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A way to valorize pomace from olive oil production: Lignin nanoparticles to biostimulate maize plants

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ABSTRACT

Agriculture will increasingly face in coming years numerous challenges, such as climate change, the growing global demand for food, its environmental impact due to greenhouse gas emissions, the release of pollutants into the environment, and the production of significant amounts of waste.

This study focused on pomace, a waste of olive pressing, as it contains substances that can be valorized. Specifically, this study aimed to obtain lignin nanoparticles (LNPs) by treating the biomass with an ionic liquid (IL) composed of triethylamine and sulfuric acid [Et_3NH][HSO_4], having different concentrations of water (IL_95, IL_90 and IL_80). The LNPs obtained were characterized by Fourier Infrared Spectroscopy (FT-IR), Field Emission Scanning Electron Microscopy (FE-SEM), and Thermogravimetric (TGA) analysis. The 95/5 IL/water ratio allowed for obtaining the best LNPs regarding purity, yields and morphology, which were further characterized by investigating Zeta Potential (ξ) and Energy Dispersive x-ray Spectroscopy (EDX). The LNPs from IL_95 were applied to maize plants at 25, 50 and 200 mg L^{-1}. Improvements in growth, physiological and biochemical traits were ascertained in the treated samples. LNPs generally induced photosynthetic activity, stomatal conductance and reduced cellular CO_2 concentration. In addition, improvements in biomass production, root morphology and pigment content were ascertained. Finally, the study of the cellular redox state showed that the lower dosages did not cause oxidative perturbations; instead, they had ameliorative effects. In conclusion, this study opens a new way to valorize olive oil pomace by obtaining nano-scaled lignin, a sustainable and biobased material with interesting biostimulant action.

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1. Introduction

Climate change is one of the factors that is affecting and will increasingly affect agricultural production, with substantial implications for food quantity and quality (Bhupenchandra et al., 2022). This prospect raises more than one concern about the possible socio-economic implications. In addition, it is also necessary to consider that the need for food for an ever-growing world population is a challenge that agricultural production systems will necessarily have to address in coming years with effective measures (Del Buono, 2021). The Food and Agriculture Organization of the United Nations (FAO) stated that by 2050 the population will have increased by more than 30% and the demand for food by 70% (Sutradhar et al., 2022). To understand the context shaping up for the years ahead, it is also necessary to consider aspects of the sustainability of current agricultural systems. A high environmental impact characterizes agriculture in terms of greenhouse gas emissions, the dispersion of pollutants into the environment, and the production of huge amounts of waste that are difficult to manage from both an economic and an environmental point of view (Del Buono, 2021; Puglia et al., 2021).

For these reasons, a very interesting strategy to reduce the environmental impact of agriculture and increase its productivity and income is the valorization of agro-industrial wastes, according to a multipurpose bio-refinery model, to obtain biobased products with added value that can be reused for different scopes (Asghar et al., 2022). Overall, this approach aims to recognize the potential of a specific waste through knowledge of its chemical composition and then explore it by extracting different types of substances to be reused, creating a system of excellence and a model of bio-economy (Rodríguez-Espíndola et al., 2022). In this context, in addition to the well-known types of biobased materials (chemicals, fuels, biomaterials), special attention is recently being paid to the valorization of agro-industrial wastes, which are rich in bioactive substances, for obtaining biostimulants (Shu et al., 2021). Biostimulants are a broad class of materials of generally natural origin that can be considered an effective tool for increasing crop productivity under both normal and stressful environmental conditions (Panfili et al., 2019). Many studies have shown the effectiveness of biostimulants in increasing crop yield and product quality, as well as crop resistance to biotic and abiotic stress (Ma et al., 2022). Despite this, the mechanisms by which biostimulants improve crop performance remain challenging to frame, given the complexity and the many substances present in a given preparation (Nephali et al., 2020).

The olive-oil supply chain generates large amounts of waste containing many potentially bioactive compounds (Otero et al., 2021). With particular attention to olive oil production, the large amounts of waste biomass produced are difficult to manage and can generate disposal problems and environmental and economic impacts (Contreras et al., 2020). In more detail, olive oil production is based on two or three-phase systems, with the latter being the most common in the Mediterranean area (55% and 82% in Italy and Greece, respectively) and leading to liquid and solid wastes: olive mill wastewater and pomace (Contreras et al., 2020). Olive pomace is mainly composed of kernel and pulp. Due to its chemical composition, pomace is a by-product that, if irrationally released into the environment, could be toxic because of its pH, high salinity, and phenols and lipids content (Cequier et al., 2019). Among the components of commercial or industrial interest, olive pomace is rich in lignin that can reach 30% content on a dry weight basis (Cequier et al., 2019). Indeed, lignin is gaining interest for recent applications as it is suitable for biofuels and bioplastics production, food industry, agriculture, and obtaining nanoparticles and nanocomposites (Contreras et al., 2020; Del Buono et al., 2021a, 2022).

Regarding applications in agriculture, recent scientific evidence has shown how this biopolymer can be used to positively influence plant growth and physiology, soil microorganisms and fertility without adversely affecting the environment, given its eco-compatibility (Savy and Cozzolino, 2022). In addition, lignin can be used to produce carriers for the controlled release of nutrients, fertilizers and chemical compounds because of its molecular structure and the functional groups it contains (Savy and Cozzolino, 2022).

Concerning the biostimulant potential of lignin, some recent studies have shown as this macromolecule and some of its derivatives can exert positive effects on the growth and development of certain crops by enhancing seed germination, root elongation and stimulating the first developmental stages (Savy et al., 2017; Spaccini et al., 2019). These studies revealed that this benefit could be related to the ability of lignin to modify the hormone balance in treated species, with a trend dependent on the dose applied. Specifically, lower doses were found to be more effective, while higher doses lost efficacy due to the possible formation of aggregates or phytotoxic effects on treated plants (Savy and Cozzolino, 2022).

