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Molecular characteristics of humic substances from different origins and their effects on growth and metabolism of *Pinus laricio callus*

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Abstract

Humic susbtances (HS) are increasingly applied as biostimulants in agriculture, though their mechanism of action is not yet completely understood due to their complex and heterogeneous composition. Here, we isolated thirteen different humic and fulvic acids from different sources, such as soils, composts, peat, leonardite and lignite, and characterized the distribution of their carbon components by ^{13}C-CPMAS–NMR spectroscopy. Callus of Pinus Laricio, as a model plant species, was treated with different humic extracts and its growth and content of carbohydrates, phenols, and enzyme related to the nitrogen metabolism (invertase, glutamine synthetase, glutamate synthase, phosphoenoly pyruvate carboxylase, malate dehydrogenase) and stress resistance (catalase) were monitored. While a multivariate statistical analysis of NMR results well-separated the HS characteristics based on their origin, humic materials generally increased callus growth, as expected, with largest effects being exerted by the mostly polar humic acids from composts. However, the rest of measured parameters were not linearly related to hydrophobicity and aromaticity of humic isolates as well as their origin, but their conformational dynamics had to be advocated to explain their effects on callus cellular components. This work confirms that HS of various origin can act as sustainable biostimulants of plant growth, though the comprehension of their effects on plants biochemical activities requires further research and additional understanding of their molecular composition and conformational behaviour.

Keywords: Humic substances, CPMAS–NMR, Callus growth, *Pinus Laricio*, Phenols, Enzymes activities, Nitrogen metabolism, Plant stress

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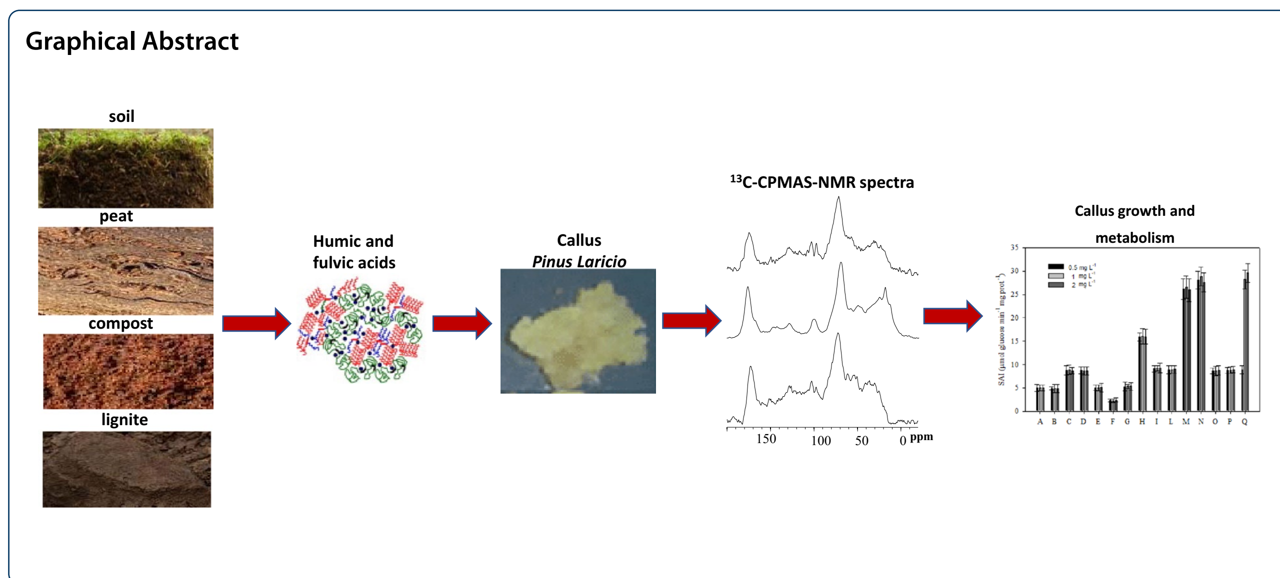
Graphical Abstract



Introduction

The interest in the bioactivity of humic substances (HS, including fulvic and humic acids) has been growing in recent years due to their increasing use as biostimulants in agriculture [12, 18] and much research has been conducted to elucidate the mechanisms of such a bioactivity [4, 28, 36, 41]. HS are extracted from soils, sediments, composted biomasses, or agro-industrial residues and might be regarded as supramolecular associations of small, heterogeneous molecules held together in metastable structures by non-covalent interactions (van de Waals, p–p, H-bonds; metal bridges) [34, 53]]. The molecular heterogeneity and conformational complexity of HS explains the range of different effects on plant metabolism [29, 30, 47]. Numerous experimental evidence showed that humified organic matter stimulates plant growth and is capable of significantly improving crops yields [1, 8]. Most studies with humic substances indicated a direct promotion of plant growth by increasing membrane permeability as enhanced ions protein carriers and by activating Krebs cycle, photosynthesis, and ATP and amino acids production [9, 25].

Nevertheless, the composition of HS which are responsible for the stimulation of plant biological activity is not yet clarified. Attempts to relate humus structure to biological activity have produced contrasting results which are both due to heterogeneity of HS and difficulty of their characterization [39]. For this purpose and to elucidate the effects of HS without environmental interference, we studied their biological activity on *Pinus laricio* callus growth and metabolism, as it is widely accepted that results obtained during in vitro studies reproduce those obtained in whole-plant experiments [15, 19, 33, 46]. Callus metabolism was evaluated by monitoring the enzymatic activities of nitrogen metabolism and respiration as well as the antioxidative defense, such as catalase.

The objectives of this work were thus: (i) apply ^{13}C-CPMAS–NMR spectroscopy to achieve information on the molecular composition of humic and fulvic acids obtained from different sources, such as soil, compost, lignites and peat, and (ii) evaluate how the different humic extracts affected callus growth and metabolism.

Materials and methods

Humic materials, origin, and extraction

The humic acids (HA) were obtained from: a North Dakota Leonardite (HA-C); an oak forest soil from Portici, Italy, classified as Typic Dystrandepts (HA-D); an industrial compost after 150 days of maturation (HA-E); a Hungarian lignite treated with gluconic acid before extraction (HA-F); an industrial compost after 30 days of maturation (HA-G); an oxidized coal (HA-H); a silver fir and beech forest soil near Trento, Italy (HA-I); a peat soil from Bientina, near Lucca, Italy (HA-L); a vermicompost of *Allolobophora caliginosa* (Sav.) and *A. rosea* (Sav.) earthworm species with a mull-type production in soil (HA-M); a Hungarian lignite (HA-N). The fulvic acids (FA) were obtained from; a loamy soil near Dugenta, near Napoli, Italy, classified as Typic Haplustaf (FA-O); a sandy loam soil near Milano, Italy, classified as Fluventic Xerochrept (FA-P); a volcanic soil near Vico, Italy, classified as Typic Melanudand (FA-Q).

Humic substances were extracted by standard procedures [37]. Original materials were shaken overnight in a 0.5 M NaOH and 0.1 M $Na_4P_2O_7$ solution under N_2 atmosphere. The HA was precipitated from alkaline

extracts by lowering the pH to 1 with 6 M HCl. The HA was separated from solution by centrifugation and extensively purified by three cycles of dissolution in 0.1 M NaOH and subsequent precipitation in 6 M HCl. The HA was then treated with a 0.5% (v/v) HCl-HF solution for 36 h, dialyzed (Spectrapore 3 dialysis tubes, 3500 MW cutoff) against distilled water until chloride-free, and freeze-dried. The FA, the supernatant left in solution after HA separation, was purified by absorbing on a Amberlite XAD8 resin, eluting by a 1 M NaOH solution, and, after adjusting the pH to 5, dialyzing in Spectrapore 3 tubes against distilled water until chloride-free, and freeze-dried. Both HA and FA were then redissolved in 0.5 M NaOH and passed through a strong cation-exchange resin (Dowex 50) to further eliminate divalent and trivalent metals and freeze-dried again. Elemental composition (C, H, N) of all humic extracts was obtained using an elemental analyzer EA 1108 Elemental Analyzer by Fisons' Instruments and is reported in Table 1. Both HA and FA samples (50 mg) were subsequently suspended in distilled water (50 mL) and titrated for 2 h to pH 7 with a CO_2-free solution of 0.5 M KOH by an automatic titrator (VIT 90 Videotitrator, Radiometer, Copenhagen) under N_2 atmosphere and stirring. The resulting potassium humates were then filtered through a Millipore 0.45 μ filter and used on the biological experiments.

^{13}C-CPMAS–NMR spectroscopy

Fine-powdered humic materials were subjected to solid-state NMR spectroscopy (^{13}C-CPMAS–NMR) using a Bruker AV300 Spectrometer equipped with a 4 mm wide-bore Cross Polarization Magic Angle Spinning (CPMAS) probe. NMR spectra were obtained by applying the following parameters: 13,000 Hz of rotor spin rate; 2 s of recycle time; ^{1}H-power for CP of 92.16 W; ^{1}H 90° pulse 2.85 μs; ^{13}C power for CP of 150.4 W; 1 ms of contact time; 30 ms of acquisition time; 5000 scans. Samples were packed in 4 mm zirconium rotors with Kel-F caps. The cross-polarization pulse sequence was applied with a composite shaped "ramp" pulse on the ^{1}H channel to account for the inhomogeneity of Hartmann–Hann condition at high rotor spin frequency. The Fourier transform was performed with 4 k data point, an exponential apodization and 50 Hz of line broadening.

