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The effects of humic substances application on the phytohormone profile in *Lactuca sativa* L.

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Abstract

Humic substances (HS) are commonly employed as plant biostimulants to enhance crop yields. However, the HS mechanisms of action, as well as the differences between radicular and foliar modes of application, remain unclear. Here, we explored the changes in phytohormonal balance as possible mechanism of HS to enhance lettuce (*Lactuca sativa* L.) growth, and the difference between both modes of application. For this purpose, BLACKJAK®, a HS-based product was applied as radicular (R) and foliar (F) at the concentrations (mL/L): 0.20 (R1), 0.40 (R2), 0.60 (R3), 0.80 (R4), 5.00 (F1), 7.50 (F2), 10.00 (F3), and 12.50 (F4). The experiment was performed in pots filled with vermiculite:perlite (3:1) and HS were applied three times with a periodicity of 10 days. Shoot and root growth parameters were measured. In addition, the phythormones indole-3-acetic acid (IAA), gibberellins (GAs), trans-zeatine (tZ), isopentenyl adenine (iP), 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA), were analysed by U-HPLC-MS. BLACKJAK® application resulted in higher shoot growth at doses R1, R2, R3, F2, and F3, whereas root biomass was increased at R2, R3, F2, F3, and F4, showing radicular better plant growth than foliar applications. Furthermore, HS changed phytohormonal balance in shoots and roots. However, it was with radicular applications, especially at R2, where phytohormonal profile was best associated with plant growth due to the increases observed in IAA, GAs, JA, SA, tZ, and decreased ABA. However, further research is needed to clarify the involvement of hormones in the growth-promoting action of HS.

KEYWORDS
biostimulant, humic substances, *Lactuca sativa* L., phytohormones, U-HPLC-MS

1 | INTRODUCTION

Humic substances (HS), a macroconstituent of soil organic matter coming from "humification" process, have been employed as plant biostimulant and as a complexer of mineral nutrients (Souri & Hatamian, 2019) to enhance crop productivity and stress tolerance (Tiwari et al., 2023). HS include humic acid (HA) and fulvic acid (FA), and chemically are comprised by functional groups (hydroxyl, carboxyl, carbonyl, amines, amides, and sulfhydryl), forming small molecules that are associated through van der Waals forces to create

supramolecular structures (Nardi et al., 2021). The majority of commercial HS are extracted from different carbon deposits, including compost, peat, coal, or leonardite (Yang et al., 2021). These HS may be applied to crops together with solution of nutrients ("root" or "radicular" HS) or directly sprayed to leaves ("foliar" HS) (De Hita et al., 2020).

The influence of HS in stimulating plant growth has been related to the enhancement of nutrients availability and uptake, as well as primary and secondary metabolism (Canellas et al., 2015; Nardi et al., 2021). In this way, Billard et al. (2014) observed that the application of HA increased *Brassica napus* L. growth by improving iron (Fe) and zinc (Zn) uptake and translocation to shoot. Similarly, Ertani et al. (2011) found that HS enhanced nitrogen (N) assimilation and crop yield in maize, whereas Man-Hong et al. (2020) showed an improvement in photosynthesis capacity in potato plants after HA application, both leading to improved plant growth. In addition, application of HA has been shown that significantly and positively influence the plant tolerance to salinity and drought stress (Amiri Forotaghe et al., 2022; Ebrahimi et al., 2021; Serri et al., 2021). The role of HS in enhancing plant growth under both optimal and stress conditions has also been attributed to changes in phytohormone levels induced by HS applications (Chen et al., 2022; De Hita et al., 2020).

Phytohormones are defined as small signalling molecules produced in low concentrations that perform their physiological functions in cells different from where they were produced (Mukherjee et al., 2022). Some hormones, such as auxins, including indole-3-acetic acid (IAA), cytokinins (CKs), that is, trans-zeatine (tZ) and isopentenyl adenine (iP), and gibberellins (GAs), regulate developmental processes as seed germination, cell division and proliferation, root elongation, and nutrient acquisition (Navarro-León et al., 2016). Other molecules such as abscisic acid (ABA), ethylene which is synthesized from 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic (SA), and jasmonic (JA) acids are known as related stress phytohormones (de la Torre-González et al., 2017).

Phytohormonal like activity has been attributed to HS as consequence of hormones such as IAA or GAs may be found embedded in their molecular structure. Nevertheless, it is less probable to find hormones in HS purified and extracted from, for example, leonardite (Mora, Baigorri, Bacaicoa, Zamarreño, & García-Mina, 2012; Nardi et al., 2018). Changes in phytohormone concentrations induced after HS application is reported in different studies as physiological mechanism of HS to enhance plant growth (Chen et al., 2022; De Hita et al., 2020; Wang et al., 2023). However, there is limited information about the differences of radicular and foliar applications, in terms of hormones concentration. For this reason, the objective of the present study is to test phytohormones accumulation changes as physiological mechanism of HS to promote plant growth, as well as compare between radicular and foliar modes of application. For this aim, HS extracted from leonardite were applied to lettuce (*Lactuca sativa* L.), and related growth parameters and hormone concentrations in plant tissues were analysed.

2 | MATERIALS AND METHODS

2.1 | Plant growth conditions

L. sativa L. cv. Capitata seeds were germinated and grown for 45 days in an experimental greenhouse located in Semillero Saliplant S.L. (Motril, Granada, Spain). The 45-day-old lettuce plants were transplanted to a controlled environmental conditions growth chamber of the Department of Plant Physiology (University of Granada, Spain) and potted in pots filled with vermiculite:perlite (3:1). The photoperiod was adjusted at 16/8 h with a photosynthetic flux density (PPFD) of 350 µmol m^{-2} s^{-1} (measured with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Besides, relative humidity and temperature were programmed at 60%–80% and 25/15°C (day/night), respectively. During the study, plants received a nutritive solution composed of: 3 mM Ca(NO$_3$)$_2$•4H$_2$O, 4 mM KNO$_3$, 1 mM KH$_2$PO$_4$, 1 mM NaH$_2$PO$_4$•2H$_2$O, 2 mM MgSO$_4$•7H$_2$O, 0.25 µM CuSO$_4$•5H$_2$O, 2 µM MnCl$_2$•4H$_2$O, 0.1 µM Na$_2$MoO$_4$•2H$_2$O, 10 µM H$_3$BO$_3$, and 5 µM Fe-chelate (Sequestrene; 138FeG100). This solution was renewed every 3 days and the pH adjusted at 5.5–6.

2.2 | Treatments and experimental design

Treatments started 7 days after transplantation and were maintained for 30 days. As source of HS, we employed BLACKJAK®, provided by Sofbey S.A. (Mendrisio, Switzerland). This biostimulant contains around 30% of organic matter and an acidic pH. A total of three applications were conducted, with a periodicity of 10 days, by the two modes of application, radicular (R) and foliar (F), at doses: R1 (0.20 mL/L), R2 (0.40 mL/L), R3 (0.60 mL/L), R4 (0.80 mL/L), F1 (5 mL/L), F2 (7.50 mL/L), F3 (10.00 mL/L), and F4 (12 mL/L). R-HS applications were made by diluting BLACKJAK® in the nutritive solution (pH = 4.5), and irrigating lettuce directly with 100 mL per pot in each application. For its part, F-HS applications were conducted by spraying BLACKJAK® diluted in distilled water onto leaves (12.5 mL per plant), 2 h after the beginning of the photoperiod. The selection of the HS doses used in this study was carried out following commercial ranges and according to a previous screening in our research group using BLACKJAK® in lettuce cultivated in the same conditions of the present experiment. Lettuces from the control treatment only received nutritional solution without HS. Hence, a total of nine treatments were employed in the present study, with eight plants per treatment and three replications each.

