CPMAS ^{13}C NMR Characterization of Leaves and Litters from the Reafforestated Area of Mustigarufi in Sicily (Italy)

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Abstract: Reafforestation is generally based on the planting of exotic fast growing tree species suitable for adapting to even harsh environments. Once the introduced plants ameliorate soil conditions, they can be progressively replaced by autochthonous plant species. Reafforestation is applied worldwide. However, only few studies on the effect of reafforestation on lands from Mediterranean regions are available. This paper reports the characterization by cross polarization ^{13}C NMR spectroscopy of fresh leaves and superficial litters from a reafforestated area in central Sicily (Italy). NMR assignment is attempted. A differentiation among the molecular systems within leaves and litters is also done on the basis of NMR assessment. Results showed that the main differences among the leaves of four forest trees (two eucalyptus spp., one cypress sp. and one pine sp.) occur in the distribution of the aromatic and alkyl carbons. In particular, the alkyl moieties in the eucalyptus spp. leaves were attributed to branched structures belonging to the eucalyptus oil, whereas linear fatty acids were more representetive in the NMR spectra of pine and cypress leaves. In addition, the aromatic carbons of the conifer leaves were assigned not only to lignin- and tannin-like structures, but also to common olefin carbons in unsaturated fatty acids and abietic acid-like systems. The spectra of the litters resembled, as expected, those of the leaves. However, the presence of very large carbohydrate NMR signals suggested that degradation processes were still ongoing in litters. A comparative evaluation of CPMAS ^{13}C NMR spectra was done by applying principal component analysis. This paper confirmed the suitability of CPMAS ^{13}C NMR spectroscopy in evaluating the differences among natural bio-masses which are the major nutrient sources for soil micro-organisms and the main input for humification processes.

Keywords: NMR, leaves, litters, reafforestation, degraded lands, soils.

INTRODUCTION

Intensive land use and management as well as inappropriate land practices have negative impacts on natural resources such as waters, soils, atmosphere, plants and animals due to nutrients decline, erosion and contamination [1, 2]. Therefore, land recovery and restoration are desirable efforts for the improvement of the ecosystem health status and sustainability [3]. Recovery and restoration are, however, complex and long processes which imply many tasks including environmental evaluations (such as pollution and/or erosion extents), strategic policies for managing degraded ecosystems, and technical accomplishments for rebuilding physical and biological ecosystem conditions [3].

One of the most used practices for restoration of the natural ecosystems on abandoned lands (i.e. lands which exhausted their natural potential for human survival) is the reafforestation [1, 4]. It consists in the planting of fast growing exotic tree species suitable to adapt to even harsh conditions, thus providing early substrates for the microbial recolonization. Then, local species can be added by enrichment planting to improve microclimate and soil conditions and to create favorable circumstances for other indigenous species invasion [5].

Reafforestation compensates for CO_2 emissions to the atmosphere through accumulation and transformation of natural organic matter into soils [2], promotes soil hydrological properties due to its impact on soil texture [4], favors mitigation of soil temperatures due to the vegetation cover [4] and re-establishes nutrient cycles thereby affecting development of soil microbial biomasses [6, 7]. Moreover, reafforestation reduces soil erosion and may have a great social impact since it increases economic potentials through enhancement of working possibilities [8].

Land restoration through reafforestation is achieved worldwide by establishing mainly eucalyptus and pine trees [1]. In fact, such plantations take deep roots and grow vigorously even in low fertility lands [4].

Only few papers up to now dealt with the effects of reafforestation on Mediterranean degraded lands [7, 9, 10]. For this reason, we have started the monitoring of the effects of reafforestation in soils of semi-arid Mediterranean areas of central Sicily (Italy) where desertification processes, related

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1874-7698/10

to intensive land uses, were ongoing. One of these areas is the regional forest property located in Mustigarufi, nearby Caltanissetta. Here eucalyptus, cypress and pine trees were planted since the late fifties and the beginning of the sixties.

In the present paper, we have concentrated our attention primarily on the molecular characterization of the fresh leaves and the superficial layers of litters of Mustigarufi forest. This because, both of them represent the major nutrient sources for soil microbial biomass and the main input for humification processes, which are very important to restore soil quality in reafforestated areas.

