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REGULAR ARTICLE

Humic-like bioactivity on emergence and early growth of maize (*Zea mays* L.) of water-soluble lignins isolated from biomass for energy

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Abstract
Background and Aims Lignin of lignocellulosic residues from biomass for energy can be exploited in sustainable agriculture as plant stimulants. Lignin monomers or their microbial bioproducts are mainly responsible for the plant growth promotion exerted by humic matter in soil. The aim of this work was to verify the humic-like bioactivity of water-soluble lignin isolated from biomass for energy towards plant growth and relate the biostimulation to the lignin molecular structure.
Methods Two water-soluble lignins isolated from giant reed (AD) and miscanthus (MG) were characterized for molecular composition by ^{1}H and ^{31}P 1D-, ^{13}C-^{1}H 2D-, DOSY-NMR spectroscopy and for conformational structure by size-exclusion chromatography. The effect of different aqueous concentrations of lignin on germination of maize seeds and growth of maize plantlets was assessed in growth-chamber experiments.
Results Both lignins showed humic-like supramolecular structures, but different conformational stability and molecular composition. Their largest bioactivity was revealed at 10 and 50 ppm of lignin organic carbon and both significantly increased length of radicles, lateral seminal roots, and coleoptiles of maize seedlings, as well as total shoot and root dry weights and root length of maize plantlets. However, differences in AD and MG bioactivity were attributed to their conformational stabilities and content of amphiphilic molecules, which may control both the adhesion to plant roots and the release of bioactive molecules upon interactions with plant-exuded organic acids.
Conclusions The humic-like bioactivity of water-soluble lignins indicated that lignocellulosic residues from energy crops may be profitably recycled in agriculture as effective plant growth promoters, thereby increasing the economic and environmental sustainability of energy production from non-food biomasses.

Keywords Plant growth promoters · Water-soluble lignins · Humic-like matter · Biomass for energy · Maize seed germination and growth · NMR · HPSEC

Introduction

Intensive tillage and monocultural practices negatively impact on soil organic matter content (Bayer et al. 2000) and, consequently, soil fertility (Chendev et al. 2015), while excessive nitrogen fertilization often results in enhanced plant susceptibility to pathogens and waters contamination (Liebman and Davis 2000). Among the practices

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proposed for sustainable intensification in agriculture, there is an increasing use of plant growth promoters based on either recycled organic matter (Al Chami et al. 2014) or endophytic microorganisms (Qiao et al. 2014), due their capacity to improve plants physiological processes (du Jardin 2012). Various natural complex materials may be classified as plant biostimulants, such as agro-industrial residues, compost, protein hydrolysates (Pecha et al. 2011) and biomass extracts (Ertani et al. 2015). They can be applied to soils to increase soil organic matter content, promote rhizobacterial activity, and provide plant nutrients (Kamble and Bhattacharyulu 2014).

Humic substances (HS) extracted from complex natural sources such as compost, lignites, and peat have been also extensively studied for their bioactivity. HS are ubiquitous in the environment and have been recently recognized to be supramolecular associations of relatively small and heterogeneous molecules, produced during the biotic and abiotic transformation of biological tissues (Piccolo 2002). HS are known to stimulate plant development and productivity (Canellas and Olivares 2014; Vaccaro et al. 2015) and alleviate biotic and abiotic stresses through different biochemical mechanisms (Kesba and El-Betagi 2012; García et al. 2012).

Humic-like materials are largely produced in or derived from paper mills industries (Restolho et al. 2009). Lignosulfonates-humates were tested on maize (*Zea mays*, L.) development and found to enhance plant physiological activity inasmuch as HS (Ertani et al. 2011a). Similarly, mixtures of phenolic residues from milling of spruce bark (*Picea abies*, L.) enhanced the development of tomato (*Lycopersicon esculentum*, L.) plantlets (Bălaş and Popa 2007). Paper mills are included in the more general concept of integrated biorefinery (González-García et al. 2001), whereby lignocellulosic biomasses become a source of lignin molecules after separation from cellulose (Cherubini 2010; Carvalho-Netto et al. 2014). Despite their similarity to humic-like materials and potential role as plant growth promoters, application of lignin residues in agriculture is still neglected. In order to obtain potentially bioactive plant growth promoters, water-soluble lignin was isolated by an oxidative alkaline extraction from biomass for energy (Savy and Piccolo 2014; Savy et al. 2015a, b). These extracting conditions not only well separated insoluble cellulose from water-soluble lignin residues, but also concomitantly depolymerised the lignin macromolecule and introduced new hydrophilic carboxyl and hydroxyl functions, thereby nearing the lignin structural features to those of bioactive humified organic matter.

The aim of this work was then to evaluate the biological activity of water-soluble lignins isolated from the non-food miscanthus and giant reed biomasses on the germination and early growth of maize (*Zea mays*, L.) seedlings, and relate their activity to the lignins molecular and conformational characteristics .

Materials and methods

Biomass

Miscanthus (*Miscanthus* χ *Giganteus* Greef et Deuter, MG) was provided by Phytatec Ltd (UK), after the February 2007 harvest in Aberystwyth, Wales, UK. Giant reed (*Arundo donax* L., AD) was cropped on January 2010 in the experimental farm of the University of Naples Federico II located near Salerno, Italy.

Alkaline oxidative hydrolysis

The alkaline oxidative isolation method has been described elsewhere (Savy and Piccolo 2014; Savy et al. 2015a, b). Briefly, 5.0 g of each lignocellulosic sample were suspended in a 150 mL of distilled water containing a 2 % H_2O_2 (v/v) solution. The mixture pH was then raised to 11.5 with a 4 M KOH solution. After stirring at 323 K overnight, the mixture was centrifuged (15,400 RCF × 20 min) and the supernatant, containing lignin and hemicellulose, was separated from cellulose by paper filtration. The pH of supernatant (containing lignin and hemicellulose) was lowered to 5.5 with HCl 5 %, and a volume of ethanol equal to three times the total supernatant volume was added to the solution to flocculate the hemicellulose, that was further separated by paper filtration. The ethanol in the filtrate was evaporated under *vacuum* and the resulting oxidized lignins from giant reed (AD) and miscanthus (MG) were dialysed and freeze-dried. Lignins

elemental composition was determined by an EA 1108 Elemental Analyzer (Fisons Instruments).