2.3 | Plant sampling

Lettuce plants from each treatment were divided into shoots and roots, washed, dried, and weighed to obtain fresh weight (FW). Half of shoots and roots were lyophilized for phytohormone analysis. Furthermore, leaf area and root surface area were measured

using a LI-COR optical reader (LI-3000A, LI-COR Inc., Nebraska, USA), whilst root length was estimated using a ruler. In addition, shoots and roots were sampled before starting application (initial time, T_i) and weighed (initial FW, FW_i). Using FW_i and FW at the end of the experiment (final FW, FW_f), RGR of shoots and roots was estimated as: $RGR = (\ln FW_f - \ln FW_i)/(T_f - T_i)$ (Navarro-León et al., 2023).

2.4 | Phytohormone extraction and analysis

Phythormones were extracted and measured as described in Ghanem et al. (2008) through U-HPLC-MS system (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) by the use of a heated electrospray ionization (HESI) interface.

2.5 | Statistical procedures

Using Statgraphics Centurion 16.1.03, data obtained were subjected to a simple ANOVA at 95% of confidence. Means were compared by Fisher's least significant difference (LSD). The significance levels were expressed as $*p < .05$, $**p < .1$, $***p < .001$, NS (not significant).

3 | RESULTS AND DISCUSSION

3.1 | Shoot and root growth

The obtained results showed that HS significantly enhanced shoot FW at doses R1, R2, R3, F1, F2, and F3, with increases of 16%, 17%, 23%, 13%, and 13%, respectively, compared to control treatment (Figure 1a). Similarly, same doses increased shoot relative growth rate (RGR) (6%, 7%, 8%, 5%, and 7%) and leaf area (11%, 10%, 11%, 10%, and 10%), with respect to control plants (Figure 1b,c). The use of plant biostimulants has been described as an environmentally friendly approach to improve plant growth through different mechanisms including the enhancement of nutrients bioavailability and uptake, nutrient use efficiency, and quality traits. In this way, different compounds are included in biostimulant definition: seaweed and plant extracts, HS, protein hydrosylates, as well as beneficial bacteria and fungi (du Jardin, 2015). The positive effect of HS on plants growth has been demonstrated in various research studies, yielding similar results to those of our experiment. In this way, Maji et al. (2017) found an increase in shoot FW in *Pisum sativum* L. plants subjected to soil HA-rich vermicompost. Wheat (*Triticum aestivum* L.) grain production and spike weight was enhanced through HA (Khan et al., 2018) and fulvic acid (FA) (Kumar Sootahar et al., 2020) application. Similarly, De Hita et al. (2020) found that foliar and radicular HA enhanced *Cucumis sativus* L. shoot growth, whereas Kazemi et al. (2023) observed that foliar HA increased shoot FW in *Physalis alkekengi* L. Hence, our data

suggest the potential of the HS employed in this study as an environmentally friendly approach to enhance yields of horticultural crops, particularly showing better results with radicular applications, especially at R3. These results pave the way for future studies using the optimal HS doses, including other vegetable species.

Concerning root growth, HS enhanced root FW at doses R2 (32%), R3 (33%), F2 (15%), F3 (16%), and F4 (21%), compared to control (Figure 2a). Likewise, root RGR was also enhanced by HS addition at the same doses (Figure 2b). As described previously, apparently root growth is more affected by HS than shoot growth (Fahramand et al., 2014; Nardi et al., 2002), which is in line with our results, especially at radicular doses (Figures 1a,c and 2a,b). Different studies also found that HS enhance root biomass production in maize (Nunes et al., 2019), cucumber (De Hita et al., 2020), rice (van Tol de Castro et al., 2021), and spinach (Turan et al., 2022). Besides, as previously demonstrated, HS may increase root plasma membrane (PM)-H^+-ATPase activity, resulting in an apoplastic pH decrease, and promoting root length (Nardi et al., 2021; Zhao & Naeth, 2022). Thereby, HS application significantly improved root length at doses R2 (28%), R4 (23%), and F3 (23%), with respect to control plants (Figure 2c). Similarly, Zhang et al. (2021) observed an increase in root length after foliar lignocellulosic-FA application to tomato, whereas same effect on root growth was found by Zhao and Naeth (2022) in alfalfa subjected to lignite-HS.

Comparing both methods of application, a largest increase in shoot and root growth was observed in lettuce plants subjected to radicular-HS. Although there are few studies that compare radicular and foliar applications, HS directly added to roots could favour the activation of root PM-H^+-ATPase, with subsequent increase in root length, nutrients uptake, and total plant growth (Canellas et al., 2015; De Hita et al., 2020; Olaetxea et al., 2018). This fact could explain that HS applied as radicular offered better plant biomass production than foliar. Different physiological mechanisms could explain the potential use of BLACKJAK® as bioestimulant. Thereby, in the present experiment we focused on phytohormonal profile changes and its correlation with plant growth.

3.2 | Changes in phytohormonal profile

As discussed above, the phytohormones IAA, GAs, and CKs regulate various developmental processes. In this way, increases in these phytoregulators are correlated with a promotion of root and shoot growth (Castro-Camba et al., 2022; Li et al., 2023; Prasad, 2022). It is well known that HS may show auxin, cytokinin, and gibberellin-like activities, emulating the physiological effects of these hormones (Jindo et al., 2020; Souza et al., 2022). In addition, and as commented previously, HS could contribute to modify hormones concentrations, affecting plant growth and development (De Hita et al., 2020; Elmongy et al., 2017; Olaetxea et al., 2019).