The distribution of carbon functions was grouped in the following chemical shift regions: alkyl-C: 0–45 ppm; methoxyl C–O and C–N: 45–60 ppm; O-alkyl-C: 60–110 ppm; aryl-C: 110–145 ppm; O-aryl-C: 145–160 ppm, and carboxyl-C: 190–160 ppm. The relative contribution of each region was determined by integration (MestreNova 6.2.0 software, Mestre-lab Research, 2010), and expressed as percentage of the total spectral area. To summarize the molecular properties, two structural indices, namely, hydrophobic index (HB) and Aromaticity (Ar), were determined by combining relative area of NMR spectral regions (Table 2) as it follows:

$$HB = \Sigma[(0-45) + (45-60) + (110-160)] / \Sigma[(45-60) + (60-110) + (160-190)]$$

$$Ar = [(110-160)/(0-190)]$$

Table 1 Percent (ash-free, moisture-free) of elemental carbon, hydrogen, and nitrogen in humic extracts

Extracts[a]	Carbon	Hydrogen	Nitrogen
HA-C	54.1	4.4	1.0
HA-D	53.4	4.4	5.1
HA-E	32.2	4.1	2.4
HA-F	56.0	4.8	1.2
HA-G	26.9	3.8	2.2
HA-H	62.9	3.0	1.9
HA-I	53.7	4.3	4.9
HA-L	57.4	3.7	6.20
HA-M	46.5	4.8	12.8
HA-N	57.3	4.2	0.9
FA-O	38.6	4.3	4.1
FA-P	22.0	4.4	3.3
FA-Q	63.5	3.9	4.6

[a] HA-C from Leonardite; HA-D from oak forest soil; HA-E from compost after 150 d maturation; HA-F from treated Lignite; HA-G from compost after 30 d maturation; HA-H from an oxidized coal; HA-I from fir and beech forest soil; HA-L from peat; HA-M from vermicompost; HA-N from a Lignite; FA-O from a loamy agricultural soil; FA-P a sandy loam agricultural soil; FA-Q from a volcanic forest soil

Growth parameters

Excised leaves of *Pinus laricio*, were grown in Petri dishes, on MS [24] basal medium, supplemented with 3% sucrose, 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.25 mg L^{-1} 6-benzylaminopurine (BAP) for 35 days to induce callusing. The pH was adjusted to 5.7 before adding 0.8% w/v Bacto agar (Oxoid 1), and the media were autoclaved for 20 min at 121° C.

Two grams of 35-day-old callus tissue was transferred for 28 days to the basal medium culture [24], pH 5.5, without (control) or with addition of either 2,4-D + BAP (0.5 + 0.25 mg L^{-1}) or humic matter at different concentrations. Filter sterilized humic solutions, were used at the concentration of 0.5, 1.0 and 2.0 mg C L^{-1}. Treated calluses were maintained in the darkness at 25 °C in a growth chamber. 28 d after the treatment's onset, the callus growth was estimated as fresh weight. Samples of callus were taken at the end of the treatment period (28 days) for enzymatic analysis.

Table 2 Relative distribution (%) of signal area over the chemical shift regions (ppm) and indexes of aromaticity (Ar) and hydrophobicity (HB), as assessed from ^{13}C-CPMAS–NMR spectra of different humic extracts

Extracts[a]	Carboxyl-C (190–160)	O-Aryl-C (160–145)	Aromatic-C (145–110)	O-Alkyl-C (110–60)	CH_3O/C–N (60–45)	Alkyl-C (45–0)	Ar	HB
HA-C	5.3	5.5	35.7	14.7	7.8	31.0	41.2	3.2
HA-D	9.7	3.2	15.7	26.0	13.0	32.4	18.9	1.4
HA-E	11.5	4.1	14.8	22.8	11.5	35.2	18.9	1.5
HA-F	5.6	4.8	64.5	6.6	2.3	16.3	69.3	6.5
HA-G	11.3	4.0	14.2	28.5	11.0	31.0	18.1	1.2
HA-H	7.7	8.6	53.1	8.8	6.1	15.8	61.7	4.1
HA-I	6.7	3.8	15.8	26.1	11.8	35.8	19.6	1.6
HA-L	9.6	5.6	20.6	16.9	10.0	37.3	26.2	2.2
HA-M	10.3	4.8	15.8	23.7	13.5	31.9	20.6	1.5
HA-N	6.7	6.1	39.9	9.2	2.3	35.7	46.1	4.9
FA-O	10.8	4.4	15.7	42.4	10.2	16.4	20.2	0.7
FA-P	15.2	3.8	7.8	32.4	13.5	27.4	11.6	0.8
FA-Q	13.0	3.7	15.0	40.2	11.3	16.8	18.7	0.7

[a] HA-C from Leonardite; HA-D from oak forest soil; HA-E from compost after 150 d maturation; HA-F from treated Lignite; HA-G from compost after 30 d maturation; HA-H from an oxidized coal; HA-I from fir and beech forest soil; HA-L from peat; HA-M from vermicompost; HA-N from a Lignite; FA-O from a loamy agricultural soil; FA-P a sandy loam agricultural soil; FA-Q from a volcanic forest soil

Extraction and determination of total phenols

Water soluble phenols were extracted from callus tissue (1:5 w/v) as follow: samples of callus tissues (about 0.5 g fresh weight) were homogenized in a pre-chilled mortar with 2 volumes of ice-cold bidistilled water. The homogenate was filtered through two layers of muslin and centrifuged at 20,000*g* for 15 min. All steps were performed at 4 °C. Total water-soluble phenols (monomeric and polyphenols) were determined using the Folin–Ciocalteu reagent, following the method of Box [6], After standing at room temperature for 10 min, the absorbance was read at 725 nm by a UV–Vis spectrophotometer (UV-2100 Shimadzu, Japan). The standard calibration curve was plotted using tannic acid and the concentration of water-soluble phenolic compounds was expressed as tannic acid equivalents (μg TAE g^{-1} fresh weight).

Soluble sugars determination

Callus tissue was extracted three times with boiling 80% ethanol (v/v). Homogenates were centrifuged at 12,000 g and the ethanolic extract was rotoevaporated under vacuum, resolubilized in distilled water, and subjected to enzymatic assay. Glucose, fructose, and sucrose were determined using, respectively, hexokinase, glucose-6-phosphate dehydrogenase, and phosphoglucose isomerase, and by the enzyme β-fructosidase after enzymatic inversion to D-glucose and D-fructose (Boehring test combination 716 260) [5]. Absorbance was detected at 340 nm by a UV–Vis spectrophotometer (UV-2100 Shimadzu, Japan).

Enzymes extraction

Enzyme extracts were obtained from callus tissue according to the method of Zhifang et al. [55] modified as it follows: samples of callus tissues (about 1 g fresh weight) were homogenized in a pre-chilled mortar with 3 volumes of an ice-cold extracting buffer containing 100 mM HEPES–NaOH (pH 7.5), 5 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). The homogenate was filtered through two layers of muslin and centrifuged at 20,000 g for 15 min. All steps were performed at 4 °C. The supernatant was used for enzyme assays by a UV–Vis spectrophotometer (UV-2100 Shimadzu, Japan).

Enzyme assays

Soluble acid invertase (SAI, β-fructofuranosidase, β-fructofuranoside fructohydrolase, EC 3.2.1.26) activity was assayed at 37 °C by adding 50 μl of extract to 50 μl of 1 M sodium acetate (pH 4.5). The enzyme reaction was started by the addition of 100 μl of a 120 mM sucrose solution. The reaction was stopped at 30 min by adding 30 μl of 2.5 M TRIS (TRIZMA base) and boiling the mixture for 3 min. The concentration of glucose liberated was determined with the glucose test kit from Sigma [56].

Glutamate synthase (GOGAT, EC 1.4.7.1) assay contained 25 mM HEPES–NaOH (pH 7.5), 2 mM L-glutamine, 1 mM α-ketoglutaric acid, 0.1 mM NADH, 1 mM Na_2 EDTA, and 100 μl of enzyme extract. GOGAT was assayed spectrophotometrically by monitoring NADH oxidation at A_{340} [3].

Glutamine synthetase (GS, EC 6.3.1.2): the mixture for the transferase assay contained 90 mM Imidazole–HCl

(pH 7.0), 60 mM Hydroxylamine (neutralized), 20 mM Na_2HAsO_4, 3 mM $MnCl_2$, 0.4 mM ADP, 120 mM Glutamine, and the appropriate amount of enzyme extract. The assay was performed in a final volume of 750 µl. The enzymatic reaction was developed for 15 min at 37 °C. The γ-glutamyl hydroxamate was determined colorimetrically by addition of 250 µl of a mixture (1:1:1) of 10% (w/v) $FeCl_3.6H_2O$ in 0.2 N HCl, 24% (w/v) trichloroacetic acid, and 50% (w/v) HCl. The optical density was recorded at A_{540} [11].

Malate dehydrogenase (MDH, EC 1.1.1.37) was assayed at 25 °C. The assay contained in 3.17 ml: 94.6 mM phosphate buffer (pH 6.7), 0.2 mM NADH, 0.5 mM oxalacetic acid, and 1.67 mM $MgCl_2$. MDH was assayed spectrophotometrically by monitoring NADH oxidation at A_{340}[5].

Phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) enzymatic activity was spectrophotometrically measured by monitoring NADH oxidation at 340 nm for 5 min at 30 °C. The assay medium (1 ml) contained 100 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH, 1.5 IU malic dehydrogenase (MDH), and 100 µl enzyme extract [32].

Total CAT (EC 1.11.1.6) activity was measured according to the method of Beers and Sizer [4], with minor modifications. The reaction mixture (1.5 ml) consisted of 100 mM phosphate buffer (pH 7.0), 0.1 µM EDTA, 20 mM H_2O_2 and 50 µl enzyme extract. The reaction was started by the addition of the extract. The decrease of H_2O_2 was monitored at 240 nm and quantified by its molar extinction coefficient (36 mM^{-1} cm^{-1}) and the results expressed as µmol H_2O_2 min^{-1} mg^{-1} protein.

Statistical analysis

All data were the means of five replicates. Results were evaluated statistically using one-way ANOVA, followed by Tukey's test to determine differences between treatments at the 0.05 level of probability [45].

Results

^{13}C-CPMAS–NMR spectra

The carbon distribution found in solid-state NMR spectra revealed three main groups of humic materials from different origins showing comparable molecular features (Table 2): geochemical sources (HA-C, HA-F, HA-H, HA-N), forest soils and composted biomasses (HA-D, HA-E, HA-G, HA-I, HA-M), fulvic acids (FA-O, FA-P, FA-Q). The humic fraction obtained from peat soil (HA-L) showed an intermediate composition between the first two groups (Table 2).

