GERAL

A demanda crescente por energia tem intensificado a remoção de resíduos da colheita de florestas plantadas para a produção de bioenergia. Tal fato tem gerado preocupação, pois ainda que a remoção desses resíduos traga bom retorno econômico momentâneo, no longo prazo, pode comprometer a matéria orgânica do solo (MOS). Apesar de a remoção de resíduos florestais uma única vez não ter resultado em redução significativa da MOS (Nave et al., 2010), a sua remoção contínua e com intervalos curtos, como é o caso da maioria das florestas plantadas de eucalipto no Brasil, pode resultar em declínio da MOS. A resiliência do sítio em relação à manutenção da MOS irá depender de vários fatores, mas as características de solo e clima (Lima et al., 2006) e a quantidade e a qualidade da fonte de C aportado (Bird and Torn, 2006; Bird et al., 2008) estão entre os mais importantes.

Nos sítios florestais, a quantidade e a qualidade do resíduo que permanece na área influenciam a entrada de C, sua taxa de decomposição e transferência para as frações da MOS. Na prática, a maior parte dos resíduos de parte aérea deixados na área após a colheita são folhas e galhos finos, uma vez que casca e galhos grossos podem ser destinados à produção de energia. A casca é um componente quantitativamente importante em sítios onde se procede o descascamento dentro do talhão. Em caso de remoção de serapilheira e de resíduos de colheita para produção de bioenergia ao final da rotação, o sistema radicular pode ter importância proporcionalmente maior que a parte aérea para a formação da MOS, como já apontado em estudos com culturas anuais (Rasse et al., 2005).

Estudos que avaliem o impacto da permanência do resíduo de colheita de eucalipto, bem como a qualidade do mesmo para a formação da MOS e para a sustentabilidade da produtividade do sistema florestal vem sendo desenvolvidos, porém há muito ainda o que se entender. Sabe-se que o material orgânico de origem vegetal é de natureza complexa, no entanto, sua composição elementar é bastante conhecida, sendo basicamente C, H, O, N, P e S os elementos que fazem parte das unidades estruturais dos tecidos. A composição bioquímica do *litter* difere entre espécies de plantas e tipos de tecidos, variando assim as proporções de proteínas, celulose, hemicelulose, amido, pectina, lipídios e lignina, e, consequentemente, o padrão de decomposição e a qualidade/quantidade da MOS formada de acordo com o resíduo. A

qualidade bioquímica dos resíduos vegetais tem forte influência sobre a sua decomposição no solo e diferentes componentes de plantas poderão apresentar níveis diferenciados em suas quantidades de C mineralizados pelos microrganismos do solo.

Dentre os diversos compostos provenientes de material vegetal, há muito a lignina tem sido considerada resistente à degradação devido a sua estrutura polimérica e complexa (Haider and Martin, 1975). Em estudos de decomposição, Williams and Gray (1974) constataram que a lignina relaciona-se inversamente com a perda de massa de litter. Mais recentemente, diversos estudos têm revelado que a preservação seletiva da lignina parece ser relevante apenas nos primeiros estágios da decomposição do *litter* (Kalbitz et al., 2006; Prescott, 2005; Sollins et al., 2006).

Alguns autores (Baldock et al., 1997; Stimler et al., 2006), mencionam que compostos alifáticos como lipídios, por exemplo, também são considerados recalcitrantes nos solos. Entretanto, a estabilização destes compostos durante o processo de mineralização da MOS pode ser devido a outros mecanismos tais como a sua interação com a superfície de minerais (Kögel-Knabner et al., 2008). A proteção física e a menor acessibilidade aos microorganismos parece ser mais importante que a recalcitrância intrínseca das moléculas devido à sua complexidade bioquímica (Dungait et al., 2012). Adicionalmente, Thevenot et al.(2013) ao compararem a lignina extraída de material vegetal de milho com a lignina extraída do solo após 9 anos de deposição de resíduos dessa cultura em campo, observaram que a lignina do solo se apresentava mais enriquecida com compostos lipídicos, sugerindo assim a formação de interações estáveis entre lignina e porções alifáticas.

Resíduos de plantas de composição variável podem apresentar diferenças na decomposição, com contribuição distinta para a MOS. Da mesma forma, frações bioquímicas de cada componente da planta podem apresentar taxas de decomposição bem como eficiência diferente para a estabilização de C nas frações da MOS. Trabalhos recentes tem proposto que resíduos ricos em compostos metabólicos e considerados de alta qualidade, mais facilmente decompostos pela microbiota do solo, são precursores mais eficientes na formação de matéria orgânica mais estável, em especial àquela fração química/ fisicamente protegida pela associação com minerais da fração silte e argila (Cotrufo et al., 2015, 2013; Haddix et al., 2016). No entanto, tal hipótese não tem sido

consensual na comunidade científica da área e tem levantado questionamentos (Castellano et al., 2015). O entendimento da contribuição relativa de cada componente bioquímico e de cada componente da planta para a MOS será útil não apenas para avançar nosso entendimento sobre a formação e estabilização da MOS, mas também poderá auxiliar na priorização da permanência em campo dos resíduos da colheita que mais contribuem para a MOS.

Dentro dos conceitos propostos, atualmente as folhas de eucalipto se encaixariam como resíduos de alta qualidade, enquanto casca, galhos e raízes teriam o comportamento esperado para resíduos de baixa qualidade. Diante disso, o presente estudo propôs determinar quais frações bioquímicas de distintos componentes dos resíduos de eucalipto (folha, galhos, casca e raízes) são mais eficientes na transferência do C para o solo e sua contribuição para frações mais lábeis e mais estáveis da MOS utilizando material vegetal previamente enriquecido com ^{13}C.

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NOT ALL MICROBIAL LABILE PLANT LITTER FRACTIONS ARE EQUALLY EFFECTIVE PRECURSORS FOR FORMATION OF MINERAL-ASSOCIATED SOIL ORGANIC MATTER

INTRODUCTION

Soil organic C is mostly derived from plant material (Kögel-Knabner, 2002). However, a large proportion of this material needs to pass through microbial biomass before being stabilized into SOM (Miltner et al. 2012; Cotrufo et al., 2013). The decomposition of plant litter and formation of soil organic matter (SOM) is largely affected by its biochemical composition (Zhang et al., 2008), which is dependent on relative concentrations of water-soluble compounds, cellulose, lignin and lipids (Cotrufo et al., 2013). Plant tissues are composed mostly by polysaccharides, lignin, tannins, and proteins (Lorenz et al., 2007). These compounds have different decomposition rates in the soil. Carbohydrates and proteins are more easily degraded, while lignin, tannin, and cuticular waxes decompose more slowly (Mikutta et al., 2006; Suseela et al., 2013). Their relative abundance in distinct plant organs is also variable and it is thought to have a large influence on litter decomposition and SOM formation (Kögel-Knabner, 2002).

