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PhD Thesis

**Organic fertilizers deriving from the sustainable valorisation of
agricultural and zootechnical waste biomass: evaluation of
promising products, including a newly-developed hydrochar**

Supervisor
Prof. Pierluigi Mazzei

Ph.D. Student
Rossella Curcio

Co-supervisor
Prof. Domenico Ronga

Coordinator
Prof. Teresa Zotta

Firmato digitalmente da: Teresa Zotta
Organizzazione: UNIBASILICATA/96003410766
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ABSTRACT

The ongoing global ecological crisis, characterized by widespread biodiversity loss and progressive soil degradation, poses urgent challenges to find and apply practices for sustainable agriculture. In this context, the management of agro-industrial residues, including horticultural and livestock wastes, is inefficient and conducted inappropriately, thus contributing to environmental pressures and threatening the sustainability of food production systems, while it could represent a fundamental initiative for sustainable strategies. Therefore, this work proposes a circular economy-based approach, aiming to convert agro-industrial residues into environmentally sustainable fertilizers, in line with European regulations and strategies, including Directive 2008/98/EC, Regulation (EC) 2092/91, and the Green Deal's Farm to Fork objectives, which aim to reduce reliance on mineral fertilizers while promoting resource recovery.

The research focused on the production, characterization, and agronomic evaluation of organic fertilizers derived from agricultural and livestock by-products, highlighting their potential as effective alternatives to conventional mineral amendments. All experimental activities were carried out at the University of Salerno, Department of Pharmacy, within the AgrichemLab - Laboratory of Agricultural Chemistry, where all analytical and agronomic investigations were performed. The Biogas plant C&F Energy (Capaccio, Italy) produced solid anaerobic digestate (AD) from buffalo manure and its vermicomposted form (VC), which were extensively characterized by using elemental and nutrients analysis and advanced techniques to evaluate the composition, including CPMAS-NMR, FT-IR and Pyr-GC-MS. AD contained a higher proportion of labile carbon, whereas VC exhibited a more recalcitrant and humified structure. In-vitro and greenhouse pot trials identified optimal doses at 40% (v/v) for most crops (20% for

VC on lettuce) and demonstrated that both materials enhanced growth and biomass production. NMR-based metabolomics revealed marked stimulation of carbohydrate metabolism and amino acid biosynthesis, with AD and VC outperforming mineral fertilizers in modulating primary metabolism.

Green compost (COM) from red chicory, escarole, spinach, and olive pruning residues, and its pelletized form (PEL), produced by OP TerrAmore (Eboli, Italy), were investigated as additional amendments. Compost was further characterized by ^{13}C CPMAS-NMR, FT-IR, and Pyr-GC-MS. Pot experiments showed that both COM and PEL improved plant growth, nutrient availability, and physiological performance, with pellets offering operational advantages. Combined COM+PEL applications further enhanced soil quality and crop yield, benefitting from the synergistic action of labile and stable organic fractions.

The most innovative aspect of this thesis lies in the development of hydrochars (HC) as novel bio-based fertilizers. This process is particularly advantageous, as it converts organic biomass into new materials potentially beneficial for soil and plants through hydrothermal carbonization, which involves the reaction of fresh biomass with water under subcritical conditions. The reaction was conducted with a 1-liter reactor, using the same biomass processed to obtain the COM, excepted the olive pruning residues, with two process conditions (180 °C and 10 Bar; 215 °C and 20 Bar). Both the resulting HC liquid and solid phases were isolated, characterized and tested as beneficial agents for soil and plants. In particular, structural analysis and greenhouse trials on lettuce demonstrated that HC180 significantly promoted root and shoot growth, achieving performance comparable to that of mineral fertilization. Metabolomic profiling showed increases in sugars and amino acids involved in energy production and stress response, indicating a rapid biostimulant effect driven by nutrient availability and soil–microbiome interactions. The corresponding liquid hydrochars (LHCs) were analyzed during

an internship at the University of Navarra. LHC180 was rich in soluble nutrients and hormone-like molecules (auxins, cytokinins, jasmonates, salicylic acid) and markedly improved seed germination, root architecture, biomass accumulation, and nutrient-use efficiency in *Lepidium sativum* and *Arabidopsis thaliana*, while LHC215 showed limited activity. LHC180 also activated nutrient transporters, iron-homeostasis regulators, and plasma-membrane H⁺-ATPases, resembling the mode of action of humic substances and supporting its suitability for sustainable fertigation strategies.

Finally, alginate-based systems (hydrogels, beads and film for a seed coating) were designed to provide slow release of water, nutrients, and humic substances extracted from AD, VC and COM. These systems were characterized for structure, water retention, ion release, and degradability. Humic-loaded coatings improved germination and early growth of watercress seeds, as confirmed by SEM and in-vitro bioassays. Iron-based beads provided effective Fe release under alkaline conditions, offering a promising approach to improve nutrient availability in challenging soils. Overall, this work demonstrates that organic fertilizers derived from agro-industrial residues, particularly hydrochar-based amendments and alginate delivery systems, represent effective, innovative, and sustainable alternatives to mineral fertilizers and synthetic biostimulants. By combining nutrient supply, biostimulant activity, and soil quality enhancement, these materials offer concrete solutions to advance sustainable agriculture and support the transition toward a circular bioeconomy.

Chapter I

Introduction

1.1 Global Agricultural Challenges and the Decline of Soil Fertility

Agriculture is the cornerstone of global food security, supports billions of livelihoods and serves as the foundation for economic stability. Over the past century, innovations such as mechanization, advanced irrigation systems, and synthetic fertilizers have significantly boosted crop yields and enabled large-scale food production. However, part of these advances have also led to critical drawbacks, including the progressive degradation of fertile soils, always accompanied by a decline in soil organic matter (SOM) which is a key indicator of soil health and productivity (**Celano et al., 2012**). This loss, driven by intensive land use and over-reliance on non-renewable mineral inputs, compromises both crop performance and soil microbial activity. As agricultural systems become increasingly intensive and resource-demanding, concerns grow about their long-term environmental and agronomic sustainability.

In this context, soil emerges as a central element in the broader challenge of sustainable agriculture. Far from being an inert substrate, soil is a dynamic, living system that plays a vital role in nutrient cycling, water regulation, carbon storage, and biological productivity. Its capacity to sustain these functions over time is captured in two often interrelated but distinct concepts: soil quality and soil health. The term soil quality, as defined by **Karlen et al. 2003**, refers to “*the capacity of a soil to function within ecosystem boundaries to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health*”. This definition focuses on the functional performance of soil in measurable terms, its physical, chemical, and biological properties.

In contrast, soil health emphasizes the biological and ecological integrity of the soil system. **Doran and Zeiss (2000)** define it as “*the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries.*” This perspective considers soil as a complex ecosystem, whose vitality depends on the diversity and activity of its microbial communities and its ability to adapt to environmental changes.

While scientists may prefer the term soil quality for its quantifiability, soil health is increasingly used (especially among farmers) to reflect the living, responsive nature of soil and the importance of maintaining its ecological balance. Unquestionably, the future of agriculture depends on the presence of a satisfying amount of quality and healthy soils.

In recent years, many fertile soils have been threatened from both natural and anthropogenic factors. Processes such as erosion, nutrient depletion, salinization, contamination, and the overuse of agrochemicals have significantly undermined soil integrity, reducing its capacity to support productive agriculture and essential ecosystem services (**Montanarella & Vargas, 2012; Keesstra et al., 2016**). Globally, it is estimated that over 33% of soils are moderately to highly degraded due to erosion, compaction, and loss of organic matter (**FAO & ITPS, 2015**).

These challenges are particularly acute in Mediterranean regions, which are recognized as among the most vulnerable in Europe to soil degradation and desertification (**Borrelli et al., 2020**). The Mediterranean climate (characterized by prolonged droughts, irregular rainfall, and high evapotranspiration) combined with historically intense land use, renders these areas highly susceptible to soil erosion, organic matter decline, and reduced biological activity (**Salvati et al., 2016**). In this context, the low levels of soil organic carbon (SOC) are of particular concern. According to **Yigini et al., 2016**, a significant portion of Southern

European soils contains less than 1.5% SOC, undermining soil structure, fertility, and water retention capacity. The causes are both climatic and related to management practices: as shown by **Lozano-García et al., 2017**, the general rise of temperatures accelerates organic matter mineralization, while the removal of crop residues from soil and the reduction in organic inputs limits carbon sequestration. These dynamics create a feedback loop of degradation that threatens long-term agricultural viability. The Mediterranean basin is also experiencing increased land abandonment, uncontrolled urban expansion and the replacement of traditional agricultural systems with intensive monocultures. These changes have contributed to the homogenisation of the landscape and the reduction in biodiversity, increased sealing of potentially fertile soils and reduced ecosystem resilience (**Zdruli 2014; Brunori et al., 2017**). Furthermore, the **IPCC (2022)** identifies the Mediterranean as a climate change hotspot, where soil degradation and water scarcity are expected to worsen, with serious repercussions on crop productivity and food security.

Within the Mediterranean, Southern Italy represents one of the most vulnerable areas, exhibiting some of the highest erosion rates recorded in the EU, with average values exceeding $20 \text{ t ha}^{-1} \text{ yr}^{-1}$, far above the tolerable limit (**Panagos et al., 2015; Borrelli et al., 2017**). Such processes are largely driven by unsustainable tillage practices, slope instability, and insufficient vegetation cover, especially in hilly and mountainous landscapes. In addition to physical degradation, soil biological fertility is also declining. Long-term dependence on synthetic fertilizers and insufficient organic input has led to reduced microbial biomass and enzyme activity, weakening soil's natural regenerative capacity (**Tsiafouli et al., 2015**). This loss has broader implications for nutrient cycling, carbon dynamics, and plant health.

The economic and agronomic consequences are substantial. Recent estimates suggest that land degradation in Italy contributes to annual losses in agricultural productivity and disaster-

related damages amounting to over €1 billion (**ISPRA, 2022**). The issue is especially pressing in the South, where agricultural output has declined and food self-sufficiency has weakened, further exacerbating socio-economic disparities between northern and southern regions.

Overall, the degradation of soils in the Mediterranean (and particularly in Southern Italy) reflects a complex interaction of climate vulnerability, intensive land use, and poor management practices. Addressing this challenge requires integrated and regenerative approaches that focus on restoring organic matter, promoting soil biological activity, and supporting sustainable nutrient management through nature-based solutions and circular agricultural inputs.

In light of these pressing challenges, safeguarding soil resources must become a strategic priority for agricultural and environmental policy in Mediterranean regions. Over the past decades, modern farming systems have become increasingly dependent on synthetic fertilizers to meet the nutrient demands of high-yield crops. While effective in the short term, these inputs often imply several inconveniences, such as nitrate leaching, eutrophication of water bodies, acidification of soils, and a general decline in soil biological activity (**Tilman et al., 2002; Sutton et al., 2013**). Additionally, many synthetic fertilizers are derived from finite resources such as natural gas and phosphate rock, raising concerns about future availability and energy costs (**Cordell et al., 2009**).

These challenges underscore the need for a systemic rethinking of nutrient management. Future strategies should be conceived to regenerate the soil's full functionality by improving its biological vitality, chemical properties, and structural integrity. This shift calls for integrated approaches that not only improve nutrient use efficiency but also promote the reintroduction of organic matter, microbial diversity, and ecological resilience. In this context,

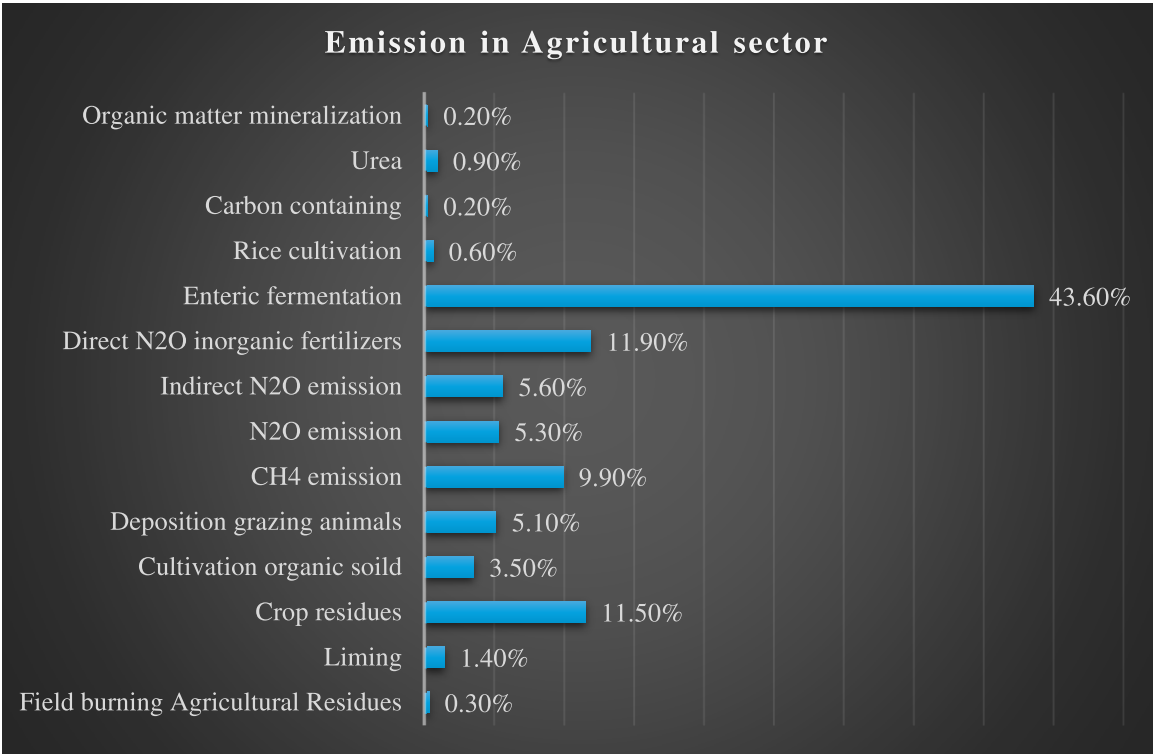
the research for alternative, more sustainable fertilization strategies has become a central focus in both research and agricultural policy.

1.2 Enabling Circular and Sustainable Agriculture: The Role of European Policy and Regulatory Frameworks

Over the last decade, European agricultural policy has undergone a substantial transformation, moving toward sustainability and circularity. A pivotal moment was marked in 2015 with the launch of the EU's strategy *Closing the Loop – An Action Plan for the Circular Economy*, which promoted the recovery and reuse of agricultural waste and by-products as inputs for new production cycles (**European Commission, 2015**). This approach recognizes the agrifood sector not only as a consumer of resources but also as a source of renewable materials that can feed into new bio-based value chains (**Diacono et al., 2019**). The transition to a circular bioeconomy aligns with a growing interest in converting residual biomass, mostly deriving from agri-food industry and livestock sector, into agricultural inputs such as soil fertilizers and plant biostimulants. International regulatory measures support and push towards these objectives: for instance, the Organic Farming Regulation (EU) 2018/848 outlines the principles for environmentally responsible agricultural production and restricts the use of synthetic substances. Environmental goals are increasingly embedded in EU strategies. From 1990 to 2019, greenhouse gas emissions in the EU have decreased by 28%, with agriculture contributing a 20.5% reduction, mostly due to a lower cattle population and reduced use of both synthetic (–25%) and organic (–13%) fertilizers, as highlighted by the **European Environment Agency (EEA, 2021) (Figure 1)**. Notably, the sub-sectors of enteric fermentation and direct emissions from agricultural soils were responsible for a 4.3% overall reduction in total emissions between 1990 and 2019. These sub-sectors have achieved the

greatest emission reductions, comparable to those recorded by the cement, aluminum, and fluorochemical industries. Nonetheless, enteric fermentation remains the dominant source, accounting for over 48% of current agricultural emissions. The rest of the emissions primarily originate from fertilizer application, soil management, and crop residue handling (Boix-Fayos et al., 2023).

Figure 1. Emission in the agricultural sector (in % of kt CO₂ eq/year) in the EU averaged from 2000 to 2018. (Data source: Environmental Agency, 2021).



On December 11th, 2019, the European Green Deal (EGD) was officially introduced as a broad policy framework encompassing a set of strategies, initiatives, and legislative measures designed to guide the European Union toward a fair, sustainable, and inclusive transformation of its economy and society. The EGD represents the EU’s new model for economic growth, aiming to turn Europe into a prosperous and equitable society supported by a competitive and sustainable economy (European Commission, 2020a). Rather than constituting a single piece of legislation, the Green Deal serves as an overarching strategic vision that defines goals and

priorities across multiple areas of environmental policy. Its implementation involves the revision of existing legal instruments as well as the development of new laws and directives. The overall framework of the Green Deal is structured around eight core thematic areas, which collectively shape the roadmap for a balanced and ecologically sound transition. These pillars not only focus on environmental protection but also seek to foster innovation, create new economic opportunities, and enhance the overall well-being of EU citizens, as illustrated in **Figure 2**.

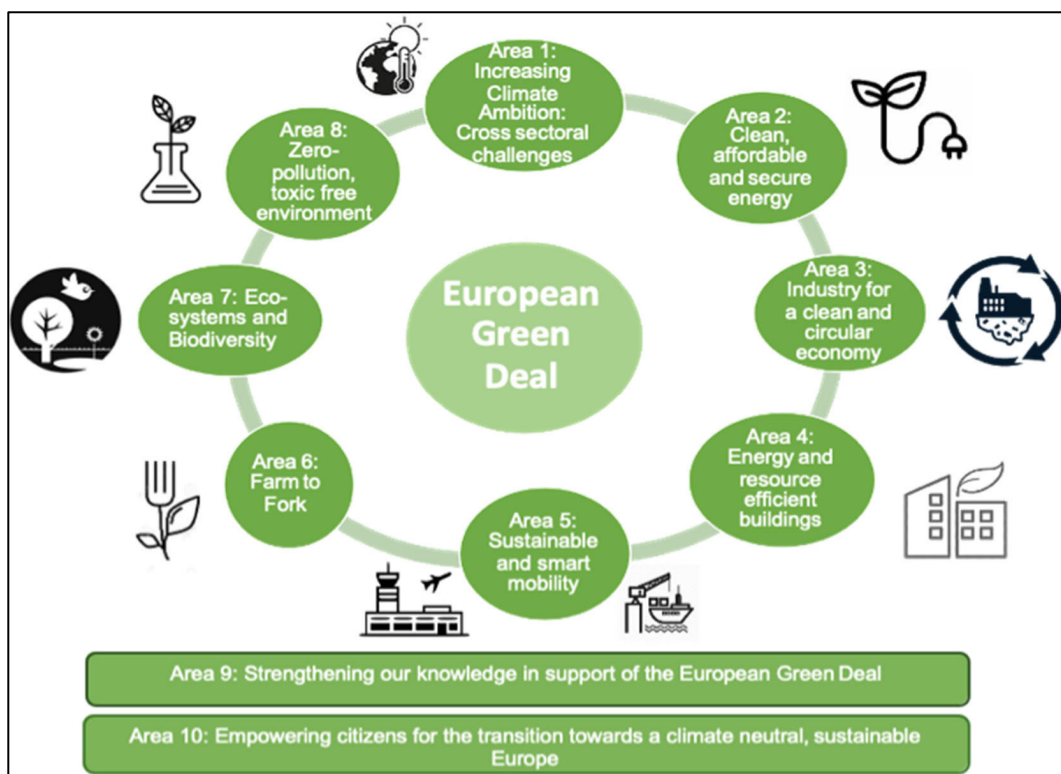


Figure 2. Key areas envisaged by the European Green Deal (Source: European Commission 2020a).

- The Farm to Fork (F2F) Strategy, which has been launched in May 2020 as a cornerstone of the Green Deal, aims to transform the EU’s food system towards fairness, health, ecological integrity, and sustainability (**European Commission, 2020b; 2020c**). It addresses the entire food value chain, spanning agricultural production, processing,

distribution, consumption, and food waste reduction. The strategy targets a comprehensive redesign of modern food systems, which contribute nearly one-third of global greenhouse gas emissions, high resource consumption, biodiversity loss, and adverse public health effects. To be achieved by 2030, its key objectives include:

- Reductions of 50 % in the use and risk of chemical pesticides and hazardous active substances (**European Commission, 2020**);
- At least 20 % reduction in fertilizer use and ≥ 50 % nutrient losses, while maintaining soil fertility (**European Commission, 2020**);
- Reduction of sales of antimicrobial products (used in livestock and aquaculture) by 50 % (**European Commission, 2020**);
- Expansion of organic farming to cover 25 % of EU agricultural land by 2030 (**European Council, 2021**);
- Halving food loss and waste across the supply and consumption chain (**European Council, 2021**);
- Promotion of healthy, sustainable diets, reduction of food fraud, improved animal welfare, and equitable economic returns to producers (**European Council, 2021**).

Achieving these targets requires not only technological innovation but also systemic shifts in production methods and consumption behaviours (**Schebesta and Candel, 2020; Naheed et al., 2024**). The strategy highlights the active role of consumers and the importance of food education, recognizing that sustainability depends on the engagement of both producers and citizens.

Financial instruments are aligned with these ambitions. The 2023–2027 Common Agricultural Policy (CAP) dedicates 40% of its budget to environmental objectives, supporting practices that improve soil health, enhance biodiversity, and contribute to climate mitigation. National

Strategic Plans now serve as the operational tools to translate EU-level objectives into tailored national actions (**European Court of Auditors, 2021**). To support the use of recycled organic materials in agriculture, Regulation (EU) 2019/1009 introduced a harmonized legal framework for fertilizer products across the EU. In force since 2022, the regulation allows fertilizers derived from compost, digestates, and food industry by-products to be placed on the internal market, provided they meet strict safety and quality standards.

The key components of Regulation (EU) 2019/1009, include:

- Product Function Categories (PFCs), classifying fertilizer products such as:
 1. Organic, organic-mineral, and inorganic fertilizers
 2. Calcination materials
 3. Soil conditioners
 4. Culture substrates
 5. Inhibitors
 6. Plant biostimulants (microbial and non-microbial)
 7. Fertilizer mixtures
- Categories of Component Materials (CMCs) define acceptable raw materials for fertilizer manufacture, comprising eleven categories:
 1. Substances and mixtures of virgin materials, including natural minerals and purified elements
 2. Plants, plant parts, or extracts contributing beneficial nutrients
 3. Compost, meeting strict quality standards to avoid harmful contaminants

4. Digestate of fresh crops derived from anaerobic digestion of plant-based organic matter
5. Digestate from other organic materials, such as agricultural residues or animal by-products, complying with safety criteria
6. By-products of the food industry, secondary materials from production processes adding agronomic value
7. Beneficial microorganisms enhancing nutrient efficiency
8. Nutritive polymers that release nutrients gradually and are biodegradable or non-toxic
9. Non-nutritive polymers used as binders or coatings, often biodegradable
10. Derived products as defined by Regulation (EC) No 1069/2009, relating to animal by-products not intended for human consumption
11. By-products as defined by Directive 2008/98/EC, including industrial process residues like biomass ash or gypsum, subject to quality and safety standards

Complementary regulations also contribute to sustainability objectives. The Packaging and Packaging Waste Regulation (PPWR, 2025) mandates recyclable or reusable packaging, widely applied in the agri-food sector. The Ecodesign for Sustainable Products Regulation (ESPR, 2024) broadens sustainable design requirements to various product categories, including agricultural equipment and inputs. At the same time, the revised Waste Framework Directive reinforces the waste hierarchy and defines conditions for materials to qualify as by-products or to cease being waste (*end-of-waste criteria*), facilitating the reuse of organic residues in agriculture without waste classification. At the national level, Italy has aligned its legislation with EU directives through the Environmental Code (**D.Lgs. 152/2006**), which sets criteria for by-product identification and end-of-waste status application. **Law 166/2016**

promotes the recovery and redistribution of surplus food and agro-industrial residues, while **Law 7/2022** introduces a prioritization scheme favoring reuse for human consumption, animal feed, industrial applications, and finally composting or anaerobic digestion.

However, regulatory gaps remain, particularly regarding animal by-products. Due to the absence of **defined end points** under **Regulation (EC) No. 1069/2009**, part of such materials are still excluded from the European fertilizer market, despite their agronomic potential, thus limiting circular economy implementation. The lack of such criteria hinders the valorization of animal-derived materials like processed manure, hydrolysates, or gelatin in agricultural applications (**Van Eetvelde et al., 2021**).

