

Article

Effectiveness of Humic Substances and Phenolic Compounds in Regulating Plant-Biological Functionality

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Received: 27 August 2020; Accepted: 9 October 2020; Published: 12 October 2020



Abstract: Significant benefit of soil organic matter (SOM) to crop productivity is scientifically well documented. The main constituents and active fractions of SOM are humic substances (HS) and phenolic compounds. Since both these two components strongly impact plant–soil relationship, it is importantly from an ecological point of view to discriminate their biological effects and relating them to their composition. In this study we compared the biological effects of HS, and the soil water soluble phenols (SWSP) on growth, antioxidant activities, carbohydrates, proteins, phenols, and vitamins of *Pinus laricio* callus. Each extract was assessed for the content of low molecular weight organic acids, soluble carbohydrates, fatty acids, and phenolic acids. Moreover, Fourier transform infrared (FT-IR) and surface-enhanced Raman scattering (SERS) spectroscopies were applied to study their molecular structure. The results showed that HS produced better callus growth compared to the control and SWSP. Carbohydrates decreased in presence of HS while proteins, vitamin C and E increased. In contrast, in callus treated with SWSP the amount of glucose and fructose increased as well as all the antioxidant activities. The data evidenced that HS rich in tartaric and fatty acids had beneficial effects on callus growth contrary to soil water-soluble phenols rich in aldehydes, and syringic, ferulic, and benzoic acids.

Keywords: callus; *Pinus laricio*; FT-IR; SERS; fatty and organic acids; antioxidant activity

1. Introduction

Soil organic matter (SOM) is a highly miscellaneous blend with well recognized effects on crop productivity. These benefits have been object of controversy for centuries and some are still debated today because of SOM is a mixture of organic material and meso–micro soil fauna and microflora with high complexity and heterogeneity. The majority of soil organic matter (~90%) is usually constituted by humus, a strongly decomposed organic material dark-colored, partly aromatic, acidic, hydrophilic, molecularly flexible polyelectrolytic matter [1]. Humus can be divided into three main groups one inactive (humins) and two actives. The active pools are constituted by a stable pool (humic substances, HS) and a labile pool of phenolic compounds. Humic substances (HS) are a durable material, specific to each type of soil that have not markedly change over decades of land use [2].

To date, the chemical structure and the nature of humus is not yet well-known and it is still controversial, while it is certain that it contains numerous and different chemical components [3]. In the past, Stevenson [2] states that humic substances are formed by polymerization and polycondensation of simple biomolecules coming from the decomposition of biological residues, later Swift [4] showed that HS have a macromolecular structure that makes random coil conformations or micelles or “pseudo micellar” structures in solution. In 2001, Piccolo [5] recognized HS as a supramolecular association of self-assembling heterogeneous and relatively small molecules (rich in carbohydrates, proteins, lipids, tannins, etc.) linked by intermolecular hydrophobic interactions and hydrogen bonds.

HS have the capacity of coordinating physical, chemical, and biological soil properties through the regulation of ions mobilization and transports in the environment, with important consequence in the rhizosphere interactions [6]. Soil fertility is dependent on the amount and characteristic of HS that with their physical-chemical and biochemical properties are able to promote plant growth and metabolism [7–12], influencing remarkably the crop efficiency. Rose et al. [13] showed, by a random-effects meta-analysis, that plants treated with HS have a significant increment (22%) in root and shoot dry weight. HS effect on plant growth were related both to their ability to boost micro and macro nutrient availability [12,14] and/or ion uptake by the activation of PMH^+ -ATPase enzyme [15–18].

To date, more than 8000 compounds belong to the phenolic class [19], ranging from simple, low molecular, or complex compounds, to highly polymerized compounds. Once integrated into the soil, phenolic compounds are important intermediates in humus formation and have a key role in controlling litter decomposition and below-ground processes, including SOM decomposition [19]. In forest soils litter decomposition free a high concentration of some soluble low-molecular-weight organic acids (e.g., citric, *p*-cumaric, *p*-hydroxybenzoic, lactic, malonic, succinic, and vanillic acid), due to a high proportion of these substances in parent plant material or to a low rate of breakage of such acids. Additionally, these soluble low-molecular-weight organic acids may be produced by plant root exudation or by mycorrhizal fungi [1]. All these soluble low-molecular-weight organic acids, are well known to affect regeneration and growth of natural forest [20] through allelopathic phenomena with an important ecological role [21].

The mechanism by which HS or phenolic compounds affect plant growth and metabolism is not well clarified and it is still under debate. Muscolo et al., 2012 [22] prepared sequential extracts, HS free from water-soluble phenols (HS-WP), and water-soluble phenols free from HS (WP-HS), to detect the biological effects of these two extracts on physiological response of different plant organs in respect to the total fraction WP and HS. The results evidenced stimulatory effects on growth and germination of HS without phenols and inhibitory effects of phenols. Since both HS and phenols strongly impact plant–soil relationship, our aim was to deepen not only the mechanisms of action at the metabolic plant level, evaluating the antioxidant system involved in the mitigation of environmental perturbations which significantly disturb metabolism, but also the chemical composition of the major SOM fractions, relating their specific activity to specific compounds. For these reasons, in this work several vibrational spectroscopic techniques have been applied for investigation of the fractions. Among these techniques, we used Fourier transform infrared (FT-IR) spectroscopy, which is a very fast and non-destructive procedure that allows the qualitative evaluation of the vibrational modes of each functional group at a specific frequency in the spectrum [23].

