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Biostimulation of humic acids on *Lepidium sativum* L. regulated by their content of stable phenolic O˙ radicals

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Abstract

Background Humic acid affects plant growth. Its source and structure may play a central role to its functionality. The relationship between humic acid and plant bioactivity is still unclear. This study investigated the biostimulation effects of two natural humic acids derived from soil (SHA) and lignite (LHA) on *Lepidium sativum* in comparison to a synthetic humic acid model (HALP) with known structure.

Results All humic acids positively affected cress seed germination and root elongation. Greater root hairs density and dry matter, compared to control, were observed using concentration of 5 mg L^{-1} for HALP, 50 mg L^{-1} for LHA, and 100 mg L^{-1} for SHA. The germination index was the largest (698% more effective than control) with 50 mg L^{-1} of SHA, while it was 528% for LHA, and 493% for HALP at 5 mg L^{-1}. SHA contained the lowest aromatic and phenolic C content, the largest pK_2 value of 9.0 (7.7 for LHA and 7.6 for HALP), the least ratio between the aromaticity index and lignin ratio (ARM/LigR) of 0.15 (0.66 for LHA and 129.92 for HALP), and at pH 6.3 the lowest amount of free radicals with a value of 0.567×10^{17} spin g^{-1} (1.670×10^{17} and 1.780×10^{17} spin g^{-1} for LHA and HALP, respectively), with the greatest g value of 2.0039 (2.0035 for LHA and 2.0037 for HALP).

Conclusions The overall chemical structure of humic acids exerted a biostimulation of cress plantlets. The level of the intrinsic stable free radicals identified by EPR in the humic acids resulted well correlated to the ARM/LigR ratio calculated by NMR. Our results suggested that HA biostimulation effect is related to its applied concentration, which is limited by its free radical content. The modulation of the humic supramolecular structure by ROS and organic acids in root exudates can determine the release of bioactive humic molecules. When the content of the intrinsic humic free radicals is high, possible molecular coupling of the bioactive humic molecules may hinder their biostimulation activity. In such cases, a low humic acid concentration appears to be required to achieve the optimum biostimulation effects.

Keywords Cress seed germination, Root growth, Supramolecular structure, Soil humic acid, Lignite humic acid, HALP, Aromaticity, Free radicals

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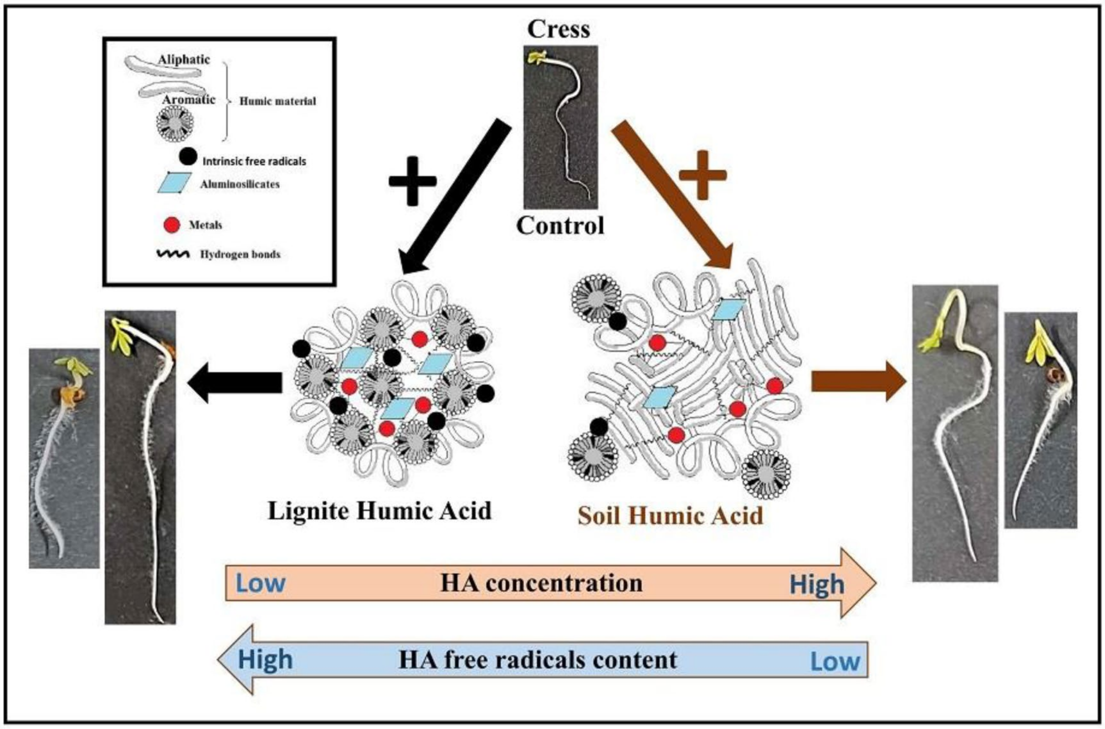
Graphical Abstract