Labile organic compounds can contribute directly to the more stable SOM pool via retention on mineral surfaces (Kramer et al., 2012), what has been treated as a critical mechanism for carbon storage in many soils and long-term stabilization against decomposition (Kaiser and Kalbitz, 2012). Selective preservation has been suggested for compounds such as lipids and lignin due to either their hydrophobicity and, or aromaticity (Song et al., 2013). Furthermore, it has been shown that the interaction of easily degradable compounds with hydrophobic organic materials in soils is efficient in reducing their mineralization (Piccolo et al., 2004). Aromatic compounds have also

been recently shown to chemically bind directly to functional groups on the surface of a Mn oxide via ligand exchange mechanism and form stable organic-mineral complexes (Johnson et al., 2015).

SOM represents a continuum of C compounds ranging from those chemically labile to those highly resistant to microbial decomposition (Ågren and Bosatta 2002; Lehman and Kleber, 2015). Therefore, even when litter chemical quality favors their decomposition, physical and chemical processes are simultaneously stabilizing these compounds in SOM (Conant et al., 2011). Despite recent advances in the understanding of SOM formation and stabilization (Dungait et al., 2012; Kleber et al., 2015; Lehmann and Kleber, 2015; Stockmann et al., 2013) the effect of plant litter quality on more stable SOM formation is still controversial (Castellano et al., 2015; Cotrufo et al., 2013). The so-called "high quality" microbial labile, metabolic organic compounds have been proposed to be more favorable precursors to mineral associated-organic matter (MAOM) of greater stability, whereas the "low-quality", more recalcitrant structural organic compounds would be less favorable to MAOM formation in detriment of particulate SOM fractions (Cotrufo et al., 2015, 2013; Haddix et al., 2016). However, using a modelling approach based on literature results it has become evident that under continuous low litter C input and thus high C saturation deficit the 'high quality" litter may have a lead on C transfer to mineral-associated organic matter (MAOM), whereas more structural, "low quality" litter may favor MAOM at intermediate litter C inputs. Accordingly, in the long-term soils nearing C saturation at continuous high C inputs may possibly make litter quality less relevant for physical SOM stabilization (Castellano et al., 2015). Such hypothesis is still in need of experimental validation. Thus, analysis of more specific compounds or fractions of plant litter may enable a

better description of litter decomposition and SOM turnover and improve our understanding of SOM stabilization (Mendez-Millan et al., 2014).

Our working hypothesis is that litter fractions more easily decomposable by the soil microorganisms, irrespective of their metabolic or structural pools, are those that contribute most for the formation of SOM, especially the MOAM fraction. In the present study, we evaluated the decomposition and ^{13}C transfer from chemically distinct labelled litter fractions to the particulate and MOAM fractions. The macrocosm incubation experiment was carried out under controlled conditions with four chemically distinct, sequentially extracted litter fractions, from plant litter components thought to have "high" (leaves) and "low" (twigs, bark and roots) quality.

MATERIALS AND METHODS

Plant labeling and litter fractionation

Two-month old clonal *Eucalyptus urophylla* X *E. grandis* hybrid plants were grown under constant aeration in a growth chamber in 3.5 L polyethylene containers supplemented with a Clark Nutrient Solution (Clark, 1975). Half of the plants were pulse-labeled with ^{13}C-CO_2 stable isotope as described by Machado et al. (2011) and the other half was submitted to the same conditions, except that no ^{13}C label was applied (unlabeled control plants).

After the labeling period (126 days), all plants were harvested and the leaves, twigs, stem and roots were separated. The stem had its bark removed and set apart too. After drying under a forced-draft oven for a week, each of these plant components was ground in a Wiley mill and stored for subsequent chemical fractionation. The debarked stem was no further used in our study. Subsamples of plant component (leaves, bark, twigs and roots) were ball milled and their relative ^{13}C enrichment was accessed by

analyzing the samples in a continuous flow isotope ratio mass spectrometer (see details below). The results indicated a substantial incorporation of the ^{13}C label in all plant components (Table 1) that was high enough to allow us to track the fate of C from litter into SOM fractions.

Table 1. Isotopic composition ($\delta^{13}C_{V\text{-}PDB}$) of eucalypt plants components enriched with ^{13}C

Plant Component	$\delta^{13}C_{V\text{-}PDB}$	
	‰	
Leaves	342	a
Twigs	349	a
Bark	320	a
Roots	398	a

Means followed by the same lower case letter in the column do not differ by the Tukey test at 5%.

In order to obtain litter fractions with distinct chemical compositions and expected to have different availability to microbial decomposition the plant material, first the unlabeled and then the labelled plant components (leaves, bark, twigs and roots), were fractioned into four operationally defined "biochemical" fractions, namely: HWE-hot water extractable (metabolic compounds); TSE-total solvent extractable (free lipids), CF-cellulosic fraction (mostly cellulose and hemicellulose), and AUR- acid unhydrolysable residue (mostly lignin and lipids); Briefly, the fractionation procedure was as follows: 2 g of each ground plant component (bark, leaves, twigs and roots) were individually Soxhlet-extracted with 150 mL of deionized water for 6 hours to obtain the HWE fraction, which was then freeze-dried and stored for later chemical analysis and use in the incubation experiment. Following, 2 g of the material free of the HWE fraction were Soxhlet-extracted with 150 mL of acetone for 6 hours thus obtaining the TSE fraction, which was also freeze-dried and stored for later use. The plant litter residue freed of the HWE and TSE fractions was subsequently used for fractionation of

the structural components in the CF and the AUR fractions in separated procedures. The CF was obtained by extracting the HWE and TSE-free residue with ethanol plus nitric acid in a 4:1 ratio (v/v) solution, and then with a 25% KOH solution (Fresenius and Dehio, 1931). Finally, the AUR fraction was obtained by treating the HWE and TSE-free residue with 72% v/v sulfuric acid solution (TAPPI, 1969). Both CF and AUR were washed extensively with ultra-pure water and then freeze-dried. The relative dry mass yield for each biochemical fraction from each plant component is shown on table 2.

Table 2. Partitioning of biochemical fractions in leaves, twigs, bark and roots of eucalyptus plants labeled with ^{13}C

BIOCHEMICAL FRACTION	PLANT COMPONENT			
	Leaves	Twigs	Bark	Roots
	% of Biochemical Fraction (Mean ± Std. Err.)			
AUR	28,97 ± 0,23	29,66 ± 0,73	28,17 ± 0,50	50,28 ± 0,59
CF	23,33 ± 0,79	41,44 ± 1,21	40,02 ± 1,28	30,04 ± 1,26
HWE	40,64 ± 0,70	27,23 ± 1,93	29,04 ± 0,72	17,28 ± 1,04
TSE	7,05 ± 0,32	1,67 ± 0,06	2,77 ± 0,14	2,40 ± 0,07

AUR- Acid Unhydrolysable Fraction, TSE – Total Solvent Extractable Fraction, HWE- Hot Water Extractable and CF- Celulosic Fraction.

Subsamples of all biochemical fractions were submitted to isotopic analysis in an isotope ratio mass spectrometer- IRMS (ANCA-GSL, 20-20, Sercon, Crewe-UK) in order to determine the $^{13}C/^{12}C$ ratio. The data were referred to a V- PDB international standard and were expressed as $\delta^{13}C$ in a per mill (‰) notation and are shown in table 3. Total C, N and H for all litter biochemical fractions were determined by dry combustion in and element analyser (Table 4).