Recently, the use of lignin nanoparticles (LNPs), obtained from commercially available lignin has been shown to positively affect maize plants, stimulating plant shoot and root development, chlorophyll content, carotenoids, anthocyanins, and soluble protein content (Del Buono et al., 2021a). Nanomaterials can exhibit interesting biological effects for their surface features that impart unique chemical and physical properties (Sohail et al., 2022). In addition, unlike synthetic nanomaterials, it is emphasized that LNPs are environmentally friendly, biocompatible and show a high penetration rate into plant cells (Schneider et al., 2021).

Although agriculture is showing increasing interest towards nanomaterials, the study of the effect of nano-scaled lignin on crops remains an almost unexplored and challenging research area, considering that it can be conveniently recovered from waste biomass, making the prospect of its valorization even more attractive.

For the above reasons, this research aimed to obtain LNPs from pomace by applying a biomass treatment method based on an ionic liquid (IL) composed by triethylamine and sulfuric acid [Et_3NH][HSO_4], employing different IL/water ratios to find the best condition for treating pomace. The approach based on this IL was selected among several presents in the literature for its effectiveness, high yields of recovered lignin, reusability of [Et_3NH][HSO_4] and low environmental impact (Brandt-Talbot et al., 2017). Subsequently, LNPs were used for the foliar treatment of young maize (*Zea mays* L.) seedlings to determine the effects on plant growth and physiological and biochemical activity.

2. Materials and methods

2.1. IL [Et_3NH][HSO_4] synthesis and LNPs obtainment from pomace

The ionic liquid (IL) - triethylammonium sulfate [Et_3NH][HSO_4] - was synthesized according to Cequier et al. (2019). 0.1 mol of triethylamine (Et_3N) was placed in a 250 mL round-bottomed flask and maintained under stirring at 60 °C. After that, 0.1 mol of H_2SO_4 was added dropwise, and the mixture was left to react for 1.5 h at 70 °C.

The pomace was characterized according to Van Soest (1963) and contained 26% lignin as mean value. The biomass extraction was carried out according to published procedures (Brandt-Talbot et al., 2017; Cequier et al., 2019) with some modifications. 2 g of the dry biomass was mixed with [Et_3NH][HSO_4] (1:10, w:w), considering three different water contents (5%, 10%, 20%): IL_95, IL_90 and IL_80. Additionally, 0.009 mol of H_2SO_4 was added to the mixture to maximize the yield of lignin extraction (Brandt-Talbot et al., 2017). The mixture was then left at 120 °C for 4 h. Once the extraction was completed, the mixture was left to cool at room temperature, and 54 mL of ethanol (EtOH) were added to the mixture, which was filtered to eliminate the solid residue. The EtOH was then evaporated, and 170 mL of H_2O were added to precipitate lignin. Finally, the lignin suspension was centrifuged and washed more times with water and then with 1% formic acid and left to dry at 60 °C.

2.2. Lignin characterization

Fourier infrared (FT-IR) spectra of lignin nanoparticles were recorded using a Jasco FT-IR 615 spectrometer (Jasco Corporation, Tokyo, Japan) in the 4000–600 cm^{-1} range, in transmission mode. The lignocellulosic materials were analyzed using KBr discs prepared by using pulverized natural materials and KBr powder.

The produced lignin nanoparticles morphology, thermal and chemical structure were investigated. The morphology of the nanoparticles was analyzed by using a Field Emission Scanning Electron Microscope (FESEM, Supra 25-Zeiss, Oberkochen, Germany). A small drop of lignin nanoparticle water suspension was deposited on silicon substrates, air-dried for 24 h, gold coated using an ion sputter coater, and observed with the gun operating at 5 kV. Analysis of thermodegradative behavior for lignin nanoparticles was performed by heating the samples from 30 to 900 °C at 10 °C min^{-1} under nitrogen flow (250 mL min^{-1}) by using a Seiko Exstar 6300 (Seiko Instruments Inc., Chiba, Japan).

The distribution of nanoparticles dimension (diameter) was obtained by analysis of FESEM images using NIS Element Basic Research Software.

Elemental composition and chemical mapping were determined using a Bruker Quantax EDX. In addition, the zeta potential value (ξ potential) of LNPs (water suspensions 0.2mg/mL) was determined with a NICOMP 380 ZLS equipped with a HeNe Laser source at 632.8 nm (Particle Sizing System Inc., Santa Barbara, CA, USA).

2.3. Maize growth conditions and LNPs treatments

Maize seeds (hybrid ISH302V) were sterilized for 3 min with a solution of NaClO (0.25%, w/v). Seeds were then rinsed several times with distilled water, placed in pots containing 40 g of perlite and 200 mL of H_2O, and left in the dark for 5 days. Subsequently, pots were placed in a growth chamber with a photoperiod of 12/12 h (day/night), light intensity at 300 $\mu mol\ m^{-2}\ s^{-1}$, at a constant temperature of 24 °C and added of a nutrient solution consisting of 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 1 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM $ZnSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 100 μM Fe-EDTA.

The seedlings were treated with LNPs by two foliar spray applications 7 and 14 days after sowing, according to a completely randomized experimental design, in which 4 replicates were performed for each experimental group. The treatments were as follows: control, 25 mg L^{-1} LNPs, 50 mg L^{-1} LNPs and 200 mg L^{-1} LNPs. These concentrations were chosen since the eventual beneficial effects of nanomaterials depend on the concentrations applied. In fact, phytotoxic effect can be provoked by excessive amounts of nanoparticles or be specie-depending (Tolisano and Del Buono, 2023).