To reach our goal we applied carbon-13 solid state nuclear magnetic resonance spectroscopy with cross polarization and magic angle spinning (CPMAS ^{13}C NMR). CPMAS ^{13}C NMR spectroscopy provides both qualitative fingerprinting of carbonaceous materials and quantitative measurements on the relative content of the different molecular moieties in very complex organic mixtures [11]. Therefore, we report the assignment of the CPMAS ^{13}C NMR spectra of fresh leaves and litter superficial layers from Mustigarufi forest as well as a comparison among the molecular characteristics of these materials. This study represents an unavoidable initial step for the integrated study of the C turnover within a reafforested semi-arid Mediterranean area.

EXPERIMENTAL

Leaf and Litter Samples

Eucalyptus camaldulensis Dehnh. (EC), *Eucalyptus occidentalis* Endl. (EO), *Cupressus sempervirens* L. (CI) and *Pinus halepensis* Mill. (PI) are tree species growing in Mustigarufi reafforested area which is located nearby San Cataldo (Caltanissetta, Sicily, Italy, 37°33'N, 13°55'E) on a hill placed at 470 m a.s.l.. In order to perform the sampling of fresh leaves and superficial litters, four distinct 20 x 20 m homogeneous squares, each including only one tree species, were selected. Composite samples of fresh leaves of each tree species were collected at random from three distinct canopies, whereas composite samples from superficial (0-3 cm) litters consisted of six sub-samples exploring the 20 x 20 m squares. It was not possible to collect the litter under *Cupressus sempervirens* L. since it appeared both heterogeneous and quantitatively insignificant. Leaves and litters, without any pre-treatment, were dried at 70°C in a vent-oven for 72 hours. Then, they were powdered in an ultra centrifugal rotor ZM200 Retsch® mill equipped with a 1 mm sieve in order to obtain solid samples for the NMR analyses.

CPMAS ^{13}C NMR Spectroscopy

CPMAS ^{13}C NMR measurements were performed on a Bruker Avance-II 400 spectrometer (Bruker Biospin, Milan, Italy) operating at 100.6 MHz on carbon-13 and equipped with a 4 mm standard bore solid state probe. Samples were packed into 4 mm zirconia rotors with Kel-F caps and the rotor spin rate was set at 13000 ± 2 Hz. A spectral width of 29761.90 Hz centered at 10061.78 Hz, an optimum contact time of 1 ms chosen after evaluation of variable contact time experiments [12], a recycle delay of 2 s, 2 k data points over an acquisition time of 35 ms and a RAMP sequence, to account for inhomogeneities of the Hartmann-Hahn condition at high rotor spin rates [11], were used. 700 scans were accumulated to obtain all the spectra. The ^{1}H 90° pulse was calibrated on each leaf and litter sample with the pulse sequence described in [13] at an attenuation level of -2.4 dB. ^{1}H 90° pulse length varied within the interval 3.60-3.85 μs depending on the sample under analysis. Spectra acquisition was achieved with Bruker Topspin® 2.0. Data elaboration was done with MestRe-C software (Version 4.9.9.9, Mestrelab Research, Santiago de Compostela, Spain). The free induction decays (FIDs) were transformed by applying first a 2 k zero filling, then a line broadening of 50 Hz and finally an automatic baseline correction with a 3rd order polynomial and Bernstein algorithm [14]. All the spectra were divided in the following regions whose assignment is fully discussed later: 0-45 (alkyl C), 45-60 (O/N alkyl C), 60-90 (O alkyls in carbohydrates), 90-120 (acetal and lignin C), 120-160 (aromatic C) and 160-180 (-COOH groups) ppm. The absolute areas (A_i) of each interval was measured and referred to the total absolute area of each spectrum (A_T) as obtained by integrating the -50 – 230 ppm interval. The -50 – 230 ppm region was accounted for to consider the effect of the spectral noise on the quantitative evaluation of the NMR spectra.

Principal Component Analysis

Principal Component Analysis (PCA) was carried out using the CPMAS ^{13}C NMR spectra obtained, after area normalization and mean-centering of the data. To improve the interpretability of the loadings, a Varimax rotation was performed. The goal of this strategy was to obtain a clear pattern of loadings, i.e., factors marked by high loadings for some variables and low loadings for others. The model validation was carried out using two different methods: full cross-validation, and segmented cross-validation with two samples per segment picked at random. The difference in the variance between the calibration and validation models was less than 5%. This analysis was performed using The Unscrambler software (CAMO Software AS, Oslo, Norway).