Surface-enhanced Raman scattering (SERS) that is a vibrational technique that can be applied to obtain characteristic structural information on HS [24]. SERS increases the sensitivity of the Raman signal due to the huge intensification of electric field on the surface of nanostructures made of several specific metals, mainly Ag or Au [25]. Additionally, the adsorption of the analyzed molecule close to the metal surface induces a fluorescence quenching of the molecule that can interfere with the Raman signal allowing the study of highly fluorescent molecules, such as HS and phenolic compounds. [26].

Infrared and SERS spectroscopies have been used in a complementary way to gain knowledge about the structure and conformation of humic substances [27]. FT-IR and SERS spectra are unique for each compound, which makes both techniques highly specific in identifying and elucidating the

structure of molecules. IR absorbance is more intense for more polar groups (carboxylate, aminol, or alcoholic functional groups), while Raman SERS is more specific for the analysis of less polar groups such as aromatic moieties and aliphatic chains.

The main aim of the present work is to highlight, deepening at the metabolic and analytical level, if differences in the biological effects of these two main fractions of SOM exist and what the extent of their activity is.

2. Materials and Methods

2.1. Extraction and Characterization of Humic Substances and Soluble Soil Phenols

2.1.1. KOH Extract, (Humic Substances, HS), and Water Extract (Soluble Phenols, SWSP)

Soil used in this study was collected from the Ah horizon (4–22 cm) under *Pinus laricio* Poiret trees, located at Monte Peripoli (San Lorenzo) Aspromonte, 1270 m above sea level, lat 38°4'00" N, long 15°52'08" E. The soil was classified as Eutric Cambisol according to FAO UNESCO System Criteria (1990). The organic carbon content in Ah horizon was 2.79%. Air-dried soil sample (30 g dry weight) was extracted with 0.1 M KOH (1:20 *w/v*) at room temperature for 16 h under a N₂ atmosphere, and then centrifuged at 7000 *g* for 20 min [12]. KOH is preferentially used as extractant if HS are used for biological experiments. Organic carbon was estimated in each soil extract by the Walkley–Black procedure [28]. Organic carbon was 2.4% in HS and 0.6% in SWSP. Total protein was analyzed adopting the method of Bradford [29] using bovine serum albumin as standard. HS contained 25 mg g⁻¹ dry soil of total protein, SWPS did not contain total protein.

Soil water soluble phenolic fraction have been extracted using 30 g of air-dried soil samples in 200 mL of distilled water shaken at 75 rev min⁻¹ for 20 h at room temperature. Solutions were filtered through Whatman No. 1 paper. The phenolic compounds were determined as reported in Muscolo et al. [22]. Samples were freeze-dried before the analyses.

2.1.2. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra of unfractionated HS and soil water soluble phenolic fraction (SWSP) in lyophilized form were registered by using a Nicolet 5700 Thermo-Corporation equipped with a diamond attenuated total reflectance (ATR) accessory (Spectra-Tech, Shelton, CT, USA) at a resolution of 4 cm⁻¹ with 64 scans. Overlapping bands were resolved using the second-derivative (2d) by using Grams/386 spectral software (Galactic Industries, Salem, NH, USA).

2.1.3. Surface-Enhanced Raman Scattering (SERS)

SERS spectra of HSs and SWSP were recorded using a silver colloid obtained by reduction with citrate as previously reported [30]. Raman spectra were obtained by adding 10 µL of HSs or SWSP solution to 1 mL of silver colloid, and finally the pH was corrected at ca. 12 with 0.1 N NaOH. The spectra were recorded with a Renishaw RM2000 Raman spectrometer (Gloucestershire, UK). The 514.5 nm excitation line provided by an Ar⁺ laser was exploited [31]. For measurement, the samples were putted in a quartz cell with 1 cm optical path length placed in a macro-sampling accessory with a focalization lens of 15 mm. The laser power on the sample was 2 mW. SERS spectra were baselined to withdraw the contribution of the fluorescence by using the algorithm provided by the Origin 6.0 Program.

2.1.4. Fatty Acids, Organic Acids, and Carbohydrates

For the detection of ester-linked phospholipid fatty acids, each extract was subjected to a mild alkaline trans-methylation procedure to produce fatty acid methyl esters [32]. These were analyzed on an Agilent 6890 GC interfaced with an Agilent 5973N Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA).