Plants were harvested at the third leaf stage (21 days after sowing) for the determination described in the following sections, and shoot height, weight and leaf area were recorded. In addition, root analyses were carried out on root-scanned images using RhizoVision Explorer v2.0.3.0, according to Seethepalli et al. (2021).

2.4. Leaves gas exchanges

Net leaf photosynthesis (Pn), stomatal conductance (gs) and sub-stomatal CO_2 concentration (Ci) were determined on four individuals for each maize treatment as described in Section 2.6. Leaf gas exchange rates were measured with a portable IRGA (ADC-LCA-3, Analytical Development, Hoddesdon, UK) and a Parkinson-type assimilation chamber. Leaves were confined in the chamber and exposed to the same light as in the growth chamber. Measurements were carried out under steady-state conditions (after about 30 s); Pn, gs and E were calculated based on the leaf area recorded for each sample investigated.

2.5. Pigment content

The content of chlorophyll a and b and carotenoids were ascertained on maize seedlings harvested 21 days after the different treatments. 0.5 g of leaf samples were extracted with 5 mL of methanol, then the suspension was filtered. The extract was analyzed spectrophotometrically at 452.5, 644, and 663 nm to obtain chlorophyll a and b as well as the carotenoid contents, according to Venkatachalam et al. (2017). In addition, the concentration of total chlorophyll (Chl a + Chl b) was estimated as the sum of Chl a and Chl b.

2.6. Oxidative status of plants (H_2O_2 and MDA) and total phenolic compounds (TPC), total flavonoid compounds (TFC) and anthocyanin

Hydrogen peroxide (H_2O_2) content was also assessed in fresh leaf shoots by extracting 0.5 g of plant tissue with 5 mL of 0.1% (w/v) TCA. That suspension was then centrifuged (10 min at 6000 rpm), and 0.5 mL of the supernatant were used to determine the H_2O_2 content, according to Velikova et al. (2000).

Malondialdehyde (MDA) content was ascertained for 0.5 g of fresh shoots extracted with 5 mL of 5% (w/v) trichloroacetic acid (TCA). The resulting suspension was centrifuged (15 min at 6000 rpm), after which 1 mL of the supernatant was incubated at 95 °C for 20 min with 2.0 mL of 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. After cooling, the MDA content was determined spectrophotometrically (Heath and Packer, 1968).

The total phenolic compounds (TPC) was determined by adding 0.5 mL of the extracts to 7 mL of distilled water and 0.5 mL of Folin–Ciocalteu's phenol reagent. After 5 min, 2 mL of 2% sodium carbonate (w/v) were added to this solution and left to react for 2 h. The TPC content was determined spectrometrically and referred to as gallic acid equivalent (GAE) g^{-1} (Paradiković et al., 2011).

Total flavonoid compounds (TFC) was determined colorimetrically, according to Atanassova et al. (2011). 0.5 g of fresh plant material was extracted with 5 mL of 80% methanol/ 20% water, centrifuged for 10 min at 6000 rpm. Then, 1 mL of supernatant was mixed, in order every 5 min, with: 0.3 mL 5% $NaNO_2$, 0.3 mL 10% $AlCl_3$, 2 mL 1M NaOH. Once the additions were complete, the volume it was brought 10 mL with H_2O. The absorbance of the samples was recorded at 510 nm. FTC are expressed in milligrams of catechin equivalents (CE) per gram fresh weight (mg EC g^{-1} fw).

For determination of anthocyanin content, the methanolic extracts were subjected to spectrophotometric determination conducted at 535 and 650 nm (Lichtenthaler and Buschmann, 2005).

2.7. Statistics

The experiments were carried using according to a completely randomized design with four treatments (control, 25 mg L^{-1}, 50 mg L^{-1} and 200 mg L^{-1} LNPs) and four replicates per treatment. Statistical analysis was performed through analysis of variance (one-way ANOVA). Significant differences were determined at $p \leq 0.05$, applying Duncan's test.

3. Results and discussion

3.1. Biomass extraction and lignin characterization

A procedure based on the use of an ionic liquid (IL) consisting of triethylamine and sulfuric acid ($[Et_3NH][HSO_4]$) at different water content (IL 80% - H_2O 20%, IL 90% - H_2O 10%, IL 95% - H_2O 5%) was adopted for treating pomace and extracting lignin. Different ILs have been used for biomass processing, and they have generally shown high lignin extraction efficiency, regardless of the biomass considered (Ocreto et al., 2022; Sai Bharadwaj et al., 2023). The effectiveness of ILs treatment lies in the capacity of the basic anion to form strong hydrogen bonds, including those with cellulose fibrils, capable of dissolving the lignocellulosic matrix (Brandt-Talbot et al., 2017). In addition, the alkyl chains (ammonium cation) have a decisive role in the efficacy of pre-treating biomass, given their capacity to create significant hydrophobic interactions (Brandt-Talbot et al., 2017).