Beyond regulations, soil biodiversity loss represents a major and multifaceted environmental challenge, with more than 40% of European soils classified as being at risk of biological degradation (**Orgiazzi et al., 2016**). As shown in **Figure 3**, this decline is particularly pronounced in intensively managed areas, where agricultural practices, such as the excessive use of synthetic fertilizers and pesticides, depletion of soil organic matter, repeated tillage, and habitat simplification, exert strong pressure on soil microbial and faunal communities. These disturbances compromise the functional diversity of soil biota, threatening essential soil-mediated processes. Preserving soil biodiversity is therefore crucial to ensure effective nutrient cycling, sustain plant health and productivity, and support long-term ecosystem resilience and sustainability (**Brussaard et al., 2007; De Corato et al., 2023**).

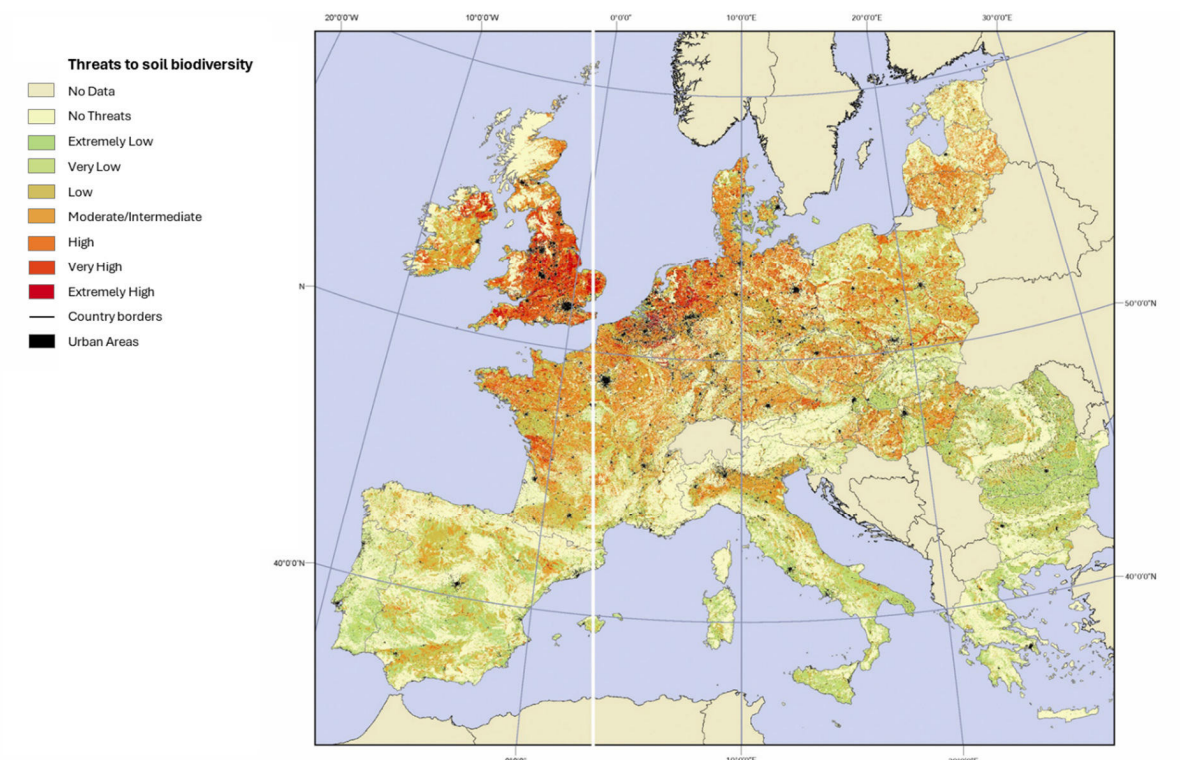


Figure 3. Map of potential threats to soil biodiversity in Europe. The map highlights areas under varying levels of pressure affecting soil biodiversity, with the highest threats observed in regions of intensive land use and urbanization. Source: **European Atlas of Soil Biodiversity, European Commission – JRC, 2010.**

Therefore, sustainable soil management and the recovery of organic matter from agro-industrial processes are increasingly viewed as ecological imperatives and regulatory priorities. Incorporating circular practices, such as the use of composts, digestates, and biologically active amendments, into conventional agriculture can restore soil functionality and contribute to the climate and biodiversity goals of the Green Deal.

1.3 Beyond Degradation: Environmental and Soil Impacts of Synthetic Fertilizers and Agricultural Waste

The management of organic residues from agro-industrial, horticultural, and livestock production systems represents both a critical environmental challenge and a strategic opportunity to advance sustainable agricultural innovation. The agri-food sector represents the leading manufacturing industry in Europe, even surpassing the automotive and chemical

sectors. In Italy it ranks as second, after the metalworking industry. The agrifood sector generates substantial amounts of organic waste, with approximately 3,000 million tonnes of material produced per year across the European Union (where 10% originates from food processing activities). Non-household organic waste alone is estimated at 30 to 50 million tonnes per year (**Ecocerved, 2013**), and these quantities continue to rise despite EU-level efforts aimed at reducing waste generation.

Globally, nearly one-third of all food produced for human consumption is lost or wasted, causing significant economic losses (up to \$936 billion per year) and accounting for nearly 8% of total anthropogenic greenhouse gas (GHG) emissions (**FAO, 2011; UNEP, 2023**). Within the EU, more than 59 million tonnes of food waste are generated annually, averaging 132 kg per person, with households responsible for over half of this amount (**European Commission, 2023**).

Improper management of these organic waste streams can adversely affect surface and groundwater quality, soil health, air quality, and human well-being. Additionally, landfill disposal increases costs for producers and contributes to climate change through GHG emissions.

In this context, the recover and the valorization of organic by-products resulting from the agrifood sector represents a sustainable solution to develop products for the agriculture, which well aligns with circular economy principles (**Aziable et al., 2017**). This approach is reinforced by environmental legislation, such as **Italian Legislative Decree No. 152/2006**, which defines “by-products” as substances or objects produced as an integral part of a production process, intended for further use without additional processing beyond normal industrial practice, and meeting health, safety, and environmental standards.

The decree also specifies conditions under which materials may cease to be classified as waste following recovery operations like recycling, provided that they are commonly used for specific purposes, have an existing market demand, comply with relevant standards, and do not pose environmental or health risks. These regulatory frameworks enable the transformation of agro-industrial residues into valuable resources that can be reintegrated into productive cycles, enhancing resource efficiency and reducing environmental impacts while supporting sustainable agricultural systems.

Conversely, the widespread and excessive use of inorganic fertilizers, mainly rich in nitrogen (N), phosphorus (P), and potassium (K), has intensified environmental problems, resulting in problems such as nutrient leaching and eutrophication (**Jwaideh et al., 2022**). In fact, due to both the high solubility of these fertilizers and the anionic form of nitrates and phosphates, a significant fraction of these nutrients escapes plant uptake and is lost through runoff or percolation, ultimately reaching freshwater and marine ecosystems (**Mahmud et al., 2021**). This nutrient overload stimulates harmful algal blooms that, upon decomposition, severely deplete oxygen levels, causing hypoxia and massive losses in aquatic biodiversity. Eutrophication thus remains a critical ecological consequence of current fertilization practices, especially in intensively farmed areas (**Wang et al., 2022**).

Furthermore, synthetic fertilizer production and application contribute substantially to greenhouse gas emissions, including CO₂, N₂O, and CH₄, thereby exacerbating climate change. Fertilizer manufacturing is energy-intensive and accounts for a significant share of global GHG emissions, while overuse of fertilizers increases nitrous oxide emissions from soils, a greenhouse gas with a global warming potential approximately 300 times that of CO₂ (**International Energy Agency, 2021; Jwaideh et al., 2022**). Consequently, reducing

dependence on synthetic fertilizers through the valorisation of organic residues is a vital strategy to mitigate agricultural GHG emissions and promote climate-smart farming.

In addition to environmental concerns, synthetic fertilizers face increasing price volatility, threatening the economic sustainability of agriculture. Global market disruptions and geopolitical tensions (like the war in Ukraine and instability in the Middle East) have severely affected supplies of key raw materials like natural gas and phosphate rock, causing a relevant increase of costs and raising long-term availability concerns (**Arndt et al., 2024**). This scenario further emphasizes the urgent need to transition toward alternative nutrient sources and resilient fertilization strategies. Moreover, the tight reliance on synthetic fertilizers accelerates the depletion of finite mineral resources, particularly phosphate rock, while intensifying disruptions of natural nutrient cycles (**Desmidt et al., 2015**). In contrast, organic amendments derived from agro-industrial residues offer a more sustainable nutrient management approach. These inputs release essential nutrients gradually through mineralization, microbial turnover, solubilization, and complexation processes, enhancing nutrient bioavailability and plant uptake efficiency while reducing losses via leaching or volatilization. They also contribute to the accumulation of soil organic carbon, improving soil structure, fertility, and long-term carbon storage (**Figure 4**). Integrating organic sources into fertilization regimes can mitigate the negative externalities of synthetic fertilizers, including eutrophication, while advancing circular and regenerative agricultural systems. Such practices not only foster environmental sustainability but also bolster agricultural resilience amid climate change and resource scarcity (**Bellamy et al., 2005**).

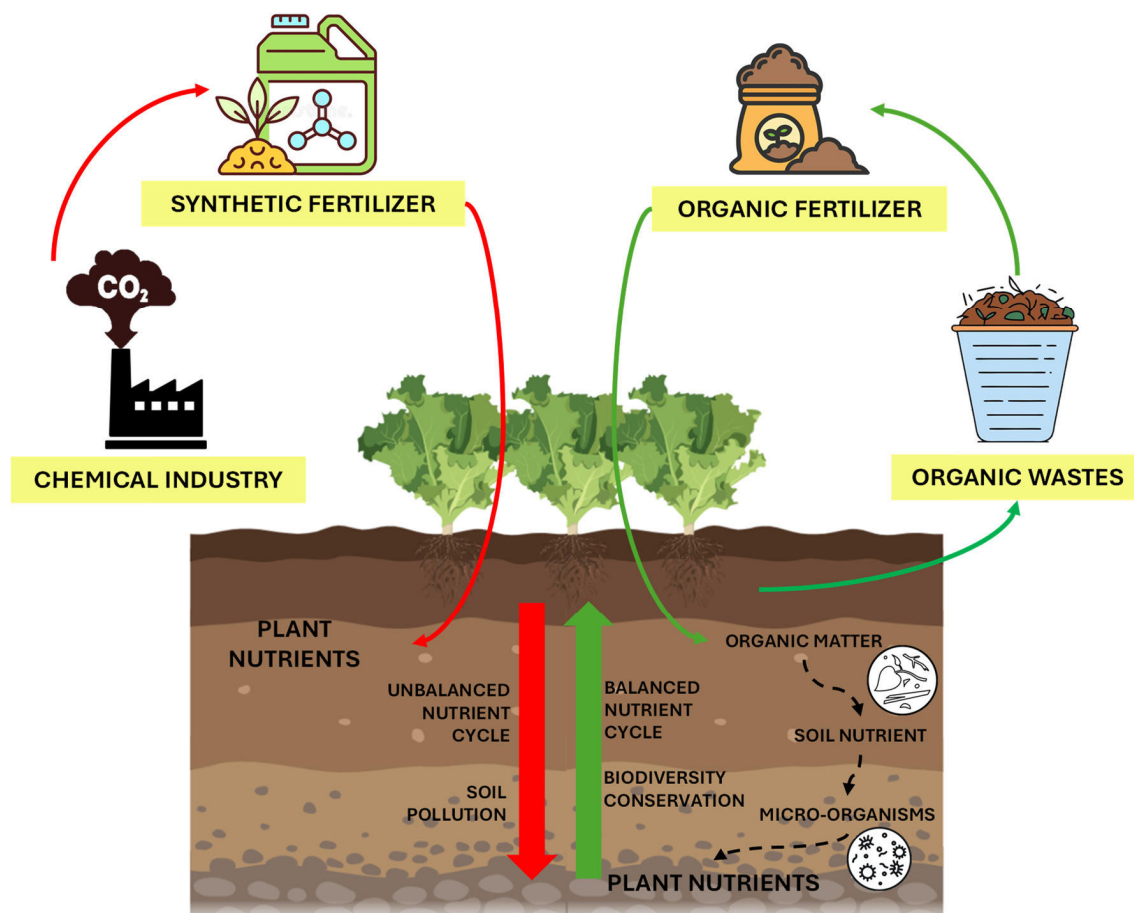


Figure 4. Comparative impact of organic and synthetic fertilizers on soil health and environmental sustainability.

1.4 From Waste to Resource: conventional Organic Fertilizers Derived from Agro-Industrial by-Products

The food and agricultural sectors are increasingly oriented towards sustainable and high-quality production systems. As a result, there is a growing demand for eco-friendly practices that minimize environmental impact, enhance resource efficiency, and support higher levels of food production (Edwards et al., 2011). In this framework, a promising strategy lies in the recovery and valorization of organic residues generated by agriculture, the food industry, and livestock farming. Through the application of advanced and suitable technologies, waste biomass can be transformed into bioenergy and value-added products that contribute to improving food quality and fostering a sustainable bioeconomy (European Council, 1991;

He et al., 2016; Greco et al., 2021). In particular, several organic materials obtained from the virtuous recycling of agro-industrial by-products, when applied to soils, provide a gradual release of nutrients, improve soil fertility and plant health, and help mitigate the ecological footprint of agricultural systems (Infascelli et al., 2010; Irshad et al., 2013; Jarwar et al., 2021). There are several techniques to transform organic waste into valuable resources, among which composting, vermicomposting, and anaerobic digestion represent the most widely applied and studied processes (Lohri et al., 2017). In addition, pyrolysis-derived biochar has gained increasing attention as a sustainable strategy for organic waste valorization, due to its potential to improve soil fertility, enhance carbon sequestration, and reduce greenhouse gas emissions (Parra-Orobio et al., 2023) (Figure 5).

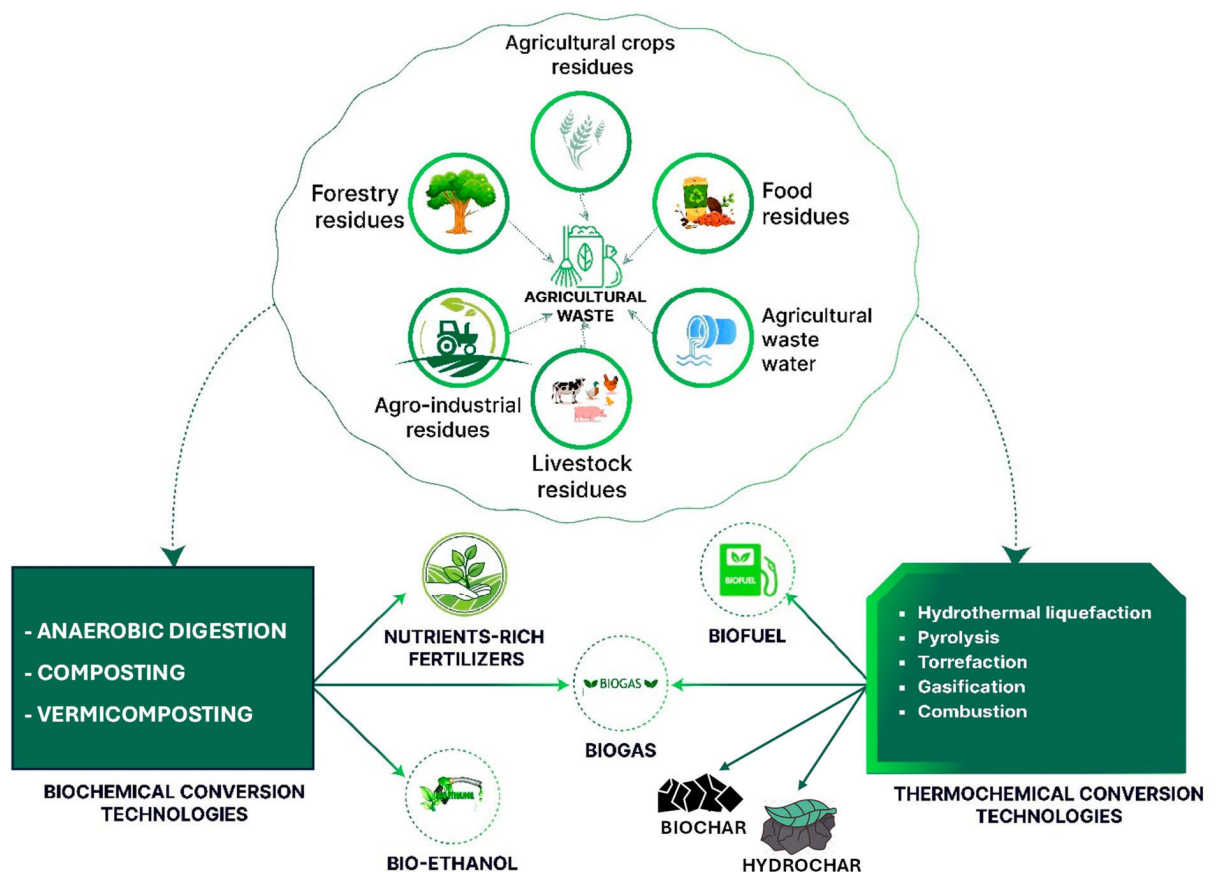


Figure 5. Schematic representation of agricultural waste valorization pathways through biochemical and thermochemical conversion technologies. Adapted from: Singh et al., 2024.

Composting is a well known aerobic biological process in which microorganisms decompose natural organic matter of plant and animal origin up to produce a stabilized and humified material in a period ranging typically within 6 and 9 months. Although the process requires that the biomass should contain a relevant fraction of labile carbon, it is necessary that part of the biomass need to be recalcitrant to serve as structuring agent. Composting consists in a succession of microbial communities that drive different phases: an initial mesophilic phase, where easily degradable compounds are rapidly metabolized and heat is generated; a subsequent thermophilic phase, during which temperatures may exceed 70 °C and pathogenic organisms and weed seeds are inactivated; and finally a maturation phase, where mesophilic microorganisms recolonize the material, promoting the stabilization of organic matter and the formation of humic-like substances (**Figure 6**) (Mustin et al., 1987; Makan et al., 2013; Oviedo-Ocaña et al., 2015).

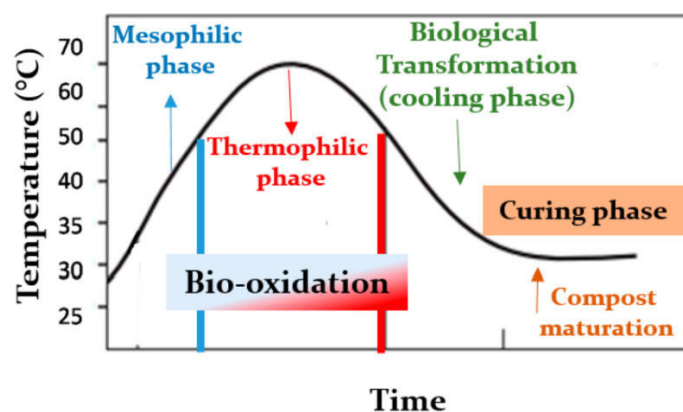


Figure 6. Temperature changes in the composting process. Source: Papale et al., 2021.

Several composting systems have been developed to optimize the process, including windrow composting, aerated static piles, in-vessel reactors, and two-stage systems (**Bernal et al.,**

2009; Lim et al., 2017). Regardless of the technology, key parameters such as aeration, moisture content, porosity, temperature, and pH must be carefully monitored and controlled to ensure process efficiency and compost quality (**Palese et al., 2020**). The resulting compost is a complex organic material that enhances soil fertility, improves physical properties such as aggregation and water-holding capacity, and supplies macro- and micronutrients mostly through a slow-release process. Despite composting releases a not-negligible amount of carbon dioxide, the compost contributes to the sequestration of soil organic carbon and to the stimulation of beneficial microbial communities, thereby reducing the need for synthetic fertilizers and lowering the environmental footprint of agricultural systems (**Pane et al., 2011; Bernal et al., 2017**). For these reasons, composting represents not only a waste management strategy but also a valuable practice for sustainable agriculture and soil health restoration.

Vermicomposting is a biological process that utilizes the combined activity of earthworms and associated microorganisms to convert organic residues into a stable, nutrient-rich product. Although it shares similarities with traditional composting as a controlled decomposition process, vermicomposting is distinguished by the predominant involvement of earthworms during the initial stages of organic matter breakdown, which significantly accelerates the stabilization process (**Alshehrei & Ameen et al., 2021**). Epigeous earthworms, naturally inhabiting the surface litter layers, are preferred due to their high consumption of organic material and rapid reproductive cycles (**Blouin et al., 2013**). In addition to physically fragmenting and digesting organic residues, the gut microbiota of earthworms plays a critical role in biochemical transformations, releasing bioactive compounds that enhance the stabilization and humification of the organic matter. Through this combined action, earthworms alter the physical, chemical, and biological characteristics of the material, gradually lowering the C/N ratio (**Domínguez et al., 2018**). The interaction between

earthworms and microorganisms facilitates the mineralization of organic matter and the formation of humified compounds with high agronomic value (**Spaccini & Piccolo et al., 2020; Pizzanelli et al., 2023**). Moreover, earthworm activity significantly improves soil fertility by increasing the availability of mineralized nitrogen for plants. The biochemical composition of earthworms, including 3% ash, 14% fat, 14% carbohydrate, and 65% protein, contributes to this nitrogen enrichment upon earthworm biomass turnover (**Edwards et al., 1976; Bhadauria et al., 2010**). Comparative studies indicate that while conventional compost tends to contain more ammonium, vermicompost is richer in nitrate, the form of nitrogen most readily available to plants (**Atiyeh et al., 2020**), and also provides other essential nutrients such as phosphorus, potassium, and magnesium (**Thakur et al., 2021**). Vermicompost is particularly valued for its high content of plant-available nutrients and its capacity to support diverse soil microbial communities. Production occurs in bioreactors where earthworms, in concert with naturally occurring microorganisms, transform organic residues into a highly stabilized product with enhanced chemical and biological properties relative to conventional compost (**Domínguez 2004; Aira et al., 2010; Edwards et al., 2011; Ndiaye et al., 2022; Piccoli et al., 2022**). As compared to traditional composting, vermicomposting is a more energy-efficient and low-emission process, as well as being much faster, typically requiring no more than 30 days to achieve satisfactory humification (**Amossé et al., 2013; Lim et al., 2017; Liu et al., 2020; Piccoli et al., 2022**). Although earthworms are sensitive to environmental stress, such conditions can be effectively managed under controlled systems (**Domínguez et al., 2004; Edwards et al., 2011**). When adequately controlled, vermicomposting yields a high-quality organic amendment that enhances soil fertility, fosters microbial diversity, and contributes to the rehabilitation of degraded soils (**Aira et al., 2010; Tammam et al., 2023**).

Anaerobic digestate (AD) is the residual material obtained from the anaerobic digestion of organic biomass, a complex multi-step microbiological process occurring in the absence of oxygen. This process proceeds through four successive stages (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) leading to the generation of biogas, primarily composed of methane (CH₄) and carbon dioxide (CO₂), along with a liquid effluent and a solid fraction. After a mechanical separation, commonly achieved with a decanter or separator, both the solid and liquid phases of digestate can be potentially exploited for agricultural applications, mostly as nutrient-rich organic amendments and biostimulant, while the produced biogas can be reused via cogeneration to develop heat and electric current, thus representing a renewable energy source (Nkoa et al., 2014; Chatterjee & Mazumder et al., 2016). The characteristics of the resulting digestate strongly depend on the feedstock type, process conditions, and microbial activity within the reactor, which in turn influence its stability, nutrient content, and chemical composition (Provenzano et al., 2014). Several studies have shown that the chemical attributes of anaerobic digestates largely reflect those of the original organic biomass, with reported variability in organic matter content (38–75%), cellulose-to-lignin ratios (0.22–1.75), oxygen uptake rates (1.129–3.774), and C/N ratios (6.2–24.8) (Möller et al., 2008). In particular, the solid fraction of digestate exerts beneficial effects on soils, acting as a nutrient-rich amendment with significant agronomic potential (Alburquerque et al., 2012). This product provides essential nutrients both in a bioavailable form and through slow-release mineralization, thereby enhancing soil fertility and contributing to the reduction of synthetic fertilizer use in a more sustainable manner (Figure 7).