The NMR spectra of humic extracts from geochemical sources, HA-C, HA-F, HA-H, HA-N, revealed a carbon distribution consisting mainly in two resonance bands centred at 32 and 128 ppm, assigned, respectively, to saturated alkyl hydrocarbons and both unsubstituted and substituted aromatic carbon structures (Figs. 1 and 2). The typical recalcitrant features of these organic materials, inherited from the intense modification and structural rearrangements associated with long-term sedimentation processes [49], are summarized by the larger values found for their hydrophobicity (HB) and aromaticity (Ar) (Table 2), that resulted from the progressive loss of polar functional groups and the preservation of less reactive linear and aromatic hydrocarbon components [36].

Conversely, a more diversified molecular composition was found for the organic fractions isolated from both forest soils (HA-D, HA-I) and composts (HA-E, HA-G, HA-M), whose NMR spectra showed different C distributions in the main chemical shift regions (Figs. 1 and 2). However, the similarity of these two groups of humic materials was suggested by comparable structural Ar and HB indexes and the common predominance of alkyl functional groups (Table 2). The wide resonances found in the alkyl-C regions (0–45 ppm), in these samples, indicated a large incorporation of alkyl chains (Figs. 1 and 2). Their intense signals around 19–21 ppm are related to terminal CH_3 and proximate CH_2 groups of aliphatic chains, while those ranging from 26 to 33 ppm are attributed to bulk CH_2 components in lipids compounds of variable structural complexity and molecular mobility, such as free lipids, waxes, and polyesters [16]. The shoulders appearing at 37 and 41 ppm may be assigned, respectively, to methylene functions close to oxygenated C, like ester and ether linkages, and to CH group in either branched or cyclic components [38]. The peak at around 57 ppm is currently assigned to the methoxyl substituent on the aromatic rings of guaiacyl and syringyl components in lignin molecules, with the possible contribution of C–N bonds pertaining to amino acid moieties [38]. The O-alkyl-C region (60–110 ppm) include carbon atoms held in the monomeric constituents of plant oligo- and polysaccharides [23]. The sharp signal around 72 ppm corresponds to the overlapping resonances of carbon 2, 3, and 5 in the pyranoside structure in cellulose and hemicelluloses, while the less intense shoulders at about in the 62–64 and 81–88 ppm ranges represent carbon 6 and 4 of carbohydrate rings, respectively, and the resonance at around 105 ppm is usually associated with the anomeric C1 of glucose units in cellulose [51]. Moreover, other O-alkyl-C nuclei in carbohydrates explain the additional signals at around 103, 101, 98 ppm and, though of lower intensity, the resonances in the 82–86 ppm interval (Figs. 1 and 2). The broad resonances at 115 and 130 ppm are assigned to

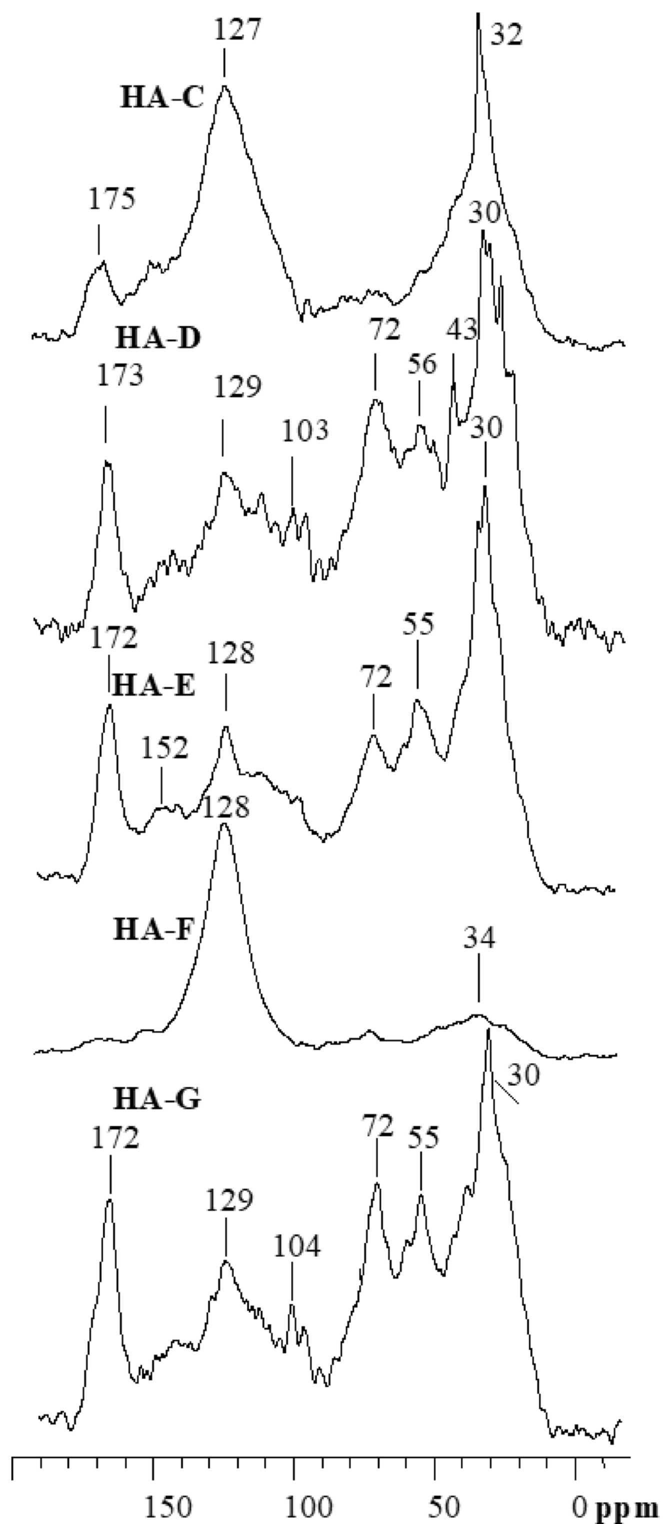


Fig. 1. ^{13}C-CPMAS–NMR spectra of humic acids: HA-C leonardite; HA-D oak forest soil; HA-E industrial compost after 150 days of maturation; HA-F Hungarian lignite treated with gluconic acid before extraction; HA-G industrial compost after 30 days of maturation

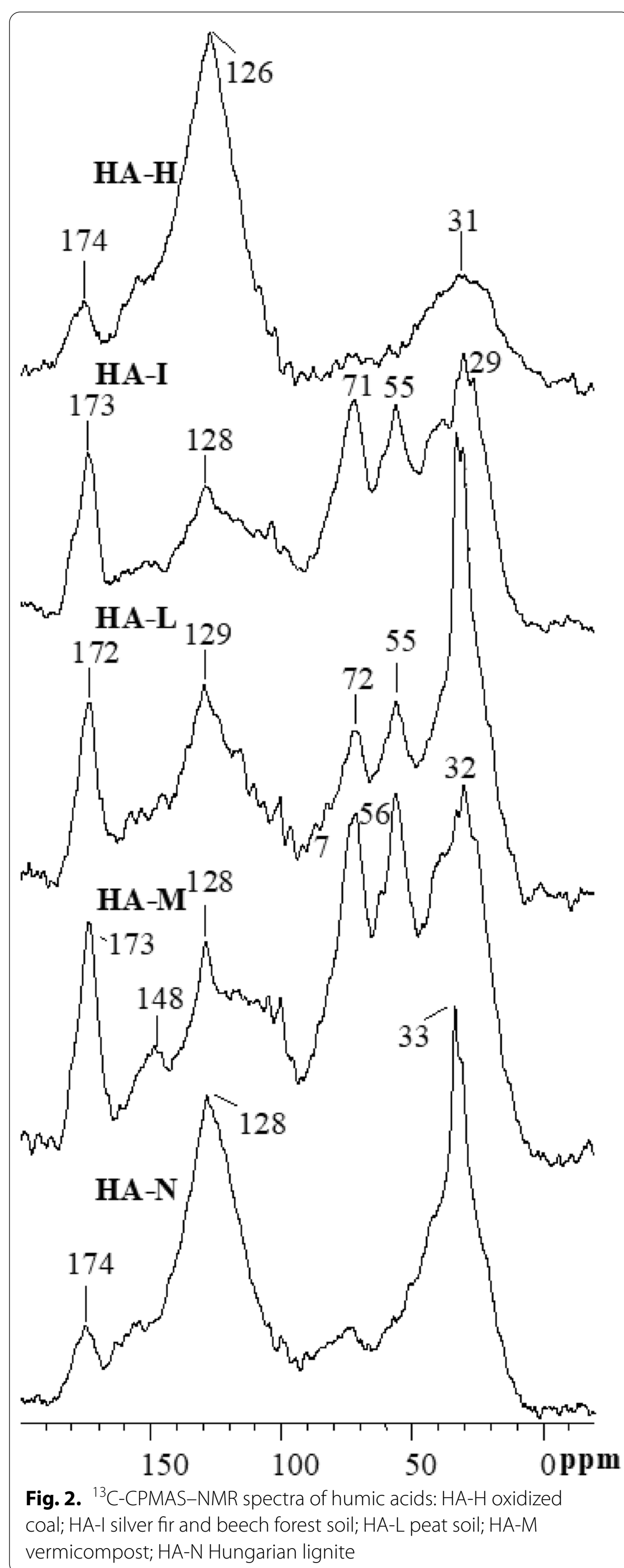


Fig. 2. ^{13}C-CPMAS–NMR spectra of humic acids: HA-H oxidized coal; HA-I silver fir and beech forest soil; HA-L peat soil; HA-M vermicompost; HA-N Hungarian lignite

both un-substituted and C-substituted phenyl carbon of lignin monomers of guaiacyl and syringyl units, while the signals shown in the O-aryl-C region (145–160 ppm) are due to O-substituted ring in lignin and polyphenol derivatives. Finally, the signals at around 175 ppm are due to carbonyl C of aliphatic acids and amino acid moieties [51].

The intermediate structural characteristics of the humic extract from a peat soil (HA-L) in respect to the other two groups of humic acids were highlighted by both HB and Ar values (Table 2). The large contribution of alkyl and aromatic carbons was accompanied by a significant percent, to total signals area, of polar components related to methoxyl/N-linked, carbohydrates and carboxylic carbons (Figs. 1 and 2). The environmental conditions of peat soils characterized by high moisture, low temperature, slow O_2 diffusion rates and acidic pH, greatly affect the modification of peat deposits and induce a progressive accumulation of fresh organic residues without a significant transformation of their molecular composition [35].

