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Decomposition of green tea and rooibos tea across three monospecific temperate forests: Effect of litter type and tree species

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ABSTRACT

We studied the effect of different forest covers on carbon (C) and nitrogen (N) dynamics of two standardised litters during decomposition in soil. For this purpose, commercially available bags containing green tea or rooibos tea were incubated in close monospecific stands of *Fagus sylvatica*, *Pseudotsuga menziesii*, and *Quercus cerris*, in the Apennines range, Italy, and then analysed at different intervals for up to two years. We also investigated the fate of various C functional groups in both types of litter under beech by nuclear magnetic resonance spectroscopy. After two years of incubation, green tea had not changed its original C/N ratio of 10, while rooibos tea had nearly halved its original value of 45, because of different C and N dynamics. Both litters progressively lost C, about fifty per cent of the initial content in the case of rooibos tea, and a little more for green tea, most of the loss occurring in the first three months. In terms of N, green tea behaved as for C, while rooibos tea in the early stage lost part of its N stock, fully recovering it by the end of the first year. Under beech, both litters showed a preferential loss in carbohydrates during the first trimester of incubation and, consequently, an indirect enrichment in lipids. Later on, the relative contribution of the various C forms remained practically constant.

Our results overall support that the decay rate and compositional changes of litter depend strongly on the litter type and little on the tree cover of the soil in which the litter is incubated.

1. Introduction

Litter decomposition has been widely studied by applying the litterbags method, where pre-dried leaves are inserted in bags of perforated material resistant to alteration and then left in the environment for a predetermined period to be finally weighed and analysed. Thanks to this method, several factors have been demonstrated to exert some influence on litter decomposition rate, and among them litter quality seems the most important one [1–3]. In general, the C/N ratio and lignin content are important indicators of litter quality, higher values of one or both implying a slower decomposition rate [4,5]. The first decomposition phase is typically characterized by the highest litter mass loss, with the rapid decrease in the content of water-soluble substances and holocellulose and

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the indirect enrichment in more resistant compounds [6]. Later, the process slows down, but how the residual litter composition influences the further stages of decay is still not well understood.

Mass loss of litters during decomposition in their home stands has been investigated by several authors [*e.g.*, 7–9]. They essentially found that decomposition is an asymptotic process mainly controlled by litter quality and only to a minor extent by other environmental conditions. Some studies [*e.g.*, 10–13] assessed the dominance of litter quality as a driving factor, also when a given litter is incubated under species different from its original one. Elucidation of the degradation pathways during natural litter decomposition was highly improved by the application of spectroscopic techniques, such as Fourier-Transform Infrared (FTIR) spectroscopy and, especially, nuclear magnetic resonance (NMR) spectroscopy [14,15].

In this work we applied elemental analysis combined with ^{13}C solid state NMR to test the changes experienced by two types of litter with significantly different composition and potential degradability – green tea and rooibos tea – during decomposition in soil under monospecific montane-temperate forest stands of *Fagus sylvatica, Pseudotsuga menziesii,* and *Quercus cerris*. To this end, commercially available teabags were used following Ref. [16]. This approach provided valuable insight into the mass loss rate and chemical changes of such litters across forested sites within the global *TeaComposition initiative* (www.teacomposition.org), a research project aimed at understanding litter decomposition across biomes. The first important finding of the *TeaComposition initiative* was that, at a global scale, litter quality is the predominant controlling factor in the early stage of litter decomposition (*i.e.*, within the first 3 months), explaining about 65% of the variability in the degradation process; the effect of climate was found to be minor and not litter specific [12]. However, when the data were aggregated at the biome scale, climate resulted to play a significant role on the decomposition of the two litter types here examined, explaining 64% of the variation for green tea and 72% for rooibos tea. More recently, using data collected within the same initiative from 394 sites after 3 months of incubation and 423 sites after 12 months of incubation across nine biomes, Ref. [17] demonstrated that the effects of climate were not litter-specific and, after 12 months of incubation, annual precipitation and temperature explained 8% and 12% of decomposition for green tea and 6% and 15% for rooibos tea, respectively.

With the aim of taking another step forward in understanding the litter decomposition process and the factors that affect it, in this work we studied the decay of green tea and rooibos tea over a two year-period at one of the locations tested within the *TeaComposition initiative*, under three different tree species. Since the soil and climatic conditions were the same, the study allowed the possible effect of forest cover to be highlighted. The structural transformations the two litters experienced in the beech stand throughout the 2-year incubation period were investigated using ^{13}C NMR spectroscopy.