Background

Humic substances (HS) are a complex mixture of diverse humified organic materials, naturally present in soils, waters, and sediments, comprising a supramolecular structure of heterogeneous molecules with carboxylic and phenolic functional groups held together by hydrogen, metal and hydrophobic bonds [1]. The principal component of soluble HS are the humic acids (HA), which are insoluble at $pH < 2$ and therefore greatly hydrophobic [2, 3]. The structure of HA can be recalcitrant to microbial degradation depending on the source of material and the extraction method, thus maintaining a great structural stability [4].

HA play a pivotal role in agricultural processes by promoting the cation exchange capacity [5], enhancing soil fertility, and converting mineral elements into forms available to plants [2]. Several studies have reported that humic acids are able to support plants in stress resistance [6, 7] and to increase roots and shoots growth [8, 9]. HA are considered to carry molecules that may act as plant hormones, such as auxin and cytokinin [10]. Nevertheless, El-Bassiouny et al. [11] and Mukherjee et al. [12] reported that use of HA did not cause significant crop growth, while Elmongy et al. [13] showed that they can be also responsible of root limitation at high concentration.

The effects of HA on plant growth are certainly linked to its structure and, hence, according to De Melo et al. [14] this should be related to its phenolic (OH) and carboxylic (COOH) content, being the most predominant functional groups. Thus, the origin of HA play an important role in their functionality [15], although a comparison of HA from different sources and the effects of these differently sourced materials on plant growth are scarce in literature [16]. The aromatic and aliphatic compounds of HA appear to be responsible for increasing the uptake of N and soluble sugars by plant, resulting in the stimulation of root growth in rice seedlings [17], as well as the yield increase in the same species [6]. On the other hand, there is not a clear picture for the responses of HA on crops [18], and there is still a lack of knowledge about the relationship between the structure of HA and plant bioactivity.

Cress (*Lepidium sativum* L., family Brassicaceae) is an important species because is rich in nutraceutical compounds, contains many bioactive constituents and possesses several pharmacological effects, such as antimicrobial, antioxidant, anticancer and cardiovascular

[19], which make its fresh leaves desirable for consumption as salad [20]. *L. sativum* is widely used as a test plant in studies of phytotoxicity and germination due to its high sensitivity to toxic substances [21, 22]. This species has also been used in bioassays for testing the biological activity of HS [23].

On this basis, the present study aimed to evaluate and clarify the biostimulation effects on cress seeds and plantlets growth by humic acids. To understand the involved structure–activity relationship, two natural HA derived from soil (SHA) and lignite (LHA), were used in seeds germination laboratory experiments, in comparison to a synthetic humic acid of known structure (HALP).

Methods

Samples

HA were obtained by a lignite sample coming from an underground mine in Ptolemaida, Greece, and by a soil collected from the top 10 cm layer of a coniferous forest soil from Parnon Mountain located in Peloponnese, Greece. The soil was a loamy Alfisol (USDA Soil Taxonomy) typical of Greek soils. Natural humic acids from both lignite and soil (LHA and SHA, respectively) were isolated using the IHSS protocol, as earlier described [24]. A synthetic polyphenolic humic acid (HALP) was used in comparison to the natural humic acids. The protocol of the HALP synthesis is described in detail by Giannakopoulos et al. [25].

^{13}C-CPMAS-NMR spectroscopy

Solid-state ^{13}C nuclear magnetic resonance (NMR) spectra of HA powder samples were recorded on a Bruker NMR spectrometer at a resonance frequency of 400 MHz, using a ramped-cross polarization MAS (CPMAS) with a spinning speed of 6.8 kHz. A contact time of 1 ms and a pulse delay of 400 ms were used. A ramped 1 H-pulse decreasing the power from 100 to 50% was used to circumvent spin modulation of Hartmann–Hahn conditions [26]. At least 50,000 single scans were collected for each sample. Based on signals area, specific indices were calculated as reported by Monda et al. [27]. Briefly, HB/HI, hydrophobicity index: the ratio of signal area in the interval of hydrophobic carbons (0–45 + 110–160 ppm) over that in the interval of hydrophilic carbons (60–110 + 160–190 ppm); A/OA, alkyl index: the ratio of alkyl-C signal area (0–45 ppm) to that of O-alkyl-C (60–110 ppm); ARM, aromaticity index: the ratio of signal area of aromatic components (110–160 ppm) to that of aliphatic compounds (0–60 + 60–110 + 160–190 ppm); LigR, lignin ratio: the ratio of signal area of methoxyl-C (45–60 ppm) to that of the phenolic-C region (145–160 ppm).