Organic acids were identified and classified in each extract as reported in Muscolo et al., (2013) [29], by using a Dionex DX-600 IC system (GP 50 gradient pump, AS 40 autosampler, CD 25 conductivity detector, IonPac AS11-HC analytical column (4 × 250 mm) and IonPac AG11-HC guard column (4 × 50 mm) (Dionex, Sunnyvale, CA, USA). Each peak was identified by retention times of known organic acid standard solutions purchased from SIGMA (ACS reagent 99%) and by spiking each known organic standard individually into each unknown sample.

Carbohydrates were identified in each extract as reported in Muscolo et al. [29], by injecting 50 µL of each sample into the Dionex IC system (autosampler AS 50, gradient pump GP 40 and electrochemical detector ED 40 with a thin-layer type amperometric cell). The analytes were detected with a quadrupole-potential waveform on a gold electrode.

2.2. In Vitro Detection of Biological Activity of Humic Substances and Water Soluble Soil Phenols

2.2.1. Callus Growth, Antioxidant Compounds and Antioxidant Activity

To commence, 35-day-old *Pinus laricio* callus tissue (1 g) [29] was moved for 28 days to the basal PGR-free medium (control) or medium with HSs or SWSP or plant growth regulators (PGRs: 2,4-dichlorophenoxyacetic acid, 2,4-D, 0.5 mg L⁻¹ + 6-benzylaminopurine, BAP, 0.25 mg L⁻¹). Each filter-sterilized soil extract was used at 1 mg C L⁻¹. The concentrations of soil extracts and hormones were selected on the basis of laboratory experiments (data not shown) and previous works evidenced that they induced better callus growth and performance. All treatments were at 25 °C in the dark [33–35]. Callus growth was determined as fresh weight at the end of the growth period (28 days). Data are the means of five replicates. After weighing, the mean relative growth rate (RGR) was calculated as

$$RGR = \ln \frac{(FW2 - FW1)}{t2 - t1} \quad (1)$$

where *FW1* is the fresh weight at the beginning of the measurements, and *FW2* is the fresh weight at the end of the experiments (28 days); *t1* is the time at the beginning of treatments and *t2* at the end of treatments [34].

The total phenolic (TP) content was determined colorimetrically, by using the Folin–Ciocalteu reagent diluted with distilled water (1:10 *v/v*) [34,35]. The optical density (OD) was measured at 760 nm. Tannic acid was used as standard. The results were expressed as µg of tannic acid equivalents g⁻¹ FW.

Ascorbic (ASC) acid was determined as reported by Muscolo et al. [36]. Calluses were homogenized with 5% metaphosphoric acid. The ASC activity was measured spectrophotometrically at 525 nm. The content of ascorbic acid was expressed as µg g⁻¹ FW.

For vitamin E analysis, (0.10 g) of callus tissue were extracted with 10 mL of hexane:isopropanol solution (3:2 *v/v*), with agitation for 5 h, and centrifuged at 1330× *g* (3000 rpm) for 10 min. The supernatant was used for the determination of vitamin E following the method of Muscolo et al. [36].

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) concentration was detected following the method of Muscolo et al. [37]. Changes in absorbance were measured at 517 nm after 30 min of incubation at 37 °C. The inhibition (%) of radical scavenging activity was calculated using the following Equation (2):

$$Inhibition (\%) = (A_0 - A_s) / A_0 \times 100 \quad (2)$$

*A*₀ is the absorbance of the control and *A*_s is the absorbance of the sample after 30 min of incubation. DPPH activity was expressed as µM of Trolox using a calibration curve (1.0 to 50 µM).

The ABTS (2,2'-Azinobis-(3-Ethylbenzthiazolin-6-Sulfonic Acid) was performed according to Muscolo et al. [36,37]. The absorbance of the samples was measured at 734 nm. The inhibition (%) of radical scavenging activity was calculated using the Equation (2), where *A*₀ is the absorbance of the control and *A*_s is the absorbance of the sample after 4 min of incubation. Results were expressed as µmol L⁻¹ Trolox equivalents using a Trolox (1–50 µmol L⁻¹) calibration curve.

The oxygen radical absorbance capacity (ORAC) assay was performed according to Dávalos et al. [38] with some modification. A 20 μL aliquot of extract was added to 120 μL of fresh fluorescein solution (117 nmol L^{-1}). After a pre-incubation time of 15 min at 37°C , 60 μL of freshly prepared AAPH solution (40 mmol L^{-1}) was added. Fluorescence was recorded every 30 s for 90 min ($\lambda_{\text{ex}} 485 \text{ nm}$, $\lambda_{\text{em}} 520 \text{ nm}$). The blank was 20 μL of methanol instead of the sample. ORAC values were expressed as $\mu\text{mol Trolox equivalents mg}^{-1} \text{ FW}$ using a 10–100 $\mu\text{mol L}^{-1}$ calibration curve.

2.2.2. Statistical Analysis

Data from all treatments were subjected to analysis of variance (ANOVA) and treatment means were compared using Tukey's test at $p < 0.05$ [39]. Statistical analysis was conducted using the Systat v. 8.0 (SPSS Inc., Evanston, IL, USA) software package.