The NMR spectra of the three fulvic acids revealed a dominant abundance of signals for O-alkyl C in carbohydrates and carboxyl C (Fig. 3), whose cumulative relative C distribution ranged from 47.6 to 52.3% of total spectral area (Table 2). Despite the lower hydrophobicity in respect to humic acids, a noticeable contribution of aromatic components was also revealed by FA-O and FA-P from agricultural soils, while FA-Q from the organic-matter-rich volcanic soil was mostly dominated by alkyl-C components (Table 2).

Elaboration of signal intensities and chemical shifts from ^{13}C-CPMAS–NMR spectra of 13 humic materials (Table 2) was subjected to PCA. The statistical analysis extracted two principal components, PC1 and PC2, which together covered up to 97% of the sample variance (Fig. 4). Although the PCA is an unsupervised method [7], and, hence, may not strictly account for the origin of humic extracts [44], it should be noted that the resulting score plots generally matched samples separation with the origin of the humic and fulvic acids applied here. A good separation between humic acids of geochemical origin, such as leonardite (HA-C), lignite (HA-N), lignite treated with gluconic acid (HA-F), oxidized coal (HA-H), and the rest of humic samples was obtained on PC1, whose loadings were large and positive for the aromatic carbon (160–110 ppm), hydrophobicity and aromaticity, and large and negative for the rest of chemical shift ranges. Conversely, the humic acids extracted from soils, composts, vermicompost and peat, were all placed in the

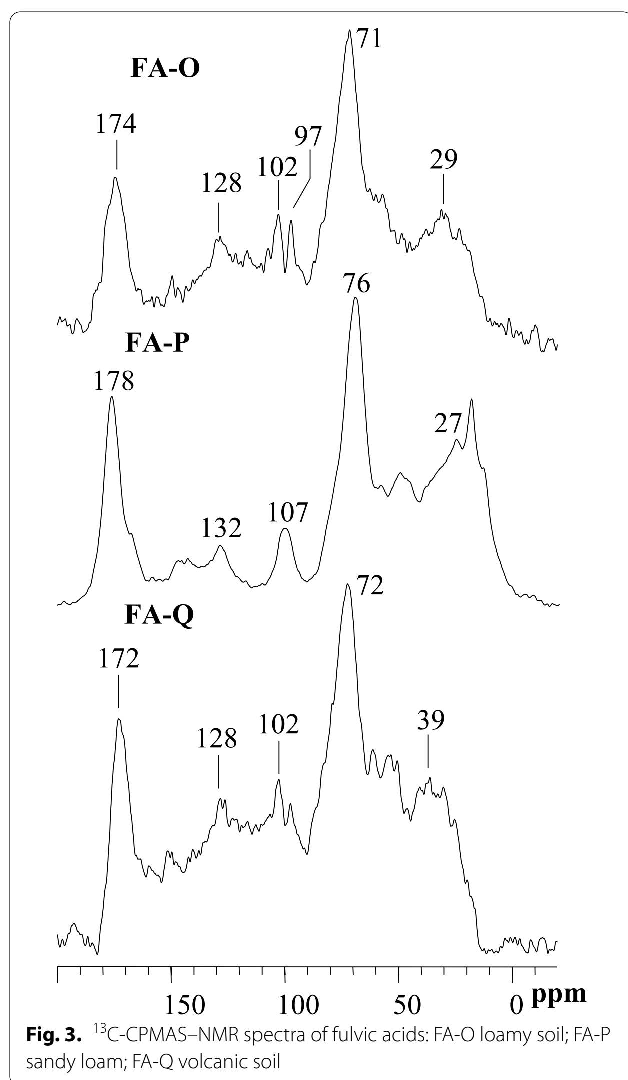


Fig. 3. ^{13}C-CPMAS–NMR spectra of fulvic acids: FA-O loamy soil; FA-P sandy loam; FA-Q volcanic soil

first quadrant of the score plot and showed positive loadings along the PC1 for alkyl (0–45 ppm) and methoxyl/N-alkyl (60–45 ppm) carbons. However, the extracts from compost at 30 d maturation (HA-G) and from peat (HA-L) were separated from the rest of humic acids extracted from fresh biomasses due to more positive values along the PC2. Finally, all fulvic acids were grouped in the third quadrant of the score plot with negative values along the PC2 for loadings of O-alkyl (110–60 ppm) and carboxyl/amide (200–160 ppm) carbons, being the FA-Q from the volcanic soils neatly separated from both FA-O and FA-P samples (Fig. 4).

Callus growth

Control callus biomass after 28 d of subculture reached 2.41 g (Table 3). Hormones (2,4-D + BAP) significantly increased the average mass of callus by about 52%, in respect to control (Table 3). Exposure to both humic and fulvic acids invariably produced a growth of *Pinus laricio* callus larger than control, though depending on type of humic matter. When treated with humic acids from compost at 30 d of maturation (HA-G), oxidized coal (HA-H) and a fir and beech forest soil (HA-I), callus growth was comparable to the hormone treatment, while the humic acid from compost at 150 d of maturation (HA-E) increased callus growth 61% more than control. However, increasing the concentration of these humic acids had no significant differences in callus growth, but the calluses always appeared white and vigorous in all treatments.

While FA-Q from the volcanic soil increased callus growth (51%) over control only at the lowest concentration of 0.5 mg L^{-1} (Table 3), increasing the concentration of FA-O and FA-P from the agricultural soils, HA-C from leonardite, HA-F from the treated lignite, HA-I from peat, and HA-M from vermicompost, produced a significant decrease in callus biomass.

Carbohydrate content

Following the 28 d of subculture, 2,4-D in combination with BAP reduced the amount of hexoses for glucose and fructose in respect to control (Tables 5, 6), while it was not different from control in the case of sucrose (Table 4). The callus treated with HA-G and HA-E from compost after 30 and 150 d of maturation, respectively, showed a general smaller content than control for all carbohydrates at all applied concentrations (Tables 4, 5, 6).

In callus tissue grown with HA-C from leonardite, FA-O and FA-P from agricultural soils, HA-D from an oak forest soil, HA-H from oxidized coal and HA-I from a fir and beech forest soil, the content of glucose, fructose and sucrose was lesser than for the control callus at each applied concentration, but it was larger than that measured in callus grown with hormones. HA-L from peat, HA-M from vermicompost, HA-F from the treated lignite and FA-Q from the volcanic soil increased the content of glucose and fructose at every used concentration in callus tissues more than for all other humic materials (Tables 4, 5, 6).

Phenol content

The content of total water-soluble phenols was about 28 μg TAE/g f.w. in both callus control and hormone-treatment (Fig. 5), while no significant differences from this value was observed in callus treated with HA-N from lignite and HA-I from a fir and beech forest soil. A slight decrease was instead noted for HA-H from oxidized coal, HA-E and HA-G from compost at 150 and 30 d maturation, respectively. An enhanced water phenol content was found in presence of HA-D from an oak forest soil, HA-F from the treated lignite, HA-L

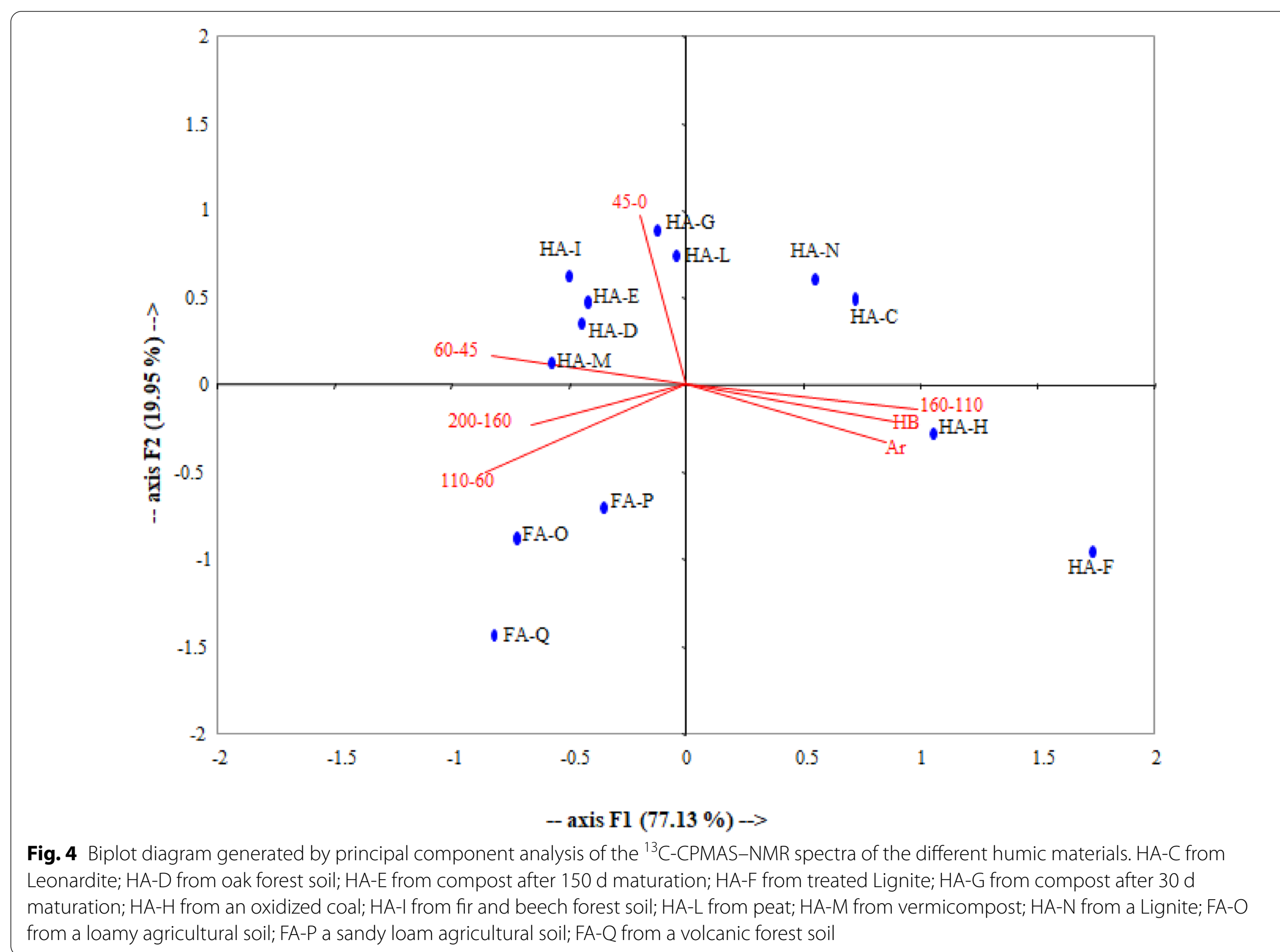


Fig. 4 Biplot diagram generated by principal component analysis of the ^{13}C-CPMAS–NMR spectra of the different humic materials. HA-C from Leonardite; HA-D from oak forest soil; HA-E from compost after 150 d maturation; HA-F from treated Lignite; HA-G from compost after 30 d maturation; HA-H from an oxidized coal; HA-I from fir and beech forest soil; HA-L from peat; HA-M from vermicompost; HA-N from a Lignite; FA-O from a loamy agricultural soil; FA-P a sandy loam agricultural soil; FA-Q from a volcanic forest soil