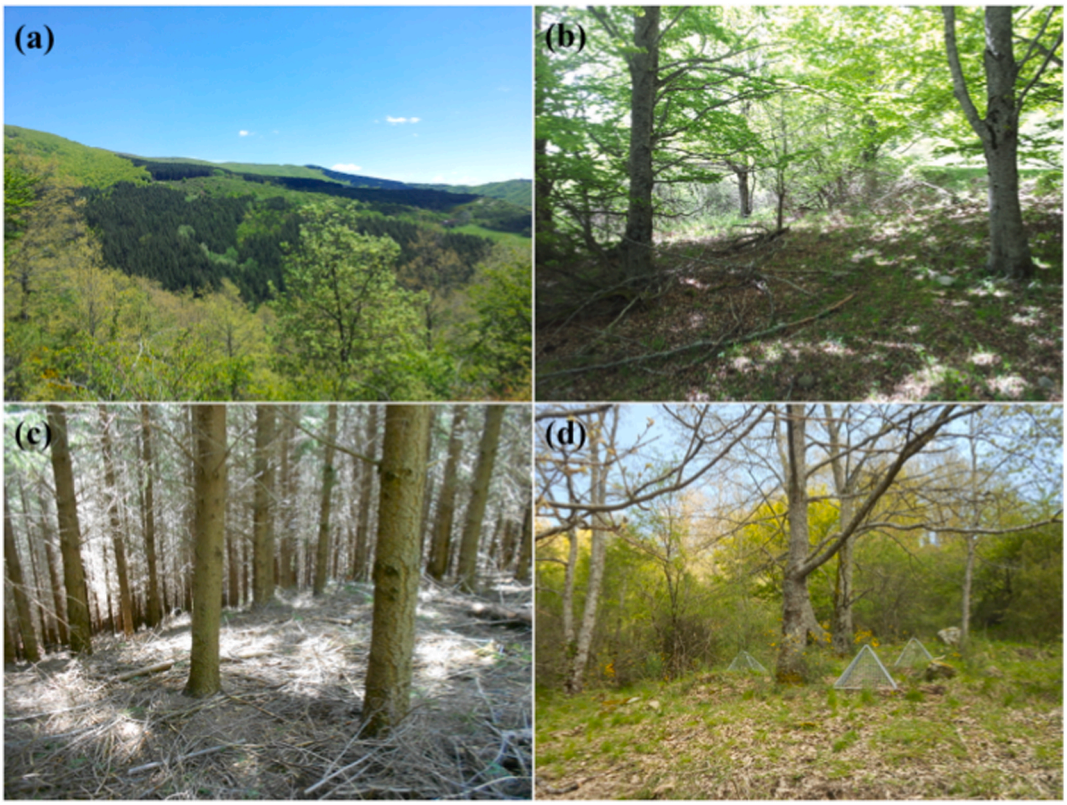


Fig. 1. General view of the study area (a). The highest forest stand on the left side in (a) is the beech study site shown in (b), the darker patch in the centre is the Douglas fir site shown in (c), while on the extreme right, partially hidden, is the oak site shown in (d). In (d) the metallic pyramids of metallic net that served to protect the litterbags from any possible damage by higher animals, ungulates in particular, are visible.

2. Material and methods

2.1. Study area

The study was conducted in the Rincine Forest (43° 51′ 48″ N, 11° 39' 40" E), 40 km northeast of Florence, central Italy, in three semi-natural, mature, monospecific stands (Fig. 1) located along a minor south-facing elevation gradient, *i.e.*, European beech (*Fagus sylvatica* L.; 1058 m above sea level), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco; 1026 m a.s.l.), and Turkey oak (*Quercus cerris* L.; 928 m a.s.l.). This area undergoes a montane-temperate climate with cold and wet winters and warm and dry summers; the modest differences in elevation between the sites guarantee that there are no significant climatic differences. The mean annual temperature is 9.2 °C, with July and August the warmest months (17.8 °C on average). The mean annual precipitation is 1273 mm, with November the rainiest month (163 mm on average) and July the driest (58 mm). Temperatures and precipitations during the experiment were recorded at a local weather station (Fig. 2). The forest soils are *Dystric Eutrudepts* and *Typic Dystrudepts* of the U.S. Soil Taxonomy [18] and formed on Oligocene sandstone chiefly composed of quartz, feldspars, and phyllosilicates, with minor amounts of carbonates.

2.2. Soil sampling and analysis

Three top (0–5 cm) mineral soil samples were taken in each stand, within a radius of no more than 3 m from where the teabags were placed. The soil was air dried at room temperature until constant weight, then sieved to 2 mm to remove rock fragments. Particle-size distribution was determined according to the hydrometer method [19], while pH was measured potentiometrically in both deionized water and 0.01 M $CaCl_2$, using a 2.5:1 liquid to soil ratio. Organic C and total N were determined by dry combustion with an elemental analyser (Perkin Elmer 2400, Series 2) on moisture-free (105 °C overnight) and pulverised aliquots treated with excess HCl to selectively remove carbonates [20]. Inorganic N was extracted by 2 M KCl with a 1:10 soil:solution ratio, following Ref. [21]. Ammonia N in the extract was measured by a colorimetric method with Na-salicylate in place of phenol in the Berthelot's reagent, according to Ref. [22]. Nitric N in the same extract was measured by the colorimetric method based on nitration of salicylic acid, as in Ref. [23]. Available phosphorus was determined by extraction with sodium bicarbonate and quantification via spectrophotometry using the ascorbic acid method, according to Ref. [24].

2.3. Litterbag experiment

For the litterbag experiment, we used tetrahedron-shaped nylon bags (mesh size 280–300 μm) containing on average 1.7 g of green tea ("Lipton Indonesian tea Sencha tradition", Lipton, European Article Number: 87 22700 05552 5; main ingredients: 89% tea, 9.3% flavouring, 1% rose petals) or on average 1.9 g of rooibos tea ("Lipton Rooibos Tea", Lipton, European Article Number: 87 22700 18843 8; main ingredients: 93% South African Rooibos, 1% arome hibiscus), also known as "South African red tea". Green tea consisted of leaves of *Camellia sinensis* that had not undergone the withering and oxidation process used to make the more common *oolong* tea, while rooibos tea is the *afrikaans* term for *Aspalathus linearis*, a broom-like member of the *Fabaceae* family of plants growing in South Africa's fynbos. Green tea can be seen as a high-quality litter, being rich in nutrients and soluble carbon, and rooibos tea as a low-quality litter, because it is poor in nutrients, especially nitrogen, and rich in lignin [16,25]. As underlined by Ref. [26], since both green tea and rooibos tea are functionally and phylogenetically distant from tree species in European forests, using them in litterbags saves from experiencing any "home field advantage", the phenomenon whereby decomposer communities specialize towards the litter they most frequently encounter.

At the beginning of May 2014, twenty-five oven-dried (60 °C for 48 h) bags of both green tea and rooibos tea were incubated in the

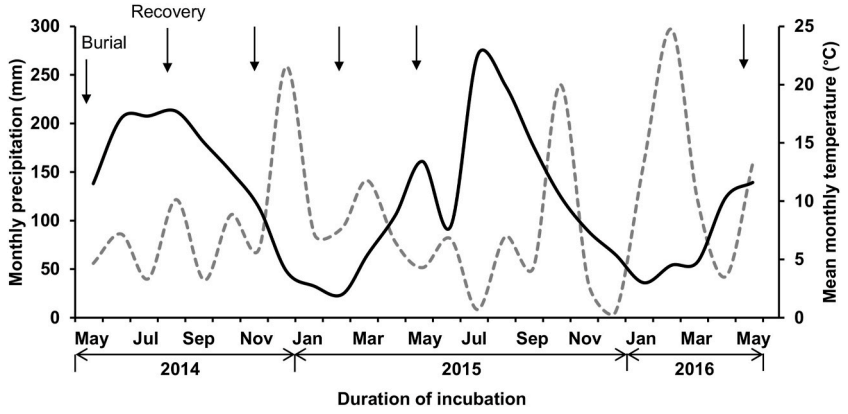


Fig. 2. Monthly precipitation (dashed line) and mean monthly temperature (solid line) at the study area during the two-year-long experiment (May 2014 to May 2016). The first downward arrow depicts the date of initial burial of green and rooibos teas, and other downward arrows depict the recovery dates of the litterbags after 3, 6, 9, 12, and 24 months of incubation.

soil of each stand, in the top mineral horizon (A), between 0 and 5 cm deep, and within about 20 cm from each other, under pyramids made of metallic net for preventing higher animals' disturbances (Fig. 1d). Five bags per litter were collected from each stand at five sampling dates: three, six, nine, twelve, and twenty-four months since burial. After externally cleaning with a brush, the bags were dried at 60 °C until constant weight; their content was then finely ground and analysed for organic C and total N by dry combustion (Fig. 3).