EPR spectroscopy

Electron paramagnetic resonance (EPR) spectra were recorded at liquid nitrogen, with a Bruker ER200D spectrometer, equipped with an Agilent 5310 A frequency counter. g-Values were calibrated versus DPPH (2,2-diphenyl-1-picrylhydrazyl), $g = 2.0036$, which was also used as spin standard for radical concentration as described earlier [28].

Biostimulation assay

Before starting the experiment, a stock solution for each HA was adjusted at the concentration of 8 g L^{-1} and pH 6.3. The effect on the germination and root initiation of cress (*Lepidium sativum* L.) was performed in a Petri dish by placing 5 mL of distilled water solution of each HA (1, 5, 50, 100 mg L^{-1}) and 10 seeds on Whatman® filter paper (Grade 1). All plates were sealed by Parafilm® and incubated in darkness for 72 h at 23–26 °C. The experiment was conducted in triplicates and sterile distilled water was used as control. The radicle length (root + hypocotyl) of each seedling was measured with a precision digital caliper (eVatmaster Consulting GmbH, Germany); the fresh and dry weights (oven-dried at 60 °C for 24 h) of shoots and roots collected from each plate were recorded.

The seed germination index (SGI) was calculated according to the following formula [29]:

$$\mathrm{SGI}(\%) = [(Gs/Gc) \times (Ls/Lc)] \times 100,$$

where: SGI is the germination index; *Gs* and *Gc* represent the average number of germinated seeds in the sample and control, respectively; *Ls* and *Lc* are the average radical length for the sample and control, respectively. A value of SGI equal or higher than 80% indicated that phytotoxicity was absent [22], while a value significantly higher than 100% indicated a biostimulant effect by humic acid.

The dry matter (DM%) was calculated according to Pane et al. [30]:

$$\begin{aligned}\mathrm{DM}(\%) &= 100 - \mathrm{RH}(\%)\\ &= 100 - [(\mathrm{FW} - \mathrm{DW})/\mathrm{FW})] \times 100,\end{aligned}$$

where RH is the relative humidity; FW and DW are the fresh and dry weights, respectively. All mean values were expressed as percentage relative to the control.

Statistical analysis

Data from the biostimulation assay were indicated as percentages relative to the control (%). Data, expressed as average ($n = 3$) ± standard deviation, were analyzed according to two-way ANOVA followed by Tukey's HSD test ($p < 0.05$). R (version 4.2.3, R Foundation for Statistical Computing, Vienna, Austria) with the software RStudio IDE (release 2023.06.0+421) to write and run R code was used.

Results and discussion

Cress plant biostimulation by humic acids

The most general trait in plant responses to humic substances application concerns growth and root arrangement, mainly influencing changes on root architecture, root size, formation of lateral roots, and/or greater density of root hairs for enlarging the surface area [31]. In the present study, the roots system of cress seedlings, germinated and grown in presence of the different humic acids, appeared more developed than control in all cases, but a different behavior was observed at the different tested concentrations (Fig. 1). Moreover, a presence of root hairs was observed in seedlings grown in humic solutions and, in particular, at concentration of 5 mg L^{-1} for HALP, 50 mg L^{-1} for LHA and 100 mg L^{-1} for SHA, respectively. On the contrary, no particular differences were highlighted in the observation of epicotyls and, for this reason, no length measurement was consequently performed (Fig. 1).

All humic acids positively affected the seed germination and root elongation because values percentage of seed germinated (G) and root length (RL), as well as the germination index (SGI), never resulted lower than 100% of control (Fig. 2 and Table 1). However, the parameters' values increased in a significantly different manner for each humic acid as the concentration increased. For example, for the solutions at 50 and 100 mg L^{-1} of HALP, 5 and 50 mg L^{-1} of LHA, and 5, 50 and 100 mg L^{-1} of SHA, the seed germination was significantly greater than control (Fig. 2a). Conversely, 5, 50 and 100 mg L^{-1} of LHA, and 50 and 100 mg L^{-1} of SHA induced a significant larger radicle elongation than control, while in the case of HALP this was reached at only 5 mg L^{-1} (Fig. 2b). Consequently, the germination index, which comprises both G and RL, showed values significantly different from those of control mainly because of RL for HALP, and of G for LHA and SHA. Noteworthy, the biostimulation by all humic acids, in terms of SGI, in concentration of more than 1 mg L^{-1} always resulted at least twice as large as control, and reached about 500% or more than control for 5 mg L^{-1} HALP and LHA, and about 700% for 50 mg L^{-1} SHA (Fig. 2c).