RESULTS AND DISCUSSION

Qualitative Interpretation of the Leaf NMR Spectra

Fig. (**1**) reports the CPMAS ^{13}C NMR spectra of the leaves sampled for the present study. Attribution of spectral regions is also indicated. According to literature [11, 16-19], six different intervals were recognized. The first one, between 0 and 45 ppm, was attributed to alkyl systems. The most important signals in this interval were located at 26, 30 and 32 ppm in the spectra of *Cupressus sempervirens* L. (CI, Fig. **1A**) and *Pinus halepensis* Mill. (PI, Fig. **1B**) and at 17, 26, 30, 32, 39 and 42 ppm in the spectra of *Eucalyptus camaldulensis* Dehnh. (EC, Fig. **1C**) and *Eucalyptus occidentalis* Endl. (EO, Fig. **1D**).

The resonance at 17 ppm was assigned to methyl groups which terminate alkyl chains [16]. Among the other remaining alkyl signals (26, 30, 32, 39 and 42 ppm), those at 26, 30 and 32 ppm, visible in all the leaf spectra (Fig. **1**), can be attributed to linear methylene (-CH_2-) chains [16, 17] belonging to lipids, cutin-like structures and other aliphatic bio-moieties [20]. The last two, at 39 and 42 ppm can be assigned to secondary methyne carbons (-CH-, signal at 39 ppm) and to fully substituted quaternary carbons (CR_4, signal at 42 ppm) [16, 19]. It is likely that the signals at 39 and 42 ppm can be generated by carbons in chlorophyll-like struc-

tures or in molecules belonging to eucalyptus-oil (a complex mixture of terpenoids) that is usually present into eucalyptus trees [21].

The second spectral interval between 45 and 60 ppm is traditionally attributed to nitrogenated and oxygenated alkyl systems (Fig. **1**). Three signals positioned at 48, 53 and 56 ppm were evidenced in the spectra of Fig. (**1**). That at 48 ppm can be assigned to N-alkyl carbons in amino acids [18, 22]. The shoulder at 53 ppm in Figs. (**1C** and **1D**) and the peak at 56 ppm in Figs. (**1A** to **1D**), can be both attributed to O-alkyl groups such as methoxyls in lignin-like structures (56 ppm) and $-CH_2O-$ systems into branched molecules as those in the eucalyptus-oil [18].

The region comprised in the chemical shift interval 60-90 ppm (Fig. **1**) is indicative of carbohydrates with the largest contribution due to celluloses and hemicelluloses [18]. In particular, the resonances at 63 and 65 ppm were due to carbon 6 in amorphous and crystalline celluloses, respectively