Table 3. Isotopic composition ($\delta^{13}C_{V\text{-}PDB}$) of biochemical fractions of eucalyptus plants components enriched with ^{13}C

BIOCHEMICAL FRACTION	PLANT COMPONENT							
	Leaves		Twigs		Bark		Roots	
	‰							
AUR	441	Aa	382	Aab	313	ABb	342	Ab
CF	502	Aa	362	Ab	368	Ab	327	Ab
HWE	324	Ba	319	Aa	276	Ba	302	Aa
TSE	504	Aa	380	Ab	334	ABb	374	Ab

Means followed by the same upper case letter in the same column or lower case letter in the same row do not differ by the Tukey´s test at 5%. AUR- Acid Unhydrolysable Fraction, TSE – Total Solvent Extractable Fraction, HWE- Hot Water Extractable and CF- Celulosic Fraction.

Table 4. Total C, N and H content and C/N and H/C atomic ratios for distinct litter biochemical fractions from eucalypt plant components.

Biochemical Fraction	Plant Component	C (%)	H (%)	N (%)	C/N	H/C
TSE	Bark	73,2	7,56	0,29	294,3	1,24
TSE	Leaves	68,7	8,42	0,46	174,3	1,47
TSE	Twigs	73,2	8,28	0,31	275,6	1,36
TSE	Roots	74,7	7,66	0,20	435,9	1,23
HWE	Bark	35,4	4,16	0,26	158,6	1,41
HWE	Leaves	38,4	4,44	0,42	106,6	1,39
HWE	Twigs	39,2	4,04	0,47	97,4	1,24
HWE	Roots	39,6	4,18	0,56	82,4	1,27
CF	Bark	40,4	6,69	0,05	943,4	1,99
CF	Leaves	48,9	6,88	0,25	228,2	1,69
CF	Twigs	40,7	6,74	0,03	1582,4	1,99
CF	Roots	40,8	6,49	0,12	396,3	1,91
AUR	Bark	57,1	6,00	1,83	36,4	1,26
AUR	Leaves	56,0	7,35	4,16	15,7	1,57
AUR	Twigs	50,5	6,41	2,24	26,3	1,52
AUR	Roots	49,6	5,47	1,60	36,2	1,32

AUR- Acid Unhydrolysable Fraction, TSE – Total Solvent Extractable Fraction, HWE- Hot Water Extractable and CF- Celulosic Fraction.

Additionally, the four litter fractions obtained as previously described had their molecular composition assessed using an off-line tetramethyl ammonium hydroxide TMAH-mediated thermochemolysis procedure based on Hatcher et al. (1995). After TMAH thermochemolysis, products were analyzed by gas chromatography-mass

spectrometry in a Shimadzu QP 2010-SE GC-MS equipped with a Rtx – 5MS column (30 m length; 0,25 mm ID; 0,25 µm film thickness). Ultrapure He was the carrier gas at a flow rate of 3 mL min^{-1}, the ion source temperature was set to 200 ^{o}C, the interface temperature to 290 ^{o}C and oven temperature was ramped from 60 ^{o}C to 300 ^{o}C at a rate of 15 $^{o}C\ min^{-1}$, with analysis initial time at 3.50 min and final time at 48.5 min. The eluted compounds above a set threshold area in the chromatograms were identified using a NIST 2011 mass spectral library (SI=85%). Lignin-derived phenols were identified based on external standards, namely: lignin; 3,5-dimethoxy-4-hydroxyacetophenone; syringaldehyde, syringic acid; ferulic acid; vanillic acid; p-coumaric acid; 3-hydroxybenzaldehyde; 4-hydroxyacetophenone; 3,4-dihydroxybenzoic acid. The quantified compounds were grouped in three distinct categories, namely: carbohydrates, lipids and aromatic compounds. A summary of the chemical groups relative abundance in the litter biochemical fractions obtained from distinct plant components is shown in table 5.

Table 5. Relative abundance (%) of carbohydrates, lipids and total aromatics determined by thermochemolysis with TMAH_GC/MS of distinct biochemical fractions from plant components

FRACTION/Precursor group	Plant component			
HOT WATER EXTRACTABLE	bark	leaves	branches	roots
Carbohydrates	64,18	38,04	44,54	11,62
Lipids	16,15	27,98	15,97	34,25
Total aromatics	2,03	24,34	30,50	29,12
SOLVENT EXTRACTABLE				
Carbohydrates	0,00	0,00	0,00	0,00
Lipids	91,10	93,60	90,36	93,79
Total aromatics	0,07	0,00	0,13	0,85
ACID UNHYDROLYZABLE				
Carbohydrates	1,00	1,50	0,00	1,40
Lipids	50,60	45,20	54,80	39,70
Total aromatics	48,50	53,20	45,20	58,80
CELLULOSIC				
Carbohydrates	51,92	50,83	56,89	40,06
Lipids	15,08	22,98	21,71	21,10
Total aromatics	0,00	1,42	0,51	0,00

Incubation experiment

The treatments were based on a 4x4 (+1) factorial scheme with 4 plant components (bark, leaves, twigs and roots), 4 biochemical fractions (HWE, TSE, AUR and CF) and one additional treatment without plant residue addition (soil only control). The experimental units were disposed in a completely randomized block design with three repetitions. Blocking was used in order to favor the timely headspace atmosphere

sampling along the incubation period (see more details below). The four litter biochemical fractions from each respective plant component were applied to the soil simultaneously, but only one biochemically ^{13}C labeled at a time so that the contribution of only one specific fraction could be accessed. The other three fractions came from control, unlabeled plants. This circumvent the limitation of applying each fraction to decompose in the soil in an isolated form. Thus, all the treatments with litter fractions addition received the same C amount and from similar plant component source.

Prior to the litter fraction incubation study air-dried soil samples (20 g) collected from the 0-20 cm layer of a sandy-loam Oxisol that was cultivated with a C-C4 tropical grass pasture (*Urochloa decumbens*) with bulk soil presenting $\delta^{13}C_{VPDB}$ = -16 ‰ were moistened to 80% of the water-holding capacity. Then the moist soil was placed inside 0.57 l air-tight glass jars with lids containing septa for headspace gas sampling. The plant litter material was added to the soil samples at a rate of 10 g/kg of soil, with a proportion of 25% (based on the C content) of each biochemical fraction. Moreover, a suspension (5: 1 water/soil ratio) made of fresh soil collected in area cultivated with eucalypt was used to inoculate the soil from pasture with the microorganisms present in the soil cultivated with eucalyptus by applying 100 µL of the suspension in all experimental units. Soils were incubated in a lab room with constant temperature (25 ± 1 °C) in the dark for 200 days. During the incubation period, soil respiration was assessed on days 1, 2, 3, 4, 7, 10, 13, 21, 28, 38, 46, 70, 80, 92, 112, 148, 178 and 200. A 100 mL subsample of jar's headspace air was taken and analyzed for determination of $^{12}C\text{-}CO_2$ and $^{13}C\text{-}CO_2$ concentrations in a cavity ring-down spectrometer (CRDS, G2131-i, Picarro, Sunnyvale, CA). After each sampling the jars were opened, completely vented with help of a small fan, and then tightly closed again.