Compared to control treatment, HS significantly enhanced IAA concentration in shoot after radicular application at R1 and R2 doses. In addition, at root level, only F2 significantly enhanced IAA (Table 1).
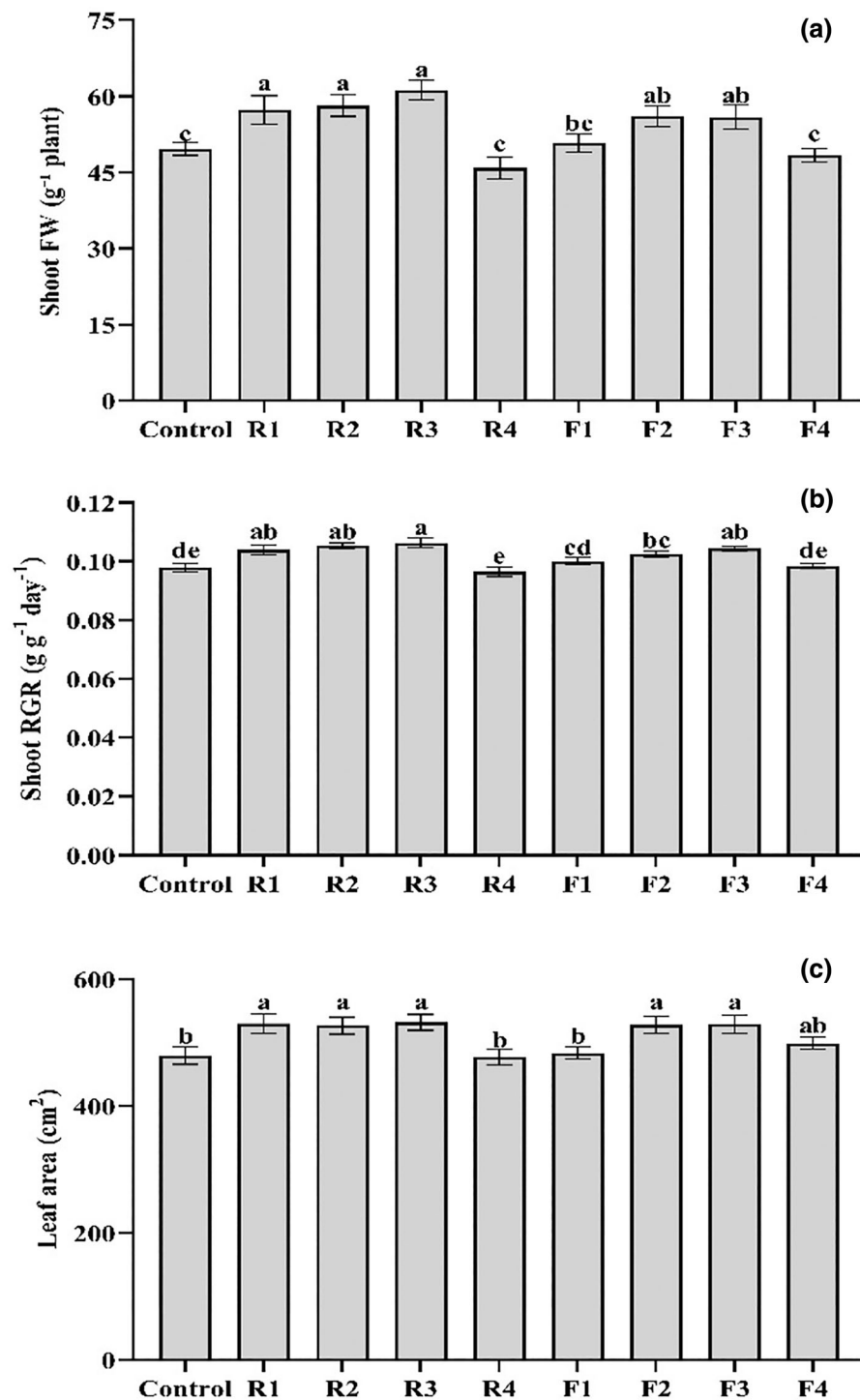
FIGURE 1 Effect of HS application on shoot FW (a), shoot RGR (b), and leaf area (c). Values are means ± standard errors ($n = 8$). Columns marked with the same letters were not significantly different based on LSD test ($p < .05$).

The involvement of IAA in plant growth has been well documented and explained through "the acid growth theory", where PM-H$^+$-ATPase is the final target of IAA. Its induction results in an acidification of cell walls, increasing their plasticity and contributing to cell growth (Canellas & Olivares, 2014). In our experiment, HS doses that significantly enhanced IAA concentration in shoot or root (Table 1) also enhanced shoot or root biomass production (Figures 1 and 2a,b). Nevertheless, F2 dose, which increased root IAA accumulation did not affect root length (Figure 2c). Similarly, some doses that enhanced plant growth, such as R3 (Figures 1 and 2), did not change IAA concentration (Table 1). Our data align with other studies where HS applied at determined doses enhance IAA concentration. Thus, Mora, Baigorri, Bacaicoa, Zamarreño, and García-Mina (2012) also found a significant accumulation of IAA at root level in cucumber plants treated with root purified HA, although this increase did not explain the morphological changes of roots. Besides, Elmongy et al. (2017) found

FIGURE 2 Effect of HS application on root FW (a), root RGR (b), and root length (c). Values are means ± standard errors ($n = 8$). Columns marked with the same letters were not significantly different based on LSD test ($p < .05$).
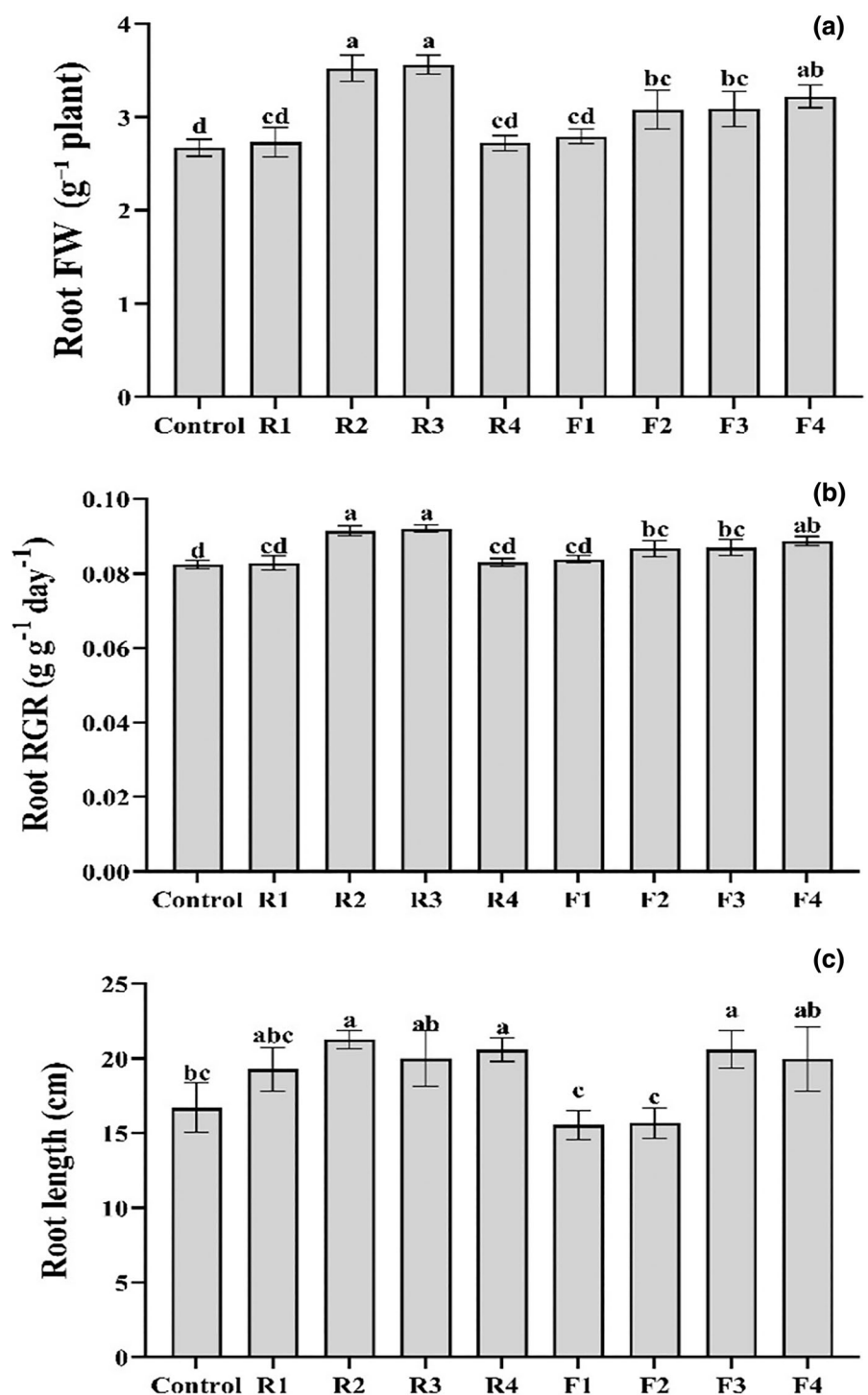
that HA added to culture medium increased IAA levels in microshoots of evergreen azalea, whereas De Hita et al. (2020) showed that foliar HA application enhanced shoot and root IAA concentration in cucumber after 48 h of the treatment.