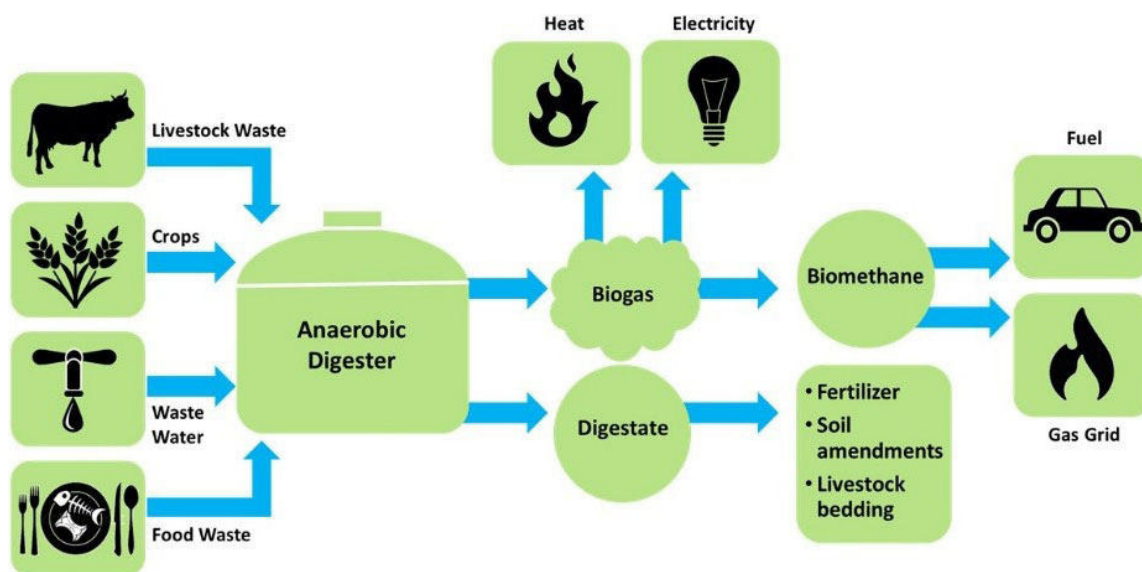


Figure 7. Diagram of organic waste conversion into energy through anaerobic digestion and biogas production. Source: Tanigawa, S. et al., 2025.

Beyond their role as nutrient suppliers, the application of organic amendments, including compost, vermicompost and anaerobic digestate, provides multiple benefits to the soil, encompassing chemical, physical, and biological aspects (Angelova et al., 2013). These practices are increasingly studied and adopted for managing agricultural waste, in part due to their relatively low environmental impact compared to conventional synthetic fertilizers. For instance, the carbon footprint of traditional mineral fertilizers ranges from 1816 to 2108 kg CO₂ eq per ton of product (El Chami et al., 2023), whereas organic fertilizers produced via composting, vermicomposting, or anaerobic digestion typically exhibit global warming potentials between 130 and 448 kg CO₂ eq per ton of fertilizer (Blengini et al., 2008; Mu et al., 2017; Martinez-Blanco et al., 2020). It is important to underline that these materials can also act as biostimulants, namely products that, when applied to plants or soil, do not directly supply nutrients but enhance the capacity of plants to acquire and utilize nutrients, improve resilience and tolerance to abiotic stresses, mitigate the effects of pathogens and diseases, and indirectly promote better physiological performance. Biostimulants influence plants at both

physiological and biochemical levels, supporting metabolic activity and fostering stronger and more sustainable growth (**González-Morales et al., 2022**). Unlike fertilizers or pesticides, biostimulants do not directly provide nutrients or combat pests, but act through biological mechanisms that facilitate plant growth and development (**European Biostimulants Industry, 2012**). They can be originated from different sources, including humic substances, complex organic matrices, beneficial chemical elements, inorganic salts, seaweed extracts, and free amino acids (**Jardin et al., 2012**). When applied to plants, seeds, soil, or other cultivation substrates, these materials have been shown to enhance multiple aspects of plant growth and development. Additionally, they can stimulate the proliferation of beneficial microorganisms, thereby improving soil physical and chemical properties and indirectly supporting plant health (**Calvo et al., 2014**).

1.5 Innovative Valorization of Agricultural Biomass: Hydrochar via Hydrothermal Carbonization

Hydrothermal carbonization (HTC) represents a thermochemical approach based on the treatment of fresh organic residues with water at subcritical conditions in order to convert them into functional materials and extract nutrients and bioactive compounds (**Fregolente et al., 2020; Bento et al., 2021**). The HTC is performed at relatively mild temperatures (170–260 °C) under autogenous pressures (ranging within 5 to 25 Bar), primarily yielding a carbon-rich material, known as solid hydrochar.

Unlike the above-mentioned biological treatments, such as composting, anaerobic digestion, and vermicomposting, the HTC relies on a thermovalorization leading to the sequential dehydration, decarboxylation, and polymerization reactions (**Pauline et al., 2020; Yu et al., 2022**). As compared to the biochar, which is another thermic process for the valorization of

organic wastes, the HTC implies a number of advantages, including its ability to process wet biomass directly, thus avoiding the energy-intensive pre-drying required for biochar production. In fact, the biochar is obtained, in oxygen-free conditions, through the slow (up to 3 hours) thermal treatment of dried and woody feedstocks. However, biocharring must be conducted at considerably higher temperatures (350-600 °C) thus requiring much more energy. The biochar and the hydrochar exhibit many divergences in physical and chemical characteristics, mostly due to the different reaction mechanisms. In fact, biochar typically results in higher aromaticity and long-term recalcitrance and stability in soil, while hydrochar is characterized a higher polar phase and a lower aromaticity and hydrophobicity (**Kambo et al., 2015**). Consequently, biochar is particularly valued for carbon sequestration and soil quality improvement, whereas hydrochar shows broader versatility in applications due to its tunable structure and reactivity (**Figure 8**).

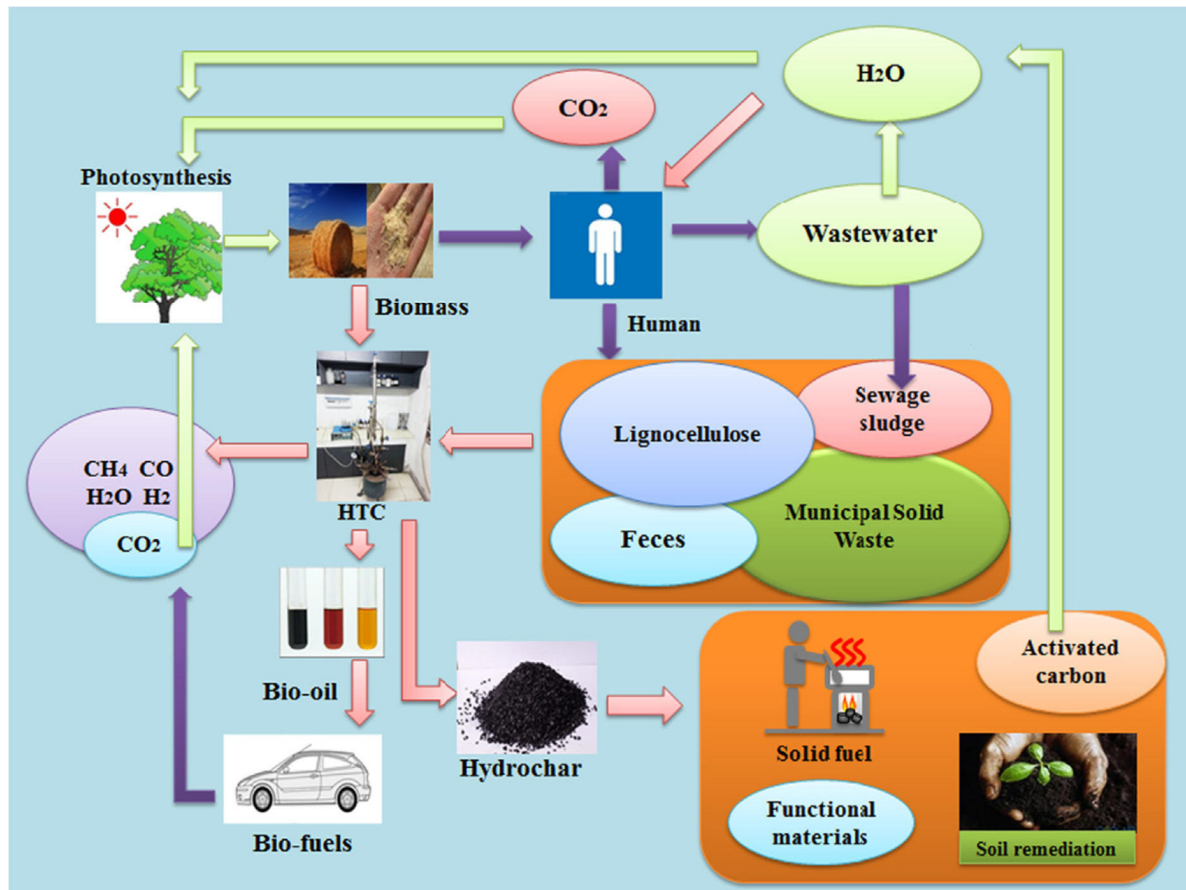


Figure 8. Schematic illustration of sustainable concepts regarding hydrochar production, applications, and impact on global climate. Source: Wang et al., 2018.

Depending on the operating conditions, the HTC, lasting from several minutes to several hours, produces three fractions: (i) a solid carbonaceous material, (ii) an aqueous phase, enriched in soluble organic and inorganic compounds and containing bioactive compounds (Islam M. A. et al., 2021), and (iii) a minor gaseous fraction, mainly composed by CO₂ (**Figure 9**).

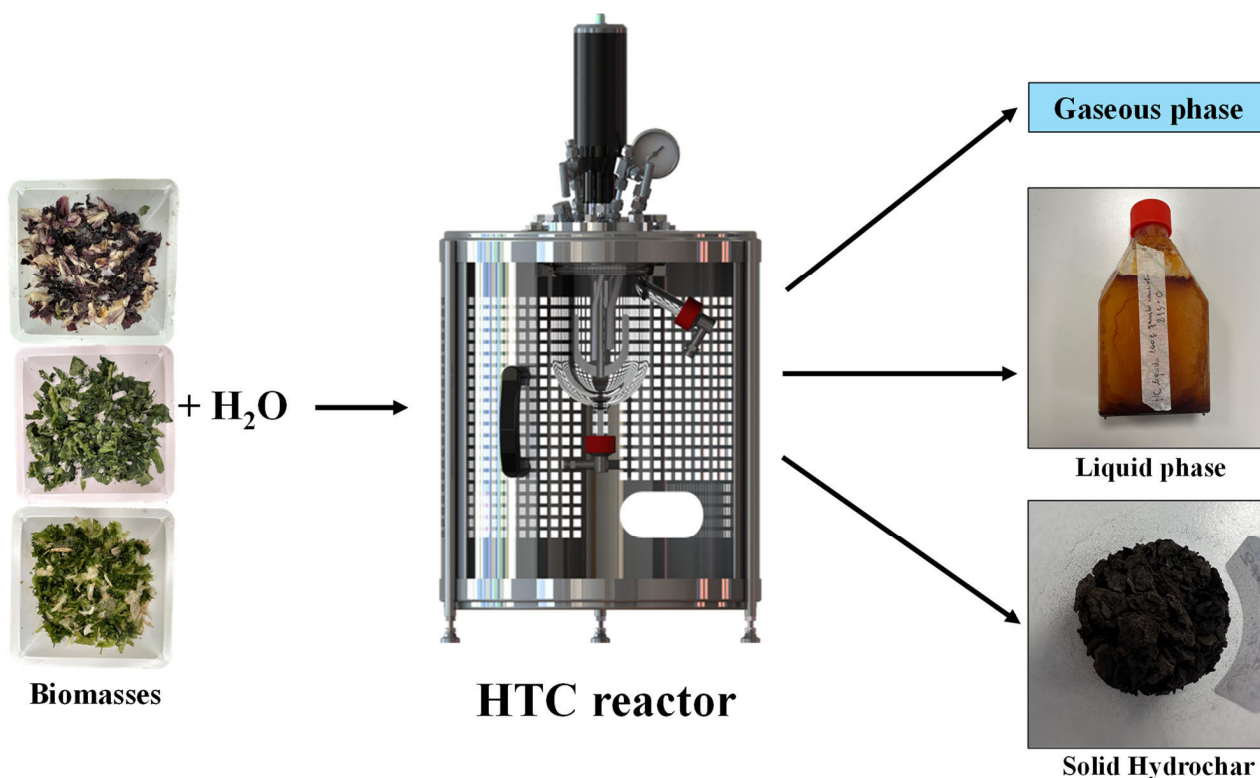


Figure 9. Schematic overview of the hydrothermal carbonization (HTC) process.

The solid hydrochar is physically separated from the liquid phase via sedimentation and centrifugation. The nature of the biomass to be processed plays a crucial role, directly affecting degradation pathways and hydrochar characteristics. Lignin and lipids contribute to higher carbon content and energy value, while hemicellulose and cellulose degrade more readily, influencing both yield and product quality (Cavali et al., 2023).

The HTC parameters mostly influencing the reaction are:

- The **temperature** affects both water properties and ionic interactions. Higher temperatures reduce water viscosity, facilitating biomass breakdown, and promoting dehydration, decarboxylation, and condensation reactions. However, excessively high temperatures may enhance the solid decomposition, increasing the gaseous and liquid fractions, and promote the hydrophobicity of residual solid phase.

- **Residence time** determines the reaction effects and extents. It dictates the intensity of carbonization, promote the condensation, as testified by the lowering of the O/C and H/C ratios. At the same time, the progressive release in the liquid phase of small fragments is time dependent. Consequently, the residence time impacts on polymers decomposition, polymerization, and the resulting morphology and structure of hydrochar particles.

- **Heating rates** also impacts on reaction dynamics. In fact, a relatively slower heating may lead to a more pronounced extration, allow for better control of product allocation, enable a more ordinated structural organization, favour the condensation processes and determine a more reproducible reaction and *viceversa* (Wang et al., 2018). Slower heating rates favor hydrochar formation over liquid products, enhancing calorific value and structural stability (Wang et al., 2020).

In hydrothermal reaction, subcritical water plays a peculiar and fundamental role. In fact, the physicochemical properties of water change markedly when approaching the critical point, with a decrease in dielectric constant, a weakening of hydrogen bonding, and an increase in ionization. As a result, subcritical water behaves like a moderately polar organic solvent, such as ethanol or propanol, allowing the dissolution of both polar and partially non-polar compounds. At the same time, viscosity and surface tension decrease while diffusivity increases, thereby improving water penetration into biomass matrices and the release of bioactive compounds. The elevated concentration of H^+ ions also promote acid-catalyzed reactions, further accelerating biomass conversion (Cheng et al., 2021). Subcritical water also drives the hydrolysis of hemicellulose and cellulose, lowering the activation energy required for their decomposition. This promotes rapid degradation and depolymerization into water-soluble products, such as monomers and oligomers. Water thus acts not only as the reaction medium but also as a facilitator of the process, combining its natural abundance in biomass

with advantages such as low cost and non-toxicity (**Kambo & Dutta 2015**). These properties explain the efficiency of HTC in converting a wide variety of substrates, including cellulose, glucose, agricultural residues, manure, food waste, municipal solid waste, sewage sludge, aquaculture residues, and algae, into valuable products, demonstrating its versatility beyond lignocellulosic biomass (**Figure 10**).

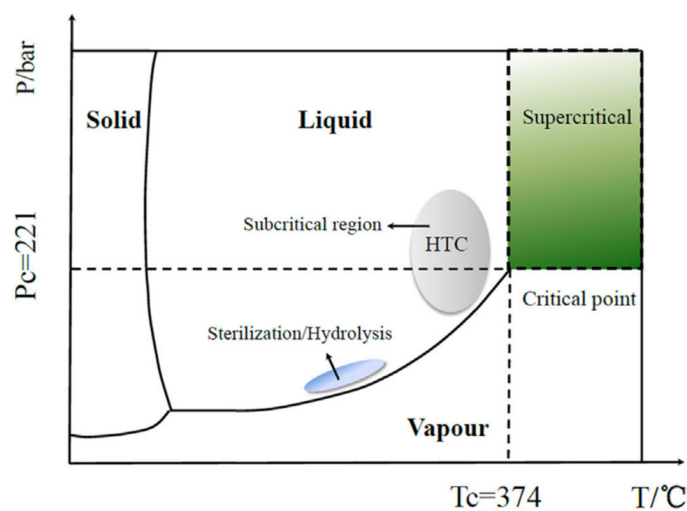


Figure 10. Variation of water properties across temperature ranges and corresponding application domains of hydrothermal processes. Source: Wang et al., 2018.

Hydrochar generally exhibits a porous structure, which can be further improved by physical or chemical activation, making it a multifunctional material with potential applications ranging from pollutant adsorption and catalyst support to wastewater treatment, renewable energy production, and agricultural use as a soil amendment (**Masoumi et al., 2021**).

When applied to soil, hydrochar can reduce bulk density, increase water retention, and influence soil pH, salinity, and nutrient dynamics in ways that vary with feedstock type, production conditions, and application rate (**de Jager et al., 2021**). It may also affect soil microbial communities, in some cases promoting beneficial microorganisms, in others exerting inhibitory effects depending on concentration and chemical composition (**Andert et al., 2015**). Recent studies have shown that hydrochar produced at lower temperatures may act

as a slow-release fertilizer, gradually enhancing soil fertility, while in other cases it has demonstrated effectiveness in improving water-holding capacity and influencing plant growth responses (Fregolente et al., 2021). Overall, HTC represents a promising strategy for the sustainable management of wet agro-industrial residues, providing both an efficient pathway for waste biomass conversion and a versatile material that contributes to carbon sequestration, resource recovery, and environmental remediation (Figure 11) (Cavali et al., 2023).

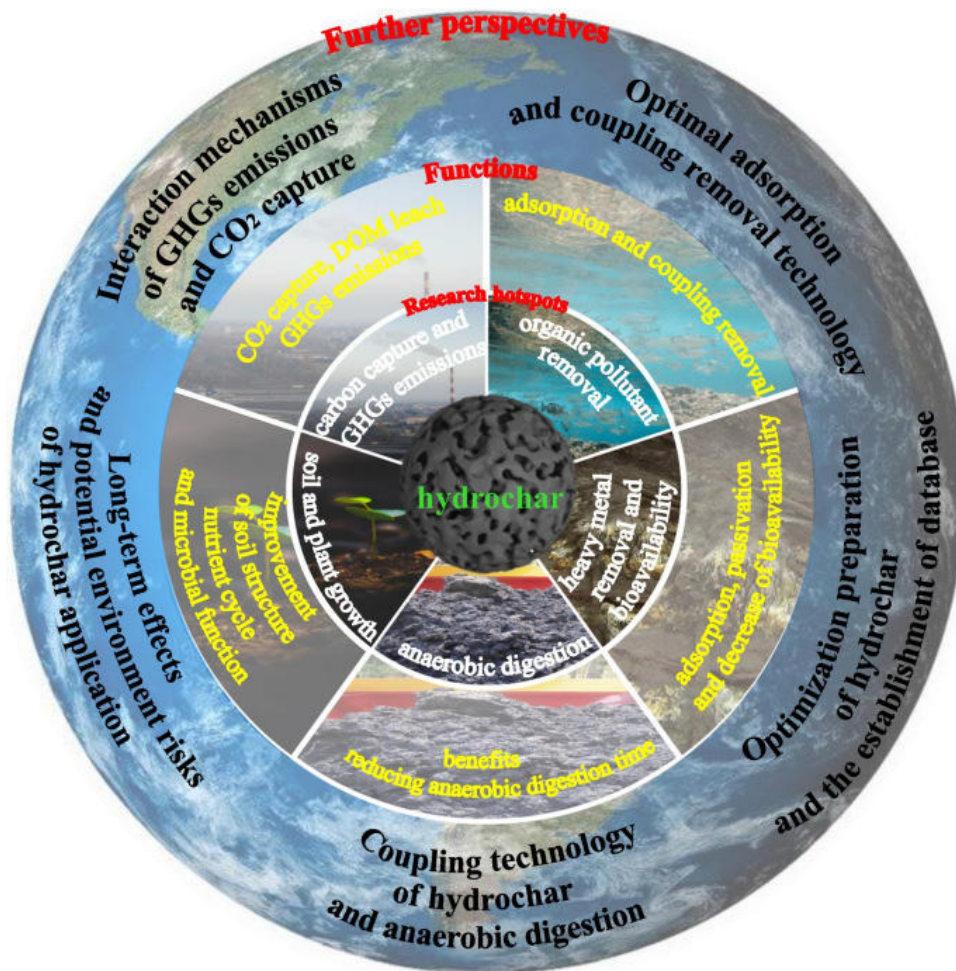


Figure 11. Large-scale effects of HC. Source: Huang et al., 2023.

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PhD Thesis Objectives

Nowadays, agriculture faces pressing environmental challenges, such as the intensive exploitation of soils accompanied by a progressive decline in fertility, the widespread reduction of organic matter in agricultural land (**Obalum et al., 2017**), with severe consequences on crop productivity and microbial quality, and the extensive use of non-renewable mineral resources. To address such complex challenges, it is increasingly necessary to identify sustainable, innovative, and cost-effective strategies capable of improving soil quality, maintaining crop yields, and reducing environmental burdens. In this framework, the recycling and valorization of agricultural and livestock by-products emerges as a particularly promising approach, fully aligned with the principles of the circular economy and the European Green Deal (**European Commission, 2019**). Within this context, the present PhD project was conceived with the overarching aim of recovering and valorizing agro-industrial and livestock residues for the production of organic amendments, including compost, anaerobic digestate, its vermicomposted form, and an innovative hydrochar. These materials were first subjected to a thorough chemical and compositional characterization, followed by bioassays on model plant seeds to evaluate their potential phytotoxic and biostimulant role. Their agronomic performance was then assessed on model crops of strategic interest under both peat-based cultivation and hydroponic systems, coupled with in-depth analyses of their metabolic and physiological responses, in order to obtain a comprehensive evaluation of effectiveness under different growth conditions. Finally, an additional objective of the work was to explore innovative formulation strategies, such as novel alginate-based delivery systems, aimed at enhancing the functional properties, stability, and applicability of the organic amendments in modern agriculture (**Qin et al., 2024**).

The specific objectives of this research are outlined as follows:

1.1 Selection and development of sustainable organic fertilizers derived from agro-industrial by-products

The present study aimed to evaluate and characterize several soil fertilizers resulting from the valorisation of (i) biomass waste from the production of ready-to-eat fresh-cut vegetables through their conversion into Green Compost (GC) and Hydrochar (HC), and (ii) waste from buffalo farms, which was converted into anaerobic digestate (AD) and then vermicomposted (VC). The goal was to evaluate the potential of each biomass source and processing method to produce fertilizers with promising chemical, physical, and functional properties. AD and VC were produced by the biogas Plant C&F Energy, sited in Capaccio (SA). GC was produced by the composting plant Terramore, situated in Eboli (SA). The same biomass material was used to synthesize Hydrochar (HC), produced at the AGRICHEMLAB of DIFARMA through hydrothermal carbonization (HTC) in a 1-liter lab-scale reactor (Buchi-Glass), yielding both a solid and a liquid fraction. The system is equipped with a cyclone to keep solid particles suspended during HTC and is connected to a manometer for continuous monitoring of the reaction pressure, as well as an external controller to regulate temperature and supply heat (**Figure 12**).

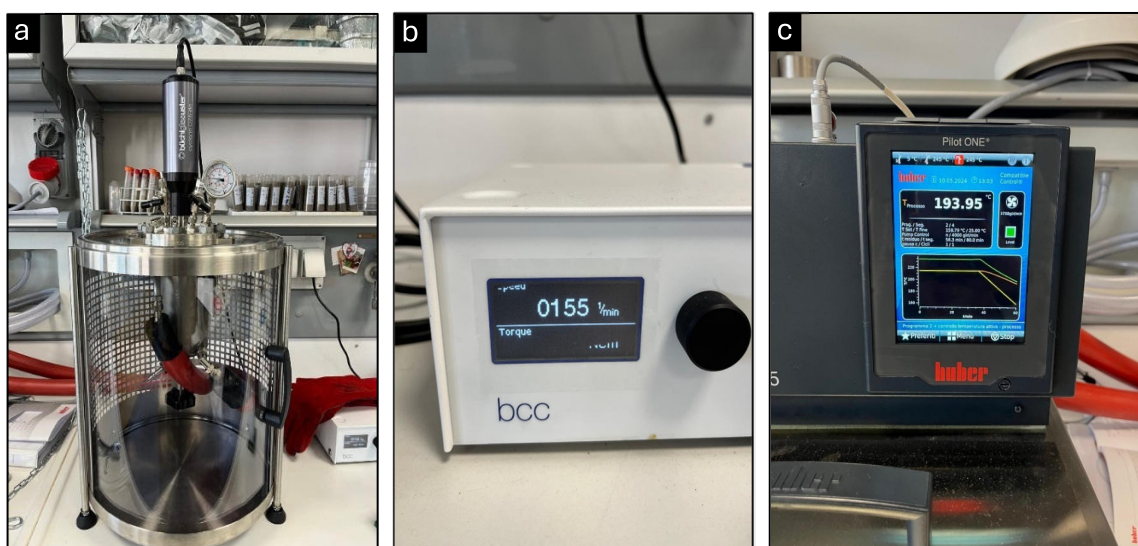


Figure 12. Lab-scale 1-L Buchi-Glass reactor used for hydrothermal carbonization (HTC) of biomass. (a) Hydrothermal reactor; (b) agitation system control (cyclone) (c) External controller used to modulate temperature and supply heat during the HTC process.