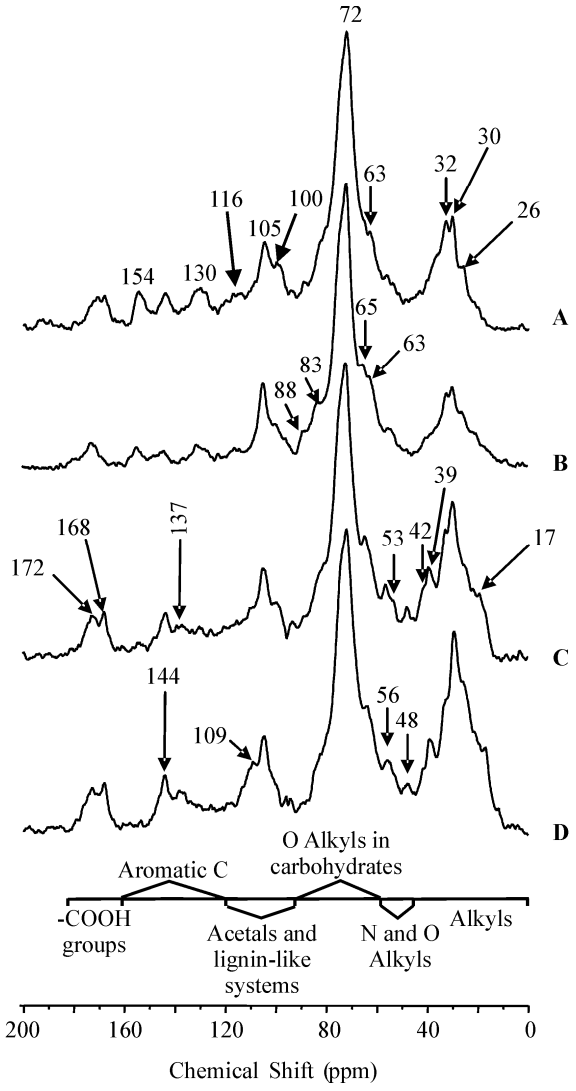

Fig. (1). CPMAS ^{13}C NMR spectra of the leaves of *Cupressus sempervirens* L. (**A**), *Pinus halepensis* Mill. (**B**), *Eucaliptus camaldulensis* Dehnh. (**C**) and *Eucaliptus occidentalis* Endl. (**D**). Attribution of the spectral intervals is also reported.

[23], while the intensity at 72 ppm was assigned to the carbons in the positions 2, 3 and 5 regardless of the cellulose nature (either crystalline or not) [24]. Carbon 4 appeared between 80 and 90 ppm.

Here, it generated a shoulder at around 83 ppm due to amorphous cellulose, hemicellulose and cellulose oligomers [25] and a signal at 88 ppm that was assigned to the ordered forms of celluloses on fibril surfaces and to "in core" paracrystalline celluloses, whose nature is still uncertain [26]. Among all the spectra (Fig. **1**), the only one where the signals at 63/65 and 83/88 ppm were clearly identifiable was generated by the pine leaves (Fig. **1B**), thereby suggesting that they may contain a larger variety of cellulose forms.

Signals of carbohydrates are also observed in the range 90-120 ppm where the large resonance at 105 ppm was attributed to C1 of cellobiose units into cellulose I, and that at around 100 ppm was assigned to the acetal carbons in the xylane systems of hemicelluloses [26]. The 90-120 ppm interval contains also other signals such as a weak one due to common olefin carbons at 116 ppm in the spectra of CI (Fig. **1A**) and PI (Fig. **1B**), and a resonance at 109 ppm associable to acetal C in cellulose II.

The spectral region, that traditionally is assigned to aromatic systems, is included between 120 and 160 ppm [16]. Three main peaks at 130, 144 and 154 ppm were revealed in the spectra of the leaves from CI (Fig. **1A**) and PI (Fig. **1B**), whereas only a signal at 137 ppm and a large resonance at 144 ppm appeared in the spectra of EC (Fig. **1C**) and EO (Fig. **1D**). All these signals are usually attributed to lignin systems [17]. In particular, p-hydroxyphenol derivative structures are assumed to give a signal at around 130 ppm, whereas O-aryl carbons from guaiacyl- and syringyl-units may give resonances at 137, 144 and 154 ppm [17]. Based on this simple attribution, we can conclude that the differences between the leaves from the two coniferous trees and the two eucalyptus plants were due to a different concentration of p-hydroxyphenol-, guaiacyl- and syringyl- structures. However, cell composition of the needles from pinus and cypress has been already well studied and it has been clarified that they contain large concentrations of acid resins made by long chain unsaturated acids and abietic acid derivatives [27, 28]. According to web data bases (such as http://riodb01.ibase.aist.go.jp/sdbs/) and to literature data [29], we attributed the resonance at 130 ppm in the spectra of CI (Fig. **1A**) and PI (Fig. **1B**) either to cis-mono-unsaturated fatty acids or to abietic acid-like structures (i.e. sp^2 carbons). The signal at 144 ppm can be, consequently, also assigned to the C13 in dehydro-phenanthrene systems [29]. According to our interpretation, the differences in the 120-160 ppm interval in the leaf spectra of the coniferous plants (Fig. **1A** and **1B**) and of the two eucalyptus trees (Fig. **1C** and **1D**) cannot be due only to a different distribution of p-hydroxyphenol-, guaiacyl- and syringyl- structures, but also to the presence of acid resins which are less abundant in the leaf samples from EC and EO.

The last spectral region between 160 and 180 ppm was attributed to –COOH and amide groups.