3. Results and discussion

3.1. Humic Substances and Soil Water Soluble Phenolic Fraction

The FT-IR spectra of unfractionated HSs and soil water soluble fraction (SWSP) and their second-derivative spectra (2d) are shown in Figure 1. The spectroscopic interpretation of the main functional groups was based on previous reports [40,41]. In Table 1 SI the attributions of main functional groups are shown.

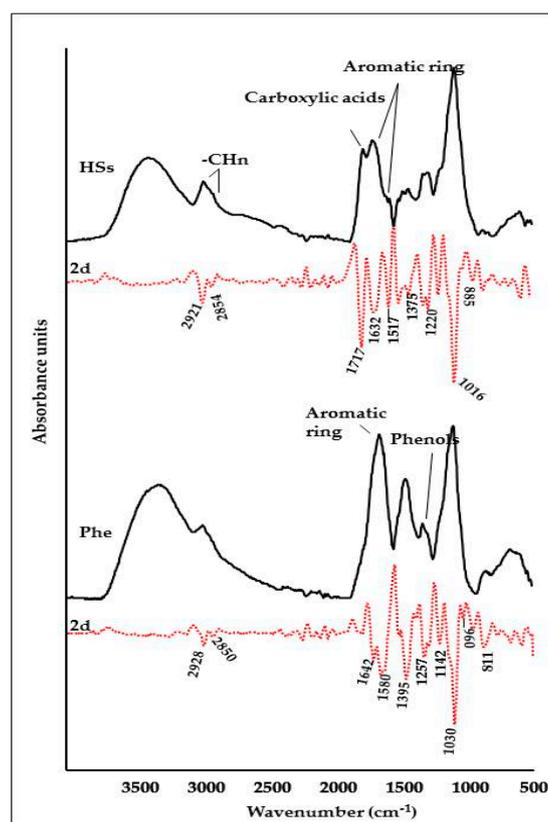


Figure 1. Fourier transform infrared (FT-IR) spectra of humic substances (HSs) and soil water soluble phenolic fraction (SWSP). Dashed lines correspond to second derivative (2d) spectra.

Table 1. The amount of phenolic acids ($\mu\text{g mg}^{-1}$ soil), aldehydes ($\mu\text{g mg}^{-1}$ soil), organic acids ($\mu\text{g mg}^{-1}$ soil), fatty acids ($\mu\text{g mg}^{-1}$ soil), and carbohydrates ($\mu\text{g g}^{-1}$ soil) in humic substances (HSs) and soil water soluble phenolic fraction (SWSP). * Different letters indicate significant differences

Class of Compounds		HSs	SWSP
Phenolic acids	Vanillic acid	0.6 ^{b*}	4.12 ^a
	Benzoic acid	5.1 ^b	21.5 ^a
	Cinnamic acid	0.2 ^a	0.06 ^b
	Salicylic acid	0.6 ^b	11.10 ^a
	Syringic acid	0.6 ^b	53.64 ^a
	Ferulic acid	0.4 ^b	28.3 ^a
	<i>o</i> -Coumaric acid	Nd †	0.30
	<i>m</i> -Coumaric acid	0.2 ^b	0.65 ^a
	<i>p</i> -Coumaric acid	0.2 ^b	2.07 ^a
	Protocatechuic acid	0.4 ^b	11.5 ^a
	Caffeic acid	0.4	Nd
	<i>p</i> -Hydroxybenzoic acid	0.9 ^b	8.4 ^a
	Gentisic acid	Nd	Nd
	Aldehydes	Vanillin	0.4 ^b
Syringaldehyde		Nd	4.1 ^a
Carbohydrates	Arabinose	Nd	0.11
	Glucose	0.02 ^b	0.25 ^a
	Galactose	0.03 ^b	0.41 ^a
	Glucuronic acid	0.01 ^b	0.14 ^a
	Mannose	0.03 ^b	0.37 ^a
	Galacturonic acid	0.01 ^b	0.06 ^a
	Total sugars	0.1 ^b	1.34 ^a
Organic Acids	Citric	Nd	Nd
	Fumaric	Nd	Nd
	Malic	Nd	Nd
	Succinic	Nd	Nd
	Oxalic	Nd	Nd
	Tartaric	77 ^a	33 ^b
	Fatty Acids	Myristic	0.07 ^a
Palmitoleic		0.08 ^a	0.02 ^b
Palmitic		1.07 ^a	0.34 ^b
Linoleic		0.30 ^a	0.2 ^a
Oleic		2.73 ^a	1.38 ^b
Stearic		0.36 ^a	0.13 ^b
Arachic		0.01 ^a	0.03 ^a
Total lipids		15.2 ^a	0.80 ^b

† N.d. = below detection limit.