HA application positively affect soil physical–chemical and plant behavior, such as nutrient availability and uptake [32]. For example, with a concomitant enhanced uptake of certain macro- and micro-nutrients, HA caused a root dry weight increase in tomato and cucumber [33], a length and dry weight increase in maize roots [34], and both fresh and dry weights increase in broad beans [35]. In agreement to these studies, seed germination, radicles length, and root dry matter were overall positively influenced in this work by the two non-synthetic humic acids LHA and SHA, which naturally contain important elements, such as N, S and Mg [24], which are not however present in the synthetic polyphenolic humic acid HALP [25].

A recent study showed that application of increased HA doses provided the enhanced content of some macro- and micro-nutrient elements in cress plants under greenhouse conditions [36]. The natural HA used in our study significantly improved germination, radicle elongation and hair root formation of cress plants, but not always in a dose-dependent manner. In fact, LHA and SHA induced the best performance of plant growth at the concentration of 5 and 50 mg L^{-1}, respectively, in respect to germinated seeds and radicle length (Fig. 2a and b). These two humic acids showed better values than the synthetic HALP in terms of germination index. In fact, as compared to control, the latter improved SGI by 493% at the smallest applied concentration, while the

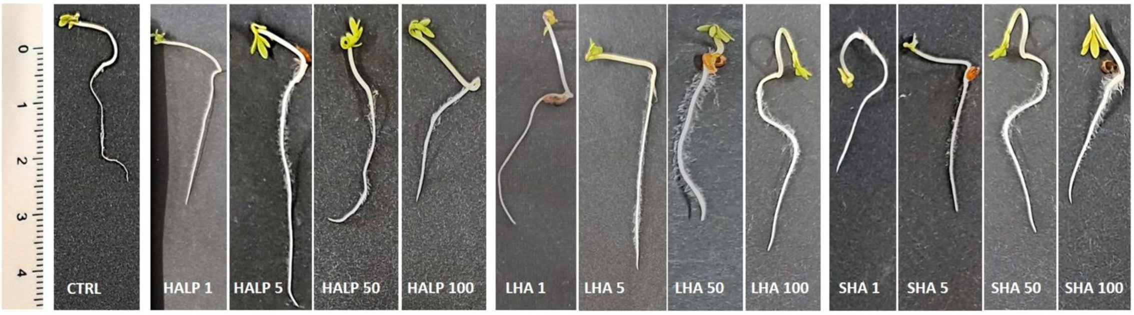


Fig. 1 Representative images of seedlings germinated in distilled water (CTRL) and in humic acids aqueous solutions at different concentrations (1, 5, 50, and 100 mg L^{-1}), after 72 h incubation in darkness

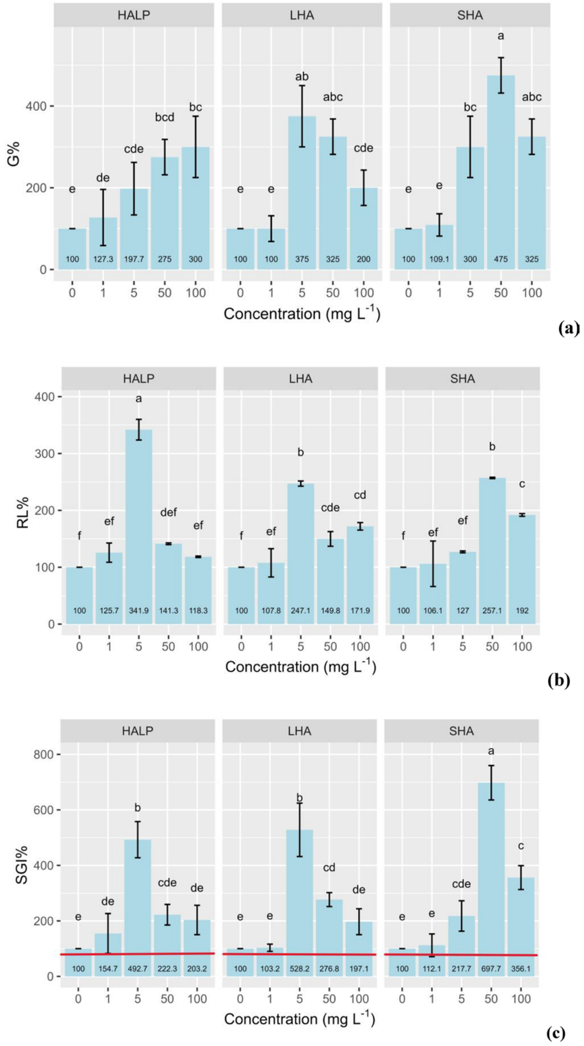


Fig. 2 Effect of humic acids at different concentrations (0, that is distilled water as control, 1, 5, 50, and 100 mg L^{-1}) on **a** germination percentage (G), **b** radicle length (RL) and **c** germination index (SGI) of cress seeds. Data are indicated as percentages relative to the control (%, with 0 = 100%), and reported as mean values ($n = 3$) ± SD. Different letters indicate significant differences according to two-way ANOVA followed by Tukey's post hoc test at $p \leq 0.05$. The red lines designate the percentage of the phytotoxicity threshold (80%), according to Luo et al. [22]

improvement by LHA, at the same concentration of 5 mg L^{-1}, was 528% and that by SHA reached 698% at 50 mg L^{-1} (Fig. 2c). However, 5 mg L^{-1} SHA improved SGI by approximately 218% in respect to control, and was lesser than the improvement by HALP. This suggests that the mineral content in humic matter should not be the main trigger of biostimulation, but it may be rather related to its intrinsic structure [37].