from peat, HA-M from vermicompost, FA-O and FA-P from agricultural soils. Conversely, a large increase in total water-soluble phenolic acids occurred for HA-N from lignite at all concentrations, and for FA-Q from the volcanic soil at greater concentrations (1 and 2 mg L^{-1}).

Enzyme activities

The catalase (CAT) activity in control callus was 45 nmol min^{-1} mg $prot^{-1}$. Hormone treatment decreased this activity compared to control (−38%). While all humic and fulvic acids provided CAT content either similar or only slightly larger than control, HA-F from treated lignite at each concentration severely decreased CAT activity (-53%) (Fig. 5). Larger levels of activity were instead observed in presence of HA-M from vermicompost and HA-N from lignite at every applied concentration, and of FA-Q from a volcanic soil when used at 1 and 2 mg L^{-1}.

The soluble acid invertase (SAI) activity is shown in Fig. 6. In callus treated with hormones, HA-E and HA-G from compost at 150 and 30 d maturation, respectively, the values of SAI were similar to control callus, while the content of SAI was even less than control for HA-F from treated lignite. The presence of HA-C from leonardite, HA-D from an oak forest soil, HA-I from a fir and beech forest soil, and HA-L from peat, at each concentration, significantly increased the amount of SAI in respect to control and hormones. Moreover, HA-H from oxidized coal, HA-M from vermicompost and HA-N from lignite, increased SAI activity strongly more than all other treatments (Fig. 6). All three fulvic acids increased slightly but significantly the SAI activity in respect to control, though FA-Q from a volcanic soil showed a much more substantial enzyme enhancement than the rest of fulvic acids at both 1 and 2 mg L^{-1}, reaching values of 29 μmol glucose min^{-1} mg $prot^{-1}$.

The activity of glutamine synthetase (GS) in callus control was 165 nmol γ-glutamil hydroxamate min^{-1} mg $prot^{-1}$ and increased up to 246 nmol NADH mg $prot^{-1}$ in the hormone treatment. Only for HA-E and HA-G from compost at 150 and 30 d maturation, respectively, the GS activity resulted larger than for the hormone treatment,

Table 3 Biomass (g) of *Pinus laricio* callus after 28 d of subculture in presence of hormones (2,4-D + BAP) or humic or fulvic acid at A = 0.5 mg.L^{-1}; B = 1.0 mg.L^{-1}; C = 2.0 mg.L^{-1}

Treatments[a]	Biomass (g f.w−1)b		
	A	B	C
Control	2.41 (0.02)^{g}A	2.41 (0.01)^{h} A	2.41 (0.02)^{m} A
2,4-D + BAP	5.08 (0.03)^{b}A	5.08 (0.01)^{b}A	5.08 (0.01)^{c}A
HA-C	4.30 (0.03)^{cd}AB	4.20 (0.05)^{d}B	4.50 (0.02)^{d}A
HA-D	4.21 (0.02)^{d}A	4.13 (0.05)^{d}A	4.00 (0.03)^{e}A
HA-E	6.10 (0.04)^{a}A	6.15 (0.04)^{a}A	6.20 (0.05)^{a}A
HA-F	3.35 (0.01)^{ef}A	3.47 (0.05)^{e}A	3.11 (0.01)^{h}B
HA-G	5.12 (0.07)^{b}B	5.01 (0.04)^{bc}B	5.55 (0.02)^{b}A
HA-H	4.90 (0.02)^{b}A	4.89 (0.06)^{bc}A	5.01 (0.02)^{c}A
HA-I	4.89 (0.05)^{b}A	4.80 (0.02)^{c}A	4.95 (0.02)^{c}A
HA-L	3.40 (0.07)^{e}A	3.20 (0.03)^{f}BC	3.09 (0.06)^{h}C
HA-M	3.21 (0.08) ^{f}A	3.01 (0.04)^{g}B	2.88 (0.05)^{i}C
HA-N	4.32 (0.02)^{c}A	3.02 (0.01)^{g}B	2.67 (0.02)^{l}C
FA-O	4.34 (0.01)^{c}A 4.19^{c} 3.80^{f}	4.19 (0.02)^{d}A	3.80 (0.01) ^{f}BC
FA-P	4.55 (0.03)^{c}A	4.15 (0.02)^{d}B	3.50 (0.01)^{g}C
FA-Q	4.90 (0.03)^{b}A	2.40 (0.03)^{h}B	2.41 (0.07) ^{m}B

[a] HA-C from Leonardite; HA-D from oak forest soil; HA-E from compost after 150 d maturation; HA-F from treated Lignite; HA-G from compost after 30 d maturation; HA-H from an oxidized coal; HA-I from fir and beech forest soil; HA-L from peat; HA-M from vermicompost; HA-N from a Lignite; FA-O from a loamy agricultural soil; FA-P a sandy loam agricultural soil; FA-Q from a volcanic forest soil

[b] Numbers in parentheses denote the standard error of the mean: n = 5. In the same column, different small letters indicate significant differences at $P \leq 0.05$ among treatments. In the same row, different capital letters indicate significant differences at $P \leq 0.05$, among the concentrations of the same treatment

Table 4 Sucrose (mg mL^{-1}) content[a] in *Pinus laricio* callus grown in 28 d of subculture in presence of hormones (2,4-D + BAP) or humic or fulvic acids[b] at A = 0.5 mg.L^{-1}; B = 1.0 mg.L^{-1}; C = 2.0 mg.L^{-1}

Treatments	A	B	C
Control	0.33 (0.02)^{l}	0.33 (0.03)^{l}	0.33 (0.02)^{m}
2,4-D + BAP	0.33 (0.02)^{l}	0.33 (0.02)^{l}	0.33 (0.02)^{m}
HA-C	4.18 (0.05)^{b}	4.38 (0.01)^{c}	4.08 (0.02)^{c}
HA-D	4.30 (0.07)^{b}	4.30 (0.02)^{c}	4.28 (0.02)^{c}
HA-E	1.03 (0.02)^{i}	1.13 (0.03)^{i}	1.00 (0.05)^{l}
HA-F	7.22 (0.02)^{a}	7.22 (0.03)^{a}	7.29 (0.02)^{a}
HA-G	3.40 (0.05)^{d}	3.30 (0.02)^{e}	3.00 (0.03)^{e}
HA-H	3.95 (0.04)^{c}	4.15 (0.04)^{c}	4.11 (0.05)^{c}
HA-I	3.95 (0.03)^{c}	3.75 (0.02)^{d}	3.72 (0.02)^{d}
HA-L	1.44 (0.02)^{g}	1.51 (0.05)^{h}	1.51 (0.02)^{h}
HA-M	2.10 (0.06) ^{e}	2.20 (0.05)^{f}	2.20 (0.04)^{f}
HA-N	1.95 (0.01)^{f}	1.85 (0.02)^{g}	1.80 (0.02)^{g}
FA-O	4.25 (0.03)^{b}	4.65 (0.02)^{b}	4.85 (0.02)^{b}
FA-P	4.10 (0.02)^{bc}	4.18 (0.07)^{c}	4.28 (0.01)^{c}
FA-Q	1.25 (0.01)^{h}	1.15 (0.01)^{i}	1.18 (0.02)^{i}

[a] Each value represents the mean ± SE of 5 replicates. In the same column, different letters indicate significant differences at $P \leq 0.05$

[b] HA-C from Leonardite; HA-D from oak forest soil; HA-E from compost after 150 d maturation; HA-F from treated Lignite; HA-G from compost after 30 d maturation; HA-H from an oxidized coal; HA-I from fir and beech forest soil; HA-L from peat; HA-M from vermicompost; HA-N from a Lignite; FA-O from a loamy agricultural soil; FA-P a sandy loam agricultural soil; FA-Q from a volcanic forest soil

while for the rest of humic material the enzyme activity remained either similar or slightly larger than for control, except for HA-M from vermicompost, HA-N from lignite and FA-Q from a volcanic soil, whereby the activity of GS decreased at the 1 and 2 mg L^{-1} concentrations (Fig. 6).

A similar behaviour was observed for Glutamate synthase (GOGAT) activity (Fig. 7), although its decrease in respect to control at larger concentrations was evident for HA-F from treated lignite, HA-L from peat, HA-M from vermicompost, HA-N from a Lignite, and FA-Q from a volcanic soil.

The phosphoenoly pyruvate carboxylase (PEPC) activity was very low in callus control (8.8 nmol NAD min^{-1} mg prot^{-1}), whereas it reached the largest level in calluses treated with hormones, and HA-G and HA-E from compost after 30 and 150 d of maturation, respectively. The values of PEPC activity were below detection limit in presence of HA-N from lignite and FA-Q from a volcanic soil when applied at greater concentrations (Fig. 7).