Qualitative Interpretation of the Litter NMR Spectra

Signal attribution of the litter spectra (Fig. **2**) is similar to that of the leaves. It has been already described in the para-

graph above. As a general remark, however, it must be noticed that all the litter NMR spectra are dominated by the signals of carbohydrates. According to Lemma *et al.* [18] and Hopkins *et al*. [30], this feature reveals that the litters are still in the early stages of decomposition. The comparison of Figs. (**1** and **2**) shows some dissimilarities between fresh leaves and leaf litters. Signal at 65 ppm (which appears as a shoulder of the signal at 72 ppm in the leaf spectra) and a different distribution of the aromatic (120-160 ppm) carbons can be observed. The signal at 65 ppm was assigned to C6 in crystalline cellulose (see above). The higher resolution of such signal can be explained considering that the first steps of the litter decomposition mechanisms involve the degradation of the amorphous celluloses [18]. As a consequence, a sharpening of the signals in the O-alkyl spectral region, which implies a better separation between the resonances at 72 and 65 ppm, is achieved. This hypothesis is further supported taking into account the disappearance of the signal at 109 ppm (in EO and EC litters) assigned to acetal carbons in cellulose II. Litter decomposition is also related to a variation of the nature of the aromatic carbons due to degradation of tannin- and lignin-like components and to a general resolution-loss of the signals ranging in the region of the alkyl moieties. Transformations of the aromatic structures turned in the coalescence of the signals at 144 and 154 ppm which was observed in the spectrum of the pinus litter (Fig. **2A**) [18]. The same signals (at 144 and 154 ppm) were, conversely, observed in the spectrum of EC litter (Fig. **2B**). They were not present in the spectrum of the EC leaves (Fig. **1C**). The presence of these two signals can be attributed to lignin-residues which were co-sampled with the leaf litter.

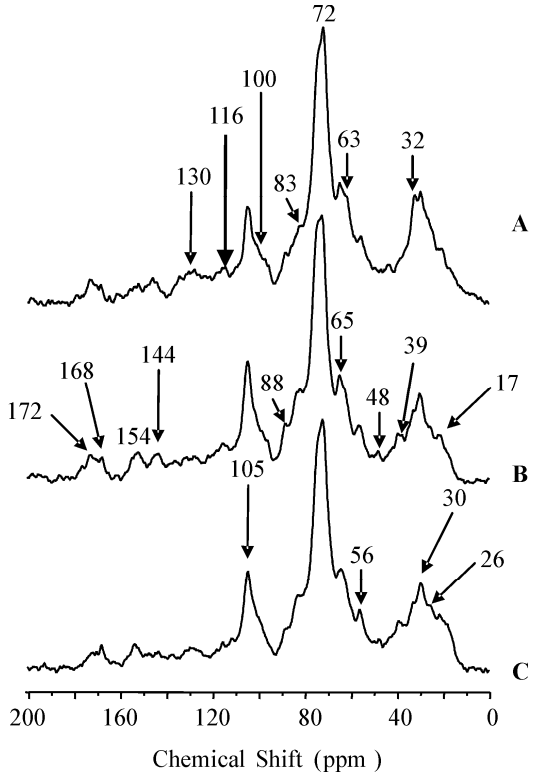

Fig. (2). CPMAS ^{13}C NMR spectra of the litters from *Pinus halepensis* Mill. (**A**), *Eucaliptus camaldulensis* Dehnh. (**B**) and *Eucaliptus occidentalis* Endl. (**C**).

Statistical Comparison of NMR Spectra

In order to evaluate the capability of the CPMAS ^{13}C NMR spectroscopy in revealing differences among leaf and litter samples, the NMR spectra were used as input data for principal component analysis (PCA). PCA is already known to be a powerful tool for the recognition of similarities and dissimilarities in NMR spectra of very complex systems such as in foods [31-36], natural organic matter [25, 37-40], living organisms [41], and environmental compartments [42-45]. The basic idea of PCA is to reduce the number of variables into just few and to seek linear combinations of those variables explaining most of the variability [15].

In the present study, PCA reduced the number of variables to only 3 (PC1, PC2 and PC3) which accounted for 83% of the total variance (Fig. **3**). Both PC1 and PC3 indicate decomposition of Eucaliptus leaves. The sole PC1, which accounted for the 35% of the variance, revealed that Eucaliptus leaves had the largest scores as compared to the Eucaliptus litters (Fig. **3A**). Moreover, this component was also able to differentiate among tree species due to the score values which varied as EO>EC≅PI>CI.