2.4. ^{13}C solid state NMR

^{13}C cross-polarization (CP) NMR spectra under magic angle spinning (MAS) were acquired on pulverised composite samples (combinations of equal amounts of the five replicates per litter type and per sampling date) from the beech stand on a Bruker AVANCE 400 NMR spectrometer, working at 100 MHz for carbon-13, equipped with a 4 mm CPMAS probe. A ramp-CP pulse sequence was used with the following acquisition parameters optimized from preliminary tests: recycle delay 2 s, ramp size 18 kHz, total contact time 3 ms and spinning rate 8 kHz. The spectra were obtained by accumulating between 6400 and 28,000 scans, depending on the sample, and were analysed by integrating the area of eight spectral regions corresponding to different C forms (in parentheses): 0–45 ppm (alkyl C), 45–60 ppm (methoxyl and *N*-alkyl C), 60–90 ppm (O-alkyl C, mainly carbohydrates), 90–110 ppm (di-*O*-alkyl C, mainly from polysaccharides), 110–140 ppm (H- and C-substituted aromatic C), 140–162 ppm (O-substituted aromatic C, mainly from lignin structures, tannins, and polyphenols), 162–190 ppm (carboxyl C from esters, acids and amides), and 190–220 ppm (aldehydes and ketones). The assignment of the specific C functional groups was made according to Refs. [27,28].

The relative populations of the different carbon forms and the carbon content at the different sampling times were used to estimate the residual content of each carbon form (as % of the original C). These values were used to estimate the initial rate (k) of mass loss for the labile fraction of each carbon type employing a simple two-pool fitting model [29], according to which the carbon mass loss has a bi-exponential trend with a fast-decaying labile fraction and a slow-decaying recalcitrant one. Given the relatively short time of the experiment, here the weight loss of the recalcitrant fraction was considered negligible and the carbon mass loss for the different carbon forms was fitted to the following equation [16]:

$$W(t) = W_l e^{-kt} + W_r \quad (1)$$

where $W(t)$ is the residual carbon fraction at time t, W_l and W_r are the initial labile and recalcitrant carbon fractions and k is the decay rate of the labile fraction.

2.5. Statistical analysis

To determine the significant differences between the means of each factor (soil property or litter chemical property), the incubation periods at each site, or the incubation sites at each incubation period, the one-way ANOVA or the non-parametric Kruskal-Wallis test was performed. The latter was used when preliminary analysis indicated that one or both assumptions of normality with Shapiro test

Fig. 3. Flow chart showing the various phases of the experiment, from placing the teabags in the soil of the three forest stands to the final analysis of the residual litter.

and homogeneity of variance with Levene test were not met. When the one-way ANOVA or Kruskal-Wallis test showed a significant difference across groups, a parametric or non-parametric post-hoc test was conducted using the "HSD.test" or "kruskal" function, respectively, in the package "agricolae" in the R Statistical Software (version 4.1.1), with $p < 0.05$ for statistical significance. For the non-parametric post hoc test, the Holm-adjusted p-value was set to $p < 0.05$ for statistical significance for multiplicity correction [30,31]. Principal Component Analysis (PCA) was performed with the package "vegan" in the R software to reduce the dimensionality of the NMR spectral regions at each incubation period for both green tea and rooibos tea [32,33], so that the changes of chemical composition over incubation period can be visually and exploratorily traced. The significant difference of chemical structural changes of the two teas during the 2-year in-situ decomposition was tested using ADONIS (PERMANOVA) with the method of Euclidean distance. The ellipses of the 95% confidence interval were also identified.

3. Results and discussion

3.1. Soil properties

The soils where the teabags were incubated were thick and well drained. The values of selected properties relative to their uppermost portion are reported in Table 1. The particle-size distribution was very similar in the three stands, all of them falling in the sandy loam texture class. Significant differences among stands were found in terms of soil pH, both in that determined in deionized water and in that in neutral $CaCl_2$ solution. In particular, the soil under fir showed pH values about one point lower than those under beech and oak, which is confidently due to the more acidic residues released to the soil by the conifer with respect to the two broad-leaved trees. The modest discrepancies between the pH values measured in the two liquid phases are however proof of low exchangeable acidity. Soil organic C content under beech was significantly lower than under fir and, especially, under oak. Differences among the three soils occurred also in terms of total N, with the soil under oak significantly richer in N than the other two. Although not major, such differences could be relevant for litter decay; in fact, Ref. [17] demonstrated that in the temperate biome, where atmospheric N deposition rates are relatively high, the 12-month mass loss of green tea significantly decreased with increasing N deposition, explaining 9.5% of the variance. As expected, inorganic N represented a minor fraction of soil total N, and occurred predominantly as ammonia, with no significant differences between the three soils. Available phosphorus was high to abundant, with no significant differences between the soils.