Synthesis of 2-chloro-4,4,5,5-tetramethyldioxaphospholane

The derivatizing phosphorous reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane was synthesized as described by Hatzakis et al. (2010). Briefly, the phosphorous reagent was obtained by mixing the following two solutions: solution (A) was prepared by dissolving 21.5 mL PCl_3 in 180 mL of dry *n*-hexane placed in a 250 mL three-necked round flask equipped with a condenser. Solution (B) was prepared by dissolving 23.7 g pinacol in a mixture of 32 mL of dry pyridine, and 150 mL of dry *n*-hexane placed in a conic flask. Solution (B) was added drop-wise to solution (A) using an addition funnel properly adjusted to the second neck of the round flask. The addition lasted 1 h under strong stirring on an ice bath, and then the mixture was left for 1 h at room temperature to complete reaction. The solution was filtered on a filter paper, while the whitish residue on the filter was rinsed with 2×100 mL of *n*-hexane and the filtrate was evaporated under *vacuum* at 30–35 °C. Finally, the phosphalane product was separated from solution by *vacuum* distillation (b.p. 76–78 °C at 4 mbar).

Lignin derivatization prior to ^{31}P-NMR spectroscopy

The amount and type of hydroxyl (OH) groups in the extracts determine the reactivity of the water-soluble lignins. Thus, they were first derivatized with a P containing compound and then ^{31}P-NMR spectra were used for their quantitative evaluation. A stock solution was made by adding, to a pyridine and deuterated chloroform solution (1.6/1 v/v), 2.92 mg mL^{-1} cyclohexanol, 10.0 mg mL^{-1} of triphenyl phosphate (TPP), as internal standard and reference peak for the ^{31}P frequency axis calibration, respectively, and 0.6 mg mL^{-1} of chromium (III) acetylacetonate as relaxation agent. Lignin samples (7.0 mg) were dissolved in 750 μL of the stock solution, and added with 50 μL of the previously synthesized 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxa-phospholane as ^{31}P derivatization agent. The NMR tube was ultrasonicated for 1 min prior to loading into the NMR magnet.

High Performance Size Exclusion Chromatography (HPSEC)

The HPSEC system was composed by a Shimadzu LC-10-AD pump equipped with a Rheodyne rotary injector and 100-mL sample loop with a UV detector (Perkin e Elmer LC295, set at 280 nm). The chromatographic column was a 300×7.80 mm Biosep SEC s2000 (Phenomenex, USA), preceded by a 35×7.80 mm Biosep S-2000 pre-column (Phenomenex, USA) and a 2 mm inlet filter. The eluent was a 0.1 mol L^{-1} NaH_2PO_4 solution, buffered at pH 6.5 with 0.1 mol L^{-1} NaOH, and added with NaN_3 up to 0.3 g L^{-1}. The eluent solution was also used to dissolve lignin at a concentration of 0.6 g L^{-1}. Another series of similarly prepared lignin solutions were added with glacial acetic acid (AcOH) to lower the pH down to 3.5 before injection into the HPSEC system. The elution profile before and after AcOH addition is a function of the stability of the lignin conformations (Piccolo et al. 1996; Piccolo 2002). All solutions were filtered through 0.45 μm Millipore filter prior to the chromatographic analyses. The elution flow rate was set to 0.6 mL min^{-1}. A Unipoint Gilson Software was used to record and elaborate chromatograms. The column calibration was obtained with the following sodium polystyrene sulfonates of known molecular masses: 123,000, 16,900, 6780 and 1200 Da. Ferulic acid (194 Da) and catechol (110 Da) were used as low molecular weight standards.

Nuclear Magnetic Resonance (NMR) Spectroscopy

A 400 MHz Bruker Avance spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm Bruker Inverse Broad Band (BBI) probe, working at ^{1}H and ^{13}C frequencies of 400.13 and 100.62 MHz, was employed to conduct liquid-state NMR measurements at a temperature of 298 ± 1 K. About 10 mg mL^{-1} of lignin samples were dissolved in 750 μL of deuterated Dimethyl Sulfoxide (DMSO) and placed into a NMR tube with 250 μL of a 0.05 mg mL^{-1} solution of a 3-(trimethylsilyl) propionic acid sodium salt standard (Merck, Darmstadt, Germany), in order to allow a semi-quantitative analysis of lignin components. ^{1}H NMR spectra were acquired by setting 2 s of recycle delay, a 90° pulse length of 12.45 μs, 32,768 time domain points, and 64 transients. The water signal ranging within 3.3–3.5 ppm was suppressed through on-

resonance presaturation technique (2 s current wave at a power level of 58 dB).

2D hetero-nuclear experiments, such as HSQC (Heteronuclear Single Quantum Coherence) and HMBC (Heteronuclear Multiple bond Correlation), were performed to identify the ^{1}H-^{13}C correlations and assign the most intense NMR signals detected in samples. Specifically, HSQC experiment was acquired by optimizing the acquisition parameters according to a J_{CH} short- range coupling value of 145 Hz and including an 80 μs length (15.6 dB power level) Waltz16 decoupling scheme. HMBC experiment, which was composed by a low-pass J-filter to suppress one-bond correlations and gradient pulses for coherence selection, was optimized as a function of a 6 Hz long-range coupling.

^{1}H DOSY (Diffusion Ordered SpectroscopY) NMR experiments were conducted by choosing a stimulated echo pulse sequence with bipolar gradients, combined with two spoil gradients and an eddy current delay. This sequence reduced signals loss due to short spin-spin relaxation times. The acquisition was conducted by setting 1400 μs long sine-shaped gradients (δ), that linearly ranged from 0.674 to 32.030 G cm^{-1} in 32 increments, and selecting a diffusion delay of 0.1 s (Δ) between encoding and decoding gradients. DOSY experiments consisted in a recycle delay of 2 s, 4096 points, a spectral width of 14 ppm (5592.8 Hz), 64 scans and 4 dummy scans. DOSY spectra were processed by Topspin software (v.3.1, Bruker Biospin, Rheinstetten, Germany). The Fourier Transform of FIDs was conducted by applying a 2-fold zero filling in F2 dimension and multiplying by a 2 Hz exponential function.