In general, both spectra showed at high frequencies the vibration of OH groups (4000 and 3000 cm^{-1}), and the asymmetric and symmetric stretching of CH_2 groups (2920 – 2800 cm^{-1}) [40,41]. At middle frequencies appeared the $\nu\text{C}=\text{O}$ vibration of COOH (1717 cm^{-1}), the $\nu\text{C}=\text{O}$ of carboxylate group (1632 cm^{-1}), $\nu\text{C}=\text{C}$ of aromatic rings and N-H deformation of amide II (1600 – 1515 cm^{-1}) [40–43], and C-H deformation and asymmetric stretching of COO^- group (1450 – 1400 cm^{-1}).

The strong peaks between 1150 and 1016 cm^{-1} were assigned to C–O stretching of polysaccharide-like components [40,41] even if a contribution of mineral compounds [44,45] may be considered.

HSs spectrum differed from the phenolic fraction for the presence of the strong bands due to methylenic chains, COOH and C–OH groups probably arising by fatty acids as also supported by the highest concentration in palmitic and oleic acids found in this sample (see Table 1 and Figure 2I,L). Additionally, the band at 1632 cm^{-1} might be due to the carboxyl groups of tartrate (Table 1; Figure 2G,H). This spectrum was also characterized by the band at 1517 cm^{-1} (Figure 1) which might be originated from lignin derivatives such as guaiacyl of softwoods [46].

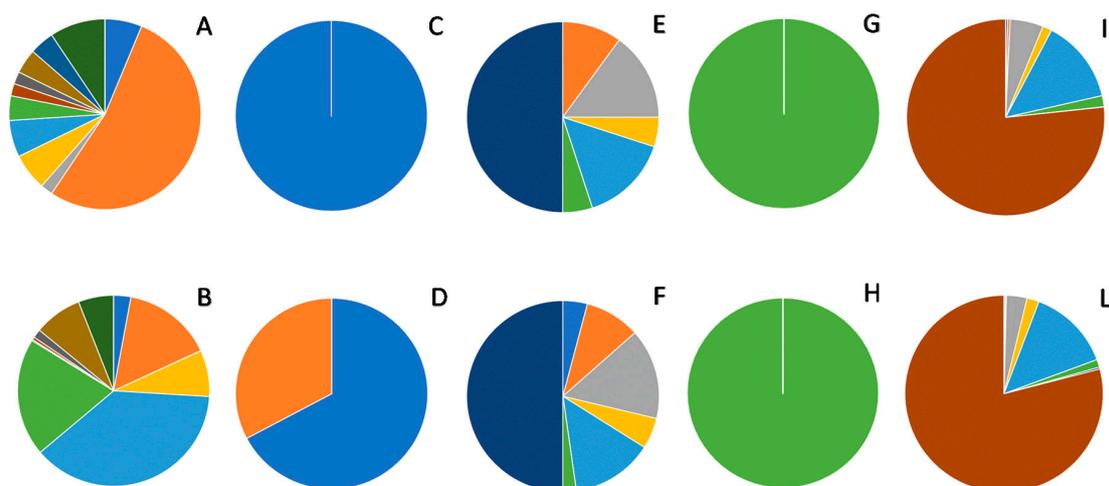


Figure 2. Pie diagrams showing the percentage distribution of selected classes of compounds in humic substances (HSs) (A,C,E,G,I) and soil water soluble phenolics (SWSP) (B,D,F,H,L) at a glance. (A,B) Phenolic acids: from benzoic (orange) to right-handed cinnamic (grey), salicylic (yellow), syringic (heavenly), ferulic (green), o-coumaric (blue), m-coumaric (red), p-coumaric (dark gray), protocatechuic (brown), caffeic (blue), p-hydroxybenzoic (dark green), gentisic (light blue); (C,D) Aldehydes: vanillin (heavenly), syringaldehyde (orange); (E,F) Carbohydrates: from arabinose (blue) to right-handed glucose (orange), galactose (grey), glucuronic acid (yellow), mannose (heavenly), galacturonic acid (green), total sugars (dark blue); (G,H) Organic acids: tartaric acid (green); (I,L) Fatty acids: myristic (blue, not visible in the pie diagram), palmitoleic (orange, not visible), palmitic (grey), linoleic (yellow), oleic (heavenly), stearic (green), arachidic (dark blue, not visible), total lipids (brown). From this figure one can see at a glance how the phenolic composition differs well in its pattern between HSs and SWSP. In the former there is a clear prevalence of benzoic (orange), while in the latter approximately 75% of the total is given by syringic (heavenly), ferulic (green) and benzoic (orange). Carbohydrates and lipids, while showing different absolute values, give instead a similar percentage composition.

In SWSP fraction the methylenic chains were not so dominant with respect to HSs. This could be justified by the low concentration of fatty acids found in this sample (Table 1; Figure 2I,L). Instead, the highest concentration in benzoic acids such as syringic and ferulic acids (Table 1; Figure 2A,B) might be justified by the strong signal observed at 1600 cm^{-1} (Figure 1).