3.2. C, N and C/N ratio dynamics in litters

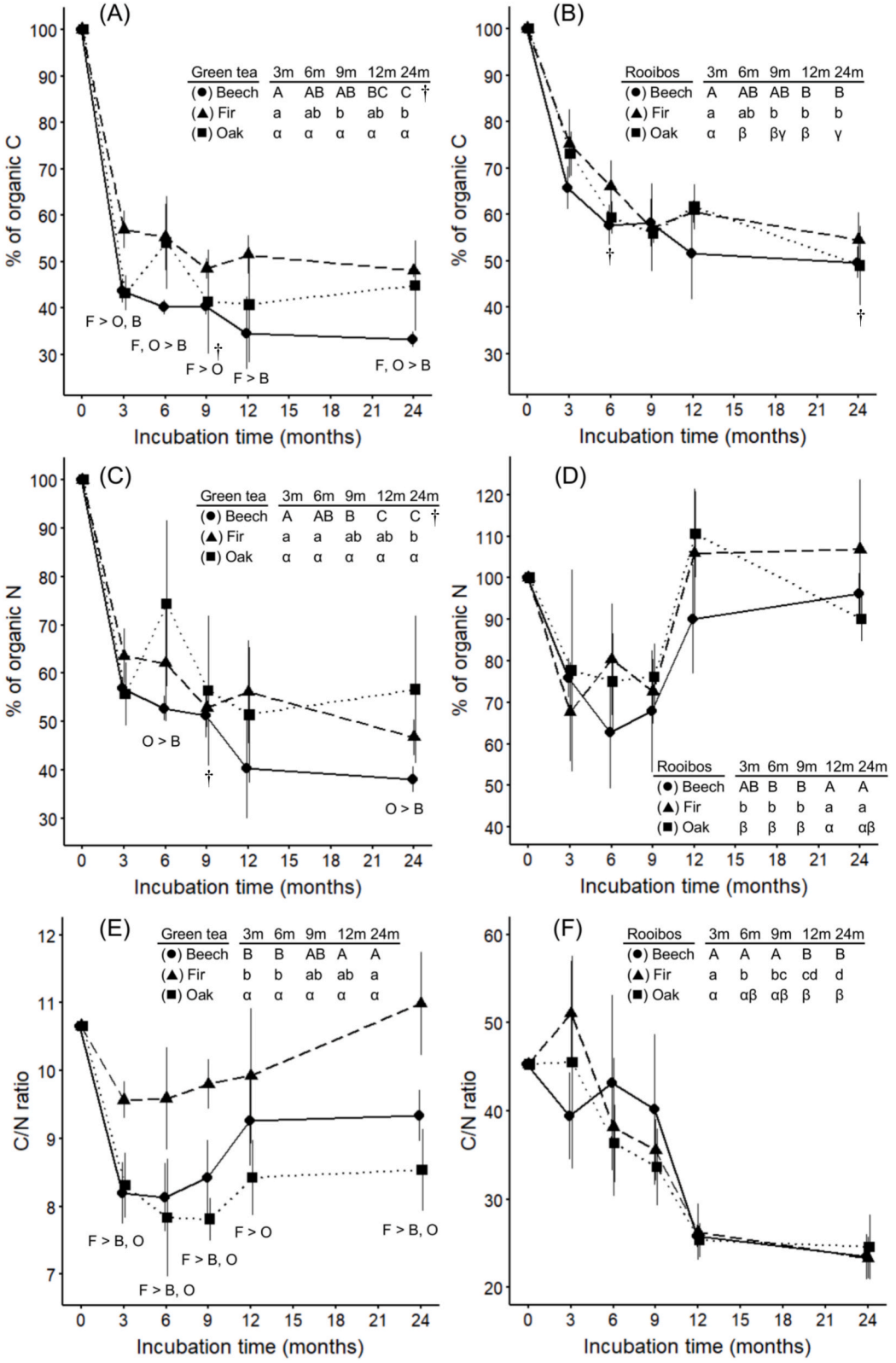
Given the proximity and modest differences in altitude, the three study sites have virtually the same precipitations and temperatures (Fig. 2), as well as the same soil types. Therefore, the only factors that may be substantially responsible for differences in litter decomposition rate in our experiment are the litter and tree cover types. Indeed, the two litters showed different decomposition pathways, as highlighted by the trends of C, N, and their ratio (Fig. 4), and in agreement with the quite different decay rates typically found, *i.e.*, high for green tea and low for rooibos tea. Compared to the mass loss, the C and N trends are not or only little affected by possible contamination from mineral particles, and partly account for the structural changes experienced by the litters during decomposition.

The initial C/N ratios of the two litters were very different, that is, 10.65 for green tea and 45.25 for rooibos tea, and suggested a much higher recalcitrance to decomposition of the latter. Starting from six months after incubation, rooibos tea experienced a progressive decrease in terms of C/N ratio, which was dramatic over the period between nine and twelve months, when, under all forest covers, it became about 26; one year later, it had slightly decreased further, to around 24 (Fig. 4). No significant differences were found between the stands. Differently from rooibos tea, green tea experienced minor changes in terms of C/N ratio throughout the whole experiment, so that two years after burial the C/N ratio was similar to the one of the initial material (Fig. 4). Nevertheless, significant differences between the stands were found at all sampling dates, with Douglas fir generally showing higher C/N values with respect to

Table 1
Basic properties of the soils (top 5 cm of mineral soil) where the teabags were incubated (n = 3; mean ± standard deviation in parentheses). Different letters indicate statistically significant differences (one-way ANOVA, or Kruskal-Wallis test only in the case of clay in particle size distribution, $p < 0.05$) between forest cover types and missing letters mean no significant differences.

Forest cover	Particle-size distribution* (g kg^{-1})	pH in H_2O	pH in 0.01 M $CaCl_2$	Organic C (g kg^{-1})	Total N (g kg^{-1})	Ammonia N (mg kg^{-1})	Nitric N (mg kg^{-1})	Available P (mg kg^{-1})
Beech	545-320-135 (10-22-14)	5.4 (0.5) a	5.0 (0.5) a	33.3 (12.9) b	2.0 (0.9) b	44.3 (22.6)	2.2 (0.3)	23 (13)
Fir	536-336-128 (39-30-8)	4.5 (0.3) b	4.0 (0.3) b	50.4 (8.6) ab	3.7 (0.6) b	60.4 (14.9)	5.4 (1.6)	29 (4)
Oak	528-373-99 (58-53-11)	5.6 (0.2) a	5.2 (0.1) a	82.1 (25.0) a	6.0 (0.9) a	59.1 (10.4)	17.6 (15.3)	59 (26)

* Sand (2000–50 μm), silt (50-2 μm), clay (<2 μm), respectively.