^{31}P-NMR spectra were acquired on samples derivatized with phospholane by applying an inverse gated pulse sequence including a 80 μs length (15.6 dB power level) Waltz16 scheme to decouple phosphorous from proton nuclei. In particular, spectra consisted in a 45° pulse length of 5.25 μs, a spectral width of 400 ppm (64,935.066 Hz), 10 s of recycle delay, 1600 transients, 8 dummy scans and 129,862 time domain points.

All spectra were processed by using both Bruker Topspin Software (v.2.1, Bruker Biospin, Rheinstetten, Germany) and MestReC NMR Processing Software (v.4.8.6.0, Cambridgesoft, Cambridge, Massachusetts, USA). Zero filling was applied during Fourier transform of free induction decays (FIDs).

Germination and seedling emergence

Maize (*Zea mays*, L. cv 3321 Limagrain) seeds were soaked in tap water overnight and fifteen (15) seeds were deposited for each replicate on round filter paper placed in a petri dish. The experiment was run in five replicates. The filters were moistened with 15 mL of aqueous solution of lignin extracts at a concentration of 0, 1, 10, 10 and 100 mg C L^{-1}. Seeds were germinated in the dark at 25 ° C for 96 h and, thereafter, were measured for length of coleoptile, radicle and lateral seminal roots. The seeds that had a disrupted testa and a radicle longer than 1 mm were considered for the evaluation of germination percentage.

Plant growth

Maize seeds were germinated in the dark at 25 °C for 4 days and then the seedlings (fifteen for each treatment) were placed in plastic tubes filled with perlite as inert solid substrate, and treated with 30 mL of a modified Hoagland solution (Hoagland and Arnon 1950) composed as it follows: 40 μM KH_2PO_4, 200 μM $Ca(NO_3)_2$, 200 μM KNO_3, 200 μM $MgSO_4$, 10 μM FeNaEDTA, 4.6 μM H_3BO_3, 0.036 μM $CuCl_2 \cdot 2H_2O$, 0.9 μM $MnCl_2 \cdot 4H_2O$, 0.09 μM $ZnCl_2$, 0.01 μM $NaMoO_3 \cdot 2H_2O$. Tubes with seedlings were placed in a climate chamber that maintained 16 h of light per day, air temperature at 26 °C and relative humidity at 50 %. After 8 days from transplanting, fifteen (15) mL of the water-soluble lignin from either AD or MG at the carbon concentrations of 0 (control), 1, 10, 50 and 100 mg C L^{-1} were added to the growing seedlings. After 96 h, plants were harvested and the plant total dry weight, dry shoot weight, dry root weight, and root length were determined. Plantlet roots from both germination and growth treatments were scanned with an Epson Perfection V700 modified flatbed scanner and length measurements were obtained by using the WinRhizo software, version 2012b (Regent Instruments, Inc.).

Statistical analysis

The percentage of germination was evaluated by the chi-square test, and all data from growth experiment were found to be normally distributed. The One-Way ANOVA and Tukey's range tests were used to

compare means among treatments. All statistical elaboration was conducted by using SPSS, version 21 (IBM SPPS Statistics), and significant difference was set at a 95 % confidence.

Results

Extraction yields and elemental analysis

The lignin extracted from the AD and MG biomasses with alkaline hydrogen peroxide was 5.1 and the 6.0 % of the initial biomass weight, respectively, as earlier reported by Savy and Piccolo (2014). The percent of elemental C, H, and N was 53.8, 5.5, 0.6 in Mg, and 50.9, 3.1, 0.4 in AD, respectively, whereas the oxygen content was calculated by difference as 33.4 % for MG and 42.3 % for AD (Supporting Material Table SM-1). The standard deviation for all elemental values was < 5 %.

High Performance Size Exclusion Chromatography (HPSEC)

The HPSEC profiles of lignins injected before and after addition of acetic acid (AcOH) to lower the solution pH to 3.5, are shown in Fig. 1, while the resulting nominal weight-averaged (M_w), number-averaged (M_n) molecular weights, and polydispersity (P) are reported in Table 1. Both substrates showed a similar chromatographic profile before AcOH addition, consisting in a diffuse absorption in the 20–29 min interval and in two further peaks at 30 and 32 min (Fig. 1 I and III). However, while the full chromatogram showed similar M_w, M_n and P values (Table 1), these parameters for the first diffuse absorption were larger for AD than for MG (Fig. 1a), while those for B and C intervals were similar in both lignins. Conversely, after AcOH addition to the same lignin solutions prior to HPSEC analysis, the M_w, M_n and P values for AD remained larger than for MG (Table 1) when calculated for both the full chromatograms and the single peak absorptions, despite the apparent chromatographic similarity (Fig. 1 II and IV). This behaviour suggested that AD had a more heterogeneous molecular composition than MG and a greater capacity of molecular aggregation into apparently larger molecular size.

NMR spectra

The ^{1}H-NMR spectra of lignins are shown in Fig. 2, while assignment of main signals is reported in Table 2. The resonances between 0.91 and 1.68 ppm were attributed to aliphatic protons, whereas the signal at 3.73 ppm

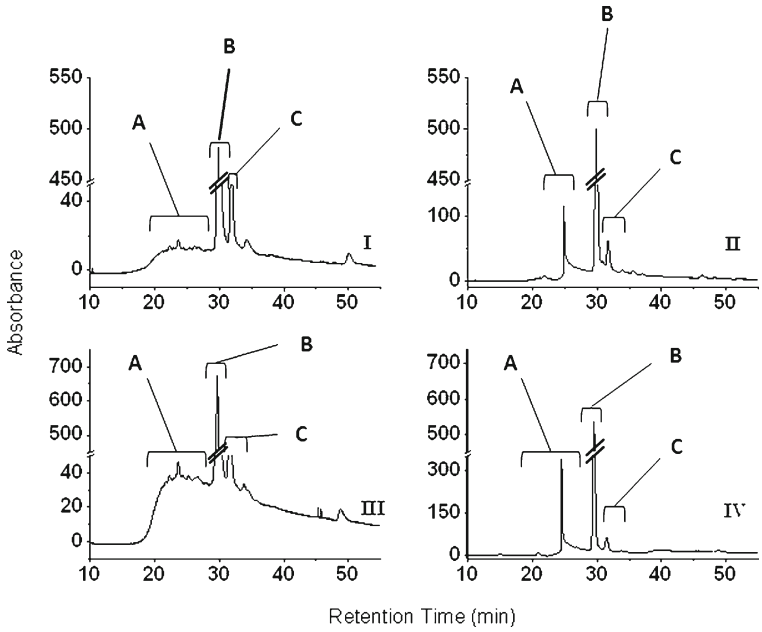

Fig. 1 UV-detected HPSEC chromatograms for water-soluble lignin isolated from giant reed (AD) and miscanthus (MG) with or without acetic acid. I: AD without acetic acid; II: AD with the addition of acetic acid until pH 3.5; III: MG without acetic acid; IV: MG with the addition of acetic acid until pH 3.5