In this study, $[Et_3NH][HSO_4]$ was chosen considering its higher extraction yield and thermal stability, and lower cost than other ILs (Cequier et al., 2019; Hart et al., 2015). Furthermore, to increase the yield of the extraction process, an additional amount of sulfuric acid was added such that a final stoichiometric ratio on triethylamine of 1.009 to 1.000 was achieved; in fact, a slight excess of sulfuric acid may increase the treatment yield in terms of lignin obtained (Brandt-Talbot et al., 2017). Concerning the IL/H_2O ratio, different lignin extraction yields were obtained. Specifically, percentage yields were 34%, 54% and 65% when IL 80% - H_2O 20%, IL 90% - H_2O 10%, IL 95% - H_2O 5%, were applied to the biomass, respectively. Regarding water content in IL for biomass treatment, studies have shown its necessity for the effective fractionation of the biomass to be treated. In particular, water participates in hydrolysis reactions with ILs, facilitating the separation of biomass components, preventing undesirable sulfation reactions and reducing solvent viscosity (Brandt et al., 2011; Brandt-Talbot et al., 2017).

FT-IR spectra suggest that $[Et_3NH][HSO_4]$ effectively dissolved the biomass allowing obtaining lignin in purity, and there were no substantial differences between the concentrations of IL used (Fig. 1). In particular, the spectra showed characteristic intense absorbance peaks at 2930 cm^{-1} and 2850 cm^{-1} assigned to C–H stretching of aromatic methoxyl,

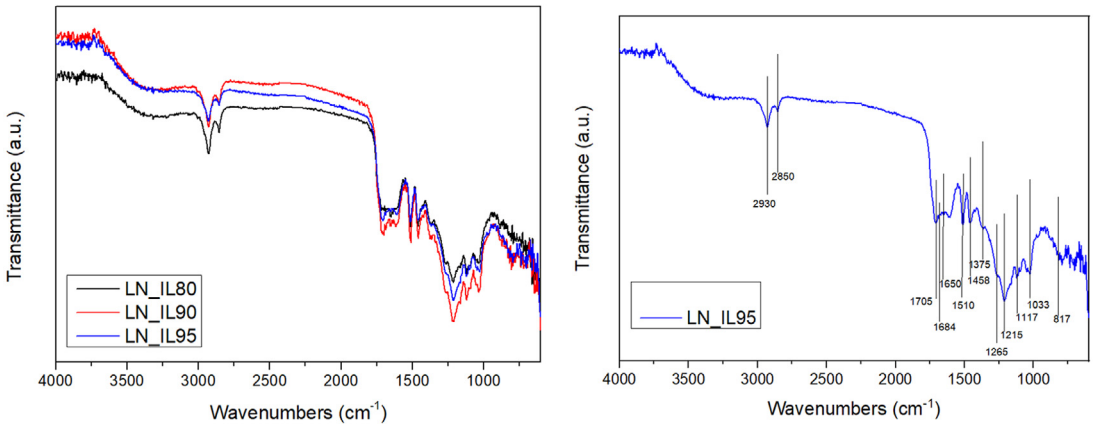

Fig. 1. FT-IR spectra of lignin obtained by extracting olive pomace with different percentages of IL ([Et_3NH][HSO_4], 80%–90%–95%). . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

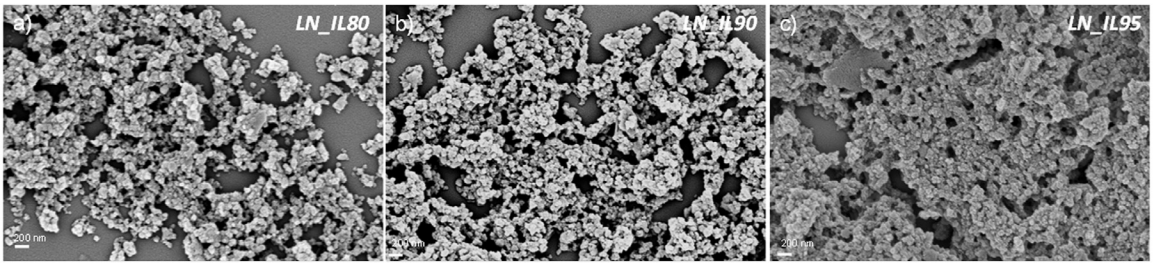

Fig. 2. FESEM images of lignin obtained by extracting olive pomace with different percentages of IL ([Et_3NH][HSO_4], 80% **(a)** - 90% **(b)** - 95% **(c)**).

methyl and methylene groups of side chains (Boeriu et al., 2004). The absorbance at 1705 cm^{-1} indicated the stretching of unconjugated carbonyls, and that at 1684 cm^{-1} was assigned to a shoulder associated with conjugated C=O and C=O stretching (Boeriu et al., 2004). The band at 1650 $cm^{-1}$was due to the presence of water in lignin (Boeriu et al., 2004; Liu et al., 2014) and that at 1510 cm^{-1} to the C=C stretching of the aromatic rings present in the structure of this macromolecule (Liu et al., 2014). The signal at 1458 cm^{-1} was associated with C–H asymmetric deformation combined with aromatic ring vibration, a typical signal common to all lignins (Boeriu et al., 2004; Liu et al., 2014). The band at 1375 cm^{-1} proved the presence of phenolic O-H and aliphatic C–H in methyl groups and that at 1265 cm^{-1} was assigned to G ring and C=O stretching (Boeriu et al., 2004; Casas et al., 2012). Both these last two bands indicated the Guaiacyl and Syringyl groups in the lignin (Liu et al., 2014). The absorbance at 1215 cm^{-1} was due to strong vibrations of C–C, C–O, C=O stretching and indicated that the condensed G is more representative than etherified G (Boeriu et al., 2004; Casas et al., 2012). The signal at 1117 cm^{-1} was due to the aromatic C–H deformation in the S ring (Casas et al., 2012). Finally, the band at 1033 cm^{-1} was assigned to the aromatic C–H in-plane deformation (G > S) (Boeriu et al., 2004; Casas et al., 2012), while that at 817 cm^{-1} was due to C–H out-of-plane deformation or vibration in position II, V, VI of G rings (Casas et al., 2012).