Soil organic matter fractionation

At the end of the incubation period (just after the last headspace gas sampling), a soil subsample was taken and the SOM was physically fractionated using a size-density combined method, slightly adapted from Denef and Galdo (2013). This procedure allowed us to separate the uncomplexed light fraction from the heavy organic matter by density, and then sand - from silt + clay-associated organic matter by size. Shortly, 5 g subsamples of air-dried soil were dispersed with a sonicator (Heinemann Branson Sonifier 250) at 240 J mL^{-1} in 25 ml of 1.80 kg m^{-3} sodium iodide solution (NaI). Then, the samples were centrifuged and the floating light fraction organic matter (LFOM) which could still contain undecomposed litter fraction components, was collected in a 2 µm pore nylon filter coupled to a vacuum system. The LFOM was rinsed with ultrapure water and the sedimented heavy fraction was rinsed with deionized water, dispersed with a sonicator in 25 ml of deionized water and then sieved through a 53 µm opening screen to separate the sand-sized (>53 µm) particulate organic matter fraction (SSOM) from the silt + clay fraction, that is, the mineral associated organic matter (MAOM).

The total content of C and the relative abundance of ^{13}C ($\delta\ ^{13}C$) of each soil organic matter fraction were measured in an IRMS after ball milling in a micromill. The litter-C contribution to each SOM fraction was assessed through a two-end members isotope mixing model (Stewart et al., 2009):

$$f_{bf} = (\delta t - \delta s)/ (\delta r - \delta s)$$

where:

f_{bf} = the proportion of a given biochemical fraction derived-C in a given SOM fraction.

$\delta t = \delta^{13}C$ of a particular SOM fraction at the end of the incubation period;

$\delta s = \delta^{13}C$ of the SOM fraction of the treatment without litter biochemical fraction addition (soil only) at the end of the incubation period;

$\delta r = \delta^{13}C$ of the labeled litter biochemical fraction in study.

A similar procedure was used to estimate the litter biochemical fraction-derived CO_2. The total amount of litter-derived CO_2 evolved during the experimental period was estimated by the difference between the total litter fractions added C and the sum of the amount of C recovered in the soil organic matter fractions.

The efficiency of SOM formation was determined according to Cotrufo et al. (2015):

$$E_{SOMF} = (LDC_{SOM})/ (CL_{litter})$$

where:

E_{SOMF} = Efficiency of soil organic matter formation
LDC_{SOM} = litter-derived carbon on soil organic matter
CL_{litter} = carbon lost as CO_2 from litter as it decomposed

RESULTS

The assessment of the isotopic composition of the CO_2 released by the soil microbial respiration enabled us to identify the decomposition dynamics of each specific biochemical fractions in the presence of the other biochemical fractions naturally found in the plant litter (Fig. 1). The $^{13}CO_2$ release pattern clearly showed that the microbial lability and thus the quality of the litter biochemical fractions was very distinct. The HWE fraction from all plant components was the most labile and presented a decomposition peak at the first incubation hours and then rapidly declined to remain constant throughout the period of incubation. The CF from all plant components presented a respiration pattern that was slower than that of the HWE fraction. Its initial decomposition lagged behind and remained higher for up to 100 days. Decomposition of the CF from leaves was greater than for those obtained from the bark, twigs and roots but, conversely, it lasted longer in the latter than in the former components. The AUR

fraction was much less labile to the soil microorganisms, and showed a long lag in decomposition once the experiment started. However, $^{13}C\text{-}CO_2$ evolution from this incubated fraction showed a clear trend to increase by the end of the experiment, irrespective of its plant component origin. The major differences in the decomposition dynamics were observed for the TSE fraction, where those from leaves and twigs seemed more recalcitrant and behaved similarly to the AUR fraction and those from bark and roots increased their respiration rates starting approximately at two months within the incubation period until reaching a plateau at the late stage of the experiment.

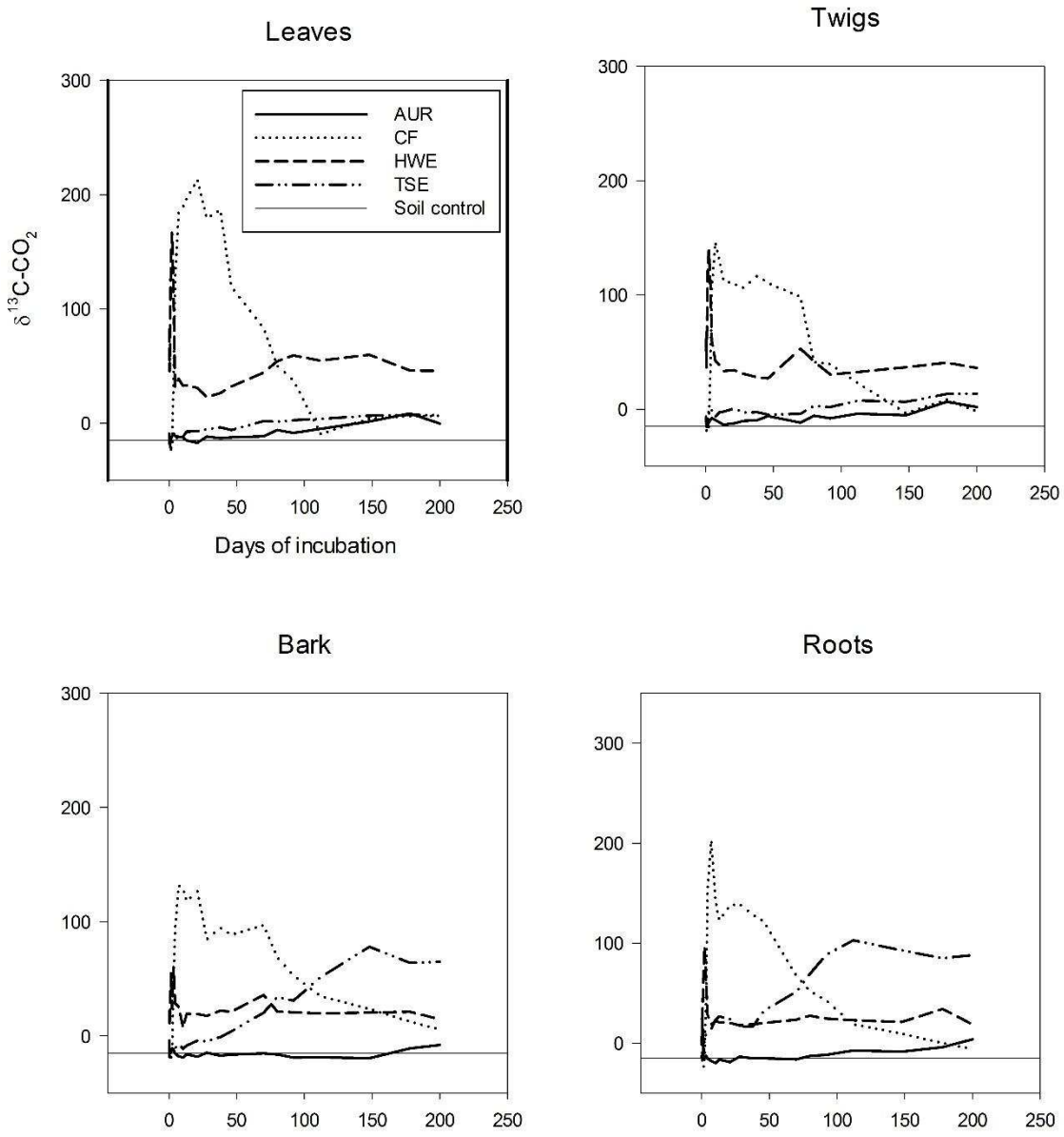

Figure 1 – ^{13}C-CO2 evolution dynamics along the incubation period of a soil treated with different biochemical fractions from distinct plant components. AUR- Acid Unhydrolysable Fraction, TSE – Total Solvent Extractable Fraction, HWE- Hot Water Extractable and CF-Celulosic Fraction.