Table 1 Weight-average (Mw), number-average (Mn) molecular weights and polydispersity (P) for different elution time (min) intervals related to full UV-detected chromatogram and single absorption bands (A, B, and C in Fig. 2) for lignin extracted from giant reed (AD) and miscanthus (MG) before and after addition of acetic acid (AcOH). Standard deviation was < 5 %

Elution time	Mw AD	Mn	P	Mw MG	Mn	P
20–32	391	77	5.0	408	78	5.2
20–29 (A)	1547	661	2.3	1344	699	1.9
30 (B)	141	139	1.0	152	150	1.0
32 (C)	75	74	1.0	74	71	1.0
	+ AcOH			+ AcOH		
24–32	270	97	2.8	168	93	1.8
24.5 (A)	852	583	1.5	608	510	1.2
30 (B)	185	183	1.0	157	156	1.0
32 (C)	94	91	1.0	79	76	1.0

was assigned to protons in methoxyl groups (Lundquist 1992). Moreover, the 4.10-5.88 ppm range was attributed to hydroxyalkyl molecules, such as carbohydrates and lignin side chains (Savy and Piccolo 2014; Savy et al.,

2015), while signals between 6.3 and 7.8 ppm were assigned to aromatic compounds (Lundquist 1992). Integration of NMR signals (Table 2) revealed that the AD lignin was richer in alkyl and methoxyl protons that

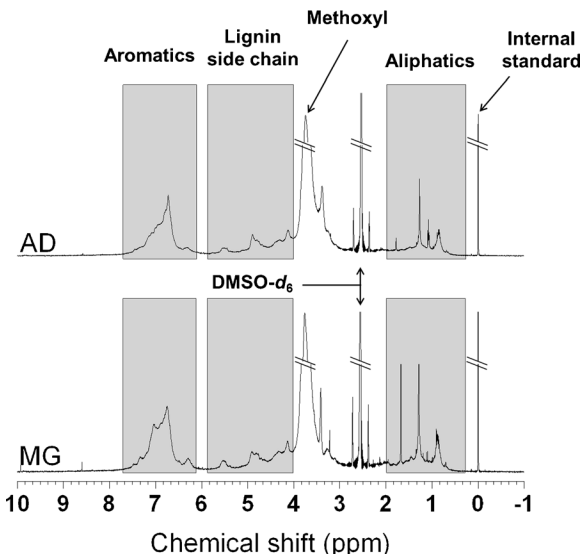

Fig. 2 ^{1}H NMR spectra for water-soluble lignin isolated from giant reed (AD) and miscanthus (MG)

Table 2 Assignment of the main signals in chemical shift ranges (ppm) of ^{1}H-NMR and ^{31}P–NMR spectra of lignins isolated from giant reed (AD) and miscanthus (MG). Proton distribution in ^{1}H-NMR spectra are reported as percent of signal areas, while OH content in lignin elaborated from ^{31}P-NMR spectra are reported in mmol g^{-1} of lignin, as related to area of an internal standard

Chemical Shift	Assignment	AD	MG
^{1}H-NMR			
0.91–1.68	Alkyl protons	10.45	9.41
3.73	Methoxyl protons	61.69	59.69
4.10–5.88	Hydroxyalkyl protons	33.97	35.14
6.30–7.30	Aromatic protons	34.11	36.26
^{31}P-NMR			
150.8–146.3	Aliphatic OH groups	5.67	5.46
143.7–142.2	Syringyl groups	0.18	0.07
140.2–138.4	Guaiacyl groups	0.63	0.46
138.6–136.9	*p*-Hydroxyphenyl groups	0.18	0.28
135.6–133.7	Carboxyl groups	1.02	0.92

MG, while the latter showed a larger content of hydroxyalkyl and aromatic protons.

Further details on lignin molecular structure can be obtained by short- and long-range correlation 2D NMR experiments. Since cross-signals found in 2D-NMR experiments for both lignins were similar, only the 2D HSQC and HMBC spectra for the AD lignin are shown here (Fig. 3). Peak assignment was based on previous literature (Savy et al. 2015a, b; Rencoret et al. 2011), and cross-peak attributions are reported in Supporting Material Table SM-2. The aliphatic oxygenated structures generated HSQC cross-signals in the range of δC/δH 50–90/2.5–6.0 ppm, which provide information on both side chain and dimeric sub-units. The most prominent signal was related to lignin methoxy groups, resonating around 55.5/3.67 ppm, while the signal at 61.1/4.03 ppm was assigned to C_γ-H_γ in cinnamyl alcohol end-groups (I) (Savy et al. 2015b). The main dimeric structures found in these lignins were the β-O-4′ and the resinol (β-β) and related substructures, as indicated by cross-peaks around 59.6/3.57, 71.7/4.7, 83.8/4.21, 84.8/4.60 and 85.8/4.03 ppm (Rencoret et al. 2011). Other dimeric sub-units, such as spirodienone (β-1′), the phenylcoumaran (β-5′), α,β-diaryl ether and tricin structures were not found in these lignins, because of possible degradation due to the alkaline oxidative treatment of original biomasses (Savy et al. 2015a).