The presence of bands appearing in the SERS spectrum of SWSP fraction (Figure 3) revealed the existence of a mixture of compounds as also corroborated in Table 1. Overall, the SERS spectrum of SWSP was better resolved than the FT-IR spectrum. (Figure 1). In particular, the SERS spectrum was dominated by two intense bands at 1590 cm^{-1} and 1320 cm^{-1} which have been attributed to the C = C stretching vibration of aromatic rings, and the C–O stretching of C–O in phenolic moieties (Figure 3). Indeed, these are the most probable structures that can be found in the SWSP fraction and can be attributed to the simplest phenolic molecules, i.e., syringic, protocatechuic and salicylic acids (Table 1; Figure 2A,B) as found elsewhere [47,48]. Other bands appearing at $1500\text{--}1400\text{ cm}^{-1}$ are also due to $-\text{OCH}_3$ groups and ring stretching of the latter compounds. The band seen at $1610\text{--}20\text{ cm}^{-1}$ can be attributed to the C = C bond of isoferulic acid. However, the contribution of isoferulic acid in the spectrum is lower, despite its relative high concentration in the SWSP fraction (Table 1). This is attributed to its lower affinity to link the surface. Another band in the $1390\text{--}1420\text{ cm}^{-1}$ can be attributed to the carboxylate symmetric stretching from benzoic acid and other fatty acids present in the SWSP fraction (Table 1; Figure 2A,B,I,L).

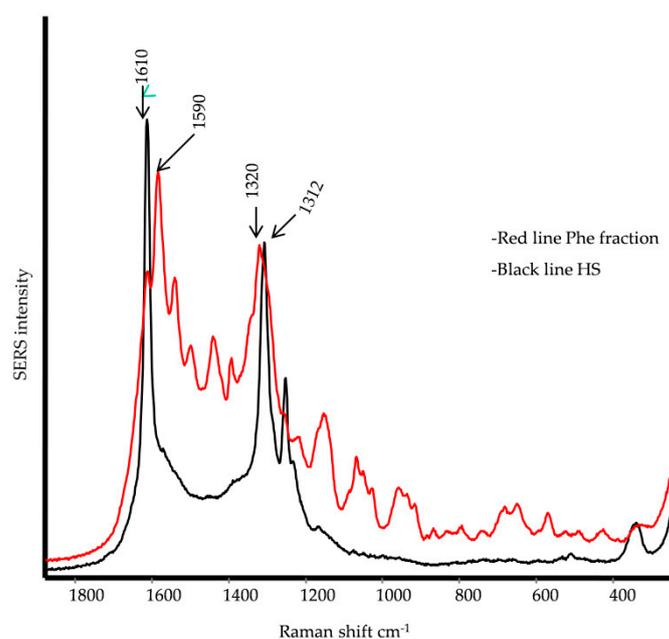


Figure 3. Surface-enhanced Raman scattering (SERS) spectra of soil water soluble phenolic (SWSP) fraction (red line) and humic substances (HSs).

The SERS spectrum of HSs was dominated by two main bands appearing at 1610 and 1312 cm^{-1} (Figure 3) associated with ring stretching of polycyclic aromatic compounds [48–50]. Other less intense bands appeared at 1250 and 320 cm^{-1} . All these bands are characteristic features of highly aromatic HSs [40,49]. Even in this case the SERS spectrum of the HSs differed from that of the FT-IR, which instead was much richer in oxygenated or polar functional groups.

3.2. Callus Growth and Metabolism

The biomass (Table 2) of control, after 28 d of subculture, was 2.55 g. Hormones (2,4-D+BAP) significantly increased callus biomass of 50% compared to control. Callus grown with humic substance always increased its weight compared to control [29]. In presence of soil water soluble phenolic fraction, callus biomass was comparable to control [7]; relative growth rate where higher in the early stage of growth (15 days) for HS treated callus and hormones compared to control and SWSP treated ones; all the calluses appeared white and in good health.

Table 2. Biomass (g) of *Pinus laricio* callus after 28 d of subculture in presence of hormones or humic substances (HSs) or soil water soluble phenolic fraction (SWSP) at 1.0 mg C L⁻¹.

Treatments	Biomass (g f.w ⁻¹)
Control	2.55 ± 0.6 c*
2,4-D+BAP	5.10 ± 0.2 a
HSs	4.30 ± 0.3 b
SWSP	2.64 ± 0.7 c

Data represent the mean ± SE (n = 5). * Different letters indicate significant differences at $p < 0.05$ [34].

Total carbohydrates (Table 3) decreased in callus grown with HSs and much more with hormones in respect to SWSP and control, suggesting that the major biomass observed in presences of hormones and HSs could be due to a greater utilize of carbohydrates, which were utilized to produce energy for the anabolic processes [18]. Sucrose, glucose, and fructose contents were in fact lower in HSs and hormones than in control and SWSP confirming the above statement (Table 3).

Table 3. Total carbohydrates, sucrose, glucose, fructose (mg mL⁻¹) content in *Pinus laricio* callus grown for 28 d with hormones or humic substances (HSs) or soil water soluble phenolic fraction (SWSP).