(caption on next page)

Fig. 4. Carbon content (% of original C), nitrogen content (% of original N), and C/N ratio at different sampling times (3, 6, 9, 12, and 24 months) of green and rooibos teas buried under European beech, Douglas fir or Turkey oak forest. Mean ± standard deviation (n = 5). Letters in the table of each panel indicate significant differences between means ($p < 0.05$) compared with five incubation periods at each forest stand. Letters under lines indicate significant differences between means ($p < 0.05$) from the three different tree covers (B for beech, F for fir, O for oak) at each sampling time. Missing letters under lines mean no significant differences between forest stands. One-way ANOVA was performed, with the only exception of the sampling times noted with the symbol †, in which Kruskal-Wallis test was used because the assumptions of normality and homogeneity of variance were not met.

the two broadleaves. These findings agree with Ref. [25], where an initial decrease in C/N ratio in green tea under both a broadleaf forest (beech) and a coniferous one (spruce) was observed, and then an almost constant value in the case of beech and an increase under spruce.

For both green tea and rooibos tea, the highest C loss occurred in the first three months of incubation, that is, from May to July (Fig. 4), when both litters were richer in soluble and/or labile compounds and when, thanks to the mild weather conditions, the soil biota was very active. Nevertheless, in this initial period, the relative C loss in rooibos tea was much less than in green tea, ranging between 25 and 35% compared to a 40–60% C loss observed for the latter. Rooibos tea was more resistant to decay than green tea also considering the whole period investigated, its C loss after two years being close to 50% (versus 50–70% of green tea). Significant differences in residual C between the home stands were observed for green tea only, and these were observed at all sampling dates (Fig. 4). In particular, green tea litter showed the lowest C loss under fir in the first 3 months and generally maintained a higher level of residual C there than under the two deciduous trees throughout the 24 months of incubation (with significant differences between oak and beech recorded only at 6 and 24 months). Rooibos tea, after the initial substantial loss, experienced few other significant C decreases: after 9 months under fir, after 12 months under beech, and after 6 months and again 24 months under oak. Our findings are at variance with those reported in Ref. [25], where higher rooibos tea degradation efficiency under beech was observed. Additionally, Ref. [26], in a teabag experiment carried out across 99 forest sites throughout France, assessed that, while green tea decomposition was chiefly related to climatic factors, rooibos tea decomposition was strongly related to edaphic factors and the identity of the dominant tree species in the stand. Anyway, based on their large dataset, these authors concluded that the relative importance of climate, soil and plant functional traits in the litter decomposition process depends on litter quality, which is the real driving factor of the decomposition rate.

Litter decay in terms of N was similar to the one in terms of C in green tea, whereas in rooibos tea the trends of the two elements were markedly discrepant (Fig. 4), and this is the main reason for the different C/N ratio trends observed for the two types of litter and discussed above. At the end of the trial, green tea had lost 40–60% of its initial nitrogen content, most of which had disappeared in the first three months, while rooibos tea on average still showed the original N content, although with high variability throughout the incubation period. In particular, in the first nine months, rooibos tea experienced a significant reduction in N, which was completely recovered by the end of the first year. In this regard, in a threemonth long pot experiment of incubation in soil, Ref. [34] recorded a similar depletion in N for green tea but, differently from us, did not observe in the same period any substantial change in N content in rooibos tea. It is known that the N dynamics in decaying litters are quite complex. After a brief leaching phase, N is usually retained relative to C loss during the early stages of litter decomposition [35], and often it does not show any net loss after one year or even more [8,36]. Based on a number of studies, Ref. [37] proposed that litter decomposition is characterized by three distinct phases in terms of N dynamics: a first phase of net N loss, especially through leaching of the soluble pool, including sugars and amino acids that are readily used by microorganisms; a second phase of indirect N accumulation, where mass is lost but without further leaching of nitrogen compounds; a final phase of loss of N, generally much slower than the initial one. Not necessarily are all phases observed in practical experiments; often, particularly in litters with high N concentration, the second phase is not detected or the third phase does not even start due to an insufficient duration of the trial. Within this theory, we could assume that in the case of rooibos tea the experiment was not long enough to include the third phase, while in the case of green tea the second phase did not occur. Neither in green tea nor in rooibos tea did we find significant differences in N content attributable to the home stand, except for those between beech and oak in green tea after 6 and 24 months. Overall, these observations somewhat reduce the influence of forest cover type on litter decay.

3.3. NMR investigation

The NMR investigation aimed at assessing the main structural changes the litters experienced during the two-year long decomposition process in the beech stand, *i.e.*, where green tea showed the highest decomposition rate. The ^{13}C CPMAS spectra, reported in Fig. 5, are quite complex since the litters are complex mixtures of numerous components (cellulose, sugars, polyphenols, etc.), often with similar functional groups. Nonetheless, the changes in signal intensities as decomposition proceeded gave indications on the progressive transformation of the chemical structure of green tea and rooibos tea.

The spectra of the two types of litter before incubation are like those reported in the literature [34], and markedly different from each other. The alkyl region (below 50 ppm) is ascribable to alkylic carbons in terpenoids, polyphenols and waxes, and is relatively more intense in green tea than in rooibos tea. With respect to green tea, rooibos tea displays a much higher carbohydrate content, which is responsible for most of the spectral intensity in the 60–110 ppm spectral region. Although rooibos tea contains much more lignin than green tea [25], the spectral intensity in the aromatic region (110–160 ppm), where most of the lignin signals are found, is higher in green tea. The reason lies in the presence in this region of signals due to polyphenols (namely, flavonoids), which, differently from rooibos tea, represent a major component of green tea [38]. Quite intense are the signals at 156.2 and 145.3 ppm, ascribable to the oxygen-bound carbons of the A ring in flavonoids and the C–OH carbons of ring B, respectively [39], and the signal at 175 ppm,