The aromatic spectral region (δC/δH 90–150/6.0–8.0 ppm) in the HSQC spectrum revealed the occurrence

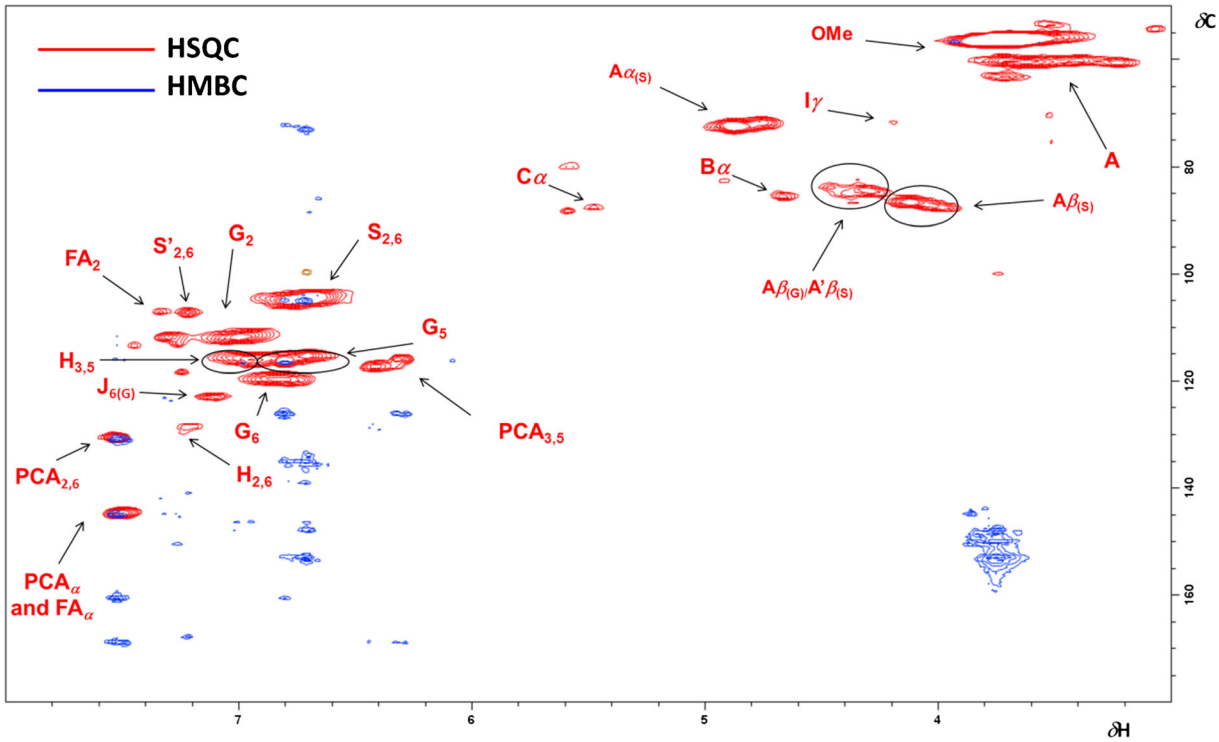

Fig. 3 Selected chemical-shift range for two-dimensional ^{1}H-^{13}C HSQC (*red*) and HMBC (*blue*) NMR spectra ($\delta C/\delta H$ 50–180/3–8 ppm) for water-soluble lignin isolated from giant reed (AD). Labels refer to lignin sub-units, whose signal assignments are reported in Tables SM-1 and SM-2 for HSQC and HMBC spectra, respectively. The molecular structures of the identified lignin sub-units are shown in Figure SM-1

of Guaiacyl (G), Syringyl (S) and *p*-Hydroxyphenyl (H) units (Fig. 3) as expected from such raw biomasses (Faix et al. 1989). The cross-peaks around 111.2/6.91, 115.3/6.76 and 119.1/6.73 ppm arose from short range correlation between C_2-H_2, C5-H_5 and C_6-H_6, in G-units, while signals at 103.8/6.63 and 106.3/7.25 ppm were assigned to the $C_{2,6}$-$H_{2,6}$ resonance in etherified S units and oxidized (C_α=O) phenolic syringyl units (S') (Savy et al. 2015b). Moreover, the ^{13}C-^{1}H correlations at around 114.8/6.63 and 127.8/7.16 ppm were attributed to $C_{3,5}$-$H_{3,5}$ and $C_{2,6}$-$H_{2,6}$, respectively. Additional cross-peaks around 116.1/6.27 and 130.0/7.44, 111.6/7.21 and 144.0/7.45 ppm were assigned to *p*-coumarate (PCA) and ferulate (FA) resonances, while the signal around 122.3/7.02 was related to cinnamyl aldehyde end-groups (J) (You et al. 2013; Savy et al. 2015a).

The information provided by the long-range ^{1}H-^{13}C HMBC-NMR spectra on the molecular nature of lignins of this study are in good agreement with those obtained by the short-range HSQC spectra (Fig. 3). The occurrence of the β-O-4′ dimer was also revealed in the HMBC spectra by the resonance of C_α-$H_{2,6}$ and C_α-H_6 in etherified S and G units at 71.8/6.7 ppm (Table SM-3), while the resinol β-β sub-structure was suggested by the cross-peak at 85.0/6.7 ppm, corresponding to the long range C_α-$H_{2,6}$ correlation (Robert 1992). The HMBC spectra allowed to recognize some correlations attributed to either PCA or FA (Table SM-3). In fact, the C_β-H_α, C_γ-H_β and C_γ-H_α signals for PCA resonated at around 115.6/7.5, 168.2/6.3 and 168.6/7.5 ppm, while the C_γ-H_α and C_γ-H_β cross-peaks in FA were located at 167.9/7.2 and 167.8/6.4 ppm (Robert 1992). The long-range HMBC spectra revealed the correlations between quaternary carbons and related protons. For example, the C_1 couplings for S and S' units (Table SM-2) are easily recognized at 130.0/7.5 and 138.0/6.7 ppm, respectively (Table SM-3). Instead, the cross-peaks at 125.1/6.3 ppm were assigned to the C_1-H_β in PCA. The long-range correlation between protons and C_3 in G units (Table SM-2) were revealed by the resonances at 145.3/7.0, 145.6/3.7, 148.9/70 and 149.6/7.2 ppm (Robert 1992), while those in S-units were found

around 147.3/3.7, 147.2/7.0, 151.8/6.7 and 152.3/7.2 ppm (Ibarra et al. 2007). Moreover, some cross-signals arose from the C_4 carbon with the related protons, since those at 134.0/6.4 and 147.0/6.7 ppm were attributed to C_4-$H_{2,6}$ in S units and to C_4-H_5 in G monolignols, respectively (Ibarra et al. 2007). Finally, the C_4 in PCA correlated with both $H_{3,5}$ and $H_{2,6}$, as indicated by the cross-peaks at 159.6/6.7 and 159.6/7.5 ppm (Robert 1992).