Although most of the studies have focused on IAA, the capacity of HS in the up-regulation of genes involved in GAs and CKs biosynthesis have also been reported (Jannin et al., 2012). In this way, the application of FA and HA increased GAs concentration in faba bean

(Ali, 2015), whereas similar results were found in evergreen azalea (Elmongy et al., 2017). In our study, shoot GAs were increased at doses R1, R2, and R4, whereas HS did not affect root GAs concentration (Table 1). These results are like those obtained for IAA, where radicular doses that enhanced shoot growth also enhanced shoot CKs accumulation, except R3 (Figure 1 and Table 1). With respect to CKs, all HS doses employed enhanced significantly tZ concentration at shoot level, showing R2 and R3 the highest values (Table 1), which is

TABLE 1 Effect of HS application on IAA, GAs, and CKs (tZ and iP) concentration in shoot and root.

	IAA		Gas		tZ		iP	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	0.016 ± 0.001c	0.012 ± 0.002b	1.641 ± 0.801de	0.943 ± 0.173abcd	0.124 ± 0.009h	1.090 ± 0.152b	0.154 ± 0.001cd	0.148 ± 0.025
R1	0.652 ± 0.366b	0.009 ± 0.001b	6.153 ± 1.210b	0.137 ± 0.004d	0.473 ± 0.037e	1.913 ± 0.030a	1.198 ± 0.430b	0.222 ± 0.008
R2	1.389 ± 0.052a	0.013 ± 0.002b	10.153 ± 1.273a	0.553 ± 0.072bcd	1.468 ± 0.018a	0.579 ± 0.001c	1.945 ± 0.344a	0.209 ± 0.048
R3	0.019 ± 0.001c	0.009 ± 0.001b	0.080 ± 0.001e	0.235 ± 0.077cd	0.896 ± 0.047b	0.300 ± 0.018d	0.231 ± 0.001cd	0.104 ± 0.014
R4	0.011 ± 0.001c	0.014 ± 0.001b	4.424 ± 0.326bc	0.507 ± 0.200cd	0.762 ± 0.007c	0.282 ± 0.012d	0.192 ± 0.022bc	0.114 ± 0.035
F1	0.053 ± 0.023c	0.009 ± 0.001b	1.936 ± 0.716de	0.346 ± 0.001cd	0.293 ± 0.017f	0.056 ± 0.003e	0.921 ± 0.001b	0.187 ± 0.004
F2	0.019 ± 0.001c	0.085 ± 0.043a	0.320 ± 0.013e	1.052 ± 0.441abc	0.329 ± 0.019f	0.404 ± 0.086cd	NS	0.272 ± 0.018
F3	0.023 ± 0.001c	0.017 ± 0.001b	0.168 ± 0.034e	1.579 ± 0.635a	0.201 ± 0.001g	0.361 ± 0.043d	0.024 ± 0.002d	0.243 ± 0.058
F4	0.014 ± 0.001c	0.025 ± 0.001c	3.521 ± 0.443cd	1.350 ± 0.192ab	0.609 ± 0.001d	0.998 ± 0.001d	0.629 ± 0.001cd	0.438 ± 0.235
p-Value	***	*	***	*	***	***	***	NS
LSD$_{0.05}$	0.367	0.043	2.110	0.825	0.068	0.182	0.584	0.349

Note: All hormones are expressed as ng g^{-1} DW. Values are means ± standard error ($n = 6$). The levels of significance were represented as * ($p < .05$), *** ($p < .001$), and NS (not significant). Values with different letters indicate significant differences.

in line with shoot growth (Figure 1). A reduction of tZ was observed in roots at all doses except for R1, which enhanced it, and F4, which did not show significant differences with respect to control. Concerning shoot iP concentration, R1, R2, and F1 increased it (Table 1). The relationship between HS application and CKs concentration has been previously described as a root to shoot distribution of these hormones. Thus, Mora et al. (2010), using radicular-HA extracted from leonardite, observed a promotion of shoot growth associated with an increase in shoot CKs concentration and a decrease in roots. Similar results were subsequently showed by Olaetxea et al. (2019) who reported that shoot CKs accumulation is crucial for the shoot-growth promoting action of sedimentary HA. In our experiment, both root and foliar HS application also caused a root to shoot distribution of tZ, in most of the doses employed (Table 1), which could contribute to plant growth, as observed for R1, R2, R3, F2, and F3 doses (Figure 1).

Our data show, taken together, that IAA, CKs, and GAs accumulation was more prolonged in lettuce plants subjected to radicular-HS, compared to foliar doses, especially at R2 (Table 1). These results align with plant growth, where root HS doses showed the most shoot-growth promotion action (Figure 1). The possible further induction of root-PM-ATPase activity, which also influences phytohormones concentration and action (Mora et al., 2010; Olaetxea et al., 2019), through radicular HS could contribute to better plant growth. Hence, our data suggest that increases in IAA, GAs, and CKs, may contribute to plant growth, although further research is needed.

ACC is the direct ethylene precursor. Thus, a correlation between ACC and ethylene is often observed in plant cells, so that an increase in ACC is accompanied by a rise in ethylene levels (Navarro-León et al., 2016). Different aspects of plant growth and development are regulated by ethylene, including senescence, abscission, fruit ripening, and response to different biotic and abiotic stress (Li et al., 2022; Navarro-León et al., 2020). In the present study, HS application at doses F2 and F4 induced an increase of ACC concentration at shoot level, whereas R4 decreased it. Besides, all HS doses (except R1) produced a significant increase of ACC levels in lettuce roots (Table 1).