Treatments	Total Carbohydrates	Sucrose	Glucose	Fructose
Control	20 ± 1.0 a*	0.33 ± 0.03 c	4.14 ± 0.3 b	10.90 ± 2 a
2,4-D+BAP	14 ± 3 b	0.33 ± 0.02 c	1.46 ± 0.4 d	2.95 ± 0.5 d
HSs	16 ± 2 b	0.48 ± 0.06 b	2.50 ± 0.5 c	3.55 ± 0.7 d
SWSP	23 ± 2 a	5.25 ± 2.00 a	4.70 ± 0.2 a	8.80 ± 3 a

Data represent the mean ± SE ($n = 5$). * Different letter indicates significant differences $p < 0.05$ [34].

Callus, being a tissue devoid of photosynthesizing cells, utilizes the products of sucrose cleavage as carbon source [51]. Glucose and fructose are generally converted to hexose phosphates and enter into respiratory pathway to provide substrates and reducing power necessary for growth. Our data evidenced the positive effects of HS compared to SWSP on growth through a stimulation of carbohydrate turnover and an increase in total proteins (Tables 3 and 4) [29].

Table 4. *Pinus laricio* callus grown parameters after 28 days of growth on hormones free culture medium (control), with hormones (2,4D-BAP) or humic substances (HSs) or soil water soluble phenolic fraction (SWSP): total protein (Prot, mg g⁻¹ DW), phenols (TP, mg g⁻¹ DW), vitamin C (Vit. C, mg 100 g⁻¹ DW), and vitamin E (Vit. E, µg g⁻¹ DW).

Treatments	Prot	TP	Vit. C	Vit. E
Control	1.24 ± 0.3 c*	1.54 ± 0.1 b	6.2 ± 2 c	2.9 ± 1 d
2,4-D+BAP	1.87 ± 0.2 a	1.13 ± 0.2 c	11.4 ± 2 b	12 ± 3 b
HSs	2.11 ± 0.2 a	1.19 ± 0.3 c	16.5 ± 2 a	20 ± 2 a
SWSP	0.99 ± 0.3 c	2.99 ± 0.2 a	6.1 ± 1 c	6.8 ± 2 c

Data represent the mean ± SE ($n = 3$). * Different letter indicates significant differences at $p < 0.05$ [34].

Callus biomass was highly and negatively correlated with glucose content ($r = -0.838$, $p < 0.001$) (Table 5), as well as fructose. Our data perfectly agree with results of Borisjuk et al. [52], evidencing that when the hexoses were low, cell division and differentiation were promoted, and processes such as cell elongation and storage of proteins were stimulated. Proteins were in fact positively correlated to callus biomass.

Considering that the growing process needs proteins the lowest amount of proteins found in callus grown with SWSP suggested a slowed down of callus metabolism. Total phenols (TP) in callus increased more with SWSP than HS, hormones, or control (Table 4), suggesting that callus tissue was suffering in presence of phenols. Phenols generally increases in plants when stress conditions occur as defensive mechanism to cope with multiple stresses [35]. Phenols play an important role in several physiological processes improving plant's defense mechanism with their antioxidant properties. Conversely, vitamin C and E were instead higher in HS and hormones than SWSP and control (Tables 4 and 5) suggesting that callus used these two vitamins to scavenge the oxygen reactive species produced during the metabolic processes that support the increased callus growth.

Correlation matrix analysis (Table 5) confirmed the above statement showing that callus biomass resulted to have a good positive relationship with vitamin C ($r = 0.77$, $p < 0.01$) and E ($r = 0.73$, $p < 0.01$). Vitamin C is able to scavenge a lot of free radicals, not only because it donates reducing equivalents but also for the relative stability of the derived monodehydroascorbate radical. In part, well-known for its antioxidant properties, vitamin C is also a cofactor for numerous enzymes involved in plant metabolism, in particular in numerous reactions of anabolic process.

Table 5. Correlation matrix for the chemical variables.

	T Carb	Sucr	Glu	Fru	Prot	TP	Vit. C	Vit. E	DPPH	ORAC	ABTS
Biomass	−0.674 *	−0.371	−0.838 ***	−0.685 *	0.887 ***	−0.574 *	0.772 **	0.732 **	−0.744 **	−0.921 ***	−0.815 ***
T Carb		0.733 **	0.953 ***	0.823 ***	−0.585 *	0.875 ***	−0.514 *	−0.451	0.875 ***	0.885 ***	0.933 ***
Sucr			0.632 *	0.437	−0.501	0.927 ***	−0.413	−0.226	0.873 ***	0.603 *	0.563 *
Glu				0.865 ***	−0.703 **	0.814 ***	−0.610 *	−0.570 *	0.883 ***	0.971 ***	0.990 ***
Fru					−0.577 *	0.568 *	−0.646 *	−0.691 **	0.636 *	0.864 ***	0.889 ***
Prot						−0.656 *	0.945 ***	0.867 ***	−0.771 **	−0.836 ***	−0.626 *
TP							−0.541 *	−0.396	0.965 ***	0.777 **	0.740 **
Vit. C								0.967 ***	−0.635 *	−0.762 **	−0.542 *
Vit. E									−0.497	−0.718 **	−0.524
DPPH										0.882 ***	0.818 ***
ORAC											0.949 ***

*, **, *** significant at ≤ 0.05 , ≤ 0.01 and ≤ 0.001 probability levels respectively; grayscale, negative correlations; green scale, positive correlations. T Carb = total carbohydrate; Sucr = sucrose; Glu = glucose; Fru = fructose; Prot = protein; TP = total phenols; Vit. C = vitamin C; Vit. E = vitamin E; DPPH = 2,2-diphenyl-1-picryl-hydrazyl-hydrate activity; ORAC = oxygen radical absorbance capacity; ABTS = 2,2'-azinobis(3-ethylbenzothiazoline 6-sulfonic acid).