The effects on shoots and roots dry matter by the different humic acids treatments are reported in Fig. 3 and Table 2. The values of shoots dry matter were not significantly different from control for LHA and SHA, while they were lower in two cases for HALP (Fig. 3a). On the contrary, HALP induced a significant increase in roots dry matter, as compared to control, at all concentrations but most notably at 5 mg L^{-1}, whereas LHA and SHA determined an increase of this value at all concentrations above 1 mg L^{-1}, but more significantly at 50 mg L^{-1} for LHA and at 100 mg L^{-1} for SHA (Fig. 3b).

In the case of SHA, the roots dry matter increased with progressive applied concentration up to the largest value at the concentration of 100 mg L^{-1} (Fig. 3b). Therefore, in agreement with Adiloğlu et al. [36] our results showed a dose-dependent increase of cress roots growth only in presence of SHA. In all cases, however, the effect of concentration on the root dry matter was related to the presence of more root hairs observed in seedlings. This may be due to a different strategy adopted by roots in different condition to increase their surface area in order to uptake more nutrients [31].

Humic acid characterization

13C-CPMAS-NMR spectroscopy

The CP-MAS ^{13}C NMR spectrum of LHA appears diverse from that of SHA (Fig. 4) especially in the alkyl-C (0–60 ppm) and aliphatic-CO (60–110 ppm) regions, where SHA showed larger resonances, whereas LHA suggested a prevalence of diverse alkyl C and aromatic C (110–160 ppm), as previously reported by Drosos et al. [24, 38]. Conversely, the synthetic humic acid (HALP), formed by radical polymerization of plain phenolic acids (gallic and protocatechuic), revealed mainly phenolic and aromatic carbons and carboxylic carbons

Table 1 Significance and probability levels resulting from two-way ANOVA for the effects of humic acid (HA), its concentration (C) and their interaction (HA*C) on germination percentage (G), radicle length (RL) and germination index (SGI) of cress seeds

Source of variation	*df*	G		RL		SGI	
		F-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value
HA	2	6.193	0.005	39.464	< 0.01	4.67	0.016
C	4	44.858	< 0.01	197.361	< 0.01	89.88	< 0.01
HA*C	8	5.703	< 0.01	70.488	< 0.01	29.993	< 0.01

df degrees of freedom

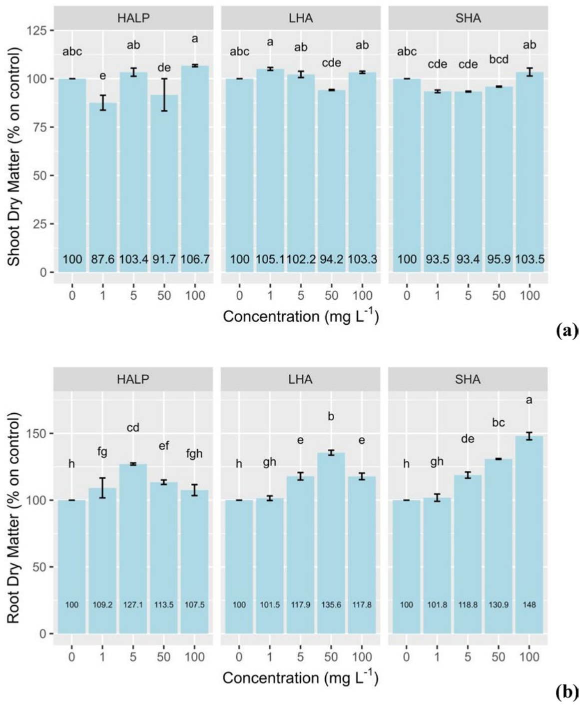


Fig. 3 Effect of humic acids at different concentrations (0, that is distilled water as control, 1, 5, 50, and 100 mg L^{-1}) on the dry matter of cress seedlings shoot (**a**) and root (**b**). Data, indicated as percentages relative to the control (%, with 0 = 100%), are reported as mean values ($n = 3$) ± SD. Different letters indicate significant differences according to two-way ANOVA followed by Tukey's post hoc test at $p \leq 0.05$