To our knowledge, there are no studies that evaluate the effect of foliar HS application on ACC accumulation. As commented, under stress conditions, a rise of ACC concentration is a typical response with negative impact on cell cycle and shoot growth (Dubois et al., 2018). It has been postulated that foliar application of HS may be perceived by plants as a stressful agent (De Hita et al., 2020). This could explain the increase of ACC levels in shoot after HS application at F2 and F4 doses (Table 2). However, these ACC increases did not negatively affect plant growth, showing F2 an increase in shoot growth (Figure 1). The main studies of HS application and ethylene accumulation are focused on root ACC levels. In this sense, Mora, Baigorri, Bacaicoa, Zamarreño, and García-Mina (2012) showed that a purified HA applied to nutritive solution significantly enhanced ethylene concentration in cucumber roots, which is exerted in an IAA-dependent pathway, as it was subsequently described (Mora et al., 2014). This result is in line with our study where most of the doses employed enhanced ACC (Table 2). However, Mora, Baigorri, Bacaicoa, Zamarreño, and García-Mina (2012) reported that this

TABLE 2 Effect of HS application on ACC and ABA concentration in shoot and root.

		ACC		ABA	
		Shoot	Root	Shoot	Root
	Control	1.73 ± 0.73cd	0.25 ± 0.01e	6.66 ± 1.41c	11.17 ± 0.43a
	R1	2.10 ± 0.50c	0.61 ± 0.08de	9.54 ± 0.01a	8.37 ± 0.62b
	R2	0.88 ± 0.01de	0.87 ± 0.01cd	2.51 ± 0.52e	7.68 ± 0.88b
	R3	1.47 ± 0.06cd	1.23 ± 0.14c	7.23 ± 0.48bc	6.64 ± 1.06bc
	R4	0.31 ± 0.04e	1.37 ± 0.16bc	7.24 ± 1.04bc	4.29 ± 0.18de
	F1	1.74 ± 0.46cd	1.84 ± 0.17ab	4.12 ± 0.61de	3.77 ± 0.27de
	F2	4.33 ± 0.04a	2.13 ± 0.07a	3.38 ± 0.14de	5.25 ± 0.52cd
	F3	1.34 ± 0.13cd	1.82 ± 0.01ab	4.54 ± 0.04d	3.12 ± 0.01e
	F4	3.11 ± 0.51b	2.18 ± 0.49a	9.08 ± 0.45ab	10.98 ± 1.11a
	p-Value	***	***	***	***
	LSD$_{0.05}$	0.91	0.56	2.02	1.99

Note: ABA is expressed as ng g^{-1} DW. ACC is expressed as µg g^{-1} DW. Values are means ± standard error ($n = 6$). The level of significance was represented as *** ($p < .001$). Values with different letters indicate significant differences.

TABLE 3 Effect of HS application on JA and SA concentration in shoot and root.

		JA		SA	
		Shoot	Root	Shoot	Root
	Control	1.88 ± 0.01bc	0.063 ± 0.002cd	3.51 ± 1.32bc	4.10 ± 0.95
	R1	1.84 ± 0.67bc	0.179 ± 0.001ab	5.45 ± 1.17ab	2.52 ± 0.19
	R2	2.98 ± 1.01ab	0.054 ± 0.029 cd	6.50 ± 0.86a	3.21 ± 0.41
	R3	4.38 ± 0.57a	0.021 ± 0.007d	5.66 ± 0.42ab	2.37 ± 0.28
	R4	0.04 ± 0.01d	0.051 ± 0.019cd	4.77 ± 1.50abc	3.33 ± 0.12
	F1	1.02 ± 0.47cd	0.045 ± 0.023cd	2.71 ± 0.62c	2.75 ± 0.01
	F2	0.23 ± 0.07d	0.106 ± 0.023bcd	2.55 ± 0.42c	4.00 ± 0.50
	F3	0.05 ± 0.02d	0.138 ± 0.001abc	3.11 ± 0.06bc	3.66 ± 1.47
	F4	0.35 ± 0.05d	0.227 ± 0.10a	2.18 ± 0.31c	3.91 ± 0.28
	p-Value	***	**	*	NS
	LSD$_{0.05}$	1.41	0.11	2.62	1.90

Note: JA and SA are expressed as ng g^{-1} DW. Values are means ± standard error ($n = 6$). The level of significance was represented as * ($p < .05$), ** ($p < .01$), *** ($p < .001$), and NS (not significant). Values with different letters indicate significant differences.

ethylene increase did not play an essential role to explain the main root morphological changes observed. Similarly, to test the implication of root ACC rise after HS application on lettuce growth, more research is needed, using specific hormonal inhibitors and analysing the expression levels of different enzymes involved in phytoregulators biosynthesis.

Moreover, ABA plays different roles in plant physiology depending on its levels. Thus, at basal levels, ABA contributes to plant growth regulating cell cycle, tissues development, chloroplast biogenesis, xylem differentiation and, ultimately, plant metabolism (Kishor et al., 2022). Additionally, it is well known that ABA is crucial in plant responses to different stresses due to the stomata closure induction (Singh & Roychoudhury, 2023). In our experiment, we observed a significant increase in ABA levels in lettuce shoots after HS application at R1 and F4 doses. Furthermore, R2, F1, F2, and F3 decreased shoot

ABA, showing R2 the lowest values. At root level, all HS doses decreased ABA concentration (except F4) (Table 2).

In contrast to ethylene, there are more studies reflecting the effect of HS application on ABA concentration in plant tissues. Thus, Mora et al. (2010) found that radicular HA application increased ABA accumulation in leaves, stem, and roots. Subsequently, Mora et al. (2014) also showed an increase in ABA concentration in roots by a nitric oxide (NO)-IAA-pathway, after HA application in cucumber. These authors suggest that the enhanced root ABA could contribute to promote shoot growth through an increase in water and nutrient uptake due to higher root hydraulic conductivity (Lp$_r$) and root aquaporins activation. Similar results were also found by Olaetxea et al. (2015). Likewise, it has been suggested that increased root ABA after HS application plays a crucial role in root growth through an enhanced Lp$_r$ (Olaetxea et al., 2019). However, our results are contrary to those
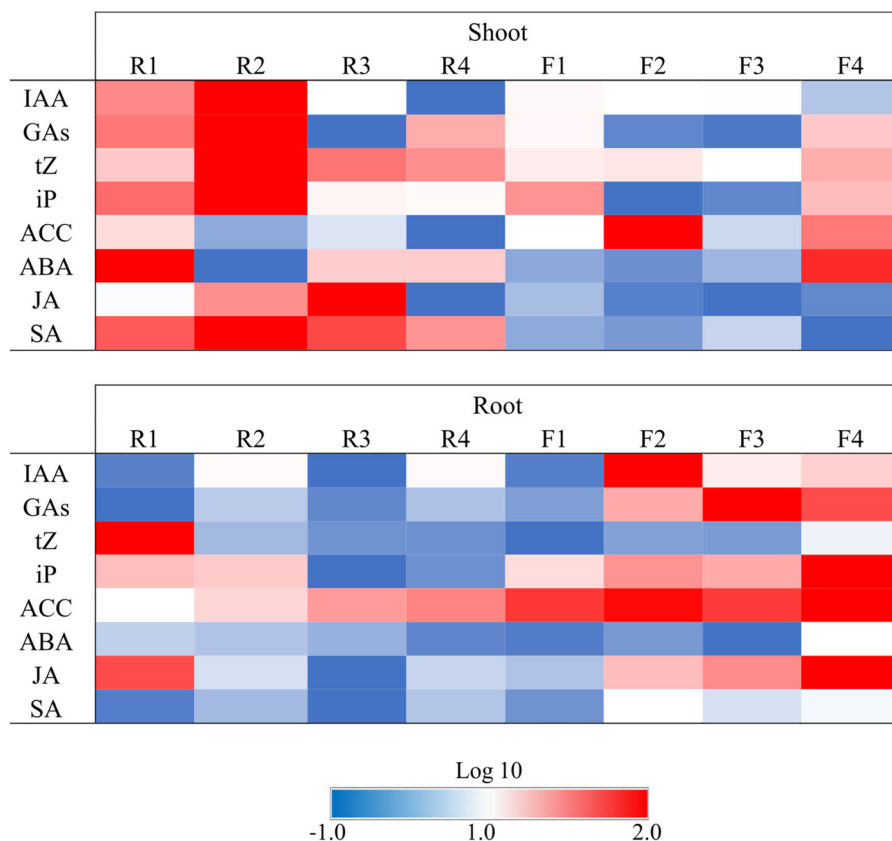
FIGURE 3 Heat map showing a summary of the changes in phytohormone profile induced by HS application in shoot and root of lettuce plants. Colour scale refers to the logarithmic transformation (log10) of measured values (higher values are shown in red, lower values in blue, and intermediate values in white colours), and it is compared to control treatment.