Fig. 2 shows the morphology of lignin obtained by extracting olive pomace with the different percentages of IL. It is possible to observe in Fig. 2a that LN_IL80 shows the presence of unreacted particles. LN_IL90 and LN_IL95 were characterized by more defined nanostructures (Figs. 2b and 2c). The amount of unreacted material decreased by increasing the IL concentration, as observed by FESEM images. Therefore, higher IL concentrations can better control the lignin particle size (Luo et al., 2021). In fact, the images underline the efficacy of the extractive method, and in particular of IL95 to obtain lignin at nanoscale dimension (mean diameter values of 40 $\pm$ 3 nm), evidencing the effectiveness of ionic liquid in allowing obtaining smaller LNPs when compared to other procedures of treating biomasses (Cheng et al., 2012).

Thermogravimetric (TG) and derivative thermogravimetric (DTG) curves of lignin obtained by extracting olive pomace with different IL/water concentrations are reported in Fig. 3. It is possible to observe that lignin decomposes in a wide range of temperatures due to its heterogeneity and lack of a well-defined primary structure (Zhang et al., 2014). The thermal decomposition of the lignin process consists of three distinct stages: drying (30–120 °C), fast degradation (150–400 °C) and slow degradation stage (500–800 °C) (Ma et al., 2016). Lignin thermal decomposition started by water release, followed by a multiple peaks event between 200 and 400 °C, with the formation of low molecular weight products. The main decomposition peak is visible in the range 300–400 °C: it should be noted that, in spite of a slight difference in the

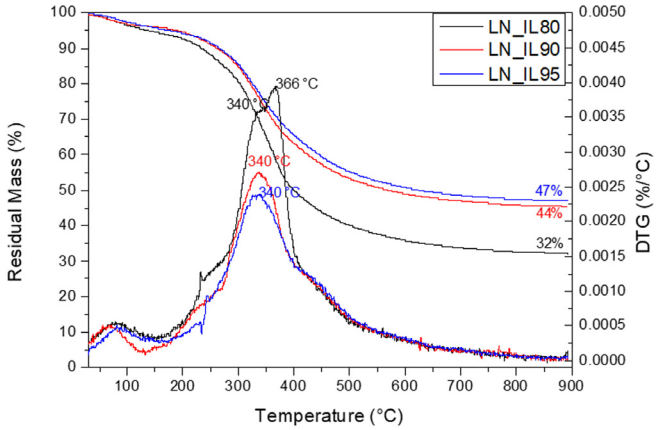

Fig. 3. TG and DTG curves of lignin obtained by extracting olive pomace with different percentages of IL ([Et_3NH][HSO_4], 80%–90% - 95%).. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DTG_{max} temperature, there was a tendency for a reduction of the heat stability as a function of IL concentration increase (Zhang et al., 2014).

A reduced amplitude of this main peak observed for IL_90 and IL_95 (centered at 340 °C) in comparison to IL_80 suggests that these systems resulted less fragmented in their inter-unit linkages (Zhang et al., 2017). The main thermal degradation peak (300-400 °C) of LN_IL80 was also characterized by the presence of double points centered at 340 °C and 366 °C. This indicates that the IL treatment broken down lignin and separated it in fractions with different chemical compositions (Zhang et al., 2014).

Finally, the cleavage of the main lignin chain was followed (above 500 °C) by condensation reactions and several rearrangements of the aromatic structure that led to the formation of char structures (Chollet et al., 2019; Prieur et al., 2017). The residual mass values at 900 °C were estimated to be 33%, 44% and 47% for LN_IL80, LN_IL90 and LN_IL95, respectively. The difference in the loss weight can be explained by considering that decrease in the hydroxyl groups from phenolic in lignins allowed the prevention of auto-condensation of this biopolymer during thermal decomposition. Therefore, IL treatment would have generated some more condensed aromatic structures leading to higher stability (Husson et al., 2019).

Since LNPs obtained using IL_95 showed a more defined nanostructure and higher yield, the nanoparticles from this treatment were furtherly characterized, determining ξ potential and EDX (Fig. 4). ξ potential mainly depends on the groups on the surface of the nanoparticles and is due to the potential created by the electrical repulsion between particles, organized to form an electrical double layer (Freitas et al., 2020). In the case of LNPs, the ξ potential depends on hydroxyl (-OH) and carboxylic groups (-COOH) (Ma et al., 2020). In general, ξ potential values for stable LNPs range between −20 and -40 mV (Ullah et al., 2022). In our study, for LNPs obtained using IL_95, a ξ potential of −23 mV was found. Therefore, our result indicates stable LNPs in line with those of Yan et al. (2023), who found for LNPs from rice straw ξ potential values ranging from −17.1 to −25.5 mV, and (Zhang et al., 2022) who found for LNPs obtained from residual biomass of paper pulp production values ranging from −20.26 mV to −28.01 mV.

EDX analysis clearly showed in LNPs the presence, as major elements, of carbon (69.3%) and oxygen (30.2%) and traces of sulfur (0.1%), chlorine (0.3%), aluminium and silicon (<0.1%) (Fig. 4). Carbon and oxygen are the main elements constituting lignin, and their wide presence can be related to its structural monolignols (Saber et al., 2022), as evidenced by FT-IR analysis. Similar results, in terms of elemental composition, were obtained by (Saber et al., 2022) who extracted lignin from wood industry waste and found a small amount of Al and Si in the material obtained. Moreover, (Hidayati et al., 2020; Rismawati et al., 2020) concerning the procedure of treating the biomass also found in lignin Al, Cl and S. However, these elements were in higher amounts compared to our results, confirming that the procedure applied based on the ionic liquid [Et_3NH][HSO_4] results in LNPs of high purity.