Diffusion-ordered NMR spectroscopy (DOSY-NMR) provides information on both dynamics and diffusivity of molecules, and, indirectly, on their size, since the larger the diffusivity constant, the faster is the molecular mobility and the smaller the molecule (Johnson 1999). ^{1}H DOSY-NMR represents a reliable technique to study the molecular diffusivity of relatively small compounds, as well as that of large biopolymers, and supramolecular associations, such as those occurring in natural organic matter (Smejkalova and Piccolo 2008; Nebbioso et al. 2014). DOSY-NMR spectra of lignins showed that the molecular diffusivity for AD (3.041×10^{-11} m^2s^{-1}) was significantly lower than for MG (6.228×10^{-11} m^2s^{-1}). This indicates that AD had a larger molecular size than MG (Fig. 4), thereby confirming the results obtained by HPSEC.

Phosphytilation of lignins with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxa-phospholane allows to run ^{31}P NMR spectra, from which a quantitative content of hydroxyl groups in lignins can be obtained (Crestini and Argyropoulos 1997). The spectra of phosphytilated lignins from AD and MG are shown in Fig. 5, while signal assignment and amount are reported in Table 2. The ^{31}P NMR spectra showed that the content of aliphatic OH (150.8–146.3 ppm) for AD lignin was slightly larger (5.67 mmol g^{-1}) than for MG (5.46 mmol g^{-1}), and so was for the content of carboxyl groups (135.6–134.7 ppm), that amounted to 1.02 and 0.92 mmol g^{-1}, respectively. Among the lignin phenolic moieties, both the phosphytilated Syringyl (143.7–142.2 ppm) and Guayacyl units (140.2–138.4 ppm) were significantly larger in AD than MG lignin, although to a different extent. In fact, while the S-units accounted for 0.18 and 0.07 mmol g^{-1}, the content of G-molecules reached 0.63 and 0.46 mmol g^{-1} for AD and MG lignins, respectively (Table 2). Conversely, the p-hydroxyphenyl units (H) in the 138.6–136.9 ppm region resulted larger in MG (0.28 mmol g^{-1}) than in AD (0.18 mmol g^{-1}) lignin.

Germination of maize seeds and seedling emergence

Treatment of maize seeds with solutions of increasing concentrations of lignin carbon showed that even though the germination rate was not affected by neither MG nor AD (data not shown), both lignins did show a positive biological activity on the emergence of maize plantlets (Table 3). Comparison among treatments were made easier by normalizing results to control values that were set to 100 in each single treatment.

Application of MG solutions determined a progressive initial increase in the length of both radicle (RR) and lateral seminal roots (LSR), up to 10 ppm of C concentration. Seeds treated with this lignin showed longer roots than control, but root elongation slightly decreased at 100 ppm C. (Table 3). The maximum stimulation of RR by MG was recorded

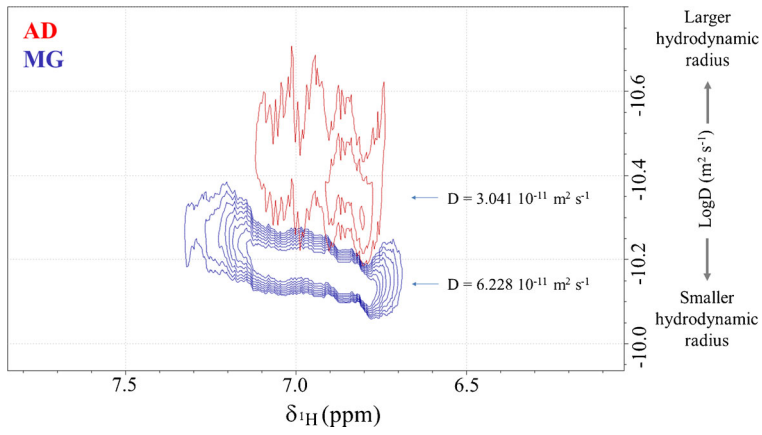

Fig. 4 Two-Dimensional ^{1}H-DOSY projections of water-soluble lignins isolated from giant reed (AD) and miscanthus (MG)

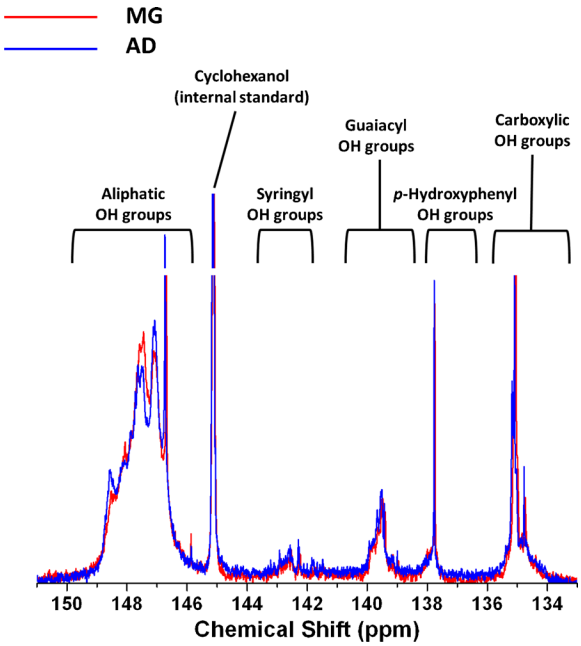

Fig. 5 ^{31}P-NMR spectra of water-soluble lignin isolated from giant reed (AD) and miscanthus (MG) biomass and derivatized with 2-chloro-4,4,5,5-tetramethyldioxaphospholane. Cyclohexanol was added as internal standard prior to derivatization

for 1 and 10 ppm C concentrations, which both caused a root elongation 41 % greater than control, whereas the 0.1 and 100 ppm C treatments did not significantly affect root length (Table 3). MG lignin enhanced the elongation of LSR at all concentrations, with most stimulation exerted by the 10 ppm C concentration (>70 % larger than control). Coleoptile development was promoted at 0.1, 1 and 10 ppm C concentrations, while the 100 ppm C solution did not affect shoot growth. However, the greatest stimulation (>40 % larger than control) was observed at 1 and 10 ppm C concentrations.

The AD lignin significantly increased radicle length at all concentrations, though to a different extent. In fact, while the 10 ppm C concentration induced the largest RR elongation (50 % greater than control), the RR length for the 100 ppm C was slightly shorter than for 1 and 10 ppm C treatments (Table 3). Similar results were found for the LSR growth, that showed an average 30 % and 50 % increase larger than control for 1 and 100 ppm C, respectively, but only a slight effect at 0.1 ppm C. Finally, coleoptile growth was significantly increased by AD lignin only at 10 ppm of C concentration (Table 3).