The hydrochar represents one of the most innovative aspects of the present work. It offers promising advantages in terms of soil fertility enhancement, nutrient retention, and biostimulant effects, while being environmentally compatible and cost-effective (**Pauline et al., 2020; Yu et al., 2022**).

This investigation is crucial for understanding how the type of biomass and the applied processing methods affect nutrient composition, functional properties, and overall agronomic value. By identifying the most effective organic amendments, including optimized hydrochar, the study seeks to deliver sustainable agricultural solutions while promoting the circular economy through the valorization of organic waste.

1.2 Compositional and chemical characterization of the obtained organic fertilizers.

The obtained organic fertilizers (AD, VC, GC, and HC) were subjected to a comprehensive chemical and compositional characterization to evaluate their nutrient content, structural features, and functional properties. Given the complexity of these materials, their characterization required the application of a wide range of advanced analytical techniques. Structural and compositional properties were investigated through solid-state ^{13}C CPMAS NMR spectroscopy, FTIR spectroscopy, and pyrolysis–GC/MS, while elemental composition was determined via HCNS or ICP-OES. This multi-technique approach provided complementary insights into the chemical composition and functional characteristics of the fertilizers, supporting the evaluation of their subsequent agronomic potential and biostimulant activity.

1.3 Evaluation of the agronomic performance.

The agronomic potential of the selected organic fertilizers was assessed through a combination of phytotoxicity, biostimulation and growth tests. Preliminary analyses were carried out using aqueous extracts of the soil improvers on cress seeds, providing information on potential inhibitory or stimulatory effects during germination (Zucconi et al., 1981). The solid organic soil improvers (AD, VC, GC and solid HC) were then tested in growth trials on peat on lettuce (*Lactuca sativa*) and radish (*Raphanus sativus*), two species widely cultivated in the Piana del Sele area, applying increasing doses of fertilisers to evaluate growth responses, nutrient uptake and biostimulant activity. This integrated approach allowed a comprehensive evaluation of the growth-promoting potential and biostimulant effects of the organic fertilizers, linking their chemical and compositional characteristics to functional agronomic performance.

1.4 Investigation of the metabolic and physiological responses of model plants to fertilizer treatments.

The metabolic and physiological responses of model plants to the selected organic fertilizers were investigated using complementary approaches. For plants cultivated in peat-based trials, metabolomic profiling was performed on harvested tissues using ^1H NMR spectroscopy of the liquid extracts, providing detailed insights into the biochemical changes induced by the different fertilizer treatments

1.5 Development of innovative formulations based on the characterized organic products.

Building on the chemical and compositional characterization of the organic fertilizers, novel formulations were developed using alginate-based systems to enhance soil properties, nutrient availability, and biostimulant potential. The investigations included hydrogel layers and beads, crosslinked with either calcium or iron (III) cations, and were loaded with bioactive water-soluble humic fractions extracted from GC, VC and AD. The formulations were characterized in terms of degradability, water and nutrient release rates, structural rigidity, and viscoelastic properties, providing insights into their stability and performance under realistic soil conditions. Preliminary tests, including seed coating experiments with watercress, demonstrated that some formulations exerted biostimulant effects on germination, while iron-based systems also offered a strategy for supplying bioavailable iron in alkaline soils, reducing the risk of nutrient precipitation. Overall, these innovative alginate-based formulations represent a promising tool for improving soil fertility, enhancing the efficiency of organic amendments, and providing a controlled, slow-release delivery of water, nutrients, and bioactive humic substances (Hu et al., 2022).

1.6 Investigation of liquid hydrochar extracts – research conducted at the Department of Environmental Biology, Biology and Agricultural Chemistry Group (BACH), University of Navarra, Spain

During the research period abroad, the liquid fraction of hydrochar (LHC) was investigated to evaluate the impact of HTC process parameters, specifically temperature and pressure, on its chemical and biological quality. LHC was characterized for its content of bioactive molecules, including polyphenols and nitrogen-containing compounds (Gimenez et al., 2020; Li et al., 2023), essential nutrients, and plant hormones (Lu et al., 2021; Chen et al., 2025). Complementary analyses of molecular features and functional groups were performed using UV–VIS spectroscopy, fluorescence spectroscopy, and UPLC-ESI-HRMS. The effects of LHC applications on *Arabidopsis thaliana* (Col-0) were assessed under controlled hydroponic conditions. Plants were treated with LHC at 100 mg OC L⁻¹, with negative and positive controls. Both vegetative growth and root development were monitored, while targeted molecular analyses evaluated the expression of genes involved in nutrient transport (N, Fe, Cu), hormonal signaling, and plasma membrane H⁺-ATPase activity using qRT-PCR on shoot and root tissues (Chen et al., 2025). This integrative approach provided a comprehensive understanding of how LHC composition, shaped by HTC process conditions, influences plant physiology, metabolism, and biostimulant activity, highlighting its potential as a bioactive amendment in sustainable horticulture and circular economy frameworks.

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Results organization

This PhD thesis consists of a collection of 5 scientific papers focused on the production and evaluation of sustainable fertilizers derived from agro-industrial waste. The articles are presented in a logical sequence to ensure a coherent and comprehensive exploration of these interconnected topics.

The first article evaluates the use of solid anaerobic digestate (AD) from buffalo manure and its vermicomposted form (VC) as sustainable fertilizers. Their chemical and structural features were analyzed using elemental analysis, Pyr-GC-MS, CPMAS-NMR, and FT-IR spectroscopy. AD showed higher labile carbon, while VC displayed more recalcitrant structure, indicating advanced humification. Greenhouse trials identified optimal doses at 40% (v/v), except for VC on lettuce, which performed best at 20%. Metabolomic profiling via ^1H NMR and chemometric tools (PCA, heatmaps) revealed a prominent effect on the plant primary metabolome, especially in terms of carbohydrate contents and amino acid biochemical pathways. These findings support the validity of strategies based on the transformation of livestock waste into effective biofertilizers.

The second article examines green compost (COM) from horticultural (red chicory, escarole, spinach), and olive pruning residues and its pelletized form (PEL). Both were chemically characterized, and compost was further analyzed by ^{13}C CPMAS NMR, Pyr-GC-MS, and FT-IR. Pot trials with lettuce and radish showed that the amendments, either alone or with mineral fertilizer, enhanced growth, yield, and physiological status of plants. Lettuce was more sensitive to nitrogen form, while radish showed improved root development. The combination of labile and stable organic fractions promoted nutrient release and soil quality, and

pelletization added practical benefits, making this a sustainable strategy to boost vegetable production and reduce synthetic inputs.

The third article focuses on the solid fraction of hydrochars (HC) produced from spinach, red chicory, and escarole as a sustainable strategy to valorize horticultural residues. Two HCs were obtained under mild (H180; 180 °C/10 Bar) and severe (H215; 215 °C/20 Bar) hydrothermal carbonization (HTC) conditions and characterized for elemental composition, nutrient content, and molecular structure. Greenhouse pot trials on baby-leaf lettuce showed that HC180 strongly promoted shoot and root growth, with results comparable to those resulting from mineral fertilization. ¹H NMR metabolomics revealed that HC180 stimulated sugars and amino acids involved in energy production and stress response, indicating a biostimulant effect driven by nutrient availability and soil–microbiome interactions. Mild HTC thus produces solid HCs with rapid biostimulant activity and a balanced composition of labile carbon, nitrogen, and plant-available nutrients, offering a tunable approach for sustainable residue management.

The fourth article is based on the liquid fraction of hydrochars associated to the abovementioned hydrochars. Part of the experimental activities have been conducted at the University of Navarra, in collaboration with Prof. Garcia Mina's group, during the foreign internship period. LHCs were produced under mild (LHC180, 180 °C/10 bar) and severe (LHC215, 215 °C/20 bar) HTC conditions and characterized for labile carbon, soluble nutrients, and bioactive molecules, including auxins, cytokinins, jasmonates, and salicylic acid. Bioassays on *Lepidium sativum* and hydroponic trials with *Arabidopsis thaliana* showed that LHC180 strongly promoted seed germination, root growth, shoot biomass, and nutrient use efficiency, whereas LHC215 had limited effects. Both LHCs activated nutrient transporters, iron homeostasis regulators, and plasma membrane H⁺-ATPases, suggesting a

hormone-like mechanism similar to that elicited by humic substances. These results highlight LHC180 as a bioactive amendment with growth-promoting and nutrient-regulating properties, providing a practical strategy for sustainable fertigation and circular management of vegetable residues.

The fifth article focuses on alginate-based hydrogels and beads designed to improve soil fertility through slow release of water, nutrients, and humic materials. Systems were produced via calcium or iron ionotropic gelation and characterized for structure, degradability, water and cation release, and rheological properties. Humic fractions were extracted from compost, vermicompost, and anaerobic digestate (described and characterized in the third and fourth articles) and analyzed by ^1H -NMR, DOSY 2D NMR and FT-IR. A film based on calcium-alginates loaded with humic substances was produced and applied on watercress seeds. The seed-coating, which was proved by SEM, was assayed in-vitro demonstrating biostimulant effects of HSVC- and HSDIG-loaded systems. Iron-based formulations (Only beads and seed-coating, as stable iron-hydrogels were not produced) represented an effective solution to release water and iron, especially in alkaline soils. These results indicate that alginate-based systems can act as slow-release carriers of water, nutrients, and humic substances, offering a promising strategy to enhance soil fertility and resilience.

Chapter II

Original research paper

“Solid anaerobic digestate from buffalo livestock wastes and its vermicomposted form as soil fertilizers and modulators of lettuce and radish primary metabolism”

Rossella Curcio^{1,2}, Annamaria Di Serio³, Maria Luisa Graziano, Diana Agrelli⁴, Mariavittoria Verrillo⁴, Riccardo Spaccini⁴, Giancarlo Cattaneo⁵, Massimo Zaccardelli,⁶ Domenico Ronga²,
Pierluigi Mazzei²

¹*DAFE - Department of Agricultural, Forest, Food, and Environmental Sciences, University of Basilicata, 10, Viale dell'Ateneo Lucano - Potenza (PZ)*

²*Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA Department of Chemistry and Biology*

³*Department of Chemistry and Biology, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Italy*

⁴*Department of Agriculture, University of Naples Federico II, Via università, 100, 80055, Portici, Italy*

⁵*C&F ENERGY Società Agricola Biogas Plant, Via Tirrena inferiore SS 18, 84047, Capaccio Paestum, Italy*

⁶*CREA Research Centre for Vegetable and Ornamental Crops, 84098 Pontecagnano Faiano, Italy*

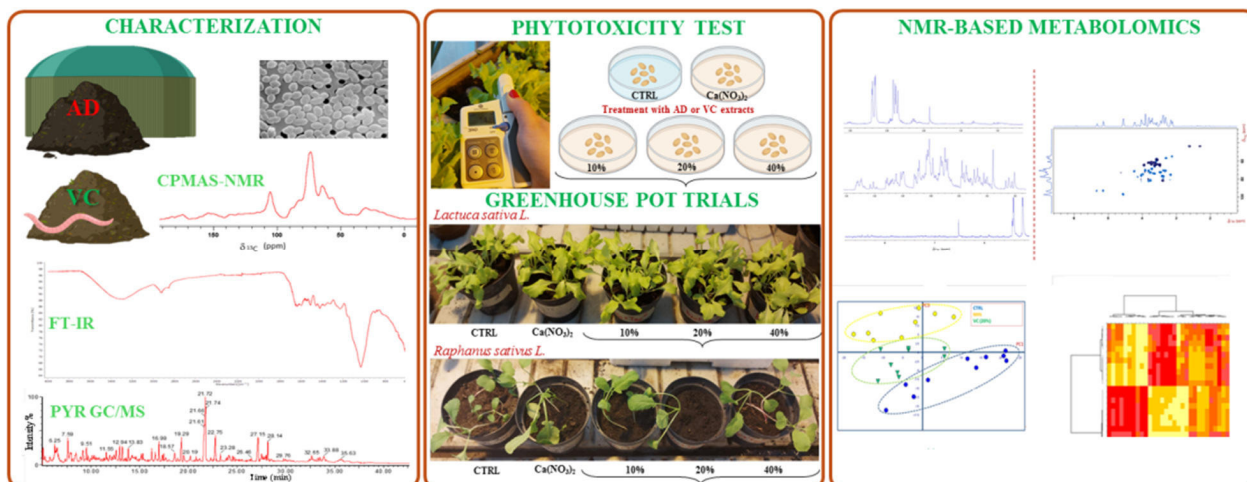
*** Corresponding author:** Pierluigi Mazzei, orcid.org/0000-0002-5312-4969,
pmazzei@unisa.it

2.1 ABSTRACT

The potential role of solid anaerobic digestate (AD) was evaluated, obtained from the elaboration of buffalo livestock wastes, and its vermicomposted form (VC) as sustainable soil improvers and plant biostimulants. Their chemical and compositional properties were characterized by elemental analysis, Pyr-GC-MS, CPMAS-NMR, and FT-IR. AD showed higher labile carbon content, while VC was characterized by a larger amount of aromatic and carboxylic groups, thus testifying a more advanced humification state. VC was characterized by relatively higher recalcitrance and hydrophobicity, suggesting its long-term persistence after application in soil. Plant responses to AD and VC were monitored, *in vitro*, through phytotoxicity test, and, via greenhouse pot trials with peat, through physiological, agronomic and qualitative analyses. The latter ones revealed that the optimal doses of AD and VC were 40%, (v/v) in all cases, except for VC on lettuce which provided the best results at a dose of 20%. (v/v) Metabolomic profiling via ^1H NMR spectroscopy indicated that the optimal doses induced the development of a peculiar metabolic trait which, at certain extents, resembled the one elicited by the mineral fertilization. Chemometrics (PCA and Heatmap clusterization) indicated that the largest involvement occurred for the metabolic pathways related to carbohydrates, the Krebs cycle, and the aromatic amino acids. AD and VC are efficient fertilizers, supplying nutrients and enhancing the plant metabolism. In line with the circular economy principles, these products represent a dual strategy to valorize buffalo manure, transforming it into high-value biofertilizers, ultimately promoting environmental sustainability and agricultural productivity.

Keywords: Biomass recycle; Biostimulant; Buffalo Livestock; Chemometry; NMR-based
Plant Metabolomics; Organic Fertilizers.

Highlights and Graphical abstract



- 1** VC boosts growth and quality; AD increases biomass but also foliar nitrate.
- 2** VC shows humified aromatics; AD is rich in aliphatic, protein, and labile C.
- 3** VC and AD modulate primary metabolism similarly to mineral fertilizers.
- 4** VC is microbiologically safe; AD requires pre-application sanitization.
- 5** VC and AD valorize buffalo slurry and support agroecological circular practices.

2.2 INTRODUCTION

Buffalo farming for the production of milk and dairy products represents a strategic economic sector for the Southern Italy, being it related to the production and the worldwide export of the "mozzarella di Bufala Campana", awarded of the prestigious label “Protected Designation of Origin product” (PDO). In fact, this industry, which involves around 250,000 buffaloes, generates over 1 billion € profits per year (**Chirone et al., 2022**). However, high animal density presents environmental and logistical challenges, encompassing the management of large volumes of manure. Buffalo manure, which is composed mainly of slurry and solid dung, is a nutrient-rich organic material which, when stabilized, has a great potential for agricultural applications, since its incorporation into soil can enhance carbon sequestration and increase the soil fertility. However, it can release large amounts of nitrogen and phosphorus as well as potentially harmful components, including pathogens (**Irshad et al., 2013**). These factors require careful management to maximize benefits and mitigate risks, according to the European Nitrates Directive (91/676/EEC) and the Italian Legislative Decree 152/2006. Since the food and agricultural sectors are ever more focused on sustainable and high-quality productions, there is a growing demand for eco-friendly practices minimizing environmental impacts, optimizing resource efficiency and improving the food production level. In this context, an effective strategy is represented by the recovery and valorization of organic residues deriving from agriculture, food industry, and zootechnical farming. By exploiting advanced and appropriate technologies and methods, waste biomass can be converted into bioenergy and materials useful to augment the quality of food, thereby promoting a sustainable bioeconomy (**Tshikalange et al., 2022**). In fact, organic biomass, such as buffalo manure, when opportunely valorized and supplied to soil, can safely enable the slow-release of nutrients, improve soil

quality and plant health, and mitigate the ecological footprint of agricultural systems (**Tambone et al., 2010; Pizzanelli et al., 2023**).

Anaerobic digestate is the residual material resulting from the digestion of organic biomass in absence of oxygen. The latter one is a multi-step microbiological process which involves four stages, consisting in hydrolysis, acidogenesis, acetogenesis, and methanogenesis, and producing gases (including methane), a liquid phase and a solid residue (**Ziemiński et al., 2012**). In particular, it has been proven that the solid anaerobic digestate (AD) can exert a beneficial effect on soil and plants, exhibiting a great potential as a fertilizer, enhancing crop yields and soil fertility (**Zamri et al., 2021; Loffredo et al., 2024**). The vermicomposting of the solid anaerobic digestate (VC) represents another valid strategy to process organic matter. The biomass is elaborated by earthworms into bioreactors which, through their digestive enzymes and in synergy with naturally occurring microorganisms, produce the vermicompost potentially useful to improve soil fertility and restore degraded soil (**Yasmin et al., 2022**). These beneficial properties are due to the fact that VC is rich in plant-available nutrients and may biostimulate plants and the soil microbial diversity. Extensive research has consistently demonstrated the effectiveness of soil and plant treatments with AD and VC as both fertilizer and agents to enhance the crop productivity (**Pizzanelli et al., 2023; Greco et al., 2021; Amuza et al., 2022; Toor et al., 2024**).

However, a characterization of AD and VC through advanced analytical techniques may provide interesting information on the molecular composition of these products, potentially useful to explain their beneficial role in soil. For example, solid-state ^{13}C CPMAS (Cross Polarization Magic Angle Spinning) Nuclear Magnetic Resonance (NMR) spectroscopy (**Pizzanelli et al., 2023**), Fourier-transform infrared spectroscopy (FTIR) (**González-Rojo et al., 2024; Verrillo et al., 2021**), and thermochemiolysis followed by GC/MS (**de Aquino et al.,**

2019), are very tailored for this purpose. These methods can help elucidating the complex chemical composition of AD and VC and provide valuable insights into the transformation dynamics, involving the organic material during the anaerobic digestion and after the bio-oxidative vermicomposting (**Pizzanelli et al., 2023**). Finally, it is of a paramount importance to investigate on the possible influence exerted by these products on the primary metabolism of plants developed on soil treated with AD and VC.

Therefore, this work aimed to characterize solid anaerobic digestate from buffalo manure and its vermicomposted form through a composite analytical approach involving microbiological, elemental, chemical and advanced compositional techniques, such as CPMAS NMR, FTIR and Thermochemiolysis followed by GC-MS. The effects exerted by AD and VC on crop productivity were assessed through phytotoxicity tests and pot trials, involving lettuce and radish model plants. Finally, the alteration in the primary metabolism of lettuce and radish plants showing agronomical benefits from the AD and VC treatments was investigated via ^1H NMR-based metabolomics.

2.3 MATERIAL AND METHODS

2.3.1 Solid anaerobic digestate and vermicompost

Solid anaerobic digestate (AD) and its vermicomposted form (VC) were supplied by the biogas plant “C&F ENERGY” situated in Capaccio Paestum (Salerno, Italy). In detail, AD was obtained through the anaerobic reprocessing of buffalo livestock wastes. The material was put into a large-scale digester, and the reaction occurred for 30 days under an agitator action, which suspended the biomass continuously to free the gases and prevent the formation of layers, at a temperature ranging from 35 to 55 °C. At the end of the process, the solid phase was isolated from the liquid one and enriched with ammonia resulting from a partial removal from the liquid

digestate via stripping. Part of the solid digestate was subsequently reprocessed into an earthworm-based bioreactor through a 14 days long process which converted it into the VC.

2.3.2 *Chemical parameters and elemental composition of AD and VC*

AD and VC samples were oven-dried at 70 °C for at least 24 hours, up to achieve a constant weight, and sieved through a 2-mm mesh. The chemical analyses of these products included pH, electrical conductivity (EC), and ash content. pH and EC ($\mu\text{S cm}^{-1}$) were measured by suspending the organic materials into ultrapure water (1:10, w/v). The supernatants were filtered under vacuum (Whatman 41 cellulose filters; ϕ of 20 μm) and examined via a two-channels HANNA HI5222 detector. Ash content was determined by incinerating 1 g of dried sample at 650 °C for 6.5 hours in a muffle furnace (SNOL E5CC) and expressed as a residual content (%) in respect of the initial dry weight.

Elemental composition (C, H, N, S) was analyzed with UNICUBE elemental analyzer (Elementar Analysensysteme GmbH, Germany), by using 2 mg of material *per* sample (**Curcio et al., 2025**). The essential plant nutrients were examined *via* Inductively Coupled Plasma - Mass Spectrometry (ICP-MS). Briefly, 0.1 g of samples were mixed with 3 mL HNO₃ 67 %, 1 mL HCl 37 % and 1 mL HF 47 %, in a Teflon vessel, and digested into a microwave system (Ultrawave, Milestone), according to the following program: temperature increase for 25 min up to achieve 240 °C followed by 10 min of steady state at 240 °C. After cooling, the digested solutions were diluted up to achieve a volume of 10 mL (depending on the nutrient concentration, the solution was further diluted up to 200 times) for the ICP-MS analyses.

2.3.3 Solid-state CPMAS NMR and FT-IR spectroscopies

The molecular composition of AD and VC samples was characterized in the solid state using the Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (CPMAS NMR) and the Fourier Transform Infrared (FT-IR) spectroscopies. ^{13}C CPMAS NMR spectra were acquired with a Bruker Avance 300 MHz wide-bore magnet (Bruker Biospin, Rheinstetten, Germany), equipped with a CPMAS probe, operating at 25 °C, with ^{13}C and ^1H frequencies of 75.47 and 300.13 MHz, respectively. Finely powdered solid samples (60–80 mg), sieved to 0.5 mm, were placed into 4 mm zirconia rotors, sealed with Kel-F caps, and spun at $10,000 \pm 1$ Hz. The ^{13}C NMR proton-decoupled spectra were obtained through the CPMAS experiment, with a setup including 1 ms of contact time, 1814 time domain points, a spectral width of 300 ppm (22,727.3 Hz), a recycle delay of 5 s, 8 dummy scans, and 8000 scans. Free induction decays (FIDs) were processed with Bruker Tospin (v 4.0.1) software, Fourier transformed using 2-fold zero-filling, applying an exponential filter function of 250 Hz, and performing a final phase- and baseline-correction. Details on method to integrate CPMAS NMR spectra and calculate related structural indexes are reported in **Supplementary Paragraph SP1**.

FT-IR analyses were carried out by using the Attenuated Total Reflectance (ATR) technique and examining dried and sieved samples (≤ 0.5 mm). Analyses were performed in duplicate, using a PERKIN ELMER Frontier spectrometer and setting 128 scans, a spectral range included within 4000 and 600 cm^{-1} , and a resolution of 4 cm^{-1} . For each acquisition set, a background spectrum was recorded and subtracted. Each spectrum was converted into absorbance mode and baseline corrected to ensure consistency and enable accurate comparison between different samples. All spectra were processed with *Spectragryph* software (v. 1.2.16.1).

2.3.4 Gas-Chromatography and mass spectrometry following thermochemiolysis (Pyr/GC-MS)

Thermochemiolysis coupled with GC–MS analysis was carried out as described previously (Monda et al., 2017). Briefly, 500 mg of each sample (AD and VC) was derivatized with 1 mL of a 25% tetramethylammonium hydroxide (TMAH) methanolic solution and placed into a quartz boat. After 2 hours, the latter one was introduced into a Pyrex tubular reactor (50 cm × 3.5 cm i.d.) and heated at 400 °C for 30 minutes using a Barnstead Thermolyne 21,100 tubular furnace. The depolymerized and derivatized volatile compounds which developed during the pyrolysis were transported by a helium stream (20 mL min⁻¹) into two dichloromethane solutions, arranged in series and kept in an ice bath. The two solutions were then combined, concentrated by rotary evaporation, and the final residue was redissolved in 1 mL of dichloromethane prior to GC–MS analysis.