Regarding the malate dehydrogenase (MDH) enzyme, the largest levels of activity were again found in callus grown up in presence of hormones, and HA-G and HA-E from compost after 30 and 150 d of maturation, respectively. The most negative effects were observed when HA-N from lignite and FA-Q from a volcanic soil were present in the medium culture at greater concentrations (Fig. 8).

Discussion

The humic extracts invariably increased the fresh weight of callus in comparison with control, but only the humic acids from compost after 150 day maturation (HA-E)

Table 5 Glucose (mg mL^{-1}) content[a] in *Pinus laricio* callus grown in 28 d of subculture in presence of hormones (2,4-D + BAP) or humic or fulvic acids[b] at A = 0.5 $mg.L^{-1}$; B = 1.0 $mg.L^{-1}$; C = 2.0 $mg.L^{-1}$

Treatments	A	B	C
Control	4.14 (0.03)^{e}	4.14 (0.01)^{ef}	4.14 (0.01)^{ef}
2,4-D + BAP	1.46 (0.02)^{l}	1.46 (0.01)^{l}	1.46 (0.02)^{i}
HA-C	4.50 (0.01)^{d}	4.30 (0.02)^{e}	4.31 (0.01)^{e}
HA-D	3.90 (0.06)^{f}	3.93 (0.03)^{f}	3.96 (0.02)^{f}
HA-E	1.12 (0.07)^{m}	1.19 (0.02)^{m}	1.03 (0.02)^{l}
HA-F	2.94 (0.03)^{h}	2.74 (0.02)^{h}	2.89 (0.03)^{h}
HA-G	1.65 (0.02)^{i}	1.75 (0.02)^{i}	1.53 (0.02)^{i}
HA-H	3.55 (0.05)^{g}	3.59 (0.06)^{g}	3.56 (0.02)^{g}
HA-I	3.75 (0.05)^{f}	3.95 (0.05)^{f}	3.96 (0.05)^{f}
HA-L	4.70 (0.03)^{d}	4.83 (0.05)^{d}	4.79 (0.02)^{d}
HA-M	5.78 (0.04)^{c}	5.90 (0.02)^{c}	5.96 (0.08)^{c}
HA-N	7.95 (0.02)^{a}	7.90 (0.08)^{a}	7.88 (0.02)^{a}
FA-O	4.00 (0.02)^{ef}	4.11 (0.03)^{ef}	4.31 (0.02)^{e}
FA-P	3.50 (0.03)^{g}	3.90 (0.02)^{f}	4.90 (0.02)^{d}
FA-Q	6.45 (0.02)^{b}	6.95 (0.02)^{b}	6.90 (0.07)^{b}

[a] Each value represents the mean ± SE of 5 replicates. In the same column, different letters indicate significant differences at $P \le 0.05$

[b] HA-C from Leonardite; HA-D from oak forest soil; HA-E from compost after 150 d maturation; HA-F from treated Lignite; HA-G from compost after 30 d maturation; HA-H from an oxidized coal; HA-I from fir and beech forest soil; HA-L from peat; HA-M from vermicompost; HA-N from a Lignite; FA-O from a loamy agricultural soil; FA-P a sandy loam agricultural soil; FA-Q from a volcanic forest soil

Table 6 Fructose (mg mL^{-1}) content[a] in *Pinus laricio* callus grown in 28 d of subculture in presence of hormones (2,4-D + BAP) or humic or fulvic acids[b] at A = 0.5 $mg.L^{-1}$; B = 1.0 $mg.L^{-1}$; C = 2.0 $mg.L^{-1}$

Treatments	A	B	C
Control	10.90 (0.07)^{a}	10.90 (0.06)^{a}	10.90 (0.08)^{a}
2,4-D + BAP	2.95 (0.01)^{h}	2.95 (0.04)^{i}	2.95 (0.01)^{l}
HA-C	5.65 (0.01)^{ef}	5.70 (0.02)f	5.50 (0.02)^{g}
HA-D	5.90 (0.02)^{e}	5.99 (0.02)^{ef}	5.85 (0.07)^{ef}
HA-E	3.10 (0.01)^{h}	3.28 (0.01)^{h}	2.34 (0.02)^{l}
HA-F	4.48 (0.02)^{g}	4.84 (0.03)^{g}	5.99 (0.02)^{ef}
HA-G	4.68 (0.03)^{g}	4.71 (0.03)^{g}	3.28 (0.01)^{h}
HA-H	8.75 (0.04)^{c}	8.91 (0.07)^{c}	4.84 (0.03)^{g}
HA-I	7.65 (0.05)^{d}	7.75 (0.02)^{d}	4.71 (0.03)^{g}
HA-L	8.65 (0.06)^{c}	8.81 (0.05)^{c}	8.91 (0.07)^{c}
HA-M	5.80 (0.05)^{ef}	6.10 (0.01)^{e}	7.75 (0.02)^{d}
HA-N	5.55 (0.05)^{f}	5.95 (0.02)^{ef}	8.81 (0.05)^{c}
FA-O	5.80 (0.05)^{ef}	6.10 (0.01)^{e}	6.50 (0.03)^{e}
FA-P	5.55 (0.05)^{f}	5.95 (0.02)^{ef}	6.63 (0.02)^{e}
FA-Q	9.65 (0.08)^{b}	9.75 (0.08)^{b}	9.77 (0.07)^{b}

[a] Values are the *means* ± SE of 5 replicates. In the same column, different letters indicate significant differences at $P \le 0.05$

[b] HA-C from Leonardite; HA-D from oak forest soil; HA-E from compost after 150 d maturation; HA-F from treated Lignite; HA-G from compost after 30 d maturation; HA-H from an oxidized coal; HA-I from fir and beech forest soil; HA-L from peat; HA-M from vermicompost; HA-N from a Lignite; FA-O from a loamy agricultural soil; FA-P a sandy loam agricultural soil; FA-Q from a volcanic forest soil

showed, at all applied concentrations, a significantly larger biomass weight than the treatment with hormones (Table 2). This significant HA-E bioactivity may be attributed to a great content of hormone-like compounds due to the vigorous microbial biomass transformation during the aerobic composting process [40], and to its relatively small Ar and HB values [50]. These bioactive functions are not exerted to the same extent by the rest of humic materials of soil or geochemical origin.

We investigated the effects of humic and fulvic extracts on nitrogen metabolism of callus cultured in vitro nurtured by the nitrogen present in the growing medium. The metabolic pathways of nitrogen and carbon are linked, since nitrogen assimilation also requires carbohydrates to produce energy and build-up the carbon skeleton [31]. Callus tissue does not have photosynthetic activity, and exploits sucrose in the medium culture as sole carbon source [14]. Since sucrose is necessary, it is rapidly hydrolysed to glucose and fructose before use in most studied species. Sucrose may be cleaved either by sucrose synthase (SuSy), a cytosolic enzyme that is crucial to sucrose utilization in fruit development [48, 52], and by soluble acid invertase (SAI), a hydrolase that is particularly active in rapidly growing tissues [21]. The products of sucrose cleavage, converted to hexoses phosphates, subsequently enter the respiratory pathway to provide the substrates and reducing power required by the growth process. The levels of SAI activity were generally increased over those of control and hormones by humic and fulvic acid treatments, thus indicating that sucrose was rapidly hydrolyzed into glucose and fructose. However, it is to be noted that the HA-M from vermicompost, HA-N from lignite and FA-Q from a volcanic soil, which showed the largest SAI activity, were also the extracts revealing the greatest content of glucose and fructose in callus, thereby indicating a similar functionality, although they belong to different classes of humic extracts, according to the PCA score plot.

Callus growth is restricted when nitrogen uptake is limited, since a shortage of available nitrogen inhibits protein synthesis and accumulation/depletion of protein and non-protein amino acids [17]. In fact, assimilation of inorganic nitrogen in the form of ammonium is a critical biochemical step for plant growth and development. This process is catalysed by glutamine synthetase (GS) and Glutamate synthase (GOGAT) in a concerted manner [22] and requires availability of carbon skeletons that increases the TCA cycle carbon flow [2]. Here we verified