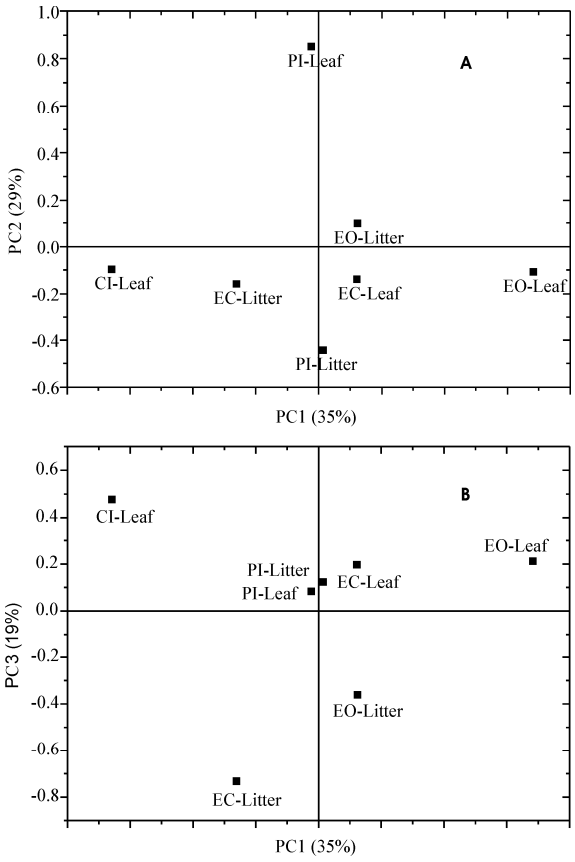

Fig. (3). Scores of rotated (varimax) PCA from CPMAS ^{13}C NMR spectra. CI=*Cupressus sempervirens* L.; PI=*Pinus halepensis* Mill.; EO=*Eucaliptus occidentalis* Endl.; EC=*Eucaliptus camaldulensis* Dehnh. **A**. PC1 *vs* PC2 plot; **B**. PC1 *vs* PC3 plot.

PC1 was characterised by high positive loadings in the region of methyl groups (< 30 ppm), with clear peaks at

16.8, 20.7, 24.0, 28.3 ppm and shoulders at around 11.9 and 18.4 ppm (Fig. **4**). According to Fig. (**3A**), methyl systems are more abundant in EO leaves (higher scores) which, in turn, are subjected to an easier decomposition than the leaves from the other tree species. PC1 also showed positive loadings (Fig. **4**) in the region of methyne and quaternary groups (39.5 and 41.8 ppm, respectively). Chlorophyll-like and oil-like structures (see above) appeared to resonate at those chemical shift values, thereby confirming that such structures are peculiar in Eucaliptus trees.

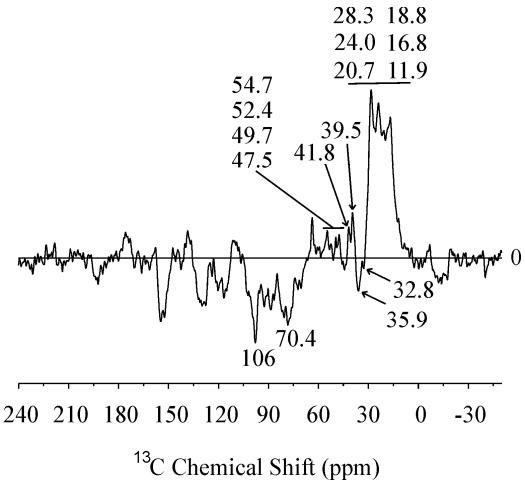

Fig. (4). Loadings of first rotated (varimax) Principal Component from PCA of CPMAS ^{13}C NMR spectra.

The negative loadings for the signals at 32.8 (weak peak, crystalline poly-methylene) and at 35.9 ppm are an indication that during the initial stage of decomposition a relative accumulation of crystalline forms of poly-methylene may occur.