Litter-derived C was recovered in soil on an average relative rate of approximately 45% and is in a similar order of magnitude observed for studies of same nature (Cotrufo et al., 2015; Haddix et al., 2016; Stewart et al., 2009). The C transfer to the total SOC as well as for the MAOM from the different plant organs (averaged across biochemical fractions) presented no significant differences (Fig. 2). However, greater proportion of C derived from leaves was retrieved in the LFOM. Small amounts of litter-derived C were transferred to SSOM fraction (close to 1% of the added C), with the highest contribution by bark (1.19%) followed by roots (1.03%), leaves (0.71%) and twigs (0.52%). An average of 55 % of the C applied to the soil was respired, with no differences observed among the plant organs.

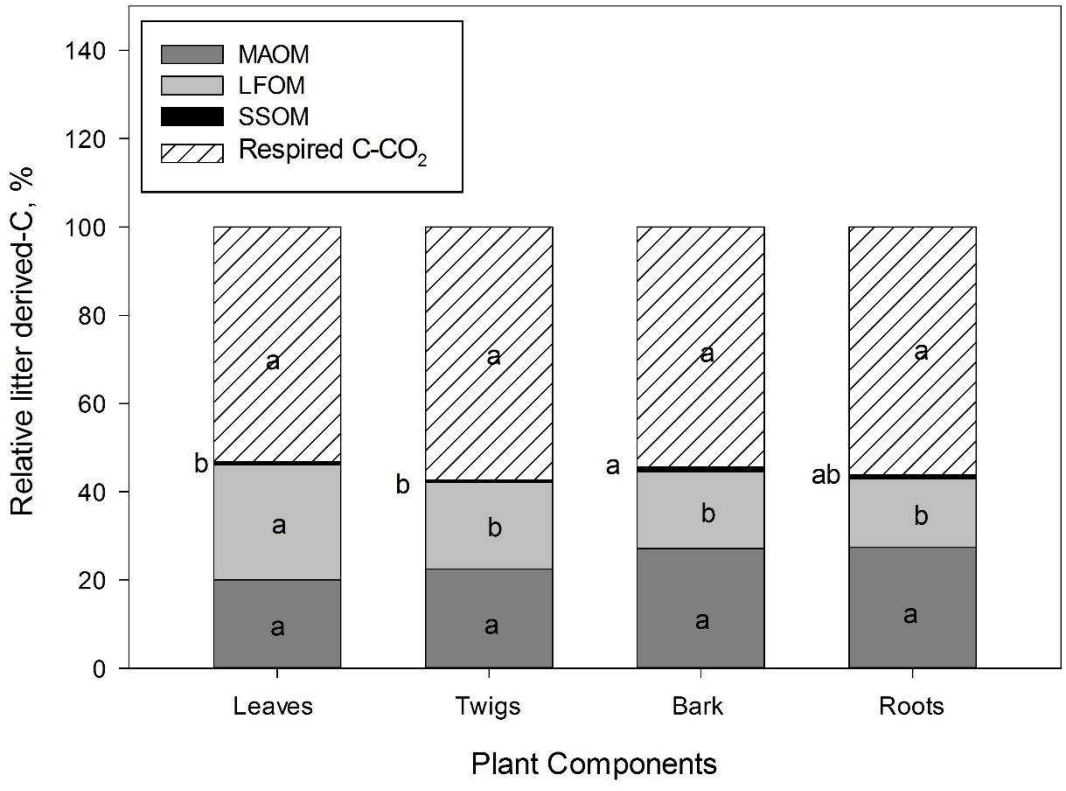

Figure 2 – Partitioning of litter derived-C among soil organic matter fractions and respired C-CO_2 as affected by the application of distinct plant organs (data are averaged across the biochemical fractions). MAOM – Mineral Associated Organic Matter, LFOM – Light Fraction Organic Matter, SSOM – Sand Sized Organic Matter.
Means followed by the same lower case letter within a SOM fraction or respired C-CO_2 derived from different plant components do not differ by the Tukey´s test at 5%.

More pronounced differences in C recovery rates were observed when the effect of biochemical fractions applied to the soil are considered separately (Fig 3). The overall means indicate that the AUR fraction had the highest proportion of C transferred to total SOC (69 %), followed by TSE (52%), HWE (38%) and CF (21%) (Fig. 3). Greater proportions of AUR and HWE derived-C were found in the MAOM. By contrast, lower amounts of C from the TSE and CF were transferred to MAOM. High percentages of AUR and TSE derived-C were still found in the LFOM, while the C derived from CF and HWE was limited to no more than 5% of the total litter fractions C applied to the soil. Much of the CF C (up to 80%) was respired by the soil microbiota, while only a smaller proportion of the AUR (33%) was released as CO_2. Intermediary CO_2 emissions were observed for the TSE (54%) and the HWE (66%) fractions.