Table 2 Significance and probability levels resulting from two-way ANOVA for the effects of humic acid (HA), its concentration (C) and their interaction (HA*C) on shoot dry matter and root dry matter of cress seedlings

Source of variation	*df*	Shoot dry matter		Root dry matter	
		F-value	*p*-value	*F*-value	*p*-value
HA	2	9.137	< 0.01	35.342	< 0.01
C	4	24.381	< 0.01	175.946	< 0.01
HA*C	8	11.303	< 0.01	51.402	< 0.01

df degrees of freedom

(160–190 ppm), as shown by Giannakopoulos et al. [25]. Nevertheless, there is also a small peak in the aliphatic C region (0–60 ppm) indicating the occurrence of ring opening reactions during polymerization [39].

Based on the areas of different chemical shift intervals, we calculated various ratios as shown by Monda et al. [27] and summarized in Table 3. Briefly, LHA revealed the greatest hydrophobicity index, and HALP the lowest. Concomitantly, LHA showed the greatest alkyl index, thereby indicating that the hydrophobic character of

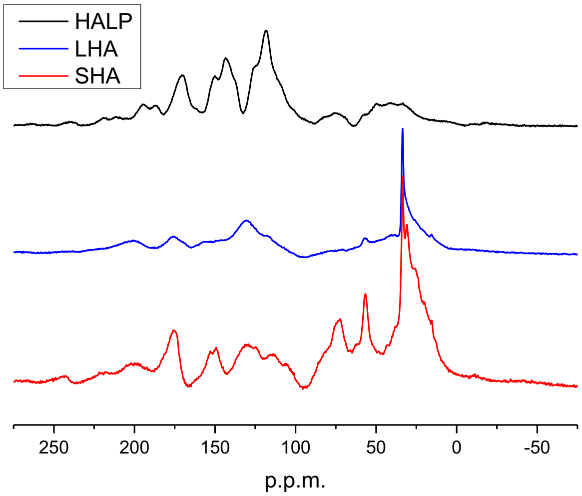


Fig. 4 Solid-state ^{13}C-CPMAS-NMR spectra of the humic materials used

LHA was mainly due to aliphatic/lipidic moieties. Due to the smallest alkyl and aromatic indices of SHA, it can be argued that this HA contained prevalently esteric-etheric and/or amidic moieties. As shown earlier [24, 40, 41], LHA is still a more aromatic material than SHA, while HALP was designed to contain most phenolic carbons [25, 39, 42], and with a lignin ratio close to zero (0.012). In fact, the polymerization mechanism did not result in methylation of the phenolic OH groups [39]. Conversely, most aromatic material of SHA was related to lignin derivatives (1.930), while LHA showed an intermediate lignin ratio between that of HALP and SHA (0.938), thus indicating a greater phenolic C and OH than in SHA [24].

EPR spectroscopy

EPR data (Fig. 5) show that at pH 6.3 the humic acids contained significant amount of indigenous stable free radicals. As earlier reported [28, 43, 44], natural humic acids were found to possess phenolic type O$^{\cdot}$ radicals, which are pH dependent. In fact, the g-value of these radicals was smaller at pH 5 and tended to upshift at higher pH values towards pH 12 (Table 3). This appears as an evidence of the quinone–semiquinone–phenol reaction occurring in the humic aromatic moieties, which is regulated by the amount of the phenolic C and the pK$_2$ value of the phenolic-OH functional groups. In fact, SHA at pH 6.3 was found to have the least amount of free radicals among the samples (0.567×10^{17} spin g^{-1}), but with the highest g value (2.0039). This result was expected due to the smallest aromatic and phenolic C of SHA, and its largest pK$_2$ value (9.0) among the three humic materials (7.7 for LHA and 7.6 for HALP) [24, 39]. On the contrary, HALP had the greatest aromaticity index, and phenolic

Table 3 Summary of the humic acids spectrometric data taken from NMR and EPR

	HB/HI[a]	A/OA[a]	ARM[a]	LigR[a]	ARM/LigR	$O^{\cdot}$ ($\times 10^{17}$ spin g^{-1})[b] pH 6.3	*g* value[b]	$O^{\cdot}$ ($\times 10^{17}$ spin g^{-1})[b] pH 12.0	*g* value[b]
HALP	2.081 ± 0.007	6.314 ± 0.010	1.559 ± 0.006	0.012 ± 0.003	129.92 ± 23.03	1.780 ± 0.010	2.0037 ± 0.0002	8.900 ± 0.010	2.0047 ± 0.0002
LHA	5.644 ± 0.010	9.361 ± 0.011	0.620 ± 0.004	0.938 ± 0.005	0.66 ± 0.02	1.670 ± 0.010	2.0035 ± 0.0002	3.221 ± 0.010	2.0039 ± 0.0002
SHA	3.066 ± 0.008	3.213 ± 0.009	0.299 ± 0.004	1.930 ± 0.008	0.15 ± 0.01	0.567 ± 0.010	2.0039 ± 0.0002	1.542 ± 0.010	2.0047 ± 0.0002