described in these studies as both root and foliar HS application, in general, decreased root ABA concentration (Table 2). Nevertheless, De Hita et al. (2020) observed that foliar HA application decreased ABA accumulation in both roots and shoots, despite the increase of plant growth. These authors suggest that decreasing ABA levels could contribute to increased growth through HA, as this hormone is associated with a decrease in plant growth when its concentration rises (Vysotskaya et al., 2017). In our study, R2 and F2 doses, which enhanced shoot growth (Figure 1) were the HS doses that most decreased shoot ABA (Table 2). Similarly, doses that decreased root ABA levels (Table 2), significantly enhanced root growth and length except F4 dose (Figure 2). Thus, and according to De Hita et al. (2020), the decrease in ABA could contribute to better plant growth after HS application, especially for R2 and F2 doses (Table 2). Nevertheless, future research is needed.

JA and SA are phytohormones involved in plant immunity responses to diseases caused by pathogens (Hou & Tsuda, 2022). Besides, both hormones participate in different types of abiotic stresses, as well as secondary metabolism modulation, being crucial as signal compounds for plant development (Lv et al., 2021; Navarro-León et al., 2016). HS application increased shoot JA accumulation at R3 dose, whereas R4, F2, F3, and F4 decreased it. Furthermore, only R1 and F4 enhanced JA at root level. Concerning SA, only R2 increased it in shoot (Table 3).

A negative crosstalk between JA and SA has been reported in several studies, as it influences plant development and immunity (Lv et al., 2021). JA regulates the response to herbivores and necrotrophic pathogens, whereas SA is involved in response to

biotrophic and hemi-biotrophic pathogens (Macioszek et al., 2023; Peng et al., 2021). Our results indicate that HS could contribute to increase lettuce tolerance to pathogen attacks at doses R2 and R3, as these doses enhanced SA and JA in shoots, respectively (Table 3). Similarly, De Hita et al. (2020) also suggested the possible implications of HA in cucumber tolerance to pathogens attacks, due to the enhanced JA in shoots after foliar HA applications. Besides, different studies have reported the potential involvement of JA in plant growth promoting action of HS (De Hita et al., 2020; Olaetxea et al., 2019; Silva et al., 2023). This fact could explain the possible relationship between JA and lettuce growth at R3 dose application (Table 3 and Figure 1). Likewise, future studies would be necessary to understand the physiological implications of JA and SA in shoot-growth promoting action of HS at doses R2 and R3, as well as their positive influence under eventual pathogens attacks. In addition, and concerning foliar applications, our data showed that all HS foliar doses (except F1) decreased shoot JA, which is contrary to those obtained by De Hita et al. (2020). A possible explanation of these differences is that, as it is well known, the physiological effects of HS depend on different aspects, including type of HS, source, dose, mode of application, and plant species (Canellas et al., 2015).

4 | CONCLUSIONS

According to our results, root and foliar applications of BLACKJAK® changed the phytohormonal concentrations in roots and shoots of

lettuce plants, showing for radicular and foliar doses higher increases in shoots and roots, respectively (Figure 3). In general, the relationship between plant growth promotion and hormones accumulation was more consistent with radicular HS application, due to the observed increase in IAA, GAs, tZ, JA, and SA, and decrease in shoot ABA, especially at R2 dose. However, although changes in phytohormonal profile could contribute to lettuce growth, it is possible that other physiological features such as nutrient uptake and photosynthesis performance may be behind the plant growth promotion of BLACK-JAK®. Likewise, future in-depth studies are needed to test the implications of hormones on the stimulatory effects of BLACKJAK®.

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REFERENCES

Ali, O. A. M. (2015). Role of humic substances and compost tea in improvement of endogenous hormones content, flowering and yield and its components of faba bean (*Vicia faba* L.). *Annals of Agricultural Sciences*, 53, 373–384.

Amiri Forotaghe, Z., Souri, M. K., Ghanbari Jahromi, M., & Mohammadi Torkashvand, A. (2022). Influence of humic acid application on onion growth characteristics under water deficit conditions. *Journal of Plant Nutrition*, 45, 1030–1040.

Billard, V., Etienne, P., Jannin, L., Garnica, M., Cruz, F., Garcia-Mina, J. M., Yvin, J. C., & Ourry, A. (2014). Two biostimulants derived from algae or humic acid induce similar responses in the mineral content and gene expression of winter oilseed rape (*Brassica napus* L.). *Journal of Plant Growth Regulation*, 33, 305–316.

Canellas, L. P., & Olivares, F. L. (2014). Physiological responses to humic substances as plant growth promoter. *Chemical and Biological Technologies in Agriculture*, 1, 3. https://doi.org/10.1186/2196-5641-1-3

Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., & Piccolo, A. (2015). Humic and fulvic acids as biostimulants in horticulture. *Scientia Horticulturae*, 196, 15–27.

Castro-Camba, R., Sánchez, C., Vidal, N., & Vielba, J. M. (2022). Plant development and crop yield: The role of gibberellins. *Plants*, 11, 20650.

Chen, Q., Qu, Z., Ma, G., Wang, W., Dai, J., Zhang, M., Wei, Z., & Liu, Z. (2022). Humic acid modulates growth, photosynthesis, hormone and osmolytes system of maize under drought conditions. *Agricultural Water Management*, 263, 107447.