3.2. Leaves gas exchanges, plant biomass, and pigment content in maize treated with LNPs

Once the LNPs were obtained and characterized, those extracted from pomace through IL_95 were applied to maize seedlings by foliar applications at three different concentrations (25, 50 and 200 mg L^{-1}) to ascertain their effects on the early developmental stages of the crop.

The physiological data reported in Table 1 show a markedly positive effect of all treatments on the photosynthetic activity (Pn) and stomatal conductance (g). To these positive effects and the induction of photosynthetic activity, lower intracellular CO_2 concentration can be related, as shown in Table 1. These results suggest that LNPs promoted beneficial effects in maize, stimulating leaf photosynthetic activity and plant growth. Furthermore, the increase in photosynthetic

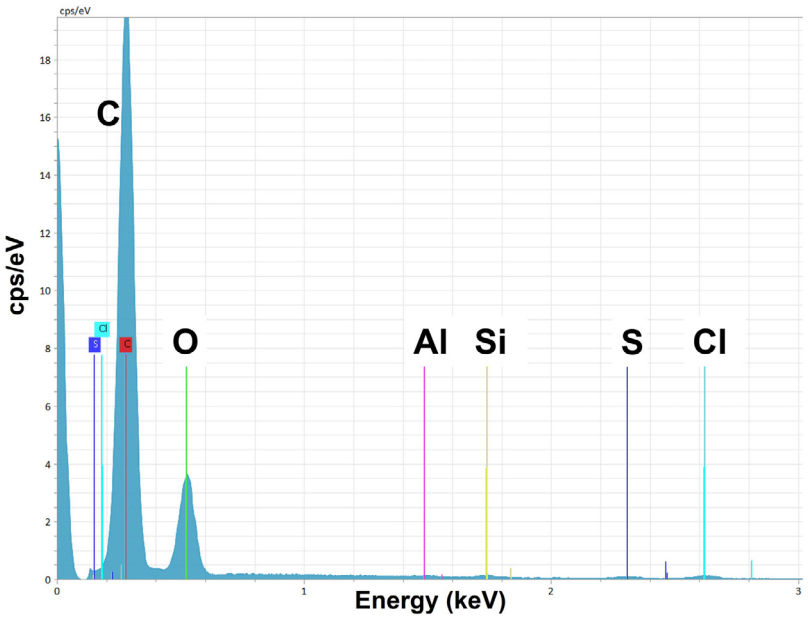

Fig. 4. EDX analysis of lignin nanoparticles obtained from pomace with IL_95 ([Et3NH][HSO4] 95%). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Photosynthesis, stomatal conductance and intracellular CO_2 of maize samples untreated (controls) and treated with different LNPs concentrations.

Treatment	Pn μmol (CO_2) m^{-2} s^{-1}	gs mmol (H_2O) m^{-2} s^{-1}	Ci μmol mol^{-1}
Control	4.34 ± 0.51 b	7.24 ± 0.50 c	526 ± 5 a
25 mg L^{-1}	6.43 ± 1.06 a	8.94 ± 0.82 ab	496 ± 4 b
50 mg L^{-1}	5.79 ± 0.34 a	9.13 ± 0.35 a	492 ± 3 b
200 mg L^{-1}	5.49 ± 0.26 a	8.25 ± 0.34 b	483 ± 3 c

Different letters are statistically significant according to Duncan's multiple comparison test ($p < 0.05$).

Table 2
Shoot analysis of maize samples untreated (controls) and treated with different LNPs concentrations.

Treatment	Shoot height (cm)	Leaf area (cm^2)	Fresh weight per plant (g)
Control	30.42 ± 4.04 b	29.75 ± 6.17 b	5.71 ± 0.31 b
25 mg L^{-1}	34.51 ± 2.74 a	34.34 ± 5.54 a	6.91 ± 0.33 a
50 mg L^{-1}	33.70 ± 1.47 a	32.17 ± 2.53 ab	6.52 ± 0.28 a
200 mg L^{-1}	34.41 ± 2.17 a	33.05 ± 5.48 ab	6.66 ± 0.59 a

Different letters are statistically significant according to Duncan's multiple comparison test ($p < 0.05$).

activity was associated with increased stomatal conductance, suggesting that lignin could enhance photosynthesis leading to a decrease in Ci. These results are in line with findings by Kok et al. (2021), which showed that these effects could be attributed to the ability of lignin to stimulate photosynthesis by increasing the amount of photosynthesis-related proteins and some regulatory proteins. In agreement with these findings, it has been shown that monolignols constituting lignin can prompt many benefits in treated plants and, among others, stimulate photosynthetic machinery (Zhu et al., 2023).

Consistently with what was observed, increased photosynthetic activity resulted in increased biomass produced at the shoot and root levels (Tables 2 and 3). In general, the length and weight of shoots were increased by all the LNPs concentrations applied. The leaf area appeared to have significantly increased by the LNPs concentration of 25 mg L^{-1}. In addition, the stimulatory effect prompted by LNPs was particularly interesting in the root system. In fact, 25 and 50 mg

Table 3
Root analysis of maize samples untreated (controls) and treated with different LNPs concentrations.