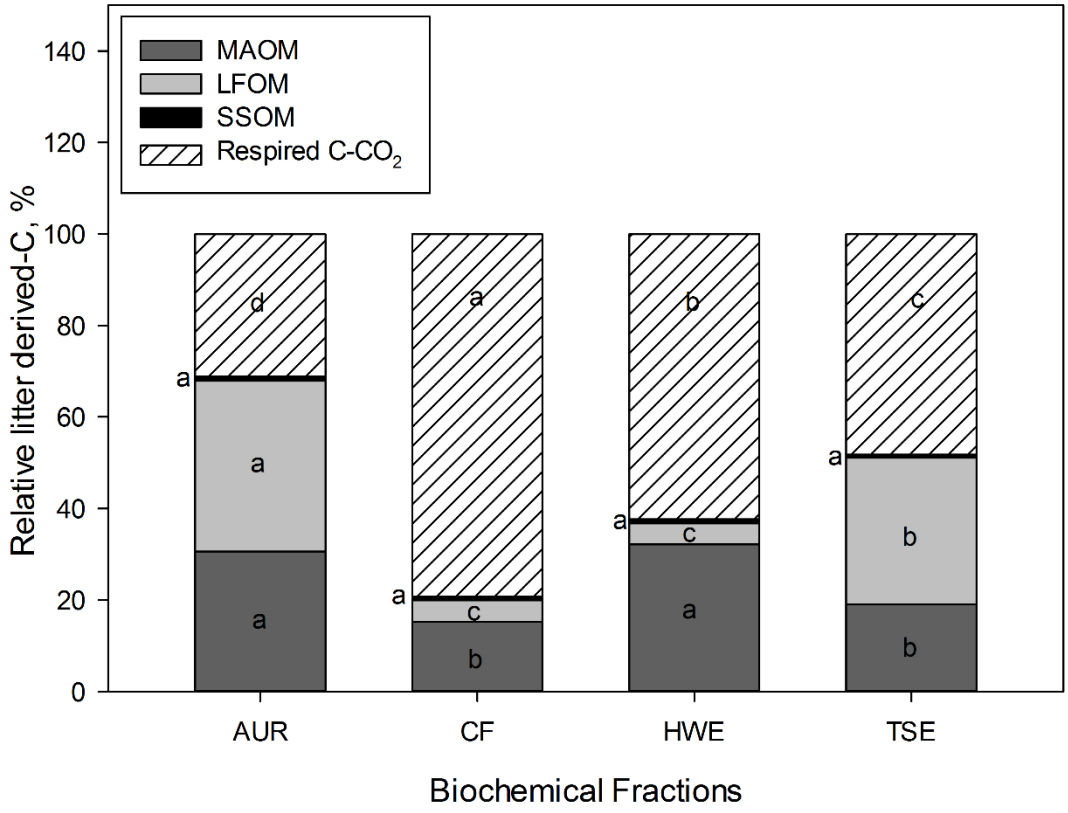

Figure 3 – Partitioning of litter derived-C among the soil organic matter fractions and respired C-CO_2 as affected by the application of distinct plant litter biochemical fractions (data are averaged across the plant components). MAOM – Mineral Associated Organic Matter, LFOM – Light Fraction Organic Matter, SSOM – Sand Sized Organic Matter, AUR – Acid Unhydrolysable Fraction, CF- Cellulosic Fraction, HWE- Hot Water Extractable and TSE – Total Solvent Extractable Fraction.

Means followed by the same lower case letter within a SOM fraction or respired C-CO_2 as affected by the application of distinct litter biochemical fractions do not differ by the Tukey's test at 5%.

In a general, similar biochemical fractions obtained from different plant components do not have significant effect on their C transfer rates to total SOC, except for TSE (Fig. 4A). The lipophilic compounds from leaves contributed more to SOM, with values up to 67% of the added C being recovered in SOM. Lower conversion of lipophilic components into SOM was observed for roots (41%) and intermediary values were found for the TSE fraction extracted from twigs (48%) and bark (52%).

On average, similar quantities of C from the AUR (31%) and HWE (32%) fractions were stabilized in the MAOM, while the same situation was observed for the TSE (19%) and CF (15%) fractions (Fig. 4B). No significant differences were observed among the AUR plant source materials, which means that AUR from leaves, twigs, bark and roots contribute at the same magnitude to SOM stabilization on the mineral fraction (Fig. 4B).

Although in different proportions, HWE and CF from different plant organs behaved similarly regarding the C transfer to MAOM (Fig. 4B). For both biochemical fractions, those extracted from roots had higher proportion of their C transferred to the MAOM, while those from bark presented intermediary values, and those from leaves and twigs were among the least efficient. More pronounced variations due to the TSE source plant component were observed for the C transfer from the TSE to the MAOM, in which more TSE C from bark (28%) and roots (24%) were preferentially retrieved in the MAOM as compared to those from leaves (10%) and twigs (15%) (Fig. 4B).

Under our experimental condition most of LFOM in soils that received application of distinct litter biochemical fractions was formed by undecomposed litter. Indeed, in soils the AUR and TSE fractions were the ones with more remaining material. On the other hand, only small amounts of C derived from the CF and HWE fractions were found in the LFOM (Fig. 4C). No significant variation was observed on the C transfer to LFOM among the distinct AUR, CF and HWE parent materials. Contrastingly, in the treatments receiving the TSE fraction it could be observed significant differences on C recovery rates depending on the plant component (Fig. 4C). Up to 56 % of leaves TSE C added was still in LFOM after 200 days of incubation, while 32; 23 and 16% of the TSE derived-C from twigs, bark and roots, respectively, was recovered in the LFOM.

Less than 3% of C from HWE and CF fractions ended up in the LFOM, with no differences among the parent plant material for both biochemical fractions (Fig.4C).

Only a small portion of the C applied to the soil was transferred to SSOM (close to 1%) and therefore, it is expected that the variations found will not affect the global fate of litter-derived-C among the different SOM fractions. The contribution of CF and HWE fractions C to sand-sized OM was independent of the source material of these fractions (Fig. 4D). The C of AUR and TSE from leaves and twigs was retrieved in smaller proportions on SSOM. The C derived from roots AUR fraction had the highest contribution to SSOM, whereas root TSE derived-C had an intermediary proportion transferred to SSOM. Soils treated with bark and root TSE showed a greater proportions of C transferred to SSOM, followed by twigs and leaves TSE-treated soils (Fig. 4D).