The chemical shift interval comprising the signals at 54.7, 52.4, 49.6, and 47.5 ppm (Fig. **4**) and the region 154.7 and 152.4 ppm revealed opposite loading signs. In fact, the former region, generally attributable either to O-alkyl or N-alkyl carbons, showed positive loadings, whereas the second one, attributed to O-aryl groups, was negative (Fig. **4**). Due to this inverse relationship, it can be concluded that the main organic systems which are subjected to decomposition are N-alkyls in peptides.

In addition, Fig. (**4**) reports negative loadings for the peaks due to crystalline cellulose (from 106 to 70.4 ppm) and positive loadings for the peak of amorphous cellulose (63.7 ppm), thereby revealing an accumulation of the crystalline cellulose, while the amorphous one is decomposed.

Finally, the aryl region showed negative loadings, probably due to a relative accumulation of lignin during the decomposition.

PC3 provide additional information concerning the alterations of Eucalyptus leaves (Fig. **3B**). In fact, Fig. (**5**) shows positive loadings from the chemical groups which are more abundant in Eucalyptus leaves and that sharply decrease in litter samples (i.e. 70 and 32 ppm). Conversely, the negative signals at 104, 75 and 21 ppm (Fig. **5**) belong to the groups which accumulate in the litter.

PC2 accounted for 29% of the total variance (Fig. **3A**). It is characterized by positive loadings in the region 60-90 ppm (data not reported). Its scores clearly reveal decomposition of amorphous cellulose in PI-leaves while the crystalline one is accumulated.

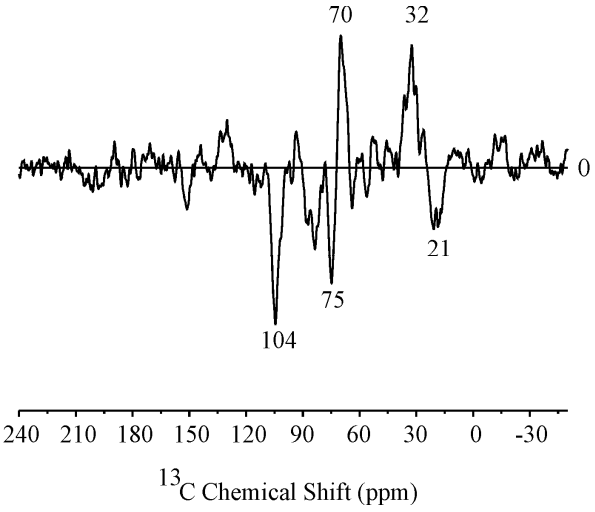

Fig. (5). Loadings of third rotated (varimax) Principal Component from PCA of CPMAS ^{13}C NMR spectra.

CONCLUSIONS

The molecular characterization by solid state ^{13}C NMR spectroscopy of leaves and litters from a reafforestated area in Sicily (Italy) is reported. Leaves from two different eucalyptus plants and two conifers (pine and cypress) appeared significantly discriminated in the alkyl and aromatic NMR regions. In fact, the eucalyptus leaves were the richest in alkyl systems, whereas the pine and cypress leaves resulted to have the largest content of aromatic carbon. The aromatic C region in the leaves of the conifers was assigned not only to lignin- and tannin-like structures as reported in literature [17, 20], but also to common olefin carbons in unsaturated fatty acids (signal at 130 ppm) and abietic acid-like systems. This suggested that the differentiation between the leaves of the eucalyptus trees and the two conifers cannot be based only on p-hydroxyphenol, guaiacyl- and syringyl- derivatives, but also on the presence of acid resins which are well known to be very abundant in conifer leaves.

The spectra of the leaf litters were dominated by the carbohydrate signals, thereby revealing that first stages of decomposition were ongoing [30].

Principal component analysis revealed that the initial stages of leaves decomposition leads to the preferential decomposition of methyl, methyne and quaternary C, N-alkyl and amorphous cellulose. On the other hand, this process leads to a relative accumulation of lignin and crystalline cellulose and crystalline poly-methylene.

CPMAS ^{13}C NMR spectroscopy turned out to be a very powerful technique for the molecular characterization of leaves and litters which, in turn, are very important for the development of microbial communities in reafforestated soils.

ACKNOWLEDGEMENTS

The NMR experiments were done at the Centro Grandi Apparecchiature (CGA) - UniNetLab – of Università degli Studi di Palermo (http://www.unipa.it/cga)

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Received: June 15, 2009 Revised: December 04, 2009 Accepted: December 07, 2009