[a] NMR spectroscopy ratios as introduced by Monda et al. [27]. HB/HI: hydrophobicity index; A/OA: alkyl index; ARM: aromaticity index; LigR: lignin ratio. [b]$O^{\cdot}$ and g value are obtained by EPR spectroscopy

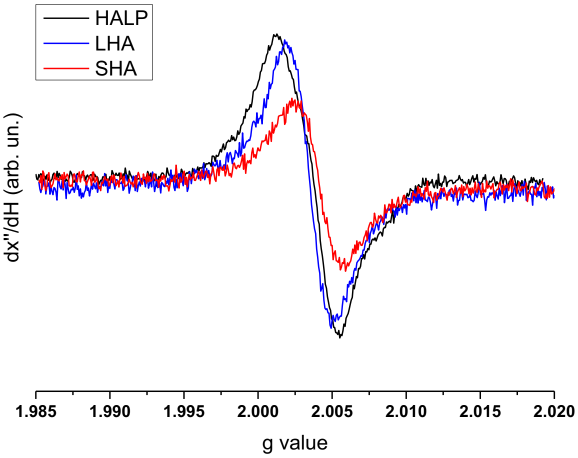


Fig. 5 EPR spectra at pH 6.3 of the humic materials used

C and OH content [24, 39]. At pH 12, the maximum amount of free radicals became 1.542×10^{17} spin g^{-1} for SHA, 3.221×10^{17} spin g^{-1} for LHA, and to 8.900×10^{17} spin g^{-1} for HALP (Table 3), thus indicating the potential intrinsic capacity of these materials to stabilize free-radicals.

Biostimulation mechanisms

A major role of root hairs is to enlarge the surface area of roots to achieve a better uptake of nutrients from the soil, and their formation, length and number is affected by genetic factors, but also by the low availability of nitrate and phosphate [45]. Here we found that root hairs were most numerous for seedlings grown in 5 mg L^{-1} of HALP, 50 mg L^{-1} of LHA and 100 mg L^{-1} of SHA (Fig. 1), thereby suggesting that the response in root hairs formation did not depend on the availability of nutrients in the growth media, and mineral components in HA.

It was previously reported that HA isolated from vermicompost determined a different adventitious rooting in the two different Brazilian red-cloak (*Megaskepasma erythrochlamys*) and sanchezia (*Sanchezia nobilis*) cuttings, as a response to several applied doses and it was attributed to different genetic factors [46]. In our study of only one species, the genetic factors should be excluded. Hence, our findings may better indicate that intrinsic structural features of HA should be held responsible for the noted biostimulant effects [37]. The EPR measurements revealed an $O^{\cdot}$ content at pH 6.3 of 1.780 ± 0.010, 1.670 ± 0.010, and $0.567 \pm 0.010 \times 10^{17}$ spin g^{-1}, for HALP, LHA and SHA, respectively (Table 3). The trend of $O^{\cdot}$ content values appears somewhat inversely related to the biostimulation effects of HAs. In fact, this is consistent with the extent of root hairs and root dry matter increase (Fig. 3b), that reached a maximum of biostimulation at 5 mg L^{-1} for HALP, 50 mg L^{-1} for LHA and 100 mg L^{-1} for SHA. However, while the $O^{\cdot}$ content for HALP and LHA was comparable, that for SHA was much lesser, thereby reflecting an inverse trend with the germination index (Fig. 2c). In fact, HALP and LHA unveiled the greatest SGI at the least concentration of 5 mg L^{-1}, whereas SHA reached the same stimulation at 50 mg L^{-1}, and even more so at the largest concentration of 100 mg L^{-1} (SGI 356% more effective than control).

Many authors have reported that the positive influence of HS on plant growth might be related to the availability of specific biostimulating molecules in the humic matrices [18, 47, 48] related to their supramolecular and metastable spatial arrangements [1]. In fact, LHA was proved to express a greater supramolecular character than SHA [41], based on results of size and polarity fractionation [40]. Conversely, the synthetic HALP is a true macromolecular humic-like material formed by the radical polycondensation of gallic and protocatechuic acid without any presence of metals or plant nutrients [25, 39]. While this material was used as a negative control to evaluate the biostimulation of natural humic acids, it was surprising to find that HALP showed a fivefold SGI increment over the control for just a 5 mg L^{-1} treatment. This result indicates that it is the overall chemical structure of the tested materials to exert a biostimulation of the plant germination and growth.