Growth of maize plantlets

The bioactivity of both AD and MG lignins was assayed during the early growth of maize plantlets. Lignin from MG enhanced the Total Dry Weight (TDW), Shoot Dry Weight (SDW) and root elongation, while the Root Dry Weight (RDW) was not affected by lignin addition (Table 4). As above, the differences among treatments were made more easily comparable by setting control values to 100, and normalizing treatments results to that. The TDW was 25 % larger than control for 50 ppm C, while it slightly decreased at greater concentrations, though TDW was still significantly larger than control at 100 ppm C. Similarly, all MG treatments significantly increased SDW, with the 50 ppm C concentration as the most stimulating solutions, that provided a 27 % greater stimulation than for control (Table 4). While RDW was not significantly altered by additions of MG lignin, total length of maize roots was significantly enhanced at 10, 50 and 100 ppm C concentrations (Table 4).

Table 3 Length (cm) of radicle root, lateral seminal root, and coleoptile for maize seedlings treated with different organic carbon (OC) concentration (ppm) of MG and AD water-soluble lignins. Values in parenthesis refer to values found for control and set as 100. Different letters indicate significant differences in column at 0.05 probability level, as revealed by the Tukey's test

Treatment (ppm of OC)	Radicle root		Lateral seminal roots		Coleoptile	
	MG	AD	MG	AD	MG	AD
0	3.08 (100)^{a}	6.00 (100)^{a}	2.59 (100)^{a}	6.72 (100)^{a}	1.29 (100)^{a}	2.75 (100)^{a}
0.1	2.89 (94)^{a}	6.36 (106)^{ab}	3.89 (150)^{bc}	7.71 (115)^{ab}	1.51 (117)^{ab}	2.61 (95)^{a}
1	4.35 (141)^{b}	7.35 (122)^{b}	4.07 (158)^{bc}	9.17 (136)^{bc}	1.89 (146)^{b}	2.64 (96)^{a}
10	4.35 (141)^{b}	8.98 (150)^{c}	4.51 (174)^{c}	10.29 (153)^{c}	1.89 (146)^{b}	3.39 (123)^{b}
100	3.43 (111)^{a}	7.33 (122)^{b}	3.47 (134)^{ab}	9.07 (135)^{bc}	1.29 (100)^{a}	2.84 (103)^{a}

Table 4 Total Dry Weight (g), Shoot Dry Weight (g), Root Dry Weigth (g) and root length (m) of maize seedlings treated with MG and AD water-soluble lignins. . Values in parenthesis refer to values found for control and set as 100. Different letters indicate significant differences in column at 0.05 probability level, as revealed by the Tukey's test

Treatment	DTW		DSW		DRW		Root Length	
	MG	AD	MG	AD	MG	AD	MG	AD
0	0.269 (100)^{a}	0.215 (100)^{a}	0.213 (100)^{a}	0.155 (100)^{a}	0.059 (100)^{a}	0.061 (100)^{a}	1.493 (100)^{a}	2.048 (100)^{a}
1	0.292 (108)^{ab}	0.243 (113)^{ab}	0.232 (109)^{ab}	0.171 (110)^{ab}	0.060 (102)^{a}	0.072 (119)^{ab}	1.836 (123)^{a}	2.593 (127)^{b}
10	0.309 (115)^{ab}	0.300 (139)^{b}	0.243 (114)^{ab}	0.231 (150)^{b}	0.065 (107)^{a}	0.068 (113)^{ab}	2.421 (132)^{b}	2.475 (121)^{b}
50	0.337 (125)^{b}	0.250 (116)^{ab}	0.271 (127)^{b}	0.169 (109)^{ab}	0.066 (112)^{a}	0.080 (133)^{b}	2.447 (133)^{b}	2.505 (122)^{b}
100	0.314 (116)^{ab}	0.255 (114)^{ab}	0.250 (118)^{ab}	0.182 (118)^{ab}	0.063 (111)^{a}	0.073 (120)^{ab}	2.429 (132)^{b}	2.023 (101)^{a}

Lignin from AD promoted a significant increase in all plant parameters (Table 4). The TDW and SDW values resulted 39 % and 50 % larger than control for 10 ppm C, respectively. Contrary to MG, lignin from AD significantly enhanced RDW that reached values 33 % greater than control at 50 ppm C. Finally, root elongation was similarly promoted at 1, 10 and 50 ppm C concentrations (21–27 % more than control) (Table 4).

Discussion

The two lignins isolated from energy crops had similar carbon, hydrogen and nitrogen elemental content, but differed in oxygen content, that was larger in AD than in MG. Despite this difference, both lignins were oxidized during extraction to ensure depolymerisation and solubilisation in water. In fact, the oxidative alkaline procedure produced water-soluble fragmented phenols through hydrolysis and Dakin-like reactions (Kadla et al. 1999), that depolymerise the cross-linked lignin polymer through the action of the hydroperoxide anion formed from H_2O_2 at alkaline pH and the production of a quinone methide (Figure SM-2).

More differences in composition were shown by the HPSEC profiles of the two water-soluble lignins (Fig. 1). In particular, while the average molecular sizes (Mw and Mn) and polydispersity (P) of both lignins appeared similar when derived from the full size-exclusion chomatogram, the values for the first diffuse absorption (peak A in Fig. 1) resulted larger for AD than MG, but became again similar for peak B and C. The AcOH treatment of lignin solutions prior to HPSEC separation resulted in a decrease of all M_w, and M_n values for both lignins, but those of AD remained larger than for MG, either for full chromatograms or separated peaks (Table 1).

The conformational size of AD lignin larger than that of MG lignin was also indicated by the DOSY-NMR spectra (Fig. 4), which revealed a molecular diffusion for AD slower than for MG. Moreover, previous NMR measurements (Savy and Piccolo 2014) showed that proton relaxation times in the solid state ($T1\rho H$) was greater for AD than for MG. Since $T1\rho H$ is directly proportional to the rigidity of a molecule or a supramolecular structure, its large values suggested that lignin separated from AD had a greater aggregation size than for MG lignin.