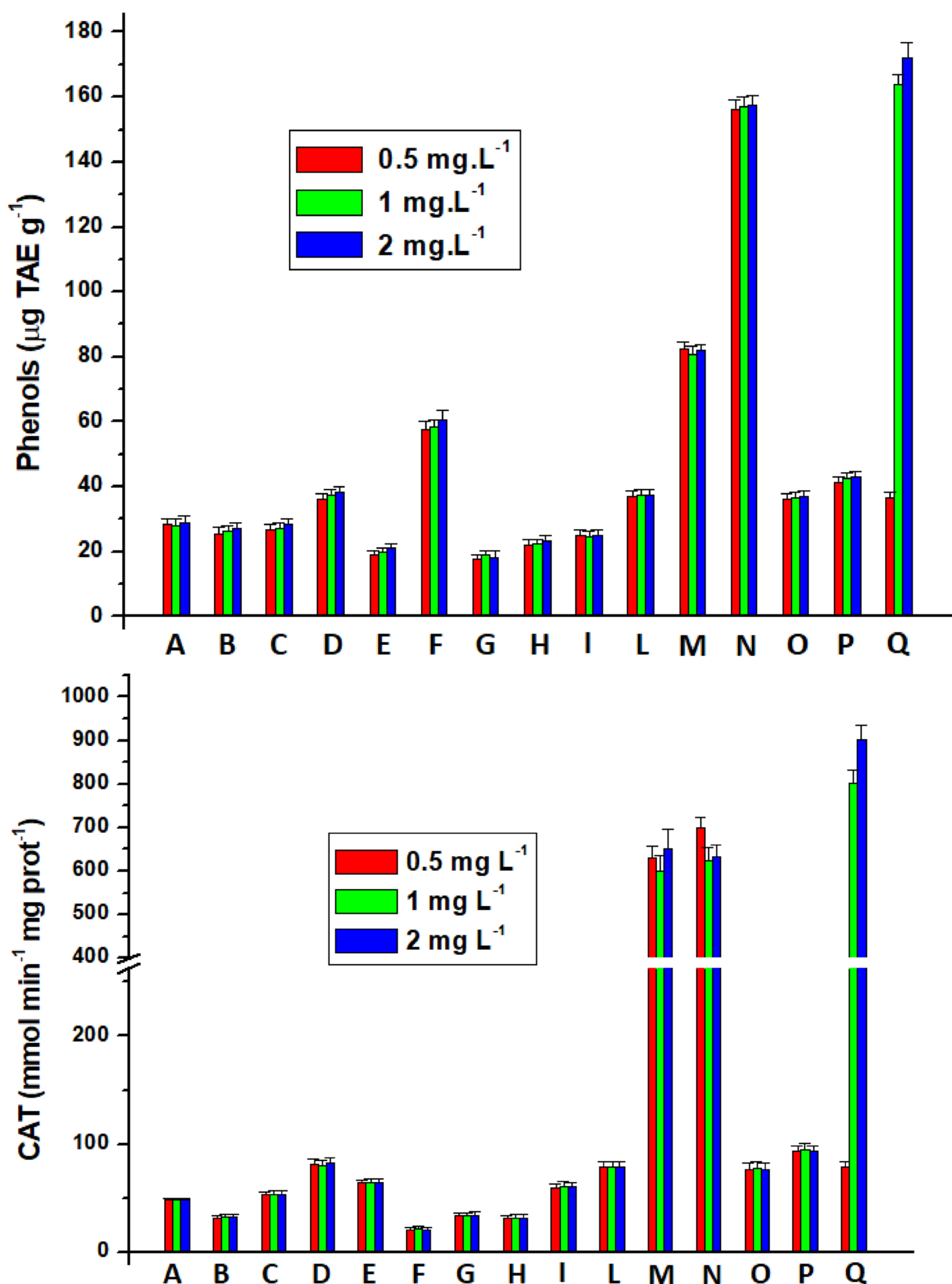


Fig. 5 Phenol content and catalase (CAT nmol min^{-1} mg^{-1} protein) activity, in *Pinus laricio* callus grown for 28 days on MS medium with hormones, or different concentrations (0.5, 1, 2.0 mg.L^{-1}) of humic or fulvic acids from different source. Bars represent standard errors (mean ± SD). A = Control (callus on MS medium); B = hormones (2,4-D + 6-BAP); C = HA-C from Leonardite; D = HA-D from oak forest soil; E = HA-E from compost after 150 d maturation; F = HA-F from treated Lignite; G = HA-G from compost after 30 d maturation; H = HA-H from an oxidized coal; I = HA-I from fir and beech forest soil; L = HA-L from peat; M = HA-M from vermicompost; N = HA-N from a Lignite; O = FA-O from a loamy agricultural soil; P = FA-P a sandy loam agricultural soil; Q = FA-Q from a volcanic forest soil

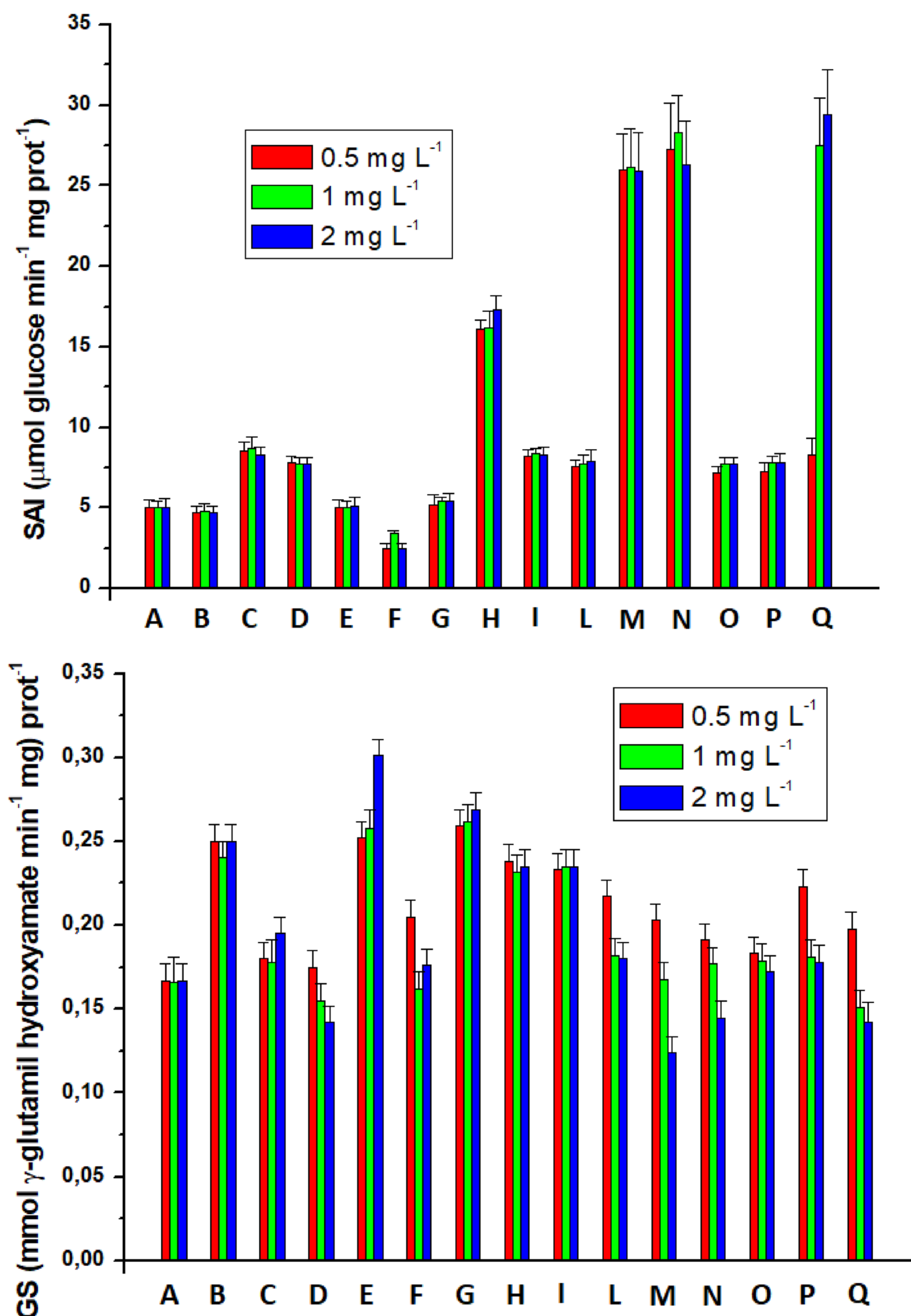


Fig. 6 Soluble acid invertase (SAI) and Glutamine synthetase (GS) activities, in *Pinus laricio* callus grown for 28 days on MS medium with hormones, or different concentrations (0.5, 1, 2.0 mg.L^{-1}) of humic or fulvic acids from different sources. Bars represent standard errors (mean ± SD). A = Control (callus on MS medium); B = hormones (2,4-D + 6-BAP);; C = HA-C from Leonardite; D = HA-D from oak forest soil; E = HA-E from compost after 150 d maturation; F = HA-F from treated Lignite; G = HA-G from compost after 30 d maturation; H = HA-H from an oxidized coal; I = HA-I from fir and beech forest soil; L = HA-L from peat; M = HA-M from vermicompost; N = HA-N from a Lignite; O = FA-O from a loamy agricultural soil; P = FA-P a sandy loam agricultural soil; Q = FA-Q from a volcanic forest soil