GC-MS analyses were performed on a Perkin Elmer Autosystem XL gas chromatograph equipped with an RTX-5MS WCOT capillary column (30 m × 0.25 mm; film thickness 0.25 µm) and coupled to a PE Turbomass-Gold quadrupole mass spectrometer *via* a heated transfer line at 250 °C. The oven temperature program consisted of an initial hold at 60 °C for 1 minute, a ramp of 7 °C min⁻¹ up to 320 °C, and a final hold at 320 °C for 10 minutes. Helium was used as carrier gas (1.90 mL min⁻¹), with the injector set at 250 °C and operating in split mode (30 mL min⁻¹ split flow). Mass spectra were acquired in electron impact mode (70 eV), scanning from 45 to 650 m/z, with a cycle time of 0.2 s. Compound identification was achieved by both comparing spectra to those of authentic standards and the NIST 05 mass spectral library, and with the support of the appropriate scientific literature (Monda et al., 2017b; Pizzanelli et al., 2023). The areas of the peaks in the pyrograms were integrated, grouped by compound class, and expressed both in absolute terms and as percentages of the

total chromatogram area. The compound classes included: fatty acid methyl esters (FAMES), branched FAMES and hydroxyacids, mono- and di-unsaturated FAMES, dioic acids, and lignin-related moieties (specifying the contribute of *p*-hydroxyphenyl - P, guaiacyl - G, and syringyl -S units).

2.3.5 NMR-based metabolomics in treated plants

AD and VC were characterized from a microbiological point of view (**Supplementary Paragraphs SP2**) and evaluated via phytotoxicity assay (more information on methods are reported in **Supplementary Paragraph SP3**). Then, these materials were applied at different concentrations to model plants (lettuce and radish) in order to assess, via greenhouse pot test wit peat, their role from an agronomic, physiological and qualitative point of view (more information on methods are reported in **Supplementary Paragraphs SP4**, respectively). Based on agronomic evaluation, several concentrations of AD and VC induced an enhanced biostimulation in radish and lettuce plants, as compared to the control. In order to decipher the metabolic aspects related to such a response, radish and lettuce plants resulting from the optimal AD and VC treatments underwent an NMR-based metabolomics and were compared with CTRL and MIN samples. Information on the method to extract the primary metabolome of lettuce leaves ad radish roots for NMR-based metabolomics, are reported in **Supplementary Paragraph SP5**.

¹H NMR spectra were acquired for radish and lettuce extracts at 25 ± 1 °C using a 400 MHz Bruker Ascend magnet (9.4 T; Bruker Biospin, Rheinstetten, Germany) equipped with a BBI probe. Each spectrum was obtained using an on-resonance presaturation pulse sequence (NOESYGPPR1D) to suppress the residual water signal, with presaturation power set to 55 ± 5 dB. The acquisition parameters included: 4 dummy scans, 96 scans, a 90° pulse ranging from

10.2 to 10.7 μ s (-11.64 dB), a spectral width of 16 ppm (6410.3 Hz), 67854 data points, and a total recycle delay of 10 s (5 s acquisition + 5 s relaxation). Spectra were processed using Fourier transform with an exponential line broadening of 0.3 Hz, without zero filling. Chemical shifts were calibrated using TMSPA as an internal reference ($\delta = 0$ ppm for both ^1H and ^{13}C). All spectra were processed using Bruker Topspin software (v. 4.1.4, Bruker Biospin, Rheinstetten, Germany). NMR data were statistically processed via ANOVA, PCA and Heatmap-clusterization. More information are reported in **Supplementary Paragraph SP6**.

2.4 RESULTS AND DISCUSSION

2.4.1 Chemical analyses of AD and VC

The most important chemical parameters of AD and VC were characterized to achieve important information, useful to comprehend and explain their potential impact on soil chemistry and plant response. The pH of organic materials conceived for soil applications is a critical parameter not only to figure out their chemical reactivity and total electrical charges, but also to predict their influence on the overall soil pH. In fact, materials characterized by extreme pH values can influence the soil pH up to exert a detrimental effect on the activity and the survival of plants and soil microbiota, other than restricting the bioavailability of selected nutrients (Lukehurst et al., 2010; Damrongrak et al., 2025). Anaerobic digestate typically exhibits a neutral to slightly alkaline pH, primarily due to the release of ammonia during the anaerobic decomposition of organic matter. In fact, the typical pH-dependent protonation of ammonia occurring within its conversion into ammonium ion, releases hydroxide ions, thus determining an increase in pH. However, such an alkalinizing effect varies according to the type of feedstock, its content in organic nitrogen and the specific conditions applied for the anaerobic digestion (Regelink et al., 2021). AD exhibited a slightly alkaline pH of 7.6, while VC a slightly more acidic pH of 6.82 (Table I). This indicated that AD may serve as slightly

alkalinizer for sub-acid soils, while VC as a slightly acidifier. Interestingly, the more acidic pH of VC resulted mostly from the earthworms activity and digestion which enriched the material in free organic acids as well as promoted the development of terminal acidic carboxyles via oxidation, thus indicating a more humified material (**Pizzanelli et al., 2023**). The measure of electrical conductivity of organic extracts is an important parameter related directly to the overall content in soluble and ionic components, including nutrients and metals. AD showed an electrical conductivity of 28.03 mS/cm which was slightly but significantly higher than that of VC (22.29 mS cm⁻¹; **Table 1**). The relatively high content in salts observed for AD was due, in part, to the pH-depending charges developed during the OM digestion which adsorbed ionic species, and, in part, to the AD morphology which may have physically entrapped several salts. Consequently, AD represents a source of soluble minerals, partially including nutrients (**Tambone et al., 2010**). In contrast, the lower salt content in VC was probably related to the biological activity of earthworms which absorbed and immobilized most of them (**Damrongrak et al., 2025**). Moreover, the vermicomposting process requires a regular input of water into the bioreactor to moisten the organic substrate and guarantee the earthworms survival. This can have determined a partial leaching of soluble materials, thus decreasing the overall salt levels.

In **Table 1** are shown both the organic and inorganic carbon contents for AD and VC. The inorganic carbon is represented mostly by the carbonates, whose content is directly related to the calcium and magnesium amount in the initial biomass. AD contained the largest amounts of carbonates and this may have concurred to its relatively higher pH and salinity. On the other hand, the lower content of carbonates in VC may be again explained by a carbonates leaching elicited during the vermicomposting process. The organic carbon was significantly different in AD and VC with values of 42.65 and 51.15%, respectively, while the nitrogen content was very

similar, with an average value of around 26%. The different C content determined the lower C/N observed for AD (**Table 1**). The relatively lower carbon content (%) in AD is due to a larger abundance of residual labile organic matter, characterized mostly by carbohydrates. In fact, the lower C content is compensated by the significantly larger oxygen content in AD (almost the double, as compared to that in VC), consistently due to the presence of polyalcohols, such as saccharides. Conversely, the vermicomposting results from an advanced humification of the material which implies a larger consumption and oxidation of labile organic material, leading to an overall decrease in oxygen, accompanied by the enrichment of metabolically derived carbon, primarily in the form of humus-rich excreta. Such an outcome permits to expect a more recalcitrant and biochemically stable organic material in VC as well as to expect a relatively longer persistence of VC in soil with a more efficient role in the carbon sequestration in soil (**Damrongrak et al., 2025**). The sulphur (S) content was slightly higher in VC, with a value 50% more abundant. According to the previous comments about the higher content in carbon and a lower EC value (**Table 1**), we can infer that the exceeding amount of S in VC was due to organic-sulphur forms rather than soluble inorganic sulphates.

The ash content in anaerobic digestate represents an important factor which depends on the type of feedstock and reflects the biogas production yields. In fact, relatively abundant contents in ashes, typically accompanied by a relatively lower content in carbon, may affect the yields. On the other hand, the proper content in mineral and non-volatile components may improve biogas production and process stability, by providing both essential trace elements for microorganisms during the digestion and a buffering capacity to the reaction mixture. Considering that, depending on the biomass type, the ash content in the solid anaerobic digestate may reach values up to 40%, the 3.58% content detected for AD can be considered as sufficiently low. Interestingly, the ashes in VC were almost 4-times more abundant. This property was mostly

due to the activity of earthworms, which, through processes of biological immobilization, led to a relative bioaccumulation of inorganic nutrients (**Pizzanelli et al., 2023**). The fact that VC exhibited a lower EC suggested that the ashes elemental composition was characterized mostly by non-volatile and non-soluble elements. The remaining essential nutrients were characterized via ICP-MS technique (**Table 1**). VC exhibited the higher content for most of examined elements, excluding sodium, potassium and boron, which were higher in AD. Interestingly, the abundance of monovalent Na and K may explain the larger levels of electrical conductivity observed for AD, which are weakly retained via ionic bonds and represent most of soluble salts. Again the larger abundance of other elements in VC may be ascribed to the earthworms-mediated bioaccumulation. The levels of these nutrients are relatively high and permit to consider both AD and VC as promising sources of macro, meso and micronutrients for plants and soil microorganisms (**Damrongrak et al., 2025**).

TABLE 1

Chemical analyses of anaerobic digestate (AD) and vermicompost (VC) including pH, electrical conductivity (mS/cm), ashes (% w/w), elemental analysis (C, H, N, S) and nutrients content (determined via Inductively Coupled Plasma Mass Spectrometry - ICP-MS).

		AD		VC	
pH		7.60	± 0.07	6.82	± 0.01
EC	mS/cm	28.03	± 0.13	22.29	± 0.52
Organic C	% (w/w)	42.65		51.15	
Inorganic C	% (w/w)	1.80		1.20	
N	% (w/w)	2.61		2.58	
H	% (w/w)	4.93		6.31	
S	% (w/w)	0.54		0.81	
O	% (w/w)	47.49		24.95	
C/N	% (w/w)	39.72		47.33	
H/C	% (w/w)	1.33		1.45	
Ashes	% (w/w)	3.58		15.41	
B	mg/kg	32.61	± 2.63	21.17	± 0.98
Na	mg/kg	2189.40	± 46.29	877.55	± 59.60
Mg	mg/kg	4238.84	± 297.35	6517.75	± 676.92
P	mg/kg	8306.09	± 1306.48	14004.04	± 1525.03
K	mg/kg	11990.81	± 332.02	5818.82	± 229.09
Ca	mg/kg	14613.76	± 693.29	24680.17	± 1576.10
Mn	mg/kg	107.17	± 0.72	155.02	± 17.40
Fe	mg/kg	2919.56	± 570.79	6056.44	± 1292.28
Cu	mg/kg	16.39	± 0.63	20.67	± 1.61
Zn	mg/kg	92.75	± 2.32	132.32	± 3.88

2.4.2 Compositional characterization of AD and VC

The compositional characteristics of AD and its vermicomposted form may offer valuable insights into their chemical nature, helping to comprehend their potential role in soil and predict the mechanisms relying on possible interactions with soil biological components. Therefore,

ATR FT-IR and solid-state ^{13}C CPMAS NMR spectroscopies along with Pyr-GC/MS were used to evaluate both materials. In **Fig. 1** it is shown a comparison between AD and VC.

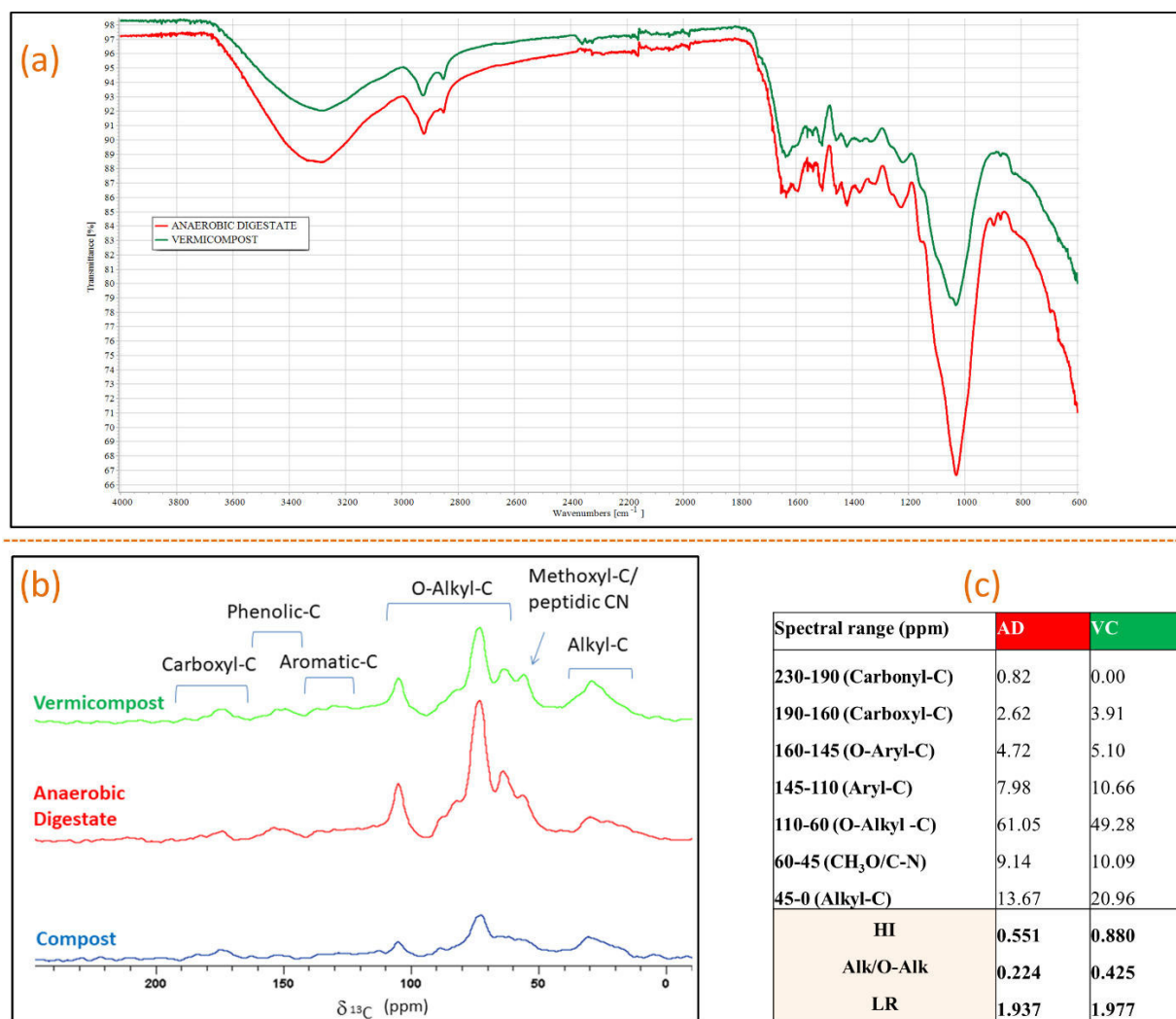


Fig. 1. Solid-state ATR FT-IR (a) and ^{13}C CPMAS NMR (b) spectra of AD (red) and VC (green); the box (c) reports the NMR integrations (normalized to the total) and the most relevant NMR-based indexes.

From a macroscopic point of view, both techniques unveiled clear differences between the two materials, likely arising from the distinct biochemical transformations involved in their production. The ATR FT-IR spectra of studied materials are shown in **Fig. 1a**. Briefly, the broad

absorption band in the 3200–3400 cm^{-1} region, was assigned to O–H stretching vibrations of hydroxyl groups, typically associated with hydrogen bonding in alcohols, phenols, and carboxylic acids. The absorption bands at 2920 and 2850 cm^{-1} were attributed to alkyl C–H stretching vibrations, indicating the presence of lipidic and aliphatic structures. The region between 2850 and 2750 cm^{-1} was ascribed to C–H stretching of methoxyl (–OCH₃) groups, mostly attributable to lignin-derived compounds. The prominent band near 1720 cm^{-1} was assigned to C=O stretching vibrations, indicative of carboxylic acids, esters, and ketones, and serving as indirect markers of the degree of organic matter oxidation. The absorption band at approximately 1620 cm^{-1} was associated to aromatic C=C skeletal vibrations. The signal in the 1030–1080 cm^{-1} range was attributed to C–O stretching vibrations of polysaccharides, while bands in the 700–900 cm^{-1} region were assigned to the out-of-plane C–H bending vibrations of aromatic rings (Curcio et al., 2025; Verrillo et al., 2021; Machado et al., 2020). The comparison of spectra showed that the two spectra were very similar to each other except for the bands 3200–3400 cm^{-1} and at 1030–1080 cm^{-1} which were markedly more intense in AD than in VC. Both indicated a higher abundance than VC of hydroxyl-containing compounds in AD, such as alcohols and carbohydrates. Such behavior is very important since suggests a more enhanced depletion of these labile structures in VC, due to their pronounced earthworm and microbial decomposition occurred during vermicomposting.

Fig. 1b and c display the solid-state ^{13}C CPMAS NMR spectra of AD and VC and the semiquantitative integration of the main spectral regions along with the NMR-based structural indexes, respectively. The region between 0–45 ppm was attributed to (CH₂)_n and CH₃ alkyl groups and represented the aliphatic chains in lipids and natural biopolymers including waxes, cutin and fatty acids of microbial origin. These carbon signals are typically associated with more recalcitrant structures (particularly long-chain lipids and plant-derived waxes) that are

resistant to microbial degradation and play a key role in the formation and persistence of stable soil organic matter. The peak in the region of 45-60 ppm may derive by both CH₃O- carbons, ascribable to aromatic methoxyls in syringyl and guaiacylic lignin units, and the C-N carbons in oligo- and polypeptides. The interval included within 60 and 110 ppm is attributed to O-alkyl carbons, predominantly associated with carbohydrates, such as cellulose, hemicellulose and other polysaccharides. Within this region, the most intense resonance (centered at around 70 ppm) is ascribed to the overlapping of C2, C3, and C5 carbons in the pyranosidic rings of cellulose and hemicellulose. The peaks observed at 65 and 80 ppm were associated to the C6 and C4 carbons, respectively, while the signals resonating in the range 100–110 ppm were assigned to anomeric (C1) carbons. In monosaccharides and polysaccharides, these carbons are typically deshielded due to their bonding with two electronegative oxygen atoms. Since most of carbohydrates represent a labile and readily biodegradable components, intense peaks in these regions reflect the presence of relatively fresh organic matter. In the 110–145 ppm range, the signals were related to unsubstituted aromatic carbons, arising mainly from lignin, phenolic compounds, or polycondensed aromatic structures. Since these compounds are relatively resistant to microbial degradation, tend to accumulate in SOM, thus serving as key indicators of advanced organic matter humification. The slightly deshielded region 145–160 ppm is generally attributed to quaternary aromatic carbons bonded to hydroxyls and methoxyl groups. The resonances between 160-190 ppm were assigned to carbonyl carbons of carboxylic acids and esters, mostly attributable to ester-bonds in natural biopolymers, small organic acids as intermediate products of anaerobic digestion, fatty acids and carboxylated saccharides. The visual comparison of AD and VC carbon NMR spectra confirmed the neat differences between these materials already observed by IR spectroscopy. In detail, VC exhibited a higher hydrophobic nature, characterized by a larger abundance of recalcitrant compounds. Such an

outcome was proved by the pronounced signals in the aromatic and the alkyl regions (21 vs 13.7%), which determined higher values for both Alk/O-Alk ratio (0.42 vs 0.22) and hydrophobicity index (0.88 vs 0.55) as well as suggested a more enhanced humification state in VC. Conversely and in agreement with the FT-IR findings, AD resulted richer in carbohydrates with intense signals in the O-alkyl-C region (61.0 vs 49.3%). This is also in line with the C/N results (**Table 1**) indicating lower C/N for AD which, although contained a relatively lower content (%) in C, was composed by residual labile organic matter. The reduction of O-alkyl-C in VC indicated extensive microbial and enzymatic degradation of carbohydrates during vermicomposting, leading to the selective preservation and accumulation of more stable carbon fractions. The low LR values shown by both samples indicated a prevalent and consistent contribution of lignin derivatives in the corresponding spectral intervals (Verrillo et al., 2021; de Aquino et al., 2019).

The Pyr-GC/MS pyrograms of AD and VC are presented in **Fig. 2**.

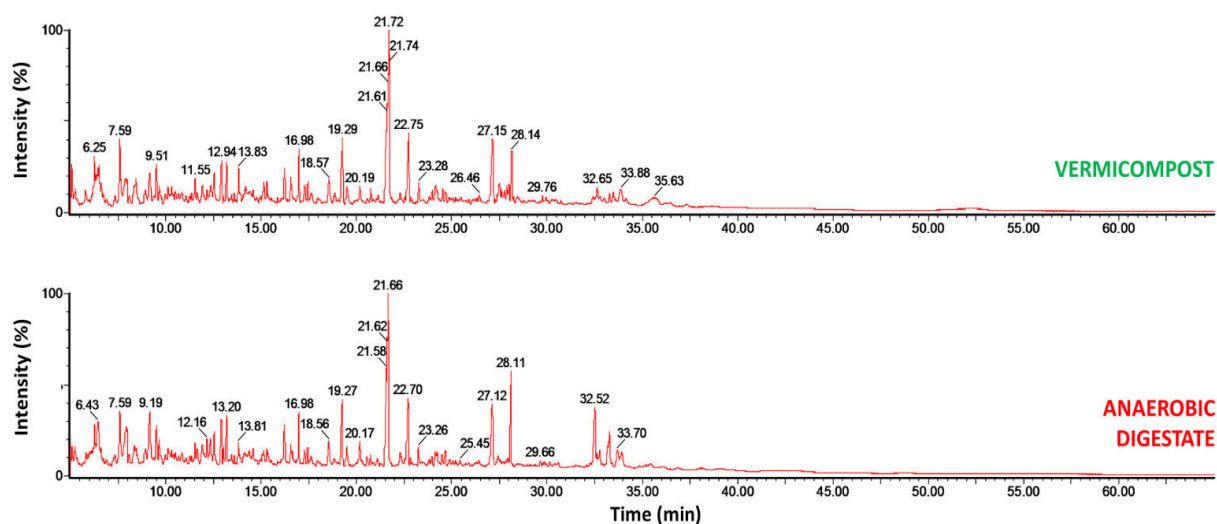


Fig. 2. GC chromatograms of AD (red) and VC (green) obtained by thermochemiolysis conducted at 400 °C with the tetramethylammonium hydroxide.

Most of peaks were identified based on the (i) a matching with NIST mass spectral library, (ii) the interpretation of the characteristic fragmentation patterns, and (iii) the comparison with authentic standards. Detailed information on the identified compounds (including class and compound name, molecular weight, and dominant fragment ions) is provided in **Supplementary Table S1**. While solid-state FT-IR and ^{13}C CPMAS NMR spectroscopies provided a broad overview of the major chemical classes present in the untreated sample, Pyr-GC/MS enabled a more detailed characterization, performed at a molecular-level, allowing for the identification and relative quantification of individual compounds, after their partial depolymerization and derivatization. Except for a few components, which were detected exclusively in one of the two materials, most of pyrolysis products were identified in both AD and VC samples. This was expected, being VC the vermicomposted form of AD. Among these, lignin-derived compounds, and in particular the p-hydroxyphenyl (P) and guaiacyl (G) units, represented the most intense signals, with P-type lignin prevailing in both materials. This is plausible since the AD resulted for the digestion of buffalo livestock wastes, containing straw and buffalo feces, resulting from the digestion of feed based mostly on hay and seeds. This means that the main source of lignin were graminaceae, which are known to be rich in P-units. Specifically, the main signals detected in both AD and VC were assigned to benzoic acid, 3,4-dimethoxy-, methyl ester (Lignin-G unit) at minute 19.2, to 2-propenoic acid, 3-(4-methoxyphenyl)-, methyl ester (Lignin-P unit) at minute 21.6, to benzoic acid, 3,4,5-trimethoxy-, methyl ester (Lignin – S unit) at minute 22.7, at 2-propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester (Lignin-G unit), and tetradecanoic acid, 10,13-dimethyl-, methyl ester (Fame methyl) at 28.11 min. An additional prominent peak was found only in the AD sample at 32.52 min and was assigned to the 9-octadecenoic acid, methyl ester (E) (mono-unsaturated FAME).

All signals detected in the pyrograms were integrated and classified into specific compound classes, as summarized in **Table 2**.

TABLE 2

Integration (normalized values, %) of GC-MS pyrograms of AD and VC. The labels FA, FAME, P, G, and S correspond to fatty acids, fatty acid methyl esters, *p*-hydroxyphenyl, guaiacyl and syringyl monomeric lignin units, respectively.