An alteration of conformational arrangement by HPSEC was reported for HS treated with acetic acid (Piccolo et al. 1996). The decrease of molecular size distribution in HS after AcOH addition was explained with the capacity of organic acids to alter the metastable supramolecular structure, in which the small but heterogeneous humic molecules self-assemble by weak dispersive forces (van der Waals, π-π, π-CH) and/or hydrogen bonds (Piccolo 2002). Organic acids treatment of complex solution was repeatedly applied in HPSEC experiments as a means to evaluate the strength of association of heterogeneous natural matrices with varying content of amphiphilic molecules (Piccolo et al. 2002, 2003; Smejkalova and Piccolo 2008; Nuzzo and Piccolo 2013).

The water-soluble lignins of this study revealed changes in chromatographic profiles upon AcOH addition that were similar to those of HS. It is hence reasonable to assume that the lignin extracts were also composed by relatively small molecules, which arrange

themselves in supramolecular associations, and whose assembling forces are also weak enough to be disrupted by AcOH. The HPSEC behaviour of the two lignins suggests that, despite the larger oxygen content in AD, the lignin components of this biomass had a greater self-assembling capacity than for MG. This property can be ascribed by a number of factors. First of all, the ^{1}H-NMR spectra indicated that AD was richer than MG in hydrophobic alkyl and methoxy functions, whereas the latter contained slightly more aromatic and hydrophilic hydroxyl-alkyl moieties (Table 2). The ^{31}P NMR spectra provided a quantitative evaluation of hydroxyl functions in various functional groups and indicated that while these OH groups were slightly greater in AD than in MG lignin, the former was significantly richer in Syringyl and Guayacyl aromatic groups than the latter (Table 2). These results were confirmed by both the 2D HSQC and HMBC spectra of AD and MG lignins where the aromatic phenolic units were clearly identified (Fig. 3).

The observed molecular and conformational differences between the two lignin extracts may explain their biological activity on maize germination and seedling growth. Even though both lignins stimulated plant growth at various concentrations, the plant response to lignin addition was not the same for AD and MG lignins (Table 3 and 4). A common positive bioactivity may be related to close similarity in their general molecular composition, as shown by NMR spectra, whereas the effects of the two lignins on specific biological parameters may be accounted to differences in conformational structure and hydrophilic components. In fact, while the MG lignin stimulated root and coleoptile elongation in both bioassays more than AD extract, shoot and root weight were found to be larger with AD than by MG additions.

These results may be accounted to the observed large size of AD, but also to its significantly larger content of hydrophobic aromatic groups, such as Syringyl and Guaiacyl units, that contribute to a more stable supramolecular association of the AD lignin. A large conformational size and relative hydrophobicity of self-assembled heterogeneous molecules, such as in humic or humic-like substances, seem to play an important role in their biostimulation of plants, since they allow a closer and more persistent interactions with root surfaces (Puglisi et al. 2009; Canellas and Olivares 2014; Ertani et al. 2015). The concomitant larger presence in the humic-like AD lignin of oxygen-containing groups may induce a more extensive alteration of conformational stability than for MG, due to a greater number of hydrogen bonds formed between the protons of organic acids exuded by seedlings and plantlets, and the complementary functions of lignin molecules. This conformational disruption of lignin may consequently cause the release of plant stimulating compounds. The formation of new strong hydrogen bonds in humic superstructures due to plant-exuded organic acids, was called upon to explain the release of bioactive molecules from altered metastable humic conformations (Piccolo et al. 2003; Canellas et al. 2010). While a similar mechanism may also explain the bioactivity of MG lignin, its less hydrophobic conformational structure may lower the adhesion of MG to plant roots and render the released bioactive molecules somewhat less effective than AD.

Phenolic acids are generally reported to produce inhibitory effect on seed germination and plant growth (Gerig and Blum 1991; Chaves et al. 2001; Djurdjević et al. 2004). However, the extent of inhibition varies significantly and depends on different factors, such as the specific structure and concentration of phenols and the plant species selected for the assay (Williams and Hoagland 1982; Almaghrabi 2012). Moreover, phenols may impact differently on plant development (Rasmussen and Einhellig 1977; Reigosa et al. 1999). The highly stimulating activity on plant growth by mixtures of natural phenols were shown by a number of experiments (Ertani et al. 2011a; Popa et al. 2008). In a comprehensive study on maize seed metabolism, it was reported that the increase of root and leaf dry weight of maize seedlings was directly proportional to the phenols contained in commercial biostimulants (Ertani et al. 2011b). Soil and foliar application of polyphenols derived from spruce bark (*Picea abes*, L.) significantly increased the dry weight of roots, stems, leaves, and seeds of sunflower (*Helianthus annuus*, L.) (Tanase et al. 2014). Moreover, photosynthetic pigments, transpiration rate and CO_2 assimilation were increased by such polyphenols, thus suggesting activity at a physiological level. Phenols and phenol-containing waste materials, such as lignosulphonates from paper mills, were also shown to behave as plant regulators due to hornome-like activities. In fact, a specific gibberellin (GA)-like activity was exerted by *p*-hydroxybenzoic, vanillic and syringic acids on seedlings of silver fir (*Abies alba*, Mill.) (Nardi et al. 2003), while gallic, protocatechuic and phenylacetic acids revealed instead an auxin (IAA)-like effect (Pizzeghello et al. 2006).

Furthermore, lignin- and phenol-based biostimulants were shown to possess both IAA- and GA-like activities (Ertani et al. 2011a, b).

Conclusions

This work showed for the first time that water-soluble lignins isolated from biomasses for energy, such as miscanthus and giant reed, stimulated the emergence of maize seedlings and their early growth. Moreover, the AD lignin was found to enhance shoots and roots dry weights more than MG, whose bioactivity was instead more effective on elongation of seedlings. The detailed molecular chracterization of these lignin extracts indicates that they possess a humic-like bioactivity towards plant growth, that is due to a combination of stability of supramolecular association and hydrophilicity of their molecular components. In particular, a somewhat greater bioactivity of AD lignin may be ascribed to a larger conformational size, although the release of bioactive molecules from the supramolecular matrix may be similar to that of MG lignin. Our results suggest that water-soluble lignins can be isolated from biomasses for energy and be used in agriculture as plant growth promoters, thereby increasing the economic and environmental sustainability of energy production from biomasses, and representing a useful alternative to the burning of lignocellulosic residues.

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