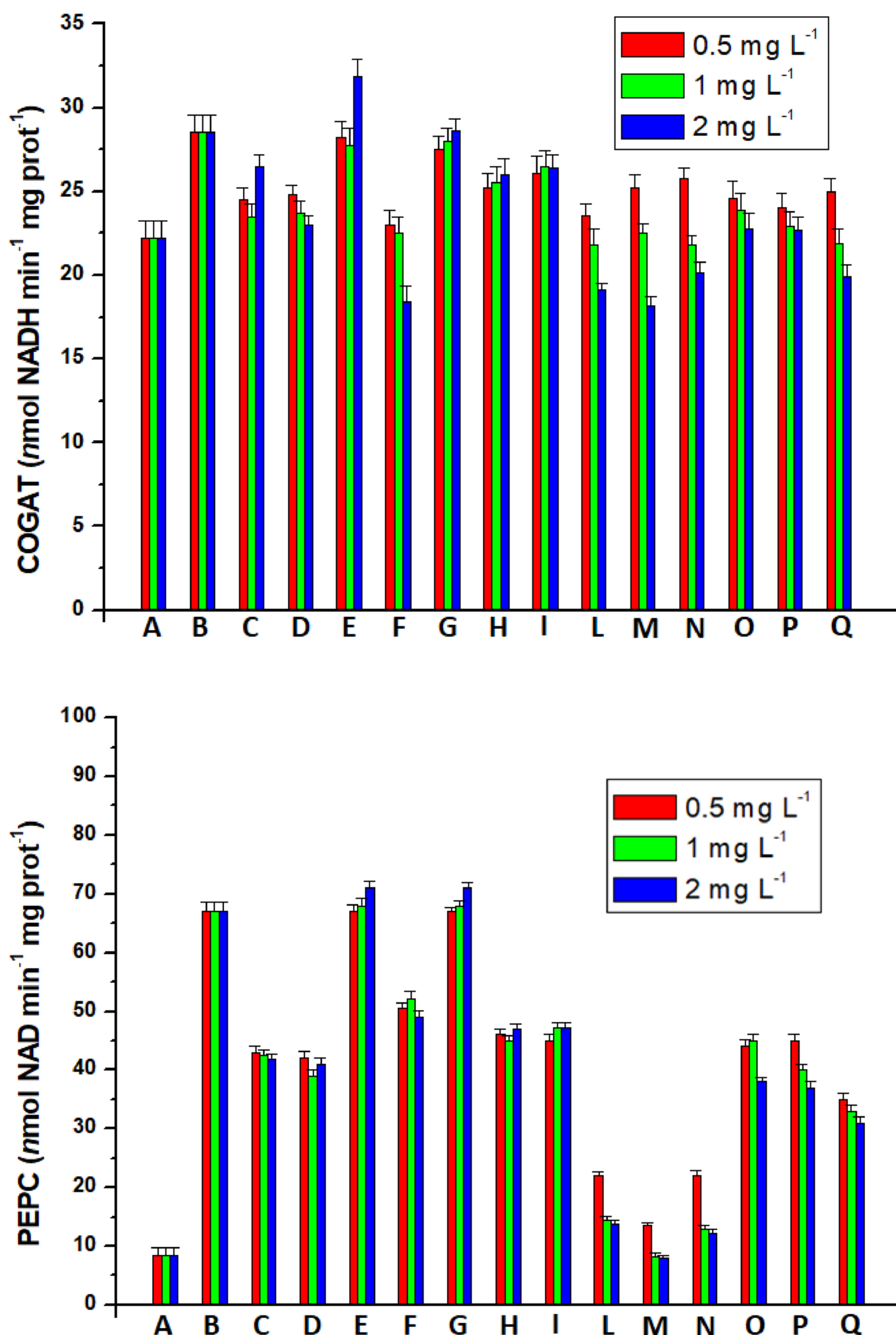


Fig. 7 Changes in Glutamate synthase (GOGAT) and Phosphoenoly Pyruvate carboxylase (PEPC) activities, in *Pinus laricio* callus grown for 28 days on MS medium with hormones, or different concentrations (0.5, 1, 2.0 mg.L^{-1}) of humic or fulvic acids from different sources. Bars represent standard errors (mean ± SD). A = Control (callus on MS medium); B = hormones (2,4-D + 6-BAP);; C = HA-C from Leonardite; D = HA-D from oak forest soil; E = HA-E from compost after 150 d maturation; F = HA-F from treated Lignite; G = HA-G from compost after 30 d maturation; H = HA-H from an oxidized coal; I = HA-I from fir and beech forest soil; L = HA-L from peat; M = HA-M from vermicompost; N = HA-N from a Lignite; O = FA-O from a loamy agricultural soil; P = FA-P a sandy loam agricultural soil; Q = FA-Q from a volcanic forest soil

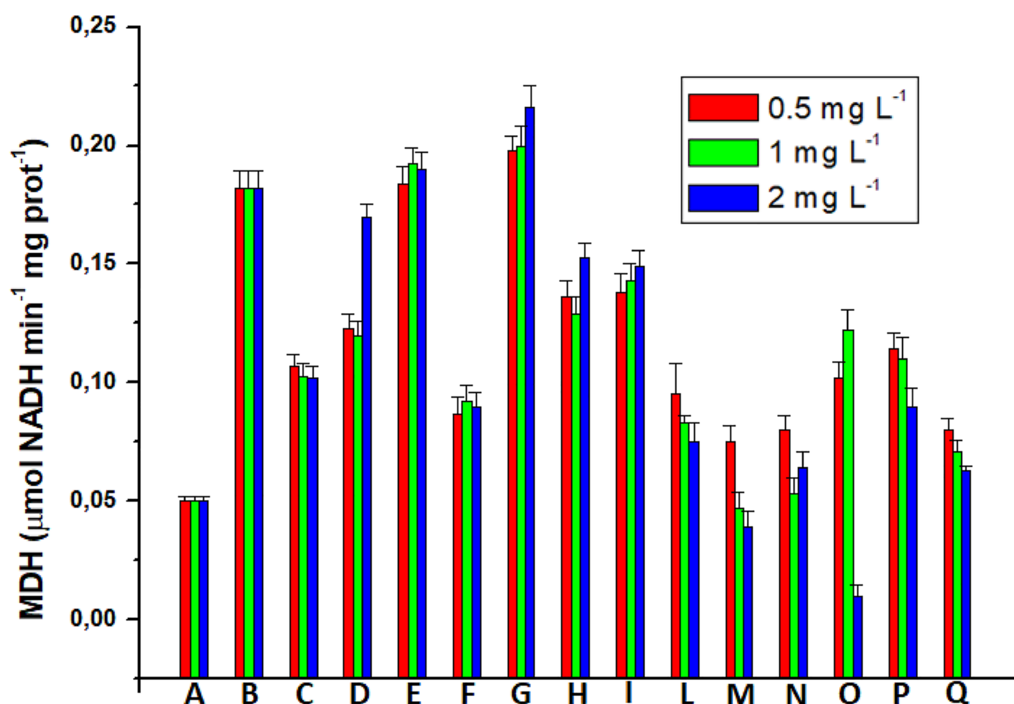


Fig. 8 Changes in Malate dehydrogenase (MDH) activity, in *Pinus laricio* callus grown for 28 days on MS medium with hormones, or different concentrations (0.5, 1, 2.0 mg.L^{-1}) of humic or fulvic acids from different sources. Bars represent standard errors (mean ± SD). A = Control (callus on MS medium); B = hormones (2,4-D + 6-BAP);; C = HA-C from Leonardite; D = HA-D from oak forest soil; E = HA-E from compost after 150 d maturation; F = HA-F from treated Lignite; G = HA-G from compost after 30 d maturation; H = HA-H from an oxidized coal; I = HA-I from fir and beech forest soil; L = HA-L from peat; M = HA-M from vermicompost; N = HA-N from a Lignite; O = FA-O from a loamy agricultural soil; P = FA-P a sandy loam agricultural soil; Q = FA-Q from a volcanic forest soil

that the activity of malate dehydrogenase (MDH), an enzyme involved in the TCA cycle, was stimulated by the presence of humic and fulvic acids, as compared to control. The most negative effects were observed when HA-N from lignite and FA-Q from a volcanic soil were added to the medium culture at larger concentrations, possibly because their supramolecular assemblies become tighter at those concentrations and inhibit the availability of the bioactive compounds contained in these humic materials [40]. Conversely, the greatest levels of activity were observed in callus grown up in presence of hormones and HA-G and HA-E from compost after 30 and 150 d of maturation, respectively. An explanation of such large positive effect of these two compost extracts may be searched in the great content of polar bioactive compounds and their increased availability to stimulate callus growth [23].

The stimulation of respiration during nitrogen uptake has been ascribed to an initial activation of PEPC, leading to an increased production of citric acid cycle carbon skeletons that is required for ammonium assimilation [20]. We found that PEPC activity was stimulated, as compared to control, by all humic and fulvic acids, even though it was a function of the molecular composition of humic extracts. In fact, the relative slight growth of calluses in presence of HA-L from peat, HA-M from vermicompost at every applied concentration and of HA-N from lignite and FA-Q from a volcanic soil at 1.0 and 2.0 mg L^{-1} (Table 3), may be related to the strong inhibition of PEPC activity observed for treatments with the same humic materials (Fig. 7). The limitation of the PEPC enzyme leads to a decreased production of the carbon skeletons required for ammonium assimilation through the GS/GOGAT pathway [43]. In fact, a reduction in the activity of the latter enzymes was noticed when callus was treated with the same humic extracts (HA-L, HA-M, HA-N, FA-Q) (Figs. 6 and 7), that may be attributed to the relatively large alkyl-C content of these materials (Table 2).

Limited nitrogen assimilation produces a plant stress that, irrespective of its nature, enhances the formation of reactive oxygen species (ROS) [54], with consequent cellular damages but also concomitant activation of protective mechanisms. Tissue damage occurs when the

capacity of antioxidative systems becomes lower than the amount of generated ROS [42]. To mitigate the oxidative damage initiated by ROS, plants have developed an antioxidant defense system, of which catalase (CAT) represents an important enzyme that serves to regulate the production of intracellular hydrogen peroxide. Moreover, phenolic compounds are also considered potent inhibitors of oxidative damage [13]. Our results revealed a strong increase in CAT activity and phenols content (Fig. 5) in calluses treated with HA-M from vermicompost and HA-N from lignite at all applied concentrations and FA-Q from a volcanic soil at greater concentrations (1 and 2 mg L^{-1}), thus suggesting that these humic and fulvic acids induce stress in callus. Again, it appears that a combination of conformational dynamics with molecular composition of these specific humic extracts, possibly related to their relatively important content of alkyl-C (Table 2, Fig. 4), is detrimental to callus growth and metabolism.

Earlier works indicated that plant growth and metabolism are enhanced by humic matter either rich in peptides, carbohydrates and organic acids, but poor in phenols [26] or in tartaric acid and fatty acids [27]. However, other studies suggest that it is not only the molecular composition that has a bearing on plant stimulation but also the conformational arrangement of the humic supramolecular assembly is essential in reaching and activating plant roots [8, 10, 40]. Here, we confirmed that the most bioactive humic samples toward callus growth were those extracted from composted organic wastes, richer in hydrophilic and oxygen-containing aliphatic compounds. However, such bioactivity did not result as straightforward when related to the callus chemical and enzymatic content. In fact, the contribution of aromatic and hydrophobic components appeared to play an increasing and significant role in determining the enzymatic activity controlling nitrogen metabolism in callus, although the precise and most effective combination of the hydrophilic and hydrophobic components remains to be elucidated [36].

Conclusions

This work related to the molecular composition of 13 different humic extracts originating from different sources, such as lignite, soil, peat and compost, to the biomass and biochemical activities of a callus of *Pinus laricio*. Humic substances contributed to increase callus biomass significantly more than the control and a treatment with hormones. However, their effects on the callus content of carbohydrates, phenols and a series of enzymes linked to the nitrogen metabolism revealed that the effect was dependent on both the different molecular compositions of humic materials and their concentrations applied to the callus. The callus growth appeared better related to humics extracted from composted fresh biomasses possessing low aromaticity and large hydrophilicity than to the materials isolated from soil and geochemical sources, such as lignite and leonardite. Less simple was the relationship between the molecular composition of humic extracts and the chemical and biochemical properties of callus, whose stimulation or inhibition could not be linearly related only to the polarity or apolarity of humic components but the conformational arrangement of the humic suprastructures had also to be called upon. More detailed experimental work, possibly with controlled mixtures of natural compounds of different degrees of polarity, is, therefore, required to reach a more advanced elucidation of the bioactivity of humic matter.

Author contributions
AM, and AP designed the experiment, analyzed the data and drafted the manuscript. ML, VC and AN contributed to the extractions of humic matter, spectral and chemical analyses of extracts, and evaluation of data. SN read and revised the manuscript. All authors read and approved the final manuscript.

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Declarations

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Consent for publication
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