Vitamin E is an important secondary metabolite, lipid soluble antioxidant, which enhances to protect plant tissues from oxidative stress. From the results obtained appeared that HS and hormones activated the same antioxidative pathway, stimulating the production of vitamin C and E, as protective action during the intense metabolic activities. Regarding the antioxidant activities (Table 6), control and SWSP treated calluses showed the highest antioxidant capacity performed as DPPH, ABTS, and ORAC. Callus biomass appeared in fact strongly and negatively correlated with ABTS ($r = -0.815$, $p < 0.001$), ORAC ($r = -0.921$, $p < 0.001$), and DPPH ($r = -0.744$, $p < 0.01$) (Table 5). There was also a close relationship between DPPH and ORAC ($r = 0.882$, $p < 0.001$) and ABTS ($r = 0.818$, $p < 0.001$); and between ORAC and ABTS ($r = 0.949$, $p < 0.001$).

Table 6. 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH, % inhibition), oxygen radical absorbance capacity (ORAC, μm of trolox equivalent g^{-1} fresh weight), 2,2-Azino-di-3-ethylbenzthiazoline sulfonate (ABTS, μm of trolox equivalent g^{-1} fresh weight).

Treatments	DPPH	ORAC	ABTS
Control	100 b*	1890 \pm 7 b	21 \pm 2 a
2,4-D+BAP	67 d	1130 \pm 9 d	5.4 \pm 2 c
HS	80 c	1319 \pm 12 c	12.5 \pm 3 b
WP	150 a	1999 \pm 6 a	22 \pm 2 a

Data represent the mean \pm SE ($n = 3$). * Different letter indicates significant differences at $p < 0.05$ [34].

The major increase in antioxidant activities in presence of SWSP, demonstrated the need of calluses to overcome the stress caused by exogenous phenolic compounds. In short, these results evidenced a different biological effect between HS and phenols, even if phenols are an active component and precursor of HS [42]. In presence of phenolic compounds, calluses shifted their metabolism from primary to secondary decreasing protein synthesis and activating the production of phenols as secondary metabolites and antioxidant enzymes. HS instead, as hormones normally do, stimulated the primary metabolism with a consequent growth of calluses, and the production of vitamin C and E as scavengers to intervene in the mitigation of oxidative stress due to the increased metabolism, which in turn led to a major production of biomass [29]. The increase in vitamin C and E contributed to naturally improving the nutritional value of calluses. The two pools of organic matter differently behaved for their different composition. The HSs rich in tartaric and fatty acids and consequently in COOH and C-OH groups had the greatest stimulatory effects on metabolism. It is well known that organic acids positively affect plant growth increasing shoot and root biomass as already demonstrated [53]. Additionally, Mei et al. [54] demonstrated in an in vitro study that the level of fatty acids was correlated with Arabidopsis growth. Conversely, the negative effects observed in presence of phenolic compounds can be ascribed to their well-known allelopathic effects, which affect photosynthesis, cell division and elongation, membrane fluidity, protein biosynthesis, and the activities of many enzymes causing failure in growth [55].

4. Conclusions

In short, it is emerged that the effects of soil organic matter change on the basis of the fractions that prevail in its composition. The prevalence of the more stable fraction (HS), and of the many compounds in it found, evidenced by using different analytical techniques, showed a more effectiveness of HS in callus growth promoting, in improving also its quality in terms of proteins and vitamins. Many compounds found in HS have been proposed as plant growth promoters and their beneficial effects on plant growth have been attributed on the basis of their mode of action. Mainly, organic acids, in particular tartaric acid, and unsaturated fatty acids have been in fact categorized in the list of biostimulants. They are reported to raise the level of free amino acids, protein, carbohydrates, phenolic compounds, pigment, and various enzymes in plants, improving the quality. Distinguishing the effects

of the two main SOM fractions, can be useful to predict the role of SOM on the biological fertility but also on plant quality.

Author Contributions: Conceptualization, A.M. and S.N.; methodology, A.M., O.F., and S.S.C.; software, D.P.; validation, D.P., formal analysis, A.M., O.F., and S.S.C.; resources, A.M., O.F., S.S.C., and S.N.; writing—original draft preparation, A.M., O.F., and S.N.; funding acquisition, O.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding

Conflicts of Interest: The authors declare no conflict of interest.

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