Treatment	Total length (cm)	Diameter (mm)	Root area (mm^2)	Volume (mm^3)	Fresh weight per plant (g)
Control	51.9 ± 5.8 b	0.446 ± 0.019 b	753 ± 92 c	1751 ± 23 c	3.73 ± 0.80 a
25 mg L^{-1}	74.6 ± 4.7 a	0.565 ± 0.058 a	1323 ± 132 a	3820 ± 78 a	4.14 ± 0.41 a
50 mg L^{-1}	70.3 ± 1.9 a	0.465 ± 0.031 b	1073 ± 59 b	2347 ± 20 b	3.74 ± 0.37 a
200 mg L^{-1}	58.3 ± 9.3 b	0.427 ± 0.063 b	796 ± 79 c	1560 ± 25 c	4.41 ± 0.68 a

Different letters are statistically significant according to Duncan's multiple comparison test ($p < 0.05$).

Table 4
Pigments content in maize samples untreated (controls) and treated with different LNPs concentrations.

Treatment	Chl a mg g^{-1} FW	Chl b mg g^{-1} FW	TotChl mg g^{-1} FW	Car mg g^{-1} FW
Control	4.71 ± 0.75 b	3.33 ± 0.45 a	8.05 ± 1.10 b	2.45 ± 0.60 a
25 mg L^{-1}	6.12 ± 0.96 a	3.93 ± 0.83 a	10.10 ± 0.78 a	2.16 ± 0.26 a
50 mg L^{-1}	5.31 ± 0.32 a	3.7 ± 0.55 a	9.00 ± 0.76 ab	2.54 ± 0.42 a
200 mg L^{-1}	4.80 ± 0.16 b	3.10 ± 0.46 a	7.88 ± 0.48 b	2.30 ± 0.59 a

Different letters are statistically significant according to Duncan's multiple comparison test ($p < 0.05$).

L^{-1} LNPs treatments increased the root length, area, and volume of the treated seedlings. In addition, 25 mg L^{-1} LNPs also increased root diameter, while 200 mg L^{-1} did not significantly affect it.

In this context, it has been shown that lignin and some of its derivatives can stimulate shoot and biomass production in common bean and maize, in a dose-dependent manner (Popa et al., 2008; Savy et al., 2018; Sutradhar et al., 2022). The positive effects on biometric parameters observed in our experiment may also be related to a different level of gas exchange, which was much more efficient following LNPs treatments. In addition, lignin and phenolic compounds it contains can stimulate plant growth (Ertani et al., 2011), exerting a hormone-like action on the early phase of seedling development. Regarding the component of lignin found in this study, the syringyl group is known to have a gibberellin-like activity which prompts seedling development (Nardi et al., 2003). Furthermore, the stimulatory effect on shoot and root development can also be ascribed to the presence of the guaiacyl group in lignin, which can exert a similar effect on plants (Savy et al., 2020). In addition, it has been documented that lignin may influence the mitotic process in the meristems or exert a hormone-mimetic activity like auxin and gibberellin (Campobenedetto et al., 2020). Likewise, these effects have been mainly attributed to the phenolic lignin groups, which may exhibit gibberellin-like activity (Campobenedetto et al., 2020; Del Buono et al., 2021a; Ertani et al., 2011). Moreover, lignin can induce root development by increasing root surface area and affecting its diameter and volume (Savy et al., 2018). This effect on roots deserves particular attention because an improved root system allows the plant to significantly increase its ability to acquire soil nutrients and establish positive symbioses with microorganisms (Cozzolino et al., 2016).

Futher studies have also documented that nanomaterials can induce in treated plants the expression of genes involved in nutrient acquisition and transport (Campobenedetto et al., 2020; Sun et al., 2020).

Finally, our results are in line with the bibliography as the effects of lignin were significant at lower dosages, while at the highest, as in the case of 200 mg L^{-1}, it can be phytotoxic or lose biological activity for the formation of aggregates that limit the plant interaction with the bioactive monolignols present in this macromolecule (Del Buono et al., 2021a).

Regarding photosynthetic pigments, an increase in chlorophyll a, at 25 and 50 mg L^{-1} LNPs, was recorded, and total chlorophyll increased in samples treated with 25 mg L^{-1} LNPs (Table 4). These effects may result from an inductive effect of LNPs on photosynthetic pigment biosynthesis (Del Buono et al., 2021a). Indeed, it is well known that phenolic compounds can stimulate pigment production by improving plant nutrition, in general, and nitrogen assimilation, in particular (Tanase et al., 2014). Finally, higher chlorophyll concentrations are also considered to represent a physiological adaptation to external stimuli to improve the photosynthetic efficiency in using light (Del Buono et al., 2021a; Peng et al., 2006). Consequently, LNPs treatment, associated with a direct effect on the photoreceptive capacity, can also ameliorate the efficiency in nitrogen acquisition, according to the increases in chlorophyll content (Ertani et al., 2011).

3.3. Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA), total phenolic compounds (TPC), total flavonoids compounds (TFC) and anthocyanin content in maize

Some parameters involved in defining the cellular oxidative status, such as hydrogen peroxide (H_2O_2) and MDA (a lipid peroxidation product), and some clusters of molecules with protective action, such as phenols, flavonoids, and anthocyanins, were investigated following the treatments with the three LNPs concentrations (Table 5). H_2O_2 is a reactive oxygen species (ROS) that, at low concentrations, functions as a signal molecule and is involved in response to various

Table 5
Cellular redox status and antioxidants in maize samples untreated (controls) and treated with different LNPs concentrations.