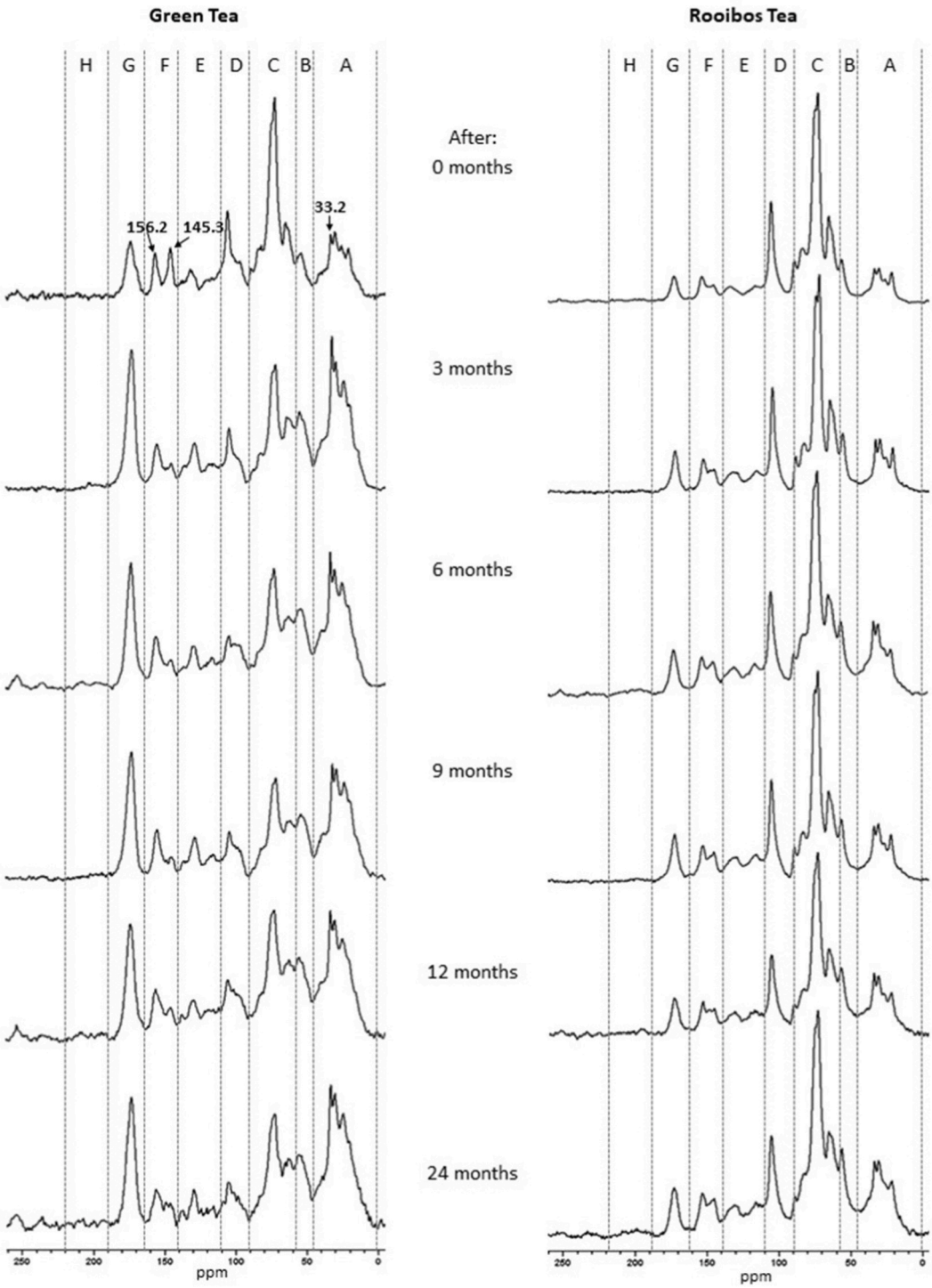


Fig. 5. ^{13}C CP/MAS NMR spectra of green tea and rooibos tea buried under *European beech* and sampled at different times: 0, 3, 6, 9, 12, and 24 months. The spectral regions are: (A) alkyl C, (B) methoxyl and *N*-alkyl C, (C) O-alkyl C, (D) di-*O*-alkyl C, (E) H- and C-substituted aromatic C, (F) O-substituted aromatic C, (G) carboxyl C, and (H) carbonyl C.

ascribable to epigallocatechin gallate, the most abundant catechin in green tea. In the case of rooibos tea, the two signals at 153 and 145 ppm are due to syringyl and guaiacyl units of lignin. No carbonyl signal was detected in the 190–220 ppm region, *i.e.*, the spectral region where aldehyde and ketone carbons are expected, nor did it appear as decomposition proceeded.

Observing the spectra at different incubation times, it can be stated that the main structural changes occurred in the first 3 months

of incubation, chiefly involving a reduction in the relative contribution of the O-alkyl and di-*O*-alkyl signals – more relevant in the case of green tea – and a corresponding increase of the alkyl C, aromatic and carboxyl components, as often found in decaying litters [40, 41]. No major changes were observed after the first 3 months of incubation. The dramatic reduction in the intensity of the signals at 156 and 145 ppm in the green tea spectrum after 3 months of incubation is due to the easy biodegradability of flavonoids, the residual intensity in the aromatic region being ascribable to lignin, which persists also after 2 years of incubation. As in rooibos tea, the two signals at 153 and 145 ppm remaining in the green tea spectra after 3 months of incubation could be assigned to syringyl and guaiacyl units of lignin. Worthy of note is the relatively high intensity of the signal at 175 ppm in green tea; this could be due to the formation of spirodienone structures or carboxyl groups in the degradation process of lignin [42], as well as to aliphatic esters, as indicated by the high intensity of the alkyl-C signal and the 33.2 ppm one, the latter arising from the resistant long-chain methylene carbons.

A quantitative analysis was performed determining the integrals of the seven spectral regions; the integrals, expressed as per cent contribution to the total area subtended by the spectrum, are reported in Table 2. In the integration procedure, the small signal at 250 ppm present in some spectra, due to spinning side bands of the aromatic signals, was neglected. In Table 2 the calculated alkyl C to O-alkyl C ratio (Ak/OAk) is also reported; this ratio is often taken as an index of decomposition since O-alkyl C is typical of the more easily degradable carbohydrate component, whereas alkyl-C derives from decomposition of carbohydrates as well as from the contribution of less easily degradable components, such as waxes (see, for example, [43,44]). The data reported in Table 2 confirm what was observed from the spectra, *i.e.*, that the major compositional changes occur within the first 3 months; afterwards, only minor increases in alkyl-C and decreases in O-alkyl-C are observed, and just in rooibos tea. This is also reflected by the trend of Ak/OAk. For green tea, this ratio abruptly and substantially increases within the first 3 months, whereas for rooibos tea it shows a more gradual and less marked change. This indicates a rapid conversion to the recalcitrant traits of green tea, consequently implying that the second phase of decomposition is not observed in this case.