% Compounds class	AD		VC	
Branched FA and hydroxyacids	6.956	± 0.0178	5.290	± 0.01
Dioic acids	1.128	± 0.0032	1.683	± 0.01
FA mono- and di-unsaturated	8.321	± 0.0173	7.016	± 0.01
FAME	0.953	± 0.0067	0.843	± 0.01
Lignin - P	20.280	± 0.0164	23.813	± 0.02
Lignin - G	29.598	± 0.0866	30.279	± 0.10
Lignin - S	8.773	± 0.0171	9.170	± 0.02
Lignin (TOT.)	58.652	± 0.1201	63.262	± 0.14

Notably, the most relevant markers of microbial biomass, including branched fatty acids and hydroxy acids as well as mono- and di-unsaturated fatty acids, were more abundant in AD. This was due to the relatively larger content in labile carbon which resulted more attractive for microorganisms. Interestingly, dicarboxylic acids — associated with plant biopolyesters such as cutin and suberin—were more abundant in VC (1.68%) than in AD (1.13%). Since the plant source of AD and VC was the same, such a difference was mostly explainable by the relative accumulation of these plant-derived components, promoted by their resistance to microbial decomposition. This finding is consistent with CPMAS-NMR data, where VC showed the highest relative abundance of carboxyl groups. As expected, only a small fraction of carbohydrates was detected in the pyrograms of both AD and VC (**Table 2**), because of the known limits of Pyr-GC/MS analysis in detecting carbohydrates (**Monda et al., 2017; de Aquino et al., 2019**). In fact, this is mostly attributed to the limited thermochemical efficiency of the technique in analyzing polyhydroxy molecules within complex matrices (**Martinez-**

Balmori et al., 2013; de Aquino et al., 2019). The fact that VC exhibited a higher lignin content than AD corroborated the structural trends observed in the CPMAS-NMR spectra, further supporting the interpretation of enhanced aromaticity in vermicompost.

2.4.3 Phytotoxicity Test and Pot Trials in Greenhouse

The biostimulant effects of VC and AD were evaluated on model plants, such as watercress, baby leaf lettuce, and radish, through germination assays and greenhouse trials. Phytotoxicity assay enabled the identification of phytotoxic and biostimulant concentrations. (more details are reported and discussed in **Supplementary Paragraph SP3**). The agronomic and physiologic results of pots trials in green house carried out on radish and lettuce plants are shown in **Table 3**. In summary, both organic amendments improved germination, growth, and physiological performance, with VC offering a more consistent, dose-tolerant effect, and AD providing strong but concentration-sensitive stimulation. In lettuce, 20% VC balanced yield and quality, whereas 40% AD maximized fresh biomass with higher nitrate levels. In radish, both amendments at appropriate doses enhanced growth and photosynthetic activity, with 40% AD producing the highest biomass. These results collectively support the strategic use of VC and AD as sustainable alternatives to mineral fertilizers, highlighting their potential to enhance crop productivity while maintaining product safety and quality, and offering practical guidance for optimizing application rates in different horticultural species. (more details are provide in **Supplementary Paragraph SP4**).

TABLE 3

Agronomic, physiological, and qualitative results of baby leaf lettuce (up) and radish root (down) collected at the harvest. The SPAD reports the leaf chlorophyll index. Different letters within each column indicate significant differences according to Tukey's test ($p \leq 0.05$).

BABY LETTUCE						
Treatments	Plant Height (cm pot ⁻¹)	SPAD (-)	Aboveground Fresh Weight (g pot ⁻¹)	Aboveground Dry Weight (g pot ⁻¹)	NO ₃ ⁻ (ppm)	
T1 - Control	20.17 b	11.64 c	13.68 g	0.60 g	195.00	h
T2 - Mineral F.	29.33 a	13.21 a	15.46 e	0.84 d	630.00	g
T3 - 10% VC	25.00 ab	12.64 b	17.94 c	0.94 b	1300.00	f
T4 - 20% VC	30.83 a	11.52 d	17.95 c	0.85 c	1650.00	e
T5 - 40% VC	26.50 a	8.71 h	20.05 b	0.84 d	2000.00	d
T6 - 10% AD	28.00 a	10.41 f	14.73 f	0.76 f	2600.00	b
T7 - 20% AD	27.33 a	10.91 e	16.61 d	0.78 e	2750.00	a
T8 - 40% AD	30.00 a	9.75 g	27.38 a	1.23 a	2250.00	c
RADISH						
Treatments	Plant Height (cm pot ⁻¹)	SPAD (-)	Radish Fresh Weight (g pot ⁻¹)	Radish Dry Weight (g pot ⁻¹)	NO ₃ ⁻ (ppm)	
T1 - Control	20.17 b	30.87 bc	2.02 d	0.17 e	350.50	f
T2 - Mineral F.	29.33 a	38.47 a	11.52 c	0.61 d	365.00	e
T3 - 10% VC	25.00 ab	30.93 bc	11.19 c	0.75 cd	350.50	f
T4 - 20% VC	30.83 a	34.80 ab	16.40 bc	0.81 bcd	415.00	cd
T5 - 40% VC	26.50 a	35.07 ab	25.46 ab	1.20 ab	405.00	d
T6 - 10% AD	28.00 a	35.90 ab	21.67 b	1.25 a	420.00	c
T7 - 20% AD	27.33 a	27.50 c	23.77 ab	1.15 abc	430.50	b
T8 - 40% AD	30.00 a	34.67 ab	32.21 a	1.36 a	450.50	a

2.4.4 Impact of AD and VC on lettuce and radish metabolomes

The mixtures of compounds resulting from the aqueous extraction of both lettuce leaves and radish root were examined through high-resolution NMR spectroscopy aiming to perform a metabolomics investigation on these horticultural products and find interesting correlations with AD and VC applications. The identification of the primary metabolome of radish has been recently carried out by our research group and discussed previously (Fasolino et al., 2025),

while that of lettuce leaves has been conducted in this work, shown in **Fig. 3** and described in **Supplementary Paragraph SP7**.

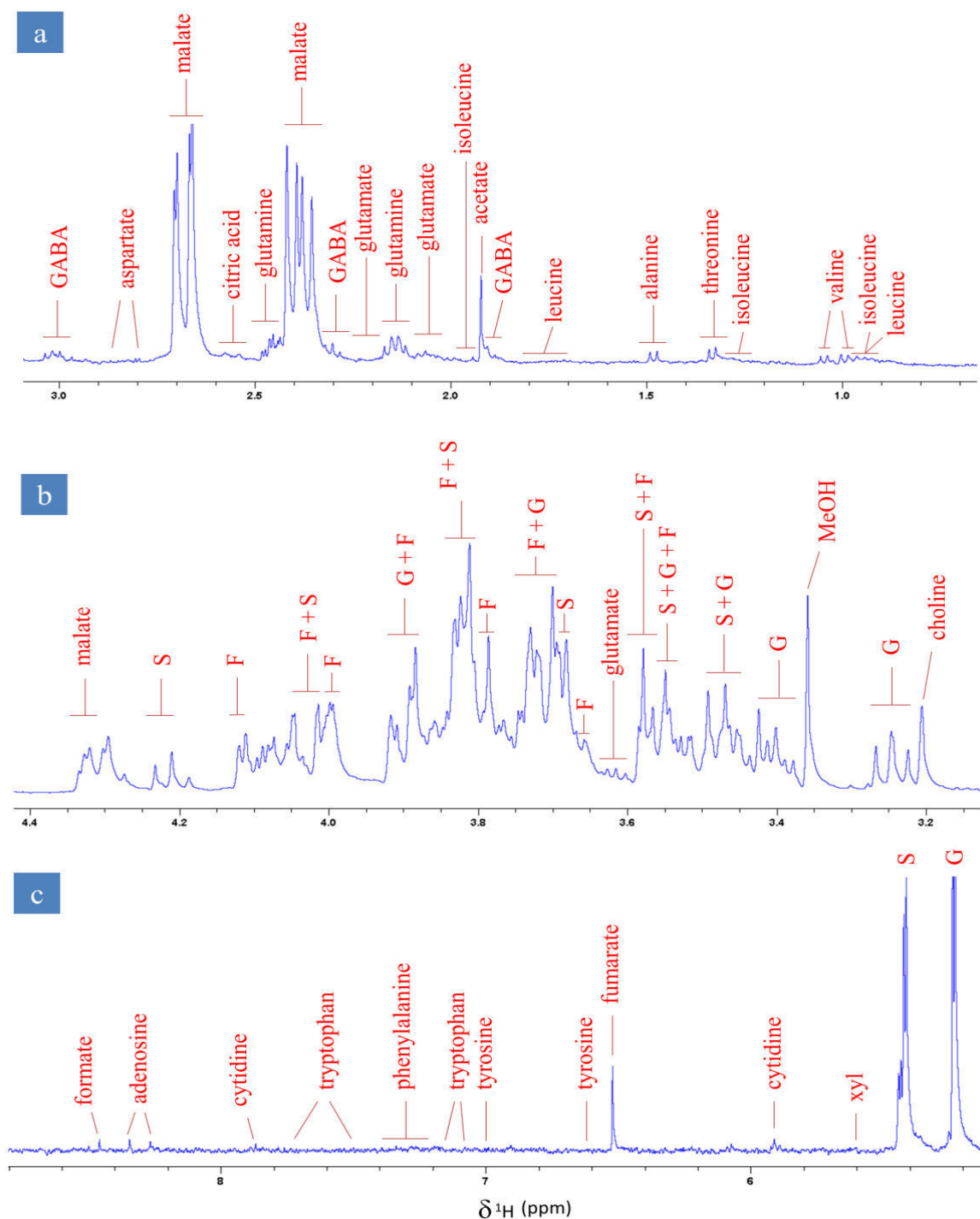


Fig. 3. Spectral regions (a, 0.65–3.1 ppm; b, 3.12–4.43 ppm; c, 5.08–8.8 ppm) of a representative high-resolution ^1H NMR spectrum of lettuce extract. The figure reports the assignments of the

most intense peaks, where the labels G, F, S, xyl and GABA stand for glucose, fructose, sucrose, xylose and γ -aminobutyric acid, respectively.

All proton spectra were analyzed through a semi-quantitative approach, aiming to elucidate the metabolic mechanisms potentially triggered (either directly or indirectly) by the application of AD and VC at the doses which induced the most favorable physiological performance in the agronomic trials. The metabolome of treated plants was compared with those resulting from both any application (CTRL) and the supply of a mineral fertilizer based on $\text{Ca}(\text{NO}_3)_2$ (MIN). The proton NMR spectra were integrated, segmented into defined spectral buckets, and compiled into a data matrix, which was subsequently processed with Principal Component Analysis (PCA) (**Fasolino et al., 2025**). As shown in the score plots in **Fig. 4**, both organic fertilizers induced a response in the primary metabolome of both lettuce and radish which was markedly different than those observed for CTRL and MIN treatments.

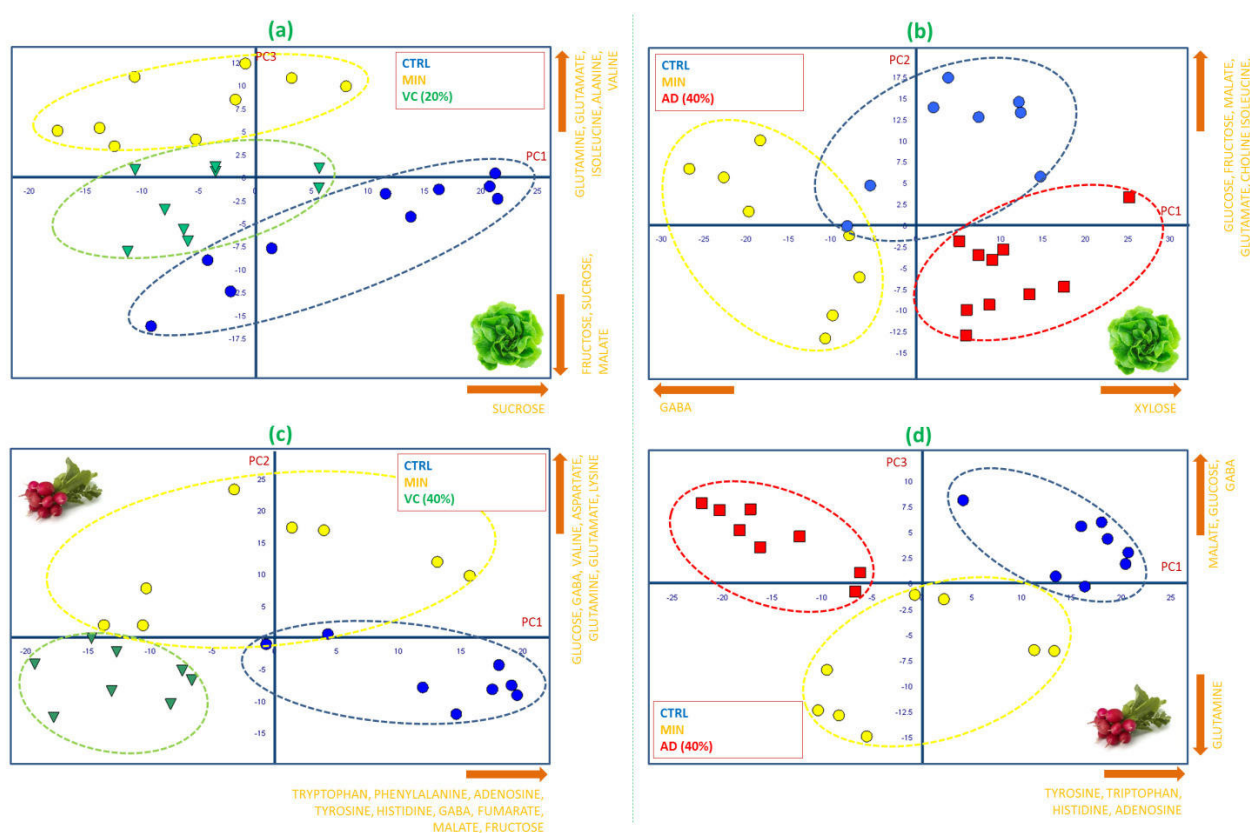


Fig. 4. PCA score plots based on ^1H NMR spectra and comparing the primary metabolome of baby lettuce (a and b) and radish (c and d) samples resulting from different treatments, including a control (CTRL, blue circles), mineral fertilization (MIN, yellow circles), vermicompost, (VC 20% and 40%, green triangles) and Anaerobic Digestate (AD 40%, red squares). The names and the orthogonal directions of the loading vectors discriminating significantly the investigated samples types are reported in yellow along the edges of each score plot.

In **Fig. 4a** is shown the PCA score plot of baby lettuce extracts treated with VC and obtained by the combination of the PC1 and PC3 axes (35.23% of the total explained variance). It revealed that most of the observations of CTRL, MIN and VC samples were collocated in separate spatial regions of the plot as well as that both axes contributed to the samples discrimination, although the most enhanced separation occurred along the PC3. MIN samples were isolated in a region characterized by positive PC3 values. Conversely, most of CTRL samples were assigned to negative PC3 values, while VC samples were collocated in a position in between MIN and CTRL. Also part of the variables associated to the PC1 provided a certain discrimination between CTRL and VC. The contextual examination of the related loading plot enabled the identification of the most represented loading vectors associated with both PC1 and PC3 which then underwent ANOVA test to select only the buckets of the metabolites which significantly discriminated the examined samples (Tukey and Benjamini-Hochberg test, at a p -value < 0.05 , and in many cases at a p -value < 0.001). In detail, samples treated with VC and MIN were characterized by a larger content than CTRL in amino acids such as glutamine, glutamate, valine, isoleucine and alanine. Conversely, CTRL exhibited a significantly larger amount of malate, fructose and sucrose. The fact that the concentration for all of these variables in VC samples corresponded to values in between those for CTRL and MIN, explained their intermediate collocation in the score plot. The larger abundance of amino acids in VC and MIN may be associated with a more efficient plant nitrogen uptake, likely due to the greater availability of nitrogen provided, in MIN case, through its direct supply in a bioavailable nitric

form, and, in case of VC (containing around 2.6% of total nitrogen), through a ionic exchange combined with slow-release process accompanying the vermicompost mineralization. The latter aspect well agrees with the nitrate content reported in **Table 3**. However, a further reason explaining the benefits observed for VC treatment, consists in the fact that VC promoted the activity of the microbial community populating the growth substrate which, in turn, provided nutrients to the plants and enriched the ryzosphere thus leading to a plant response even better than the control. The heatmap technique, which is another “unsupervised” method, was applied to process all the NMR variables that contributed significantly to discriminate the examined samples. The clustered heatmap is very advantageous because clearly and directly highlights the semi-quantitative response (from red to yellow) of the examined variables. Moreover, it reports a clusterisation of both variables (dendrogram on the left-hand side of the figure) and samples (dendrogram on the top of the figure) based on their mutual similarity, providing several tree structures where objects are linked to one another depending on their progressively decreasing similarity. Consequently, for sample clusterisation, the more similar the values for the compared variables, the closer the observations are placed in this structure. In **Fig. 5a** is displayed the clustered heatmap considering the most significant NMR signals. Focusing on the sample dendrogram on the top of the figure, it is interesting to note that the first knot clearly separated into two distinct macro-clusters all the CTRL samples from those of VC and MIN. Heatmap confirmed that the discrimination was mostly due to the larger content in sucrose, fructose and malate in CTRL. The second and the third knots assessed the differences between VC and MIN. In fact the former exhibited an intermediate content of the above-mentioned metabolites, as compared to CTRL and MIN, accompanied by a relatively lower abundance than MIN in valine, isoleucine, alanine, glutamate and glutamine. However, the sample clusterization indicated a certain similarity between MIN and VC, suggesting that the response

induced by VC was, for some traits, similar to that induced by a mineral fertilization, and in line with plant height and aboveground dry weight (**Table 3**). This permits to conclude that VC, at the dose of 20%, triggered the same physiological mechanisms activated by MIN, since the metabolites composing the primary metabolome of lettuce were the same of those modulated by MIN, although at a lower extent. In **Fig. 4b** is shown the score plot comparing the primary metabolome of lettuce samples undergone CTRL, MIN and AD treatments. The score plot obtained by the combination of the PC1 and PC2 axes (50.27% of the total explained variance) revealed that, depending on the specific treatment, the 3 samples groups were collocated into distinct spatial regions. This suggests that each treatment determined the insurgence of a peculiar metabolic trait. In particular, it occurred the discrimination of MIN along the PC1 which was ascribed to the lowest content in xylose associated with the highest content in GABA. Conversely, along the PC2 it occurred a separation between CTRL and AD samples because the former ones were characterized by the higher concentration in glucose, fructose, malate, glutamate, choline and isoleucine. The heatmap clusterization showed in **Fig. 5b** confirmed the results already emerged by PCA and ANOVA. It is interesting to note that, expected for the content in xylose and GABA, the primary metabolomes for MIN and AD were very similar to each other, being both markedly different than that of CTRL. This is proven by the fact that 5/10 MIN observations were separated by AD in correspondence of the third, rather the second sample cluster knot, (**Fig. 5b**), thus indicating that part of MIN samples exhibited similar traits with AD groups. Again this suggests that AD elicited a metabolic response similar to that triggered by MIN.

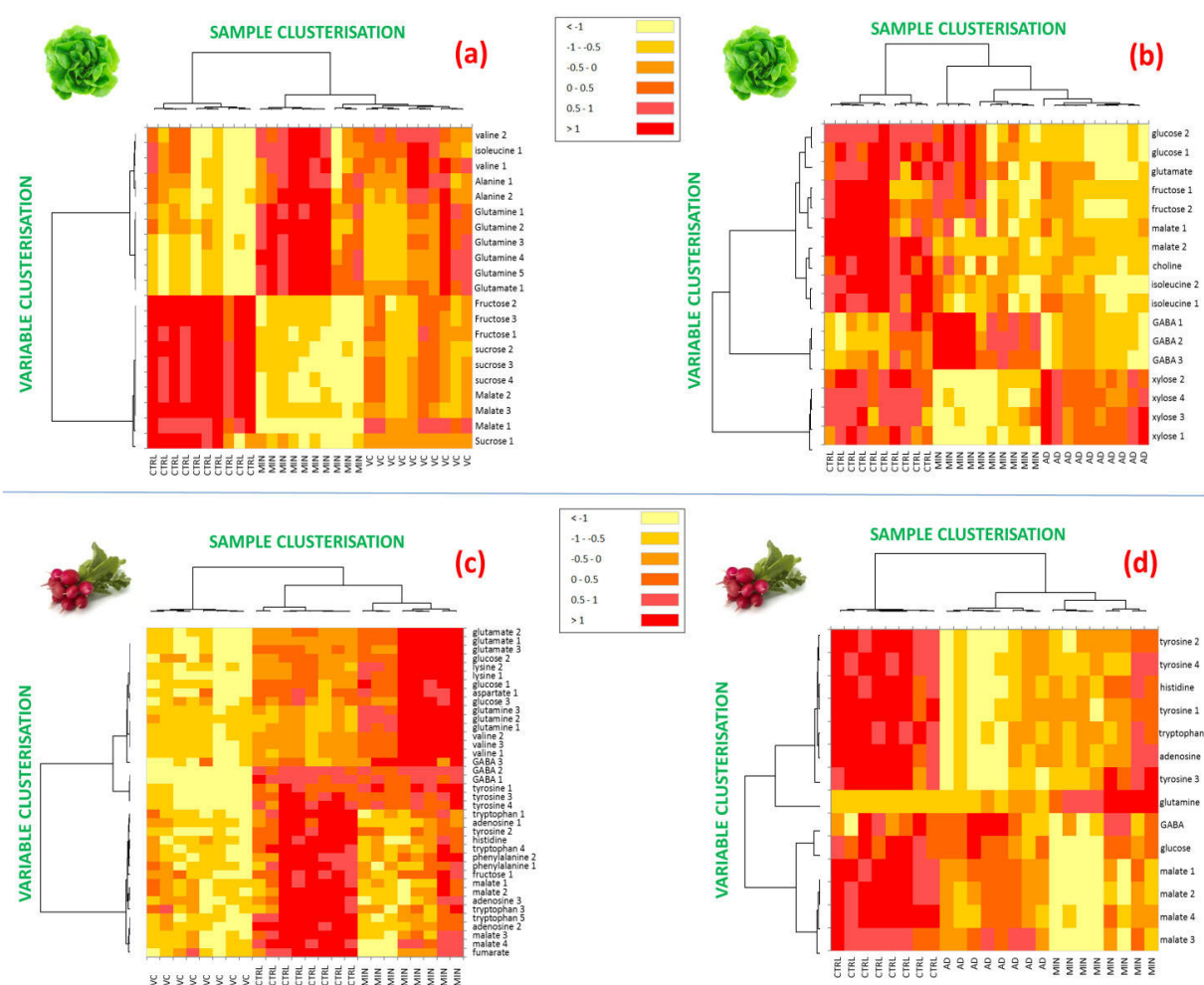


Fig. 5. Clustered heatmap based on the NMR-variables significantly involved in the discrimination among the primary metabolomes of baby lettuce (a and b) and radish (c and d) samples resulting from different treatments, including a control (CTRL), mineral fertilization (MIN), vermicompost, (VC) and Anaerobic Digestate (AD). The values were centered, scaled, and displayed according to a color gradient ranging from red (relatively high values) to pale yellow (relatively low values), with orange representing intermediate values.

This is important because it means that both AD and VC, when applied at the proper doses, can exert a role comparable to the one attainable by the supply of fertilizers on lettuce. The metabolomic analysis on lettuce trials revealed clear distinctions among treatments, particularly in metabolites related to nitrogen and carbohydrate metabolism. Untreated control plants

exhibited an accumulation of carbohydrates (glucose, fructose), malate, choline, glutamate, and isoleucine. This profile likely reflects a metabolic adaptation to nutrient-limiting conditions, where anabolic processes are slowed and metabolites involved in respiration (malate, Krebs cycle) (Gao et al., 2022), osmotic balance and membrane stability (choline) (Zhang et al., 2021), and nitrogen storage or signaling (amino acids) (Yu et al., 2018) are accumulated. Plants treated with MIN showed significantly higher levels of GABA, glutamate, glutamine, and isoleucine, as compared to CTRL. This metabolic pattern indicates enhanced nitrate availability from MIN, an easily bioavailable nitrogen source that directly activates nitrogen assimilation pathways, often reducing carbon allocation to sugar reserves. Notably, the GABA, deriving from glutamate decarboxylation, typically accumulates under high nitrogen conditions and is known to modulate nitrate uptake and assimilation, facilitating the synthesis of organic nitrogen compounds such as glutamine and glutamate (Yu et al., 2018). Thus, its accumulation in this context suggests the activation of nitrate assimilation and broader metabolic reprogramming to optimize nitrogen use. Additionally, GABA serves key roles in cytosolic pH regulation and protection against oxidative stress, acting as a signaling molecule and also as a modulator of energy metabolism (Mazzei et al., 2016; Rastegar et al., 2025). Plants treated with VC 20% occupied an intermediate position between CTRL and MIN in the multivariate model, indicating a balanced metabolic response. The metabolic profile suggests a slower, more gradual nitrogen release compared to the mineral form, yet sufficient to support the synthesis of nitrogenous compounds. This moderate availability may promote a more balanced interplay between primary and secondary metabolism, sustaining long-term growth. This is corroborated by previous agronomic trials, where plants treated with VC exhibited greater fresh weight than both CTRL and MIN (Cappelini et al., 2023; Savarese et al., 2022). In lettuce plants treated with AD, NMR analyses revealed elevated levels of xylose, a pentose sugar commonly

associated with hemicellulose degradation. This result may reflect a metabolic contribution from the digestate itself, which can contain partially degraded lignocellulosic compounds reutilized by the plant (Lu et al., 2019).