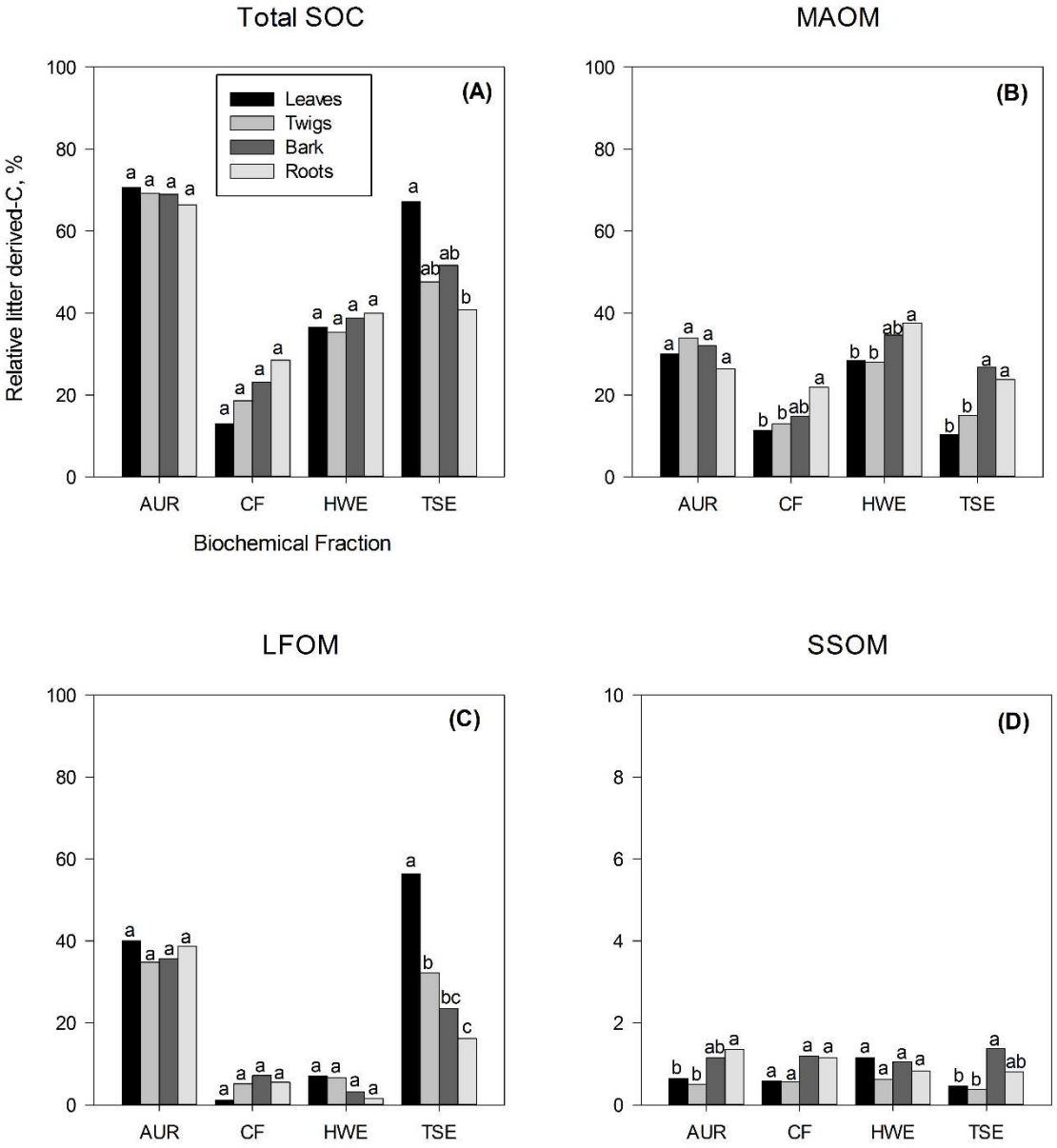

Figure 4 – Partitioning of litter derived carbon to total SOC (A); MAOM (B); LFOM (C) and SSOM (D) of soil samples treated with different biochemical fraction from distinct eucalyptus plant organs. SOC – Soil Organic Carbon, MAOM – Mineral Associated Organic Matter, LFOM – Light Fraction Organic Matter, SSOM – Sand Sized Organic Matter, AUR – Acid Unhydrolysable Fraction, TSE – Total Solvent Extractable Fraction, HWE- Hot Water Extractable and CF- Celulosic Fraction.

Lower case letter compares carbon proportion of C derived from same biochemical fraction of distinct plant organs inside the same SOM fraction. Bars represents means. Means followed by the same lower case letter do not differ by Tukey`s test at 5%.

The efficiency of SOM formation (which is the ratio of litter derived-C on SOM divided by C lost from the litter (Cotrufo et al., 2015)) was not affected by the litter biochemical fractions when averaged across plant components (Fig. 5A). Conversely, significant differences were observed on the efficiency of SOM formation when the distinct biochemical fractions were considered separately. The AUR was the fraction presenting an average (across plant components) higher efficiency, while the TSE was intermediate, and the HWE and CF were less efficient in forming SOM (Fig. 5B). No significant differences on the efficiency of SOM formation were found for similar litter biochemical fraction from distinct plant components, except for the TSE in which the fraction obtained from leaves formed SOM more efficiently (Fig. 5C).

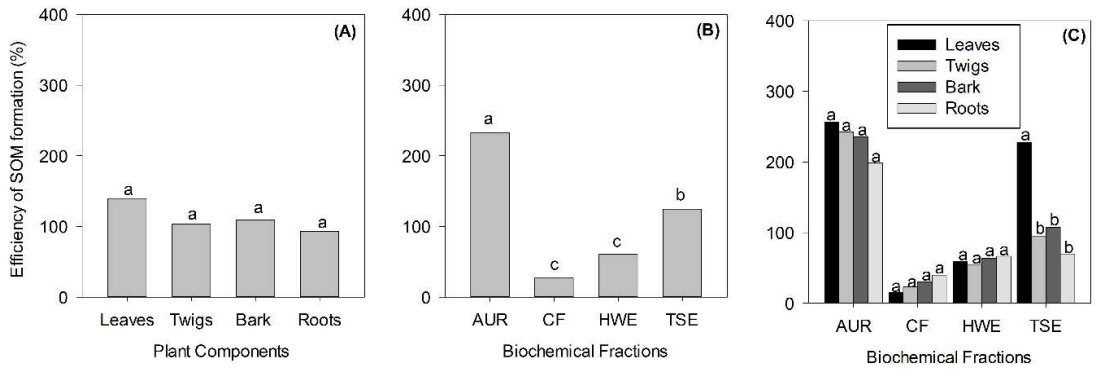

Figure 5 – Efficiency of SOM formation by different plant components, averaged across biochemical fractions (A); different biochemical fractions, averaged across plant components (B) and different biochemical fractions extracted from distinct eucalyptus plant components (C). AUR – Acid Unhydrolysable Fraction, TSE – Total Solvent Extractable Fraction, HWE- Hot Water Extractable and CF- Celulosic Fraction.
In 5A and 5B graphs lower case letter compare SOM formation efficiency of plant components and biochemical fractions, respectively. On 5C graph lower case letters compare SOM formation efficiency of different plant componensts within the same biochemical fraction. Bars represents means. Means followed by the same lower case letter do not differ by Tukey test at 5%.

DISCUSSION

The complex nature of plant litter led us to fractionate distinct plant components (leaves, bark, twigs and roots) to study their decomposition, but more importantly, evaluate the efficiency with which the molecularly distinct fractions would contribute to the formation of SOM, especially the more protected mineral-bound fraction. Our results indicate that we were able to successfully label the distinct plant organs (Table 1) and litter biochemical fractions (Table 3) with ^{13}C to an enrichment level high enough to track down the fate of litter C into SOM fractions following their incorporation to the soil. The results also confirm that our operationally defined litter fractions were indeed molecularly distinct, with variable proportions of the so-called metabolic and structural components, being more polar or apolar, more aliphatic or aromatic, according to the fraction and plant organ of origin (Table 5). This variation in biochemical composition indeed affected their availability to soil microorganism and thus determined distinct patterns of decomposition following their application to the soil (Fig. 1). As expected, the biochemical fractions with less structural C and more easily extractable hydrophilic compounds were more rapidly decomposed (Fig. 1), even though fractions dominated by structural compounds such as de CF were not resistant to microbial decomposition (Fig. 1). However, the fate of litter fractions C to the distinct SOM fractions was surprisingly distinct from our initial hypothesis. Recent research has suggested that plant litters richer in metabolic compounds are more readily used by soil microbes and are more favorable precursors to the formation of more stable silt and clay minerals-bound SOM (Cotrufo et al., 2015, 2013; Haddix et al., 2016). Also, the importance of the soil C saturation deficit and the magnitude of litter C inputs, besides litter quality, have been put forward in order to more accurately understand and predict SOM

stabilization in soils (Castellano et al., 2015), but because they were not factors under study and for clarity they will not be discussed further.