Although after 3 months the composition of the two litters did not change any further, mass continued to be lost. Considering the C mass loss, it can be easily inferred that all functional groups declined throughout the experiment, especially the O-alkyl ones (50% for rooibos tea and over 70% for green tea after two years). This is highlighted in Fig. 6, where the residual contents of the different forms of carbon are reported together with the fitting curves obtained using Eq. (1). The model, although simple, gave a good reproduction of the decreasing trends of C, yielding the decay rate of the labile fraction, k, and the initial fractions of recalcitrant and labile C, W_r and W_l for each carbon type; the fitting parameters are reported in Table 3. Considering the few data points defining the fast initial decay, the values of k were affected by large uncertainties; nonetheless, mass loss was substantial for some carbon functional groups. In particular, labile O-alkyl C was selectively degraded compared to the other components: 79% of its mass was lost in green tea and 62% in rooibos tea in the two years of incubation. On the contrary, alkyl C, which is mainly composed of saturated hydrocarbons, decreased much less: 45% of its mass in green tea and 15% in rooibos tea. The aromatic carbon mass, which mainly comprises lignin and flavonoids, reduced to 50–60% over two years. These findings are in line with those of Ref. [13] on the decomposition of green tea and rooibos tea under different hemiboreal coniferous stands. Worthy of note is the higher content of the recalcitrant fraction in rooibos tea for all types of carbon. This is due to the much higher lignin content in rooibos tea, also considering that, contrarily to green tea, the commercial product comprises branches as well. It must also be considered that lignin, besides being recalcitrant, could protect cell-wall polysaccharides and proteins from decomposition [45]. In fact, in rooibos tea, the carbohydrate component, although decreasing with incubation time, persists in a relatively high amount even after 2 years. A slower decomposition rate for rooibos tea compared to green tea, at least in the first few months, has been described in various ecosystems by Refs. [12,16].

Principal component analysis as an exploratory tool for ^{13}C NMR spectra also points out the differences in the changing pattern of chemical compositions (Fig. 7). When dimension was reduced to two with PC1 and PC2, their percentage of total variance was 69% and 17%, respectively. As aforementioned, the increase of Alkyl, the decrease of O-Alkyl and the consequent increase of Alkyl/O-Alkyl with time, were noticeable for both types of tea. In rooibos tea, but not in green tea, a gradual increase of aromatic(Ar) and polyphenol (Ph) content with time was observed, though their absolute changes were not so high (Table 2). The aromatic component was little

Table 2
Relative intensities, expressed as per cent of the total spectral area, of the seven chemical-shift regions of the ^{13}C NMR spectra of green tea and rooibos tea (intervals are in ppm) and alkyl C to O-alkyl C ratio (Ak/OAk). Experimental errors are within 10%, while the error on the Ak/Oak ratio is calculated from the experimental data and errors. Seven regions: Alkyl C (Ak), methoxyl and *N*-alkyl C (Mt), O-alkyl C (OAk), di-*O*-alkyl C (DiOAk), H- and C-substituted aromatic C (Ar), O-substituted aromatic C (Ph), mainly from lignin structures, tannins, polyphenols, and carboxyl C (Carbx).

Material	Months	Ak 0–45	Mt 45–60	OAk 60–90	DiOAk 90–110	Ar 110–140	Ph 140–162	Carbx 162-190	Ak/OAk
Green tea	0	19	6	39	12	8	8	8	0.49 ± 0.07
	3	31	10	22	9	8	7	13	1.4 ± 0.2
	6	30	10	23	10	9	7	11	1.3 ± 0.2
	9	30	10	23	10	9	7	11	1.3 ± 0.2
	12	30	10	23	10	9	7	11	1.3 ± 0.2
	24	30	10	23	9	9	7	12	1.3 ± 0.2
Rooibos tea	0	11	6	53	13	7	6	4	0.21 ± 0.03
	3	15	7	46	11	9	7	5	0.33 ± 0.04
	6	17	8	42	11	10	7	5	0.40 ± 0.05
	9	17	8	42	11	10	7	5	0.40 ± 0.05
	12	18	9	41	11	10	7	5	0.45 ± 0.06
	24	19	9	40	11	10	7	5	0.49 ± 0.07

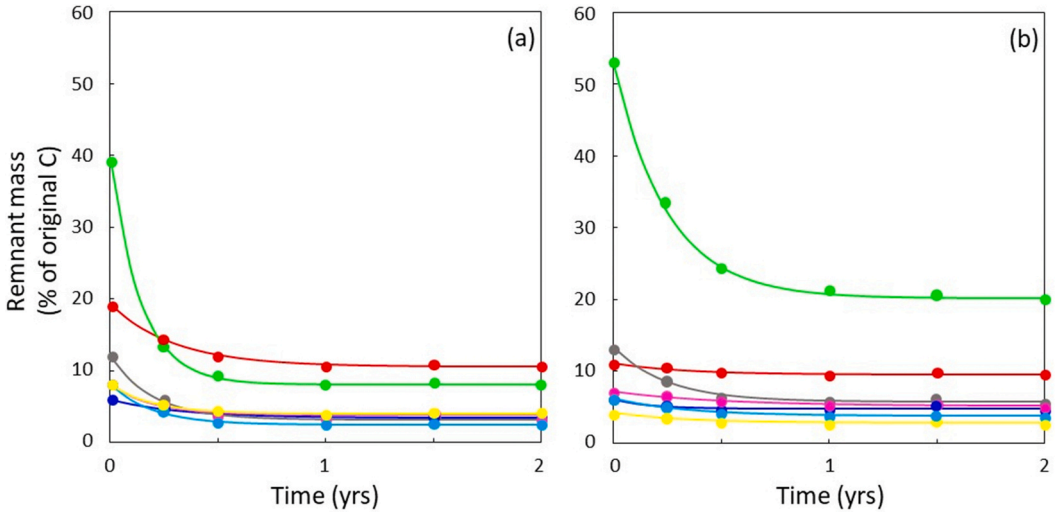


Fig. 6. Remnant mass of the different types of C at different times for (a) green tea and (b) rooibos tea buried under *European beech* determined from ^{13}C CPMAS. Carbon types are alkyl (red), methoxyl and *N*-alkyl (blue), O-alkyl (green), di-*O*-alkyl (grey), H- and C-substituted aromatic (magenta), O-substituted aromatic (cyan), and carboxylic (yellow). The lines are the fitting curves obtained using Equation (1) and the values of relative intensities of the seven chemical shift regions of the ^{13}C NMR spectra. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Relative weights of labile (W_l) and recalcitrant (W_r) fractions with respect to the initial total carbon mass, and decay rate (k) of the labile fraction for the different carbon types. The errors are obtained from the fitting procedure.