De Hita, D., Fuentes, M., Fernández, V., Zamarreño, A. M., Olaetxea, M., & Garcia-Mina, J. M. (2020). Discriminating the short-term action of root and foliar application of humic acids on plant growth: Emerging role of jasmonic acid. *Frontiers in Plant Science*, 11, 493. https://doi.org/10.3389/fpls.2020.00493

de la Torre-González, A., Navarro-León, E., Albacete, A., Blasco, B., & Ruiz, J. M. (2017). Study of phytohormone profile and oxidative metabolism as key process to identification of salinity response in tomato commercial genotypes. *Journal of Plant Physiology*, 216, 164–173. https://doi.org/10.1016/j.jplph.2017.05.016

du Jardin, P. (2015). Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae*, 196, 3–14.

Dubois, M., Van Den Broeck, L., & Inzé, D. (2018). The pivotal role of ethylene in plant growth. *Trends in Plant Science*, 23, 311–323.

Ebrahimi, M., Souri, M. K., Mousavi, A., & Sahebani, N. (2021). Biochar and vermicompost improve growth and physiological traits of eggplant (*Solanum melongena* L.) under deficit irrigation. *Chemical and Biological Technologies in Agriculture*, 8, 19.

Elmongy, M. S., Zhou, H., Cao, Y., Liu, B., & Xia, Y. (2017). The effect of humic acid on endogenous hormone levels and antioxidant enzyme activity during *in vitro* rooting of evergreen azalea. *Scientia Horticulturae*, 227, 234–243.

Ertani, A., Francioso, O., Tugnoli, V., Righi, V., & Nardi, S. (2011). Effect of commercial lignosulfonate-humate on *Zea mays* L. metabolism. *Journal of Agricultural and Food Chemistry*, 59, 11940–11948.

Fahramand, M., Moradi, H., Noori, M., Sobhkhizi, A., Adibian, M., Abdollahi, S., & Rigi, K. (2014). Influence of humic acid on increase yields of plants and soil properties. *International Journal of Farming and Allied Sciences*, 3, 339–341.

Ghanem, M. E., Albacete, A., Martínez-Andújar, C., Acosta, M., Romero-Aranda, R., Dodd, I. C., Lutts, S., & Pérez-Alfocea, F. (2008). Hormonal changes during salinity-induced leaf senescence in tomato (*Solanum lycopersicum* L.). *Journal of Experimental Botany*, 59, 3039–3050.

Hou, S., & Tsuda, K. (2022). Salicylic acid and jasmonic acid crosstalk in plant immunity. *Essays in Biochemistry*, 66, 647–656.

Jannin, L., Arkoun, M., Ourry, A., Laîné, P., Goux, D., Garnica, M., Fuentes, M., San Francisco, S., Baigorri, R., Cruz, F., Houdusse, F., Garcia-Mina, J.-M., Yvin, J.-C., Etienne, P., Etienne, P., Jannin, L., Arkoun, M., Ourry, A., Laîné, P., ... Yvin, J. (2012). Microarray analysis of humic acid effects on *Brassica napus* growth: Involvement of N, C and S metabolisms. *Plant and Soil*, 359, 297–319.

Jindo, K., Pasqualoto Canellas, L., Albacete, A., Figueiredo dos Santos, L., Frinhani Rocha, R. L., Carvalho Baia, D., Oliveira Aguiar Canellas, N., Luc Goron, T., & Lopes Olivares, F. (2020). Interaction between humic substances and plant hormones for phosphorous acquisition. *Agronomy*, 10, 640.

Kazemi, S., Pirmoradi, M. R., Karimi, H., Raghami, M., Rahimi, A., Kheiry, A., & Malekzadeh, M. R. (2023). Effect of foliar application of humic acid and zinc sulfate on vegetative, physiological, and biochemical characteristics of *Physalis alkekengi* L. under soilless culture. *Journal of Soil Science and Plant Nutrition*, 23, 3845–3856.

Khan, R. U., Khan, M. Z., Khan, A., Saba, S., Hussain, F., & Jan, I. U. (2018). Effect of humic acid on growth and crop nutrient status of wheat on two different soils. *Journal of Plant Nutrition*, 41, 453–460.

Kishor, P. B. K., Tiozon, R. N., Fernie, A. R., & Sreenivasulu, N. (2022). Abscisic acid and its role in the modulation of plant growth, development, and yield stability. *Trends in Plant Science*, 27, 1283–1295.

Kumar Sootahar, M., Zeng, X., Wang, Y., Su, S., Soothar, P., Bai, L., Kumar, M., Zhang, Y., Mustafa, A., & Ye, N. (2020). The short-term effects of mineral-and plant-derived fulvic acids on some selected soil properties: Improvement in the growth, yield, and mineral nutritional status of wheat (*Triticum aestivum* L.) under soils of contrasting textures. *Plants*, 9, 205.

Li, Y., Han, S., & Qi, Y. (2023). Advances in structure and function of auxin response factor in plants. *Journal of Integrative Plant Biology*, 65, 617–632.

Li, D., Mou, W., Van De Poel, B., & Chang, C. (2022). Something old, something new: Conservation of the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid as a signaling molecule. *Current Opinion in Plant Biology*, 65, 102116.

Lv, Z. Y., Sun, W. J., Jiang, R., Chen, J. F., Ying, X., Zhang, L., & Chen, W. S. (2021). Phytohormones jasmonic acid, salicylic acid, gibberellins, and abscisic acid are key mediators of plant secondary metabolites. *World Journal of Traditional Chinese Medicine*, 7, 307–325.

Macioszek, V. K., Jęcz, T., Ciereszko, I., & Kononowicz, A. K. (2023). Jasmonic acid as a mediator in plant response to necrotrophic fungi. *Cells*, *12*, 1027.

Maji, D., Misra, P., Singh, S., & Kalra, A. (2017). Humic acid rich vermicompost promotes plant growth by improving microbial community structure of soil as well as root nodulation and mycorrhizal colonization in the roots of *Pisum sativum*. *Applied Soil Ecology*, *110*, 97–108.

Man-Hong, Y., Lei, Z., Sheng-Tao, X., McLaughlin, N. B., & Jing-Hui, L. (2020). Effect of water soluble humic acid applied to potato foliage on plant growth, photosynthesis characteristics and fresh tuber yield under different water deficits. *Scientific Reports*, *10*, 7854.

Mora, V., Bacaicoa, E., Baigorri, R., Zamarreñ, A. M., & García-Mina, J. M. (2014). NO and IAA key regulators in the shoot growth promoting action of humic acid in *Cucumis sativus* L. *Journal of Plant Growth Regulation*, *33*, 430–439.

Mora, V., Bacaicoa, E., Zamarreñ, A.-M., Aguirre, E., Garnica, M., Fuentes, M., & García-Mina, J. M. (2010). Action of humic acid on promotion of cucumber shoot growth involves nitrate-related changes associated with the root-to-shoot distribution of cytokinins, polyamines and mineral nutrients. *Journal of Plant Physiology*, *167*, 633–642.

Mora, V., Baigorri, R., Bacaicoa, E., Zamarreño, A. M., & García-Mina, J. M. (2012). The humic acid-induced changes in the root concentration of nitric oxide, IAA and ethylene do not explain the changes in root architecture caused by humic acid in cucumber. *Environmental and Experimental Botany*, *76*, 24–32.