Treatment	H_2O_2 (nmol g^{-1} FW)	MDA (nmol g^{-1} FW)	TPC (mg g^{-1} FW GAE)	TFC (mg g^{-1}FW)	Anthocyanin (μg^{-1} FW)
Control	88.3 ± 4.0 b	24.5 ± 3.5 a	0.77 ± 0.06 a	0.76 ± 0.09 a	11.04 ± 1.76 c
25 mg L^{-1}	74.3 ± 0.7 c	18.9 ± 0.6 b	0.69 ± 0.04 ab	0.65 ± 0.06 ab	14.88 ± 1.47 ab
50 mg L^{-1}	82.3 ± 7.2 bc	19.4 ± 0.8 b	0.69 ± 0.06 ab	0.65 ± 0.06 ab	16.34 ± 3.00 a
200 mg L^{-1}	97.2 ± 3.9 a	21.7 ± 1.2 ab	0.62 ± 0.08 b	0.61 ± 0.10 b	11.88 ± 2.25 bc

Different letters are statistically significant according to Duncan's multiple comparison test ($p < 0.05$).

stimuli; however, high concentrations can be toxic and impair cellular functions (Del Buono et al., 2021b). MDA is a product of lipid peroxidation that accumulates during oxidative perturbations, although it is commonly produced in mitochondria and chloroplasts, where metabolic processes characterized by high electron flux occur (Del Buono et al., 2021b).

Our study revealed that the contents of H_2O_2 in samples treated with 25 mg L^{-1} LNPs, and MDA in samples treated with 25 and 50 mg L^{-1} LNPs were lower than in controls. These results show that LNPs did not affect the cellular oxidative status in the treated samples at the above dosages. On the contrary, LNPs reduced the content of H_2O_2 and MDA, and these results are in line with the effect shown by other substances with biostimulatory action, or lignin-based hybrid nanoparticles, which were found to improve oxidative status in treated crops (Del Buono et al., 2022; Panfili et al., 2019; Parađiković et al., 2019). In contrast, the highest dosage 200 mg L^{-1} LNPs determined an increase in H_2O_2 content. This result could indicate that the highest dosage started producing oxidative perturbations.

Phenolic compounds and flavonoids are among the most abundant secondary metabolites in plants and perform several biologically active functions, permitting plants adaptation to adverse environmental conditions, given their ability to scavenge ROS (Alharby et al., 2021; Liu et al., 2022). Lignin treatment did not generally change TPC and TFC, except for 200 mg $L^{-1,}$ which decreased the content of these classes of molecules (Table 5). The response to treatments with the two lower dosages indicated that LNPs did not perturb the cellular redox state, in agreement with the other stress-related parameters studied in this research (H_2O_2 and MDA). On the other hand, 200 mg L^{-1} LNPs may have interfered with the metabolic pathways leading to the synthesis of these class compounds. From this, it follows that the reduced content of TPC and TFC may explain the increase in H_2O_2, recorded at 200 mg L^{-1}, given their involvement in coping with oxidative perturbations and scavenging ROS (Liu et al., 2022).

Finally, our results show an increase in anthocyanins content in samples treated with 25 and 50 mg L^{-1} LNPs, while 200 mg L^{-1} did not affect them (Table 5). Anthocyanins show multiple functions as they act as antioxidants against ROS and protect plants from photoinhibition due to light stress (Regni et al., 2022). In particular, anthocyanins exert their protective function mainly in the vacuole, and their production could be increased to prevent photo-oxidative injury (Regni et al., 2022). At the same time, a significant negative correlation has been shown between oxidative stress indicators such as H_2O_2 and MDA, and anthocyanins levels since they have an essential role in protecting plants from oxidative injuries, preventing ROS generation and lipid peroxidation (Singh et al., 2022). Our findings showed that LNPs could modulate the antioxidant system in treated plants to maintain cellular redox homeostasis by increasing the anthocyanins content. These results agree with other published studies that have shown that lignin nanoparticles and other nanomaterials can induce the production of antioxidants. In particular, such inductive effects have been attributed to the ability of monolignols constituting lignin to stimulate anthocyanin biosynthesis processes (Del Buono et al., 2021a), or in other cases, to the electronic structure of nanomaterials that can prompt photo-protective responses in plants (Regni et al., 2022).

4. Conclusion

Recently, a growing interest has been oriented to recovering lignin from different biomasses to produce nanomaterials. In particular, LNPs, which are non-toxic, have a low production cost and are easily biodegradable in the environment, represent an environmentally friendly way of valorizing waste and renewable resources with applications in various technological fields. Furthermore, strategies to exploit and valorize waste from agro-industrial processes represent a cost-effective approach with interesting applications to be also explored in the agricultural sector.

The present research applied this green chemistry concept through the valorization of pomace, a waste from olive oil production that otherwise could be difficult to manage and highly impactful if irrationally dispersed in the environment. Specifically, LNPs were obtained using an IL composed by $[Et_3NH][HSO_4]$; then, they were tested on maize seedlings to ascertain their biostimulatory potential. The LNPs at the lower doses, and particularly at 25 mg L^{-1}, prompted some physiological and biochemical benefits on maize plants, thus highlighting the ability of such nanoparticles to biostimulate this crop, inducing plant growth and biomass production. In light of the above, this work proposes for the first time an innovative way to valorize olive pomace through the extraction from this biomass of nano-scaled lignin to be employed as a biostimulant, paving the way for further future studies to extend this approach to other biomasses.

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CRediT authorship contribution statement

Ciro Tolisano: Formal analysis, Investigation, Data curation, Writing – original draft. **Francesca Luzi:** Formal analysis, Investigation, Data curation. **Luca Regni:** Formal analysis, Investigation, Data curation. **Primo Proietti:** Conceptualization, Methodology, Writing – original draft. **Debora Puglia:** Conceptualization, Methodology, Data curation, Funding acquisition, Writing – original draft. **Giovanni Gigliotti:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing. **Alessandro Di Michele:** Formal analysis, Investigation, Data curation. **Dario Priolo:** Formal analysis, Investigation. **Daniele Del Buono:** Methodology, Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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