The lack of significant effects in the litter C recovery rate on total SOC depending on the plant component was expected since the biochemical fractions from distinct plant components were added at equal amounts (25% on a C basis). Plant organs like twigs, bark and roots that are naturally richer in more recalcitrant material and soils treated with these plant components had the proportion of these compounds reduced relative to their natural abundance in plants, while the proportions of more labile compounds was increased when incubating the biochemical fractions in fixed proportions. Conversely, in the treatments with leaves which naturally have proportionally more labile components became more recalcitrant as a whole once had the proportions of material resistant to decomposition increased. This was a compromise in order to make all the added materials very similar among the plant organs, allowing to scrutinize the role of the chemical characteristic intrinsic of each biochemical fraction on decomposition and C recovery in SOM fractions.

The different proportions of C transferred to SOM depending on the litter biochemical fraction reveals the importance of litter quality on SOM formation. Surely, the chemical characteristics of the input material will determine its fate in the soil. Biochemical recalcitrant material such as lignin and lipids tend to be preserved in soils (Feng and Simpson, 2008), what is thought to be related due to their hydrophobicity (Song et al., 2013).

The separation of plant litter into different biochemical fractions was a useful approach to study their dynamics in soil once they behave differently during the decomposition process. The metabolic compounds (e.g. HWE) are more easily decomposable and

treated as the labile pool of respiration models, while the structural compounds (cellulose and lignin) have been considered as recalcitrant (Haddix et al., 2016). In the current study the CF fraction, despite usually being included in a structural pool of plant litter (e.g. Haddix et al. 2016) was not resistant to microbial decomposition irrespective of the plant component origin. Therefore, the subdivision we carried out for the structural component in CF and AUR was important to better understand the fate of their different constituents in soil. In this sense, the TSE and the AUR presented more resistance to soil microbial degradation, as indicated by lower ^{13}C-CO_2 release and their high contents found in the LFOM. These findings corroborate earlier observations that lipidic and aromatic structures are usually recalcitrant since they are much more resistant to microbial attack as compared to compounds such as proteins and carbohydrates (Gleixner et al., 2002; Melillo et al., 2002, 1982) and for this reason they tend to be preserved in the soil (Feng and Simpson, 2008), at least in the short-term.

Our findings that plant structural components in the AUR as well as free lipids in the TSE fraction were effective precursors for the MAOM fraction contrast with recent findings (Haddix et al., 2016) that mineral associated OM is originated mostly from metabolic plant components and that decomposition of structural litter components only leads to C mineralization and formation of particulate light fraction of SOM (Cotrufo et al., 2015; Haddix et al., 2016).

More labile compounds are rapidly assimilated by the microorganisms (Jones and Murphy, 2007) as observed for HWE and cellulosic compounds in the CF which started being mineralized by microorganisms at early stages of incubation, with respiration peaks during the first incubation hours for HWE and first incubation days for cellulosic compounds (Fig. 1).

The rapid respiration of the cellulosic compounds (CF) combined with the low contents of the CF fraction found in soil confirm their rapid mineralization found in earlier studies (Otto and Simpson, 2006). What is intriguing, however, is that while the HWE fraction respiration was faster and larger, its contribution to the formation of MAOM was significantly greater than that of CF (Figure 3). Metabolic compounds such as free amino acids and organic acids in the HWE fraction have charged functional groups and could be adsorbed onto soil mineral surfaces suggesting a possible direct contribution to the more stable SOM pool (Kramer et al., 2012). It is more likely, however, that ^{13}C in labile compounds is being rapidly assimilated into microbial structures which in turn are being cycled and transferred to the MAOM fraction (Rubino et al., 2010; Soong and Cotrufo, 2015) and supports the hypothesis that substantial amounts of labile litter derived-C may favor microbial mediated mineral-organic associations at the early decomposition stages. Despite being labile, the CF fractions is much less effective for SOM formation and we cannot offer any plausible explanation for such distinct behavior and this needs further investigation in the future.

The high contents of AUR derived-C remaining on LFOM and low ^{13}C-CO_2 evolution shows the resistance of these compounds to microbial degradation. During litter decomposition lignin polyphenols, tannins, and other recalcitrant materials are partly solubilized (Kirk and Farrell, 1987; Shevchenko and Bailey, 1996), originating carboxyl-rich ring structures more resistant to microbial degradation (Kalbitz et al., 2006). Nevertheless, these aromatic fragments may behave as dissolved organic matter (DOM) and bind directly to mineral surfaces or pre-existing mineral-organic complexes (Kramer et al., 2012). This stabilization mechanism was probably responsible for the high AUR derived-C transferred to the MAOM fraction. The soil used in the current

experiment is a tropical soil with abundant Fe and Al oxy-hydroxides. Thus, it is possible to have occurred a strong carboxylate binding of aromatic/phenolic components of the AUR fraction to the oxy-hydroxides surfaces forming stable complexes, as proposed recently for a Mn oxide-DOC complex (Johnson et al., 2015). Additionally, the presence of substantial amounts of lipid compounds besides phenolics in the AUR does not rule out the possibility that additional contribution to SOM formation by the AUR may come from suberin-derived compounds (Hamer et al., 2012) that may well resist to the acid hydrolysis procedure. Given the low C/N ratio of the AUR fraction, there is the possibility of formation of N-bonded aromatics mediated by abiotic reactions (Gillespie et al., 2014) that were transferred to the MAOM fraction. This would conciliate our finding for high efficiency of SOM formation by the AUR fraction (similar to metabolic HWE and greater than the highly microbial labile CF) even though it was found highly resistant to microbial decomposition.

The TSE fate in soil reveals a relatively high resistance of these compounds against degradation with its greater proportion being found on LFOM. A satisfactory explanation for the TSE behavior in soil is based on its biochemical composition once the long-chain aliphatic compounds found in cuticular waxes, cutin, and suberin are known to be more slowly degraded (Lorenz et al., 2007; Mikutta et al., 2006) and thereby effective in C storage in SOM (Jandl et al., 2012). The high hydrophobicity of the compounds limits their hydration and microbial enzymes activity, and their interaction with the silt and clay minerals through hydrophobic interactions could favor their stabilization in the MAOM.

Hydrophobicity is not solely the main characteristic governing the protection of these compounds against decomposition in soil since differences on decomposition rates of

TSE from the different plant organs were observed. These differences may indeed be due to the variable proportion of the long and short chain lipids in TSE among the distinct plant organs. TSE from leaves were more resistant to decomposition (Fig. 4) probably because of its proportionally higher contents of long chain lipids due to the presence of cuticular waxes. Long chain lipids are known to be more resistant to microbial degradation than short chain lipids (Jandl et al., 2005).

CONCLUSIONS

The current study with ^{13}C-enriched litter fractions containing biochemically variable compounds was effective in accessing the contribution of litter chemistry/quality on SOM formation. Not all litter fractions that are easily decomposed are equally effective in promoting the formation of organic-mineral associations. Even materials relatively more resistant against decomposition such as lignin and lipids were more effective precursors of the more stable silt and clay minerals-bound SOM fraction (MAOM). Our findings led us do refuse our initial hypothesis that microbial labile, whether metabolic or structural, litter fractions are more effective at promoting MAOM formation. They also do not support the more recent propositions that mineral-bond SOM is mainly dependent on labile compounds in metabolic fractions and that recalcitrant materials are mineralized or contribute mostly for the particulate organic matter.

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