The biostimulation activity of humics has been reported to be either related to gibberellin activity [23, 49, 50], or to auxin activity [23, 50, 51]. Such hormone-like activity of humic matter was found responsible for the stimulation of lateral roots growth [31, 52], as proved also in the present study. However, N-containing molecules such as auxin were absent in the case of HALP, thereby implying that for this macropolymeric material it was not the hormone-like activity of specific molecules to biostimulate seeds germination but rather its inherent capacity to scavenge the reactive oxygen species (ROS) produced by the cress plantlets.

ROS production has been reported to be related to lateral roots growth by the activation of the respiratory NADPH oxidase in plants [53]. Among ROS, there are singlet O_2 species, hydroxyl radicals, superoxide radicals and hydrogen peroxide [54]. The formation of ROS can in turn produce reactive carbonyl species that enable the modulation of auxin signaling pathways [55]. Gramss and Rudeschko [56] reported that cress plantlets exudate hydrogen peroxide that may contribute to oxidize soil organic matter, thereby disrupting humic suprastructures and allow specific humic components to exhibit hormone-like activity. However, since humic matter contains a significant amount of inert stable free radicals, these can be also gradually exposed and react with ROS exuded by plantlets. Unbound molecules bearing free radicals may also couple to other bioactive molecules and deactivate their hormone-like activity or directly inhibit the root hair formation, thereby resulting in a decrease plant growth or even toxicity.

The HALP made of a known gallic-protocatechuic polymeric structure, may well serve as a model humic material to compare with the behavior of natural humic acids and verify the hypothesis of an involvement of free-radicals in modulating the plant biostimulation by humic acids. In fact, gallic-type $O^{\cdot}$ radicals have been confirmed that are the main type of radicals present in natural humic acids [43]. Ishikura et al. [57] found that gallic acid is responsible for seeds germination inhibition. On the other hand, Widhalm and Dudareva [58] reported that protocatechuic acid acts as a free radical scavenger. Protocatechuic acid was found to have either beneficial or detrimental allelopathy in plants depending on its concentration [59] and to exert at the 1.5 mg L^{-1} concentration a 215% stimulation of IAA production in rhizobial culture [60].

Conclusion

Our study has shown that while natural humic materials can influence seeds biostimulation due to their content of hormone-like molecules, a synthetic humic-like polycondensate of protocatechuic and gallic acids enabled an even larger biostimulation due to the content of free-radicals. In fact, it may be envisaged that the exudation of ROS by plants may disrupt the soil humic suprastructures from which specific molecules may then be released to exert biostimulation activity to plants. However, the amount of the intrinsic free radicals present in natural humic matter may concomitantly reduce the disruption of humic structures by reacting with ROS species, thereby limiting the release of bioactive specific molecules and resulting in a progressive inhibition of the plant stimulation effect. Based on our results, we reasoned that a value larger than 4×10^5 spin L^{-1} in humic compound may convey such inhibition. Moreover, we found that the level of the intrinsic stable free radicals in the HA of this study was well related to the ARM/LigR ratio. For an ARM/LigR of more than 1, a humic concentration of no more than 10 mg L^{-1} should be considered for optimum plant stimulation results. Nevertheless, since our findings were based only on the sole cress species, future work should be planned on other crops to verify this proposed general mechanism. Moreover, applications of different humic substances with a broad variation of their intrinsic stable free radicals can provide the required information on free radicals content and humic acid concentration to reach the optimum plant biostimulation effect.

Abbreviations

HALP	Humic acid like polymer
LHA	Lignite humic acid
SHA	Soil humic acid

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Not applicable.

Author contributions

AV analyzed and interpreted the biostimulation data and was a major contributor in writing the manuscript. LC analyzed and interpreted the biostimulation data and performed statistical analysis. GV performed solid-state ^{13}C-NMR analysis. NM was responsible for the conceptualization and the supervision of the biostimulation experiments carried out. YD performed the free radical analysis of the materials used. EG synthesized HALP together with MD. DR and AP contributed to the writing and edited the manuscript. AS was responsible for the conceptualization and the supervision of the performed research. MD provided the humic materials, taken part on the conceptualization and supervision of the research and was a major contributor in writing and editing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

More data on LHA and SHA can be found as LHA3 and SHA3, respectively, in Drosos et al. [24]. They can be also found as Unfractionated Lignite Humic Acid and Unfractionated Soil Humic Acid, respectively, in Leenheer [40]; and as parental lignite humic acid ($L_{parental}$HA) and parental soil humic acid ($S_{parental}$HA) in Drosos et al. [41]. More data on HALP can be found in Giannakopoulos et al. [25]; and as HALP_0 in Drosos et al. [39]. Additional information on HALP and LHA can be found in Cao et al. [42]. Finally, additional information on LHA can be found as LHA2 in Drosos et al. [38].

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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