In **Fig. 4c** is displayed the PCA score plot comparing the primary metabolome of radish samples undergone CTRL, MIN and VC treatments. The score plot obtained by the combination of the PC1 and PC2 axes (57.39% of the total explained variance) led to a clear separation along PC1 between VC and CTRL samples, positioned in the negative and positive PC1 regions, respectively. Such a response occurred because CTRL samples were characterized by a significantly larger amount of the metabolites tryptophan, phenylalanine, adenosine, tyrosine, histidine, GABA, fumarate, malate and fructose. On the other hand, MIN samples were differentiated along the PC2, where were associated to PC2 positive values. Such an outcome was due to a larger amount in MIN of glucose, GABA, valine, aspartate, glutamine, glutamate and lysine. As observed by the relative heatmap clusterization (**Fig. 5c**), the clusters separation among the treatments was neat. The metabolites represented by the PC1 exhibited progressively decreasing lower values from CTRL to MIN to VC. Conversely, the difference between VC and MIN was ascribed to a lower content in VC of glutamate, glutamine, lysine and valine. The score plot shown in **Fig. 4d** reports the comparison among CTRL, MIN and AD samples and was built up by combining the PC1 vs PC3 (53.46% of the total explained variance). Again, a clear discrimination among all the treatments, and developed mainly along PC1. In particular, CTRL and AD samples were located at positive and negative PC1 values, respectively, with MIN observations which were situated in intermediate values. Such a disclocation was attributed to the significantly highest concentration in CTRL of tyrosine, tryptophan, adenosine and adenosine metabolites, whose concentrations progressively decreased in MIN and finally in AD. However, by the exam of the loading vectors associated to the PC3, it emerged that MIN

samples were also characterized by a significantly higher abundance in malate, glucose and GABA, accompanied by the lowest content in glutamine. All these peculiar traits in the different radish types were confirmed by the related heatmap clusterization (**Fig. 5d**) which confirmed three neatly different metabolomes. The metabolomic and agronomic results observed in radish suggest significant differences in the efficacy and mode of action of the different fertilization treatments, related to the nature and availability of the nutrients supplied. The untreated CTRL exhibited an accumulation of metabolites such as malate, fumarate, aromatic amino acids (tryptophan, phenylalanine, tyrosine), and energy-related compounds (adenosine, GABA). This profile indicates a metabolic state typical from plants under limited nitrogen availability, where photosynthesis and carbon metabolism remain relatively active, but protein synthesis and growth are slowed down, leading to the accumulation of metabolic intermediates (**Fasolino et al., 2025; Park et al., 2022; Gao et al., 2022; Yu et al., 2018**). The treatment with MIN observed in radish, promotes a rapid supply of available nitrogen, stimulating amino acid synthesis (glutamate, glutamine, lysine, valine, aspartate) (**Park et al., 2022**), supported by increased metabolic activity related to energy and carbon metabolism (glucose). This reflects a metabolic response typical of accelerated growth and increased protein synthesis, sustained by an immediate and direct nutrient supply. Treatments with VC and AD, on the other hand, provide nutrients in more organic and gradual forms, resulting in metabolic profiles intermediate to those found in CTRL and MIN plants. This slower and modulated availability of nitrogen and other nutrients allows sustained growth over time without sudden metabolic peaks, favoring a balance between amino acid synthesis and the management of carbohydrate reserves and metabolic intermediates (**Fasolino et al., 2025**). In agronomic terms, this translates into a more marked increase in fresh edible biomass with the VC, likely due not only to nutrient supply but also to an improved soil structure and increased microbial biodiversity (factors that

promote more efficient nutrient assimilation and healthier growth) (Calderon et al., 2021). AD shows positive effects as well, although slightly lower than the first, while MIN, although accelerating growth, does not reach the same biomass levels, probably due to the lack of beneficial effects on soil and overall plant physiology. It is important to note that these results align with preliminary findings from molecular composition analyses of AD and VC, which highlighted the higher presence of aromatic and complex substances, more mature and stable in VC (as indicated by signals in CPMAS NMR and pyr-GC/MS), providing a more modulated nutrient source with slow mineralization. Conversely AD, with a higher presence of less stabilized lignocellulosic components and signals indicative of carbohydrates such as hemicellulose (detected by CPMAS NMR and pyr-GC/MS), appears to supply nutrients in an organic but less evolved form, which may explain the accumulation of pentose sugars (xylose) in plants. In conclusion, the use of organic amendments such as vermicompost and anaerobic digestate, whose differing chemical characteristics influence nutrient availability and dynamics, ensures a more balanced and sustainable nutrition as compared to mineral fertilizers, promoting more robust plant growth and balanced metabolism in accordance with metabolomic and agronomic data.

2.5 CONCLUSIONS

This study provides a comprehensive evaluation of solid anaerobic digestate derived from buffalo manure and its vermicomposted form, informing on chemical, compositional, microbiological features of these materials, as well as on agronomical and metabolomic responses elicited by their application. Our results permitted to highlight their potential as sustainable soil amendments, serving as source of nutrients and plant biostimulants. We proved that these materials represent a viable strategy within the circular bioeconomy framework. These organic amendments not only contribute to waste reduction and nutrient recycling, but

also offer tangible benefits for plant health and productivity, supporting their adoption in environmentally conscious agricultural practices. Future promising perspectives include application of their hydrosoluble extracts and their encapsulation into innovative polysaccharides-based formulations capable to modulate their slow-release in soil.

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Supporting Information

Supporting Tables

Supporting Table S1. List of compounds identified in the GC-MS pyrograms of AD and VC, including compound class compound name, molecular weight (MW), the most intense and diagnostic signals and the retention time. The labels FA, FAME, P, G, and S correspond to fatty acid, fatty acid methyl ester, *p*-hydroxyphenyl, guaiacyl and syringyl monomeric lignin units, respectively, whereas the symbol >> specifies that the specific mass signal is predominant in the mass spectrum).

Compounds identified in GC-MS pyrograms of AD and VC	Compound name	MW	Intense and diagnostic signals					
			AD		VC			
			RT	ABS	RT	ABS		
BRANCHED FA	METHYL 5,9 DI METHYL DECANOATO	214	214, 87, 74, 171	0.0000				0.0000
BRANCHED FA	TETRADECANOIC ACID, 10,13-DIMETHYL-, METHYL ESTER	222	74, 87, 270, 227, 143, 129	27.455	0.0066	27.497		0.0091
BRANCHED FA	TETRADECANOIC ACID, 10,13-DIMETHYL-, METHYL ESTER	222	74, 87, 129, 143, 270	28.108	0.0518	28.147		0.0257
BRANCHED FA	HEXADECANOIC ACID, 14-METHYL-, METHYL ESTER	284	74, 87, 143, 241, 284	29.855	0.0008	29.758		0.0045
BRANCHED FA	HEXADECANOIC ACID, 14-METHYL-, METHYL ESTER	284	74, 87, 143, 241, 284	29.947	0.0009	29.883		0.0021
BRANCHED FA	HEXADECANOIC ACID, 15-METHYL-, METHYL ESTER	284	74, 87, 143, 241, 284		0.0000	30.727		0.0024
BRANCHED FA	HEXADECANOIC ACID, 16-METHYL-, METHYL ESTER	298	74, 87 255, 298, 143		0.0000	33.482		0.0090
BRANCHED FA MONO	11-HEXADECENOIC ACID, 15-METHYL-, METHYL ESTER	282	69, 97, 110, 152, 208, 225, 250, 282	30.6	0.0094			0.0000
TOT			BRANCHED FA	0.0696				0.0529
DIOIC	HEXADECANEDIOIC ACID, DIMETHYL ESTER			36.084	0.0079	36.455		0.0130
DIOIC	HEXADECANEDIOIC ACID, DIMETHYL ESTER	314	74, 84, 98, 241, 283, 314	36.876	0.0034	37.321		0.0038
TOT			DIOIC	0.0113				0.0168
FA DI-UNS.	9,11-OCTADECADIENOIC ACID, METHYL ESTER, (E,E)-	294	67, 81, 95, 294, 263, 150	34.872	0.0025			0.0000
FA DI-UNS.	10,13-EICOSADIENOIC ACID, METHYL ESTER	322	67, 81, 95, 291, 322	35.449	0.0170	35.614		0.0375
FA MONO	8-OCTADECENOIC ACID, METHYL ESTER, (E)-				0.0000	32.418		0.0061
FA MONO	8-OCTADECENOIC ACID, METHYL ESTER, (E)-	296	55, 74, 97, 111, 180, 222, 364, 296	32.63	0.0065	32.646		0.0187
FA MONO	9-OCTADECENOIC ACID, METHYL ESTER, (E)-	296	55, 74, 97, 111, 180, 222, 364, 296	32.516	0.0471			0.0000
FA MONO	9-OCTADECENOIC ACID, METHYL ESTER, (E)-	296	55, 74, 97, 111, 180, 222, 364, 296	32.769	0.0102	32.789		0.0079
TOT			FA MONO E DI-UNS.	0.0832				0.0702
FAME	METHYL TETRADECANOATE	242	74, 87, 242, 211		0.0000			0.0000
FAME	PENTADECANOIC ACID, METHYL ESTER	256	74, 87, 256, 213	24.673	0.0095	24.668		0.0084
TOT			FAME	0.0095				0.0084
LIGNIN - G	PHENOL, 3,4-DIMETHOXY-	154	154, 139, 111, 93	12.164	0.0148	12.17		0.0048
LIGNIN - G	PHENOL, 2,6-DIMETHOXY-	154	154, 139, 111, 93	12.55	0.0184	12.555		0.0175
LIGNIN - G	BENZENE, 4-ETHENYL-1,2-DIMETHOXY-	164	164, 149, 121, 103, 77	12.924	0.0242	12.937		0.0279
LIGNIN - G	1,2,4-TRIMETHOXYBENZENE	168	168, 153, 125	15.137	0.0121	15.146		0.0119
LIGNIN - G	PHENOL, 2-METHOXY-4-(1-PROPENYL)-, (E)-	164	164, 149, 131, 77	15.299	0.0124	15.3		0.0095
LIGNIN - G	BENZALDEHYDE, 3,4-DIMETHOXY- + ALCANO	166	166, 95, 77	16.228	0.0220	16.236		0.0207
LIGNIN - G	BENZENE, 1,2-DIMETHOXY-4-(1-PROPENYL)-	178	178, 163, 107, 91, 77	16.565	0.0080	16.573		0.0177
LIGNIN - G	BENZOIC ACID, 4-HYDROXY-3-METHOXY-, METHYL ESTER	182	182, 151	17.288	0.0064	17.293		0.0095
LIGNIN - G	HOMOVANILLYL ALCOHOL	168	137>> 168, 94, 77		0.0000	17.649		0.0101
LIGNIN - G	ETHANONE, 1-(3,4-DIMETHOXYPHENYL)-	180	165, 180, 122, 77		0.0000	18.566		0.0251
LIGNIN - G	BENZENE, 4-BUTYL-1,2-DIMETHOXY-	194	151>>, 194, 77	18.554	0.0223	18.874		0.0094
LIGNIN - G	BENZOIC ACID, 3,4-DIMETHOXY-, METHYL ESTER	196	196, 165, 79	19.267	0.0491	19.282		0.0543
LIGNIN - G	PHTHALIDE, 4,6-DIMETHOXY-	194	165, 194, 77	21.124	0.0059			0.0000
LIGNIN - G	2-PROPENOIC ACID, 3-(2,3-DIMETHOXYPHENYL)-, (E)-	208	208, 177, 77	23.263	0.0091	23.278		0.0105
LIGNIN - G	2-PROPENOIC ACID, 3-(3,4-DIMETHOXYPHENYL)-, METHYL ESTER	222	222, 191, 151, 119, 91, 77	24.493	0.0043	24.525		0.0057
LIGNIN - G	ESTER	222	222, 191, 207, 77, 109	27.121	0.0611	27.142		0.0583
LIGNIN - G	BENZENE, 4-BUTYL-1,2-DIMETHOXY-	194	151>>, 194, 107, 77	20.177	0.0259	20.188		0.0098
TOT			LIGNIN - G	0.2960				0.3028
LIGNIN - P	1,4-BENZENEDICARBOXYLIC ACID, DIMETHYL ESTER	194	163, 194, 135, 103	16.646	0.0063			0.0000
LIGNIN - P	BENZENEPROPANOIC ACID, 4-METHOXY-, METHYL ESTER	194	121>>, 194, 134, 77	17.442	0.0068	17.454		0.0079
LIGNIN - P	2-PROPENOIC ACID, 3-(4-METHOXYPHENYL)-, METHYL ESTER	192	161, 192, 133, 118, 89, 77	21.663	0.1805	21.711		0.2129
LIGNIN - P	BENZOIC ACID, 4 (TRIMETHYLSILYL)OXY, METHYL ESTER	224	224, 209, 181, 165	24.236	0.0092	24.166		0.0174
			LIGNIN - P	0.2028				0.2381
LIGNIN - S	2',3',4' TRIMETHOXYACETOPHENONE	210	195, 210, 152, 77		0.0000			0.0000
LIGNIN - S	BENZOIC ACID, 3,4,5-TRIMETHOXY-, METHYL ESTER	226	226, 211, 195, 183, 155	22.709	0.0519	22.749		0.0554
LIGNIN - S	2,3,4,6-TETRAMETHOXYSTYRENE	224	224, 209, 151		0.0000			0.0000
LIGNIN - S	1,2,3 trimethoxybenzene	168	168, 153, 110	11.35	0.0045	11.352		0.0039
LIGNIN - S	1,2,4-TRIMETHOXYBENZENE	168	168, 153, 125, 110	13.034	0.0063	13.04		0.0022
LIGNIN - S	BENZENE, 1,2,3-TRIMETHOXY-5-METHYL-	182	182, 167, 139, 107	13.812	0.0139	13.59		0.0211
LIGNIN - S	PHENOL, 2,6-DIMETHOXY-4-(2-PROPENYL)-	194	194, 179, 151, 119, 77	20.547	0.0070	20.559		0.0035
LIGNIN - S	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	208, 193, 133, 105, 79	20.749	0.0041	20.757		0.0057
TOT			LIGNIN - S	0.0877				0.0917

Supporting Table S2. Microbiological analyses of anaerobic digestate (AD) and the vermicompost (VC). The results are expressed as CFU (colony-forming units) or MPN (Most probable number) per gram of dried material.

	Total bacteria (CFU/g)	<u>Siderophore producing bacteria</u> (CFU/g)	<u>Spore- forming Bacteria</u> (CFU/g)	<u>Yeast</u> (CFU/g)	<u>Mold</u> (CFU/g)	<u>Lactic Acid Bacteria</u> (CFU/g)	<u>Acetic Bacteria</u> (CFU/g)	<u>Escherichia coli</u> (CFU/g)
AD	4.13x10 ⁶	4.6x10 ⁶	4.13x10 ⁶	3.0x10 ³	6.7x10 ³	2.7x10 ⁴	2.5x10 ⁴	4.98x10 ⁴
VC	7.0x10 ⁵	5.6x10 ⁵	2.47x10 ⁶	1.25x10 ⁵	2.82x10 ⁵	1.58x10 ⁵	1.23x10 ⁶	absent
	Fecal Streptococci (MPN/g)	Total Coliform (MPN/g)	Fecal Coliform (MPN/g)					
AD	4.0x10 ²	2.5x10 ⁵	2.5x10 ⁵					
VC	absent	1.1x10 ¹	absent					

Supporting Table S3. Germination Index (GI) resulting from seed germination and phytotoxicity tests conducted on watercress, baby lettuce and radish seeds. Different letters within each column indicate significant differences according to Tukey's test ($p \leq 0.05$). CTRL: control; T1: 100% peat; T2: mineral fertilizer; T3: 10% VC; T4: 20% VC; T5: 40% VC; T6: 10% AD; T7: 20% AD; T8: 40% AD.

	GI watercress (%)		GI baby lettuce (%)		GI radish (%)	
Control - water	100.00	i	100.00	bc	100.00	h
T1 – Peat extract	144.90	c	125.70	ab	191.78	f
T2 - Mineral F.	109.00	g	83.40	c	130.10	g
T3 - 10% VC	116.00	e	151.70	a	195.40	e
T4 - 20% VC	156.50	b	140.20	a	325.10	a
T5 - 40% VC	167.40	a	123.90	ab	259.40	d
T6 - 10% AD	105.00	h	158.60	a	264.40	c
T7 - 20% AD	125.20	d	141.20	a	292.20	b
T8 - 40% AD	114.90	f	147.90	a	87.70	i

Supporting Paragraphs

SP1. Methods to integrate CPMAS NMR spectra and calculate indexes

Each ^{13}C CPMAS NMR spectrum was divided into the following resonance regions: 0–45 ppm (Alkyl-C), 45–60 ppm (methoxyl-C and N-alkyl-C), 60–110 ppm (O-alkyl-C), 110–145 ppm (aryl-C), 145–160 ppm (phenol-C), 160–190 ppm (carboxyl-C), and 190–230 ppm (quinone-C and ketone-C). Each interval was integrated and reported as percent of the total spectral range. Important indexes, such as the hydrophobicity index (1), the lignin ratio (2) and the Alk/O-Alk ratio (3) were calculated based on specific ^{13}C NMR intervals, according to the following equations (de Aquino et al., 2019):

$$HB = \frac{[(0 - 45) + (45 - 60) + (110 - 145) + (145 - 160)]}{[(61 - 90) + (90 - 110) + (160 - 190)]}$$

$$LR = \frac{(45 - 60)}{(145 - 160)}$$

$$\text{Alk/O-Alk Ratio} = \frac{(0 - 45)}{[(61 - 90) + (90 - 110)]}$$

SP2. Methods and results for Microbiological analyses

Microbiological analyses involved the determination of the following microbial groups: total bacteria, siderophore-producing bacteria, spore-forming bacteria, lactic and acetic acid bacteria, mold and yeast, *Escherichia coli*, fecal streptococci, total and fecal coliforms.

The extraction of microorganisms from AD and VC was performed by shaking, for 30' at 120 rpm in a 100 ml flask, the equivalent of 5 g of dried and 2-mm sieved fertilizer, sieved at 2 mm with an equivalent of 45 ml of 0.85% NaCl solution. After the shaking, the

suspension was left to decantate for 5 min. Then, 1 ml of the suspension was sampled and subjected to serial dilutions with water, up to dilute the sample 1:100'000'000 times. For each dilution and for each microbial group, three aliquots of 10 ul were plated on the proper substrate, which varied as a function of the examined microbial group. In detail, total bacteria were counted on soil extract agar (**Subba Rao, 1977**); siderophores forming-bacteria were counted on a selective agar medium without iron (**Scher and Baker, 1982**); spore-forming bacteria were counted by plating, on Nutrient Agar, ten-fold dilutions of a suspension previously heated at 90 °C for 10 min (**Sadfi et al., 2001**); lactic bacteria were counted on MRS Agar (**de Man et al., 1960**); acetic acid bacteria were counted on WL Agar (**Green and Gray, 1950**); mold and yeast were counted on Rose-Bengal Agar (**Jarvis, 1973**); *Escherichia coli* was counted on Tryptone Bile X-Gluc Agar (**Delisle and Ley, 1989**).

For the determination of fecal streptococci and total and fecal coliforms, serial decimal dilutions of each sample were inoculated into tubes containing the specific growth substrate and it was applied the multiple tube method (MPN = most probable number) (**Bonadonna et al., 2002**).

All agar plates or tubes were incubated for 2-5 days at 44 °C for *Escherichia coli* and fecal coliforms; at 37 °C for fecal streptococci and total coliforms; at 25 °C for molds and yeast; at 28 °C for all the other microbial groups. Results were reported as CFU (colony-forming units) or MPN (most probable number) per gram of dried fertilizer, where the MPN was determined via Mc Crady tables.

In **Supplementary Table 2** are shown the results of microbiological analyses of AD the VC. They were examined microorganisms groups, including spore-forming and siderophore-producing bacteria, lactic acid and acetic acid bacteria, and yeasts, with important beneficial roles about protection of the plants against phytopathogenic fungi and adverse bacteria and

about ability to promote plant growth. All of these groups were identified in AD and VC and were characterized by concentrations ranging from 3×10^3 CFU per g (yeasts in AD) to 4.13×10^6 CFU per g (total bacteria in AD).

Excepted for spore-forming bacteria which had the similar content in AD and VC, interesting differences were observed for the other groups. In fact, the concentration of total bacteria was one order of magnitude higher in AD than in VC, while the concentrations of yeasts and filamentous fungi (molds) were two orders of magnitude lower in AD. While siderophore-forming bacteria were more abundant in AD, lactic acid and acetic bacteria were significantly more numerous in VC. These data suggest that, both AD and VC, have a promising potential in terms of carrier of beneficial microorganisms, although VC has a higher microbiological quality for application in agriculture.

Regarding the presence of undesirable microorganisms for health and hygiene standpoint (*Escherichia coli*, fecal streptococci, total and fecal coliforms), as expected, VC was found significantly safer than digestate. In fact, VC was completely free of *Escherichia coli*, fecal streptococci and fecal coliforms, while AD contained high concentrations of all the four groups of undesirable microorganisms. The absence of undesirable microorganisms in VC demonstrates how the complex transformations that occur during the vermicomposting processes can impact positively on the product safety. On the other hand, AD should undergo to post-process management for sanitization and sterilization (i.e. *via* acid/alkaline hydrolysis) before further applications in soil, to limit the introduction of these adverse microorganisms, potentially capable to contaminate food and pose indirect risks for human health.

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SP3. Methods and detailed discussion of Phytotoxicity Test

To assess the potential role of the tested products as soil fertilizers, a preliminary phytotoxicity assay was performed using seeds of watercress (*Lepidium sativum* L.), radish (*Raphanus sativus* L. cv. "Gigante Siculo"), and baby lettuce (*Lactuca sativa* L. cv. "Chiara"). The seeds were exposed to aqueous extracts of either AD or VC, and the effects were evaluated on seed germination and early root growth.

For this experiment, a commercial and neutralized peat (Mannaflor Tray 80/20 0.4^(R); pH 5.6, EC 0.625 dS m⁻¹, N 60 mg L⁻¹, P 40 mg L⁻¹, K 60 mg L⁻¹ enriched with 0.40 kg m⁻³ of PG-Mix 18-10-20^(R)) was used. All the seeds and the peat were supplied by the company Spazio Verde s.r.l. (Eboli, Italy).

AD and VC extracts were obtained by mixing 12.5 g of peat with increasing amounts (1.25, 2.5, and 5.0 g) of either anaerobic digestate (AD) or vermicompost (VC), corresponding to 10, 20

and 40% w/w in peat, respectively. Then, the resulting solid mixtures were suspended with 250 mL of ultrapure water which were kept under stirring for 30 minutes. The suspensions were then separated by the coarse solid phase and filtered under vacuum. The pH and electrical conductivity were evaluated for all the aqueous extracts (HANNA HI5222 detector; two replicates per parameter).

A germination bioassay was conducted by placing a single layer of filter paper (Whatman) into 9-cm diameter Petri dishes, soaking the paper with 4 mL of aqueous extract (ultrapure water as control, or AD and VC extracts at increasing product concentrations), and placing 20 seeds per species in each dish. Prior to the assay, seeds were surface-sterilized by immersion in a 1.5% sodium hypochlorite solution for 30 seconds, followed by three rinses with ultrapure water, each lasting 30 seconds. Dishes were sealed with Parafilm and incubated in the dark for 72 hours at 25 °C. Two Petri dishes were prepared for each single treatment (40 total seeds per treatment). After the incubation period, germination was assessed by counting the number of sprouted seeds and measuring root elongation via a precision digital caliper (eVatmaster Consulting GmbH, Germany). The Germination Index (GI) was calculated according to the method described by **Zucconi et al., 1981**

$$GI = 100 \times \frac{(G_t)}{(G_c)} \times \frac{(R_t)}{(R_c)}$$

where G_t and G_c indicated the germinated seeds for the specific treatment and the control, respectively, while R_t and R_c indicated the average root length (mm) for the specific treatment and in the control, respectively.