Material	Carbon type	k (yr^{-1})	W_l	W_r
Green tea	alkyl	3 ± 1	8.6 ± 0.3	10.4 ± 0.2
	methoxyl and *N*-alkyl	3 ± 1	2.5 ± 0.1	3.4 ± 0.1
	O-alkyl	7 ± 1	31.0 ± 0.1	8.0 ± 0.1
	di-*O*-alkyl	5 ± 1	8.7 ± 0.1	3.1 ± 0.1
	H-aromatic	5 ± 1	4.2 ± 0.1	3.8 ± 0.1
	O-aromatic	5 ± 1	5.6 ± 0.1	2.4 ± 0.1
	carboxyl	5 ± 2	4.0 ± 0.3	4.0 ± 0.2
Rooibos tea	alkyl	3 ± 1	1.6 ± 0.2	9.4 ± 0.2
	methoxyl and *N*-alkyl	6 ± 4	1.4 ± 0.2	4.5 ± 0.1
	O-alkyl	4 ± 1	32.6 ± 0.7	20.3 ± 0.5
	di-*O*-alkyl	4 ± 1	7.5 ± 0.1	5.6 ± 0.1
	H-aromatic	2 ± 1	2.1 ± 0.1	4.9 ± 0.1
	O-aromatic	3 ± 1	2.5 ± 0.1	3.5 ± 0.1
	carboxyl	3 ± 1	1.5 ± 0.1	2.5 ± 0.1

associated with O-Alkyl and Alkyl (*i.e.*, almost perpendicular on PCA coordinate), whereas Methoxyl and Carboxyl components were in highly positive relation with the Alkyl component, therefore in negative relation with the O-Alkyl component. These general relations of chemical compositions in decaying litters were found in a wide range of vegetation types, including several ones of Mediterranean and temperate environments [46]. In our trial, the fact that from 6 months to 24 months of incubation, the relative distances on PCA coordinate were very small compared to those from 0 month to 6 months, is a confirmation that the relative changes of chemical components after 6 months were minimal.

4. Conclusions

The decomposition of green tea and rooibos tea, incubated in litterbags in the soil of three monospecific forest stands of a montane-temperate environment, was studied over two years. The two litters followed quite different decomposition patterns. Carbon loss occurred predominantly in the first three months but was much more relevant in green tea, where it accounted for a halving of the initial material. The decay of the two litters differed even more in terms of N. In fact, at the end of the experiment the rooibos tea residue had substantially the same N content of the original material, while green tea had lost half of its initial N stock. This implied that in two years the C/N ratio almost halved in rooibos tea and remained practically unchanged in green tea. Overall, the home stand species did not much affect the fate of litters; significant and durable differences in terms of C were only observed for green tea between the beech and fir stands and, to a lesser extent, in terms of C/N between the fir and both broadleaf species stands. The NMR

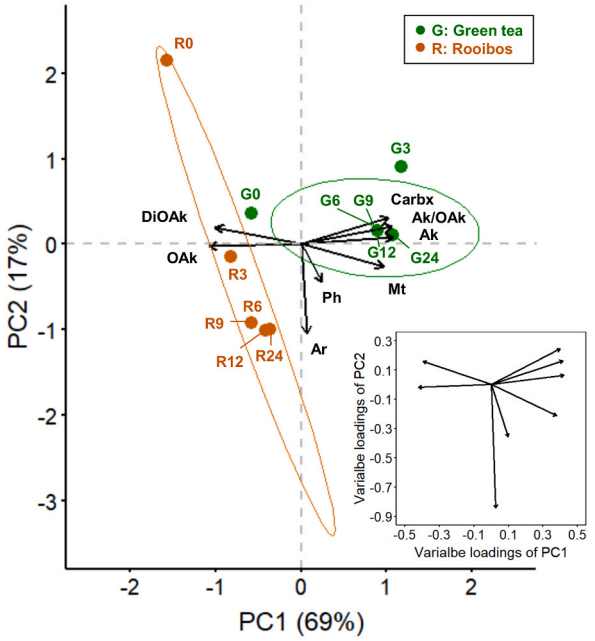


Fig. 7. Score plot of the first two principal components by PCA showing the dissimilarity of chemical structural changes, based on eight CP/MAS ^{13}C NMR spectral regions for green tea (G) and rooibos tea (R) at initial (G0, R0), 3 months (G3, R3), 6 months (G6, R6), 9 months (G9, R9), 12 months (G12, R12), and 24 months (G24, R24). Eight regions: Alkyl (Ak), methoxyl and *N*-alkyl (Mt), O-alkyl (OAk), di-*O*-alkyl (DiOAk), H- and C-substituted aromatic (Ar), O-substituted aromatic C (Ph), carboxyl (Carbx), alkyl to O-alkyl ratio (Ak/OAk). Ellipses indicate 95% confidence interval and ADONIS test with Euclidean distance shows that the dissimilarity of chemical structural changes between the two teas is significant ($p = 0.001$). Percentages on the axes of each principal component (PC) are the explained variance of each PC. Loadings of variables, *i.e.*, seven CP/MAS ^{13}C NMR spectral regions, and the ratio of Ak to OAk are also shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

investigation clearly showed that, during the first three months of decomposition, green tea and rooibos tea – but especially the former – experienced a drastic loss in carbohydrates associated to an indirect enrichment in lipids and lignin, while later on the relative content of the various C forms remained practically stable.

We believe it is important in the future to extend this type of teabags-based research to many different multi-specific forest ecosystems, to define what is the effect of tree community diversity on litter decay and the biogeochemical cycle of the principal elements.

Author contribution statement

G. Certini: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. T. Kwon: Analysed and interpreted the data; Wrote the paper. B. Rompato: Conceived and designed the experiments; Performed the experiments. I. Djukic: Contributed reagents, materials, analysis tools or data. C. Forte: Performed the experiments; Analysed and interpreted the data; Wrote the paper.

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Data availability statement

The datasets generated during the current study are available from the corresponding author on reasonable request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper

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