Mukherjee, A., Gaurav, A. K., Singh, S., Yadav, S., Bhowmick, S., Abeysinghe, S., & Verma, J. P. (2022). The bioactive potential of phytohormones: A review. *Biotechnology Reports*, *35*, e00748.

Nardi, S., Pizzeghello, D., & Ertani, A. (2018). Hormone-like activity of the soil organic matter. *Applied Soil Ecology*, *123*, 517–520.

Nardi, S., Pizzeghello, D., Muscolo, A., & Vianello, A. (2002). Physiological effects of humic substances on higher plants. *Soil Biology and Biochemistry*, *34*, 1527–1536.

Nardi, S., Schiavon, M., & Francioso, O. (2021). Chemical structure and biological activity of humic substances define their role as plant growth promoters. *Molecules*, *26*, 2256.

Navarro-León, E., Albacete, A., de la Torre-González, A., Ruiz, J. M., & Blasco, B. (2016). Phytohormone profile in *Lactuca sativa* and *Brassica oleracea* plants grown under Zn deficiency. *Phytochemistry*, *130*, 85–89.

Navarro-León, E., Grazioso, A., Atero-Calvo, S., Rios, J. J., Esposito, S., & Blasco, B. (2023). Evaluation of the alkalinity stress tolerance of three *Brassica rapa* CAX1 TILLING mutants. *Plant Physiology and Biochemistry*, *198*, 107712.

Navarro-León, E., Javier López-Moreno, F., Atero-Calvo, S., Albacete, A., Ruiz, J. M., & Blasco, B. (2020). CAX1a TILLING mutations modify the hormonal balance controlling growth and ion homeostasis in *Brassica rapa* plants subjected to salinity. *Agronomy*, *10*, 1699.

Nunes, R. O., Domiciano, G. A., Alves, W. S., Melo, A. C. A., Nogueira, F. C. S., Canellas, L. P., Olivares, F. L., Zingali, R. B., & Soares, M. R. (2019). Evaluation of the effects of humic acids on maize root architecture by label-free proteomics analysis. *Scientific Reports*, *9*, 12019.

Olaetxea, M., Mora, V., Bacaicoa, E., Baigorri, R., Garnica, M., Fuentes, M., Zamarreño, A. M., Spíchal, L., & García-Mina, J. M. (2019). Root ABA and H⁺-ATPase are key players in the root and shoot growth-promoting action of humic acids. *Plant Direct*, *3*, e00175.

Olaetxea, M., De Hita, D., Garcia, C. A., Fuentes, M., Baigorri, R., Mora, V., Garnica, M., Urrutia, O., Erro, J., Zamarreño, A. M., Berbara, R. L., & Garcia-Mina, J. M. (2018). Hypothetical framework integrating the main mechanisms involved in the promoting action of rhizospheric humic substances on plant root- and shoot-growth. *Applied Soil Ecology*, *123*, 521–537.

Olaetxea, M., Mora, V., Bacaicoa, E., Garnica, M., Fuentes, M., Casanova, E., Zamarreño, A. M., Iriarte, J. C., Etayo, D., Ederra, I., Gonzalo, R., Baigorri, R., & García-Mina, J. M. (2015). Abscisic acid

regulation of root hydraulic conductivity and aquaporin gene expression is crucial to the plant shoot growth enhancement caused by rhizosphere humic acids. *Plant Physiology*, *169*, 2587–2596.

Peng, Y., Yang, J., Li, X., & Zhang, Y. (2021). Salicylic acid: Biosynthesis and signaling. *Annual Review of Plant Biology*, *72*, 761–791.

Prasad, R. (2022). Cytokinin and its key role to enrich the plant nutrients and growth under adverse conditions — An update. *Frontiers in Genetics*, *13*, 883924.

Serri, F., Souri, M. K., & Rezapanah, M. (2021). Growth, biochemical quality and antioxidant capacity of coriander leaves under organic and inorganic fertilization programs. *Chemical and Biological Technologies in Agriculture*, *8*, 33.

Silva, R. M., Peres, A. N. A., Peres, L. E. P., Olivares, F. L., Sangi, S., Canellas, N. A., Spaccini, R., Cangemi, S., & Canellas, L. P. (2023). Humic substances isolated from recycled biomass trigger jasmonic acid biosynthesis and signalling. *Plants*, *12*, 31–48.

Singh, A., & Roychoudhury, A. (2023). Abscisic acid in plants under abiotic stress: Crosstalk with major phytohormones. *Plant Cell Reports*, *42*, 961–974.

Souri, M. K., & Hatamian, M. (2019). Aminochelates in plant nutrition: A review. *Journal of Plant Nutrition*, *42*, 67–78.

Souza, A. C., Lopes Olivares, F., Eustáquio, L., Peres, P., Piccolo, A., & Canellas, L. P. (2022). Plant hormone crosstalk mediated by humic acids. *Chemical and Biological Technologies in Agriculture*, *9*, 29.

Tiwari, J., Ramanathan, A. L., Bauddh, K., & Korstad, J. (2023). Humic substances: Structure, function and benefits for agroecosystems—A review. *Pedosphere*, *33*, 237–249.

Turan, M., Ekinci, M., Kul, R., Kocaman, A., Argin, S., Zhirkova, A. M., Perminova, I. V., & Yildirim, E. (2022). Foliar applications of humic substances together with Fe/Nano Fe to increase the iron content and growth parameters of spinach (*Spinacia oleracea* L.). *Agronomy*, *12*, 2044.

van Tol de Castro, T. A., Berbara, R. L. L., Tavares, O. C. H., da Graca Mello, D. F., Pereira, E. G., de Souza, C. C. B., Espinosa, L. M., & García, A. C. (2021). Humic acids induce a eustress state via photosynthesis and nitrogen metabolism leading to a root growth improvement in rice plants. *Plant Physiology and Biochemistry*, *162*, 171–184.

Vysotskaya, L. B., Arkhipova, T. N., Kudoyarova, G. R., & Veselov, Y. (2017). Dependence of growth inhibiting action of increased planting density on capacity of lettuce plants to synthesize ABA. *Journal of Plant Physiology*, *220*, 69–73.

Wang, Y., Lu, Y., Wang, L., Song, G., Ni, L., Xu, M., Nie, C., Li, B., & Bai, Y. (2023). Analysis of the molecular composition of humic substances and their effects on physiological metabolism in maize based on untargeted metabolomics. *Frontiers in Plant Science*, *14*, 1122621.

Yang, F., Tang, C., & Antonietti, M. (2021). Natural and artificial humic substances to manage minerals, ions, water, and soil microorganisms. *Chemical Society Reviews*, *50*, 6221–6239.

Zhang, P., Zhang, H., Wu, G., Chen, X., Gruda, N., Li, X., Dong, J., & Duan, Z. (2021). Dose-dependent application of straw-derived fulvic acid on yield and quality of tomato plants grown in a greenhouse. *Frontiers in Plant Science*, *12*, 736613.

Zhao, Y., & Naeth, M. A. (2022). Soil amendment with a humic substance and arbuscular mycorrhizal fungi enhance coal mine reclamation. *Science of the Total Environment*, *823*, 153696.