The results of germination assay are reported in **Supplementary Table S2**, where the test with only peat (T1) served as a positive control, due to the benefits deriving from the release of bioavailable nutrients and labile organic matter. In watercress, VC consistently promoted seed germination and growth, with the highest germination index (GI) observed at 40% VC (167.4%), followed by 20% VC (156.5%). In contrast, AD treatments produced moderate responses, with 20 and 40% AD yielding GI values of 25.2 and 14.9% more than CTRL, respectively. These results clearly indicated a strong and dose-tolerant biostimulant effect of VC on watercress, whereas AD gave the highest response at the dose of 20%, with decreasing effects at higher concentrations. The germination test on lettuce indicated that both AD and VC led to a response even better than the control, with the largest enhancement obtained at the doses of 10 and 20% (**Supplementary Table S2**). The assay on radish confirmed that the fertilizers induced a relevant biostimulant effect than the control, with the best results obtained at the doses of 20% AD and VC.

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SP4. Methods and detailed discussion for Greenhouse Pot Trials

The potential role of AD and VC as soil fertilizers was investigated through pot trials conducted at the greenhouse of the University of Salerno. During the whole experiment, light conditions were controlled using lamps (Indio 1158 Sap-T 400 Cnr-L Graphite Disano, 250W) set to a photoperiod of 16 hours light and 8 hours darkness. Greenhouse temperature and relative humidity were maintained within the ranges of 25–28 °C and 37–50%, respectively.

The pots (100 mm in diameter, 80 mm in height, approximately 0.5 L volume) were prepared by individually mixing either AD or VC with peat at different concentrations (0, 10, 20, and

40% v/v). The experimental design also included a control treatment, with peat only (CTRL), and a reference treatment where $\text{Ca}(\text{NO}_3)_2$ was added as a mineral fertilizer (0.145 g at sowing and 0.145 g ten days after sowing) (MIN). Ten replicate pots were prepared for each treatment. Each pot was manually sown either with 15 lettuce seeds (*Lactuca sativa* L.) or 2 radish seeds (*Raphanus sativus* L.). Throughout the entire experimental period, pots were irrigated regularly with 125 mL of water per pot, three times a week. The irrigation water had a pH of 7.3 and an electrical conductivity of 0.518 dS m^{-1} . Lettuce and radish plants were harvested 27 and 58 days after sowing, respectively. Part of lettuce leaves and the radishes were immediately stored at -80°C for metabolomic analyses.

To assess the impact of AD and VC amendments on lettuce and radish, both non-destructive and destructive analyses were performed to evaluate plant growth, physiological status, morphological and qualitative responses.

Non-destructive measurements were conducted periodically from 1 to 50 days after sowing, enabling continuous monitoring of treatment effects without altering plant integrity. Plant height was recorded with a standard measuring meter and chlorophyll content was estimated with a SPAD meter (Konica Minolta, Japan). Destructive measurements were conducted at harvest to obtain quantitative insights into biomass allocation and morphological traits. Fresh and dry biomass were determined using an analytical balance (KERN) and a forced-air drying oven. Nitrate (NO_3^-) concentration was determined using a LAQUAtwin ion-selective meter (Horiba, Kyoto, Japan).

This integrated methodological approach allowed for a comprehensive evaluation of the amendments' agronomic and physiological effects.

The pot test trials conducted in greenhouse on baby leaf lettuce confirmed the promising role of both organic fertilizers, since the proper doses exerted a significant biostimulant effect. Treatments with 20% VC and 40% AD achieved the greatest plant height, with values significantly higher than the control and comparable to those resulting from mineral fertilization. Chlorophyll content, which was based on SPAD measurements, resulted higher under mineral fertilization. Moreover, it exhibited relatively higher values at the doses of 10 and 20% of VC, which were similar or even higher than the control, thus indicating the role of vermicompost in determining a physiological performance comparable to that induced by synthetic nutrients. However, AD decreased the chlorophyll level at values even lower than the control. Dry and fresh biomass were higher after VC treatment, with values of fresh weight even higher than the positive control. AD gave the significantly higher responses at the highest dose. These observations highlight the potential of VC to enhance plant development efficiently while minimizing excessive input. Finally, nitrate accumulation in lettuce followed a treatment-dependent pattern for VC while, in case of AD the highest content was recorded for the dose of 20%. Interestingly, AD applications provided the highest levels, thus indicating a larger amount of bioavailable nitrogen. Although 40% AD produced the highest fresh weight, nitrate concentrations remained below the maximum residue limits (3000–5000 ppm), highlighting a trade-off between yield and nutritional quality. Overall, 20% VC provided a balanced response, enhancing dry biomass while maintaining lower nitrate content, whereas 40% AD maximized fresh yield but increased nitrate accumulation. This balance between growth stimulation and product safety emphasizes the importance of selecting appropriate amendment concentrations.

Radish exhibited the most pronounced biostimulant response among the tested species. At harvest, 20% VC and 40% AD achieved the highest plant heights, much higher than control and comparable to mineral fertilization. SPAD values followed a similar trend, with mineral

fertilization leading but VC and AD treatments maintaining near-optimal chlorophyll content. Fresh and dry weights were highest under 40% AD, significantly exceeding other treatments, including mineral fertilizer. VC enhanced biomass without excessive nitrate accumulation, while AD promoted rapid vegetative growth due to its richer nitrogen content.

These findings confirm the species-specific and dose-dependent biostimulant potential of VC and AD. VC consistently provided safe and effective stimulation across all tested species, even at higher doses, whereas AD exhibited positive effects at low and medium concentrations but signs of reduced performance or potential phytotoxicity at higher doses, particularly in radish. The results align with previous phytotoxicity assays (Zucconi et al., 1981; Vitti et al., 2021) and underscore the importance of dose optimization for maximizing biostimulant efficacy while minimizing risk during early plant development.

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SP5. Method to extract and identify the primary metabolome of lettuce and radish for NMR-based metabolomics.

For NMR-based metabolomics fresh lettuce samples (150 mg, $n = 10$ per treatment) were accurately weighed and ground in liquid nitrogen to obtain a fine powder. The powdered tissue was then subjected to a preliminary extraction with 1.6 mL of chloroform, in order to remove

most of the lipid components. The suspension underwent ultrasonication (10 min at 22 °C), followed by agitation on a linear shaker (10 min at 350 rpm). After centrifugation (13,000 rpm, 10 min, 4 °C), the supernatant was discarded, and the pellet was dried under a gentle nitrogen stream, to remove the residual chloroform. A second extraction was performed using 1.6 mL of a 1:1 mixture of ultrapure water and methanol (v:v), followed by the same ultrasonication and agitation steps. The supernatant was collected after centrifugation and evaporated to dryness using a rotary evaporator at 40 °C. The resulting extract was redissolved in 1 mL of a deuterated solution prepared by diluting (1:4; v:v) a phosphate buffer solution (brought at pH 6 and obtained with K_2HPO_3 and KH_2PO_3 salts) with deuterated water (99.8% D_2O/H_2O ; Eurisotop) and adding, as internal standard, the 3-Trimethylsilylpropionic Acid (TMSPA) up to attain a final concentration of 0.3 mg/mL. The mixture was processed via ultrasonication (10 min, 22 °C), agitation (10 min, 350 rpm), and centrifugation (13,000 rpm, 10 min, 4 °C). The recovered supernatant was transferred into 5mm high-precision NMR tubes, degassed and finally loaded into the NMR magnet. In the case of radish, the extraction procedure was slightly different. Fresh samples (150 mg, $n = 8$ per treatment) were accurately weighed and ground in liquid nitrogen to a fine powder, then directly extracted with 1.6 mL of the deuterated solution containing the internal standard TMSPA (0.3 mg/mL). The mixture was processed via ultrasonication (10 min, 22 °C), agitation (10 min, 350 rpm), and centrifugation (13,000 rpm, 10 min, 4 °C). The recovered supernatant was transferred into 5mm high-precision NMR tubes, degassed and finally loaded into the NMR magnet.

For both crop types, the identification of the primary metabolome was carried out by assigning most of the detected proton signals through the use of a composite approach, based on (i) the interpretation of the 1D and 2D NMR spectra, both homo- and hetero-correlated, of representative lettuce and radish extracts, (ii) the spectral examination of the detected proton

signals, (iii) the evaluation of inherent literature (**Fasolino et al., 2025; Lanzotti et al., 2022; Chadwick et al., 2024; Sobolev et al., 2005; Feizi et al., 2020**) and (iv) the comparison of the extract's spectra with those of certified standards. Two-dimensional homo- and heteronuclear spectra were acquired using a 500 MHz Bruker spectrometer (11.75 T, Bruker Oxford/Spectrospin Cryomagnet) equipped with a BBO probe. The 2D experiments included ^1H - ^1H COSY, TOCSY (mixing time: 0.08 s), and NOESY (mixing time: 0.8 s), as well as ^1H - ^{13}C HSQC (optimized for $J_{\text{CH}} = 145$ Hz) and HMBC (long-range $J_{\text{CH}} = 8$ Hz). Acquisition parameters for 2D spectra included: 16 dummy scans, 48 scans, spectral widths of 16 ppm (^1H , 6410.3 Hz) and 300 ppm (^{13}C , 30186.8 Hz), with 2048 points in F2 and 256 increments in F1.

SP6. Multivariate analysis of NMR data

The spectral range included within 10 and 0.7 ppm of 1D ^1H NMR spectra of radish and lettuce extracts, was divided into n -parcels (buckets) of equal size (0.02 ppm, each corresponding to about 8 Hz) thus generating a matrix composed by 484 buckets per spectrum/replicate. 1D proton spectral bucketing was conducted using MestReC software (v. 4.9.9.9). Each bucket was then integrated and the resulting values were divided by the area of the internal standard TMSPA (semi-quantitative standardisation).

Principal Component Analysis (PCA) was used to evaluate data, identify similarities and dissimilarities among samples and find variables (loading vectors) discriminating the different types of radishes and lettuce. One-Way ANOVA (ANalysis Of VAriance) test was used to examine and select the most important and significant loading vectors associated with the explored principal components of PCA. In all cases, only loading vectors capable of contributing significantly (p value at least < 0.05 for both Tukey and Benjamini-Hochberg tests;

α confidence level of 0.05) to the discrimination between the different types of investigated radishes and lettuce, were considered. The clustered heatmap was performed by considering only the NMR variables which significantly contributed to discriminate the studied treatments. The heatmap was developed by centring and reducing the values and displaying them according to a colour scale which ranged from dark red (relatively higher/more concentrated) to light yellow (relatively lower/less concentrated) through orange (intermediate values). Samples were clustered using Euclidean's distance and the Ward's aggregation method. All the statistical applications reported in this study were executed through the XLStat software (v. 2016, Addinsoft, Paris, France).

SP7. Identification of NMR-based lettuce leaves primary metabolome

The analyses were performed immediately after the sample extraction and preparation, in order to minimize possible degradative and oxidative phenomena. The identification of the primary metabolome of radish has been recently carried out by our research group and discussed previously (**Fasolino et al., 2025**). In case of lettuce leaves, different spectral regions of the proton spectrum of a representative lettuce extract are shown in **Fig. 3**. The primary metabolome of the lettuce revealed the presence of 23 compounds, including amino acids, organic acids, carbohydrates, alcohols, and nucleosides. Most of the peaks detected in the alkyl and hydroxyalkyl region (ranging between 0.65 and 3.1 ppm; **Fig. 3a**) were attributed to GABA, aspartate, glutamine, malate, glutamate, acetate, threonine, citrate, leucine, valine and isoleucine; the signals observed in the hydroxyalkyl region between 3.12 and 4.43 ppm (**Fig. 3b**) were assigned to glucose (G), fructose (F), sucrose (S), methanol and choline while the signals appearing in the region of double bonds and aromatic compounds, included within 5.8 and 8.8 ppm (**Fig. 3c**), were ascribed to xylose, cytidine, formate, fumarate, tryptophan,

phenylalanine and tyrosine. The most intense and predominant signals observed in the lettuce metabolome were represented by the multiplets of carbohydrates. This was expected, as carbohydrates are the most abundant compound class in lettuces, with an average content ranging within 2.5 and 3%, where about half is composed by fibers. The cytidine is a nucleotide consisting of cytosine and ribose which is relatively abundant in the polar fraction of the lettuce leaves. It is noteworthy that the obtained aqueous extract was also rich in malate, which is the most abundant organic acid in lettuce, as well as contained a relevant content in both citrate and fumarate.

Chapter III

Original research paper

“The combination of green compost and its pelletized form as a strategy to improve lettuce and radish productivity”

Annamaria Di Serio¹, Rossella Curcio^{2,3}, Tommaso Nappo³, Mariavittoria Verrillo⁴, Silvana Cangemi⁴, Riccardo Spaccini⁴, Pierluigi Mazzei³, Domenico Ronga³

¹*Department of Chemistry and Biology, University of Salerno, Via Giovanni Paolo II 132,
84084 Fisciano, Italy*

²*DAFE - Department of Agricultural, Forest, Food, and Environmental Sciences, University
of Basilicata, 10, Viale dell'Ateneo Lucano - Potenza (PZ)*

³*Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084,
Fisciano, SA Department of Chemistry and Biology*

⁴*Department of Agriculture, University of Naples Federico II, Via università, 100, 80055,
Portici, Italy*

*** Corresponding author:** Pierluigi Mazzei, orcid.org/0000-0002-5312-4969,
pmazzei@unisa.it

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3.1 ABSTRACT

The valorization of organic waste through composting and pelletization represents a sustainable strategy to enhance soil fertility and reduce dependence on synthetic fertilizers. In this study, a green compost produced from escarole, red chicory, spinach, and olive pruning residues was characterized through advanced chemical and molecular analyses (elemental composition, ATR FT-IR, and ^{13}C CPMAS NMR), revealing a nutrient-rich matrix containing a balanced mixture of labile and stable organic fractions.

The agronomic performance of both the compost and its pelletized form was evaluated on lettuce (*Lactuca sativa* L.) and radish (*Raphanus sativus* L.) grown in pots, either alone or in combination with synthetic fertilizer, at equivalent nitrogen rates. Non-destructive physiological measurements (chlorophyll content index, NDVI, and leaf fluorescence) were recorded during plant growth, while biometric and productive traits (leaf number, leaf area, root length, fresh and dry biomass, and color parameters) were determined at harvest.

Both compost and pellets significantly enhanced vegetative growth and total biomass compared to the unfertilized control and the mineral-fertilized treatment. On average, fresh biomass increased by 35–45% in lettuce and by 40–50% in radish relative to the mineral control. The combined application of compost and pelletized compost showed synergistic effects, further increasing yield by up to 60% and improving physiological indicators of plant vigor. Overall, the results demonstrate that both compost and its pelletized form act as effective, nutrient-rich organic fertilizers. Their combined use enhances plant productivity, promotes physiological efficiency, and supports a more sustainable horticultural production system, providing a promising alternative to conventional mineral fertilization.

Keyword: CPMAS NMR spectroscopy; PyrGC/MS; Sustainable organic amendment; biomass recycle

3.2 INTRODUCTION

The increasing global population, the challenges posed by climate change and developments in energy policy necessitate a substantial rethinking of agricultural production systems, aiming to satisfy the global food demand in line with the concept of sustainability on limited land resources (**Hunter et al., 2017**). To achieve this goal, agroecological approaches, valorizing natural nutrient cycles and promoting circular economies, are considered a valuable alternative to the intensive use of energy-demanding chemical fertilizers. The sustainable management of organic wastes not only must aim to foster circular processes, but also to reduce the environmental impact of agriculture. Importantly, the administration of essential macronutrients, such as nitrogen and phosphorus, represents a limiting factor for vegetable production. On this basis, the identification of organic fertilizers can overcome these problems, mitigating some critical issues, such as the virtuous recycle of plant residues, the disposal of ingent waste biomass, the supply of nutrients, including N, P and S, through slow-release, the improvement of the chemic fertility of soils. Composting and pelleting constitute effective strategies to valorize plant residues, transforming them into organic amendments that enhance soil fertility and reduce dependence on synthetic fertilizers (**Sung-inthara et al., 2024**). **Ronga et al. (2020)** have demonstrated that composting and pelleting can provide a feasible and sustainable solution to mitigate potential risks associated with weed seeds and harmful microorganisms, while concentrating nutrients and enabling more efficient and sustainable management of organic resources. Currently, composting is one of the main technologies for managing and valorizing organic residues, allowing their transformation into amendments that improve the chemical, physical, and biological properties of soils (**Setti et al., 2019**). Composting is a biological process in which aerobic microorganisms partially decomposes and stabilize, from a biochemical point of view, organic matter under controlled

conditions, producing a nutrient-rich material. Compost quality depends on several factors, including the feedstock type, carbon-to-nitrogen and carbon-to-phosphorus ratios, the length of compost maturation, the pH and the process temperature. Chemical and compositional characterization of compost is essential for assessing its quality, predict and explain its effectiveness as a soil fertilizer, providing information on nutrient availability, potential contaminants and biostimulant components (**Verrillo et al., 2021**).

Recent studies have demonstrated that compost derived from organic waste can effectively enhance the growth and quality of various crops, including baby leaf lettuce and radish. **De Falco et al. (2021)** and **Giménez et al. (2020)** reported that compost-based substrates improved root and shoot development, yield, and disease suppression in baby leaf lettuce. Similarly, **Almaramah et al. (2024)** found that food waste compost significantly promoted radish growth, supporting its use as a sustainable alternative to chemical fertilizers. Long-term field trials by **Zaccardelli et al. (2021)** confirmed that compost from municipal solid waste could partly replace mineral fertilizers while maintaining soil fertility and reducing nitrate leaching.

However, compost often retains high moisture content (>30%) and may not fully eliminate pathogens or weed seeds (**Brito et al., 2018; Olsen et al., 1987**). Pelletization has therefore emerged as a promising approach to reduce moisture ($\approx 7\%$), inactivate biological contaminants, and improve handling, storage, and nutrient release (**Pulvirenti et al., 2020; Zajonc et al., 2014; Mujtaba et al., 2021**). Recent formulations combining compost, water, and biochar optimized pelletization efficiency, lowering energy demand and producing denser, more stable pellets (**Sung-inthara et al., 2024**). These compost-based pellets have shown positive effects on nutrient availability, carbon retention, and soil biological activity,

thereby supporting sustainable agriculture (San Martin et al., 2016; Souri et al., 2018; Sánchez-Méndez et al., 2025).

On this basis, the present study focused on a green compost produced from a mixture of escarole, red chicory, spinach, and olive pruning residues. The compost was thoroughly characterized to evaluate its quality indexes, elucidate its chemical composition, and assess its suitability as a soil amendment. Comprehensive analyses were performed, including chemical determinations and advanced spectroscopic and pyrolytic techniques (^{13}C CPMAS NMR, ATR FT-IR, and Pyr/GC-MS). In addition, a pelletized form of the compost was developed and evaluated through greenhouse pot trials on two model species, lettuce and radish. Both destructive and non-destructive measurements were conducted to assess and compare the effects of the compost and its pelletized counterpart (applied individually or in combination) on plant growth, yield, and physiological traits. It is noteworthy that, although several studies have demonstrated the beneficial impact of compost and related pellets on horticultural crops, none have provided a comprehensive evaluation integrating agronomic performance with detailed chemical characterization.

3.3 MATERIALS AND METHODS

3.3.1 Green Compost and Pellet Production

The company “TerrAmore” (sited in Eboli, Italy) produced and supplied a green compost produced by processing the waste biomass consisting of radish, escarole, spinach and olive pruning residues (weight distribution of 35:35:10:20, respectively). The fresh organic biomass was shredded and homogenized, then subjected to controlled aerobic composting, with periodic monitoring of temperature, moisture content, and C/N ratio to ensure optimal maturation. The compost reached the maturation after 6 months. Then, part of the compost was converted into pellets by using a roller press pelletizer (Metalmecc MT-130). During

pelleting, moisture content (approximately 15–20%) and pressure were optimized, following the recommendations of **Hettiarachchi et al. (2019)**, to produce compact pellets with high mechanical strength and uniform dimensions.

3.3.2 Chemical parameters and elemental composition of green compost and pellets

The green compost was oven-dried at 70 °C for at least 24 hours until a constant weight was reached and sieved through a 2 mm mesh screen. The chemical analyses of this product included pH, electrical conductivity (EC), and ash content. pH and EC were measured by suspending the organic material in ultrapure water (1:10, w/v). The supernatant was vacuum-filtered through Whatman 41 cellulose filters (20 µm pore size) and analyzed using a dual-channel HANNA HI5222 meter. Ash content was determined by incinerating 1 g of dried sample at 650 °C for 6.5 hours in a muffle furnace (SNOL E5CC) and expressed as residual content (%) relative to the initial dry weight.

Elemental composition (C, H, N, and S) was determined via UNICUBE elemental analyzer (Elementar Analysensysteme GmbH, Germany). For each sample, 2 mg of material were analyzed following the procedure described by **Savarese et al. (2022)**. The essential nutrients and several metals were examined via Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Briefly, about 0.1 g of samples were mixed with 3 ml HNO₃ 67 %, 1 ml HCl 37 % and 1 ml HF 47 %, in a Teflon vessel, and digested into a microwave system (Ultrawave, Milestone) according to the following program: increase for 25 min up to achieve 240 °C followed by 10 min of steady state at 240 °C. After cooling, the digested solutions were diluted up to achieve a volume of 10 ml (depending on the nutrient concentration, the solution was further diluted up to 200 times) for the ICP-MS analyses. The following isotopes were monitored in KED (Kinetic Energy Discrimination) mode and used as on-line internal standards: ⁷Li, ⁹Be, ¹¹B, ²³Na, ²⁴Mg, ²⁷Al, ³¹P, ³⁹K, ⁴⁴Ca, ⁵¹V, ⁵²Cr, ⁵⁵Mn, ⁵⁷Fe, ⁵⁹Co, ⁶⁰Ni,

⁶⁵Cu, ⁶⁶Zn, ⁷⁵As, ⁷⁸Se, ⁸⁸Sr, ⁹⁵Mo, ¹¹¹Cd, ¹²¹Sb, ²⁰²Hg, ²⁰⁵Tl, ⁽²⁰⁶⁺²⁰⁷⁺²⁰⁸⁾Pb, ²⁰⁹Bi, and ⁴⁵Sc, ⁷²Ge, ⁸⁹Y, ¹⁰³Rh, ¹⁹³Ir.

3.3.3 *Solid-state CPMAS NMR and FT-IR spectroscopies*

The molecular composition of SPIN, REDCH, ESCAR and Green compost was characterized by using the solid state ¹³C Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (CPMAS NMR) and the Attenuated Total Reflectance Fourier Transform Infrared (ATR-FT-IR) spectroscopies. ¹³C CPMAS NMR spectra were acquired with a Bruker Avance 300 MHz wide-bore magnet (Bruker Biospin, Rheinstetten, Germany), equipped with a CPMAS probe, operating at 25 °C, with ¹³C and ¹H frequencies of 75.47 and 300.13 MHz, respectively. Finely powdered solid sample (60–80 mg), sieved to 0.5 mm, was placed into 4 mm zirconia rotors, sealed with Kel-F caps, and spun at 10,000 ± 1 Hz. The ¹³C NMR spectra was obtained through the CPMAS experiment, with a setup including 1 ms of contact time, 1814 time domain points, a spectral width of 300 ppm (22,727.3 Hz), a recycle delay of 5 s, 8 dummy scans, and 8000 scans. Proton decoupling was performed using the TPPM15 (time proportional phase modulation) sequence. Free induction decays (FIDs) were processed with Bruker Tospin (v 4.0.1) software, Fourier transformed using 2-fold zero-filling and an exponential filter function of 250 Hz was applied, followed by phase- and baseline-correction. ¹³C CPMAS NMR spectrum was divided into specific resonance regions, whose areas were integrated and expressed in absolute values per 100 mg of sample: 0–45 ppm (Alkyl-C), 45–60 ppm (methoxyl-C and N-alkyl-C), 60–110 ppm (O-alkyl-C), 110–145 ppm (aryl-C), 145–160 ppm (phenol-C), 160–190 ppm (carboxyl-C), and 190–230 ppm (Carbonyl-C). Important indexes, such as the hydrophobicity index (1), the lignin ratio (2) and the Alk/O-Alk ratio (3) were calculated based on specific ¹³C NMR intervals, according to the following equations (Monda et al., 2017; de Aquino et al., 2019):

$$HB = \frac{[(0 - 45) + (45 - 60) + (110 - 145) + (145 - 160)]}{[(61 - 90) + (90 - 110) + (160 - 190)]}$$

$$LR = \frac{(45 - 60)}{(145 - 160)}$$

$$Alk/O-Alk\ Ratio = \frac{(0 - 45)}{[(61 - 90) + (90 - 110)]}$$

ATR FT-IR was carried out on dried and sieved samples (≤ 0.5 mm). Analyses were performed in duplicate using a PERKIN ELMER Frontier spectrometer, setting up 128 scans per sample, a spectral range included within 4000 and 600 cm^{-1} , and a resolution of 4 cm^{-1} . For each acquisition set, a background spectrum was recorded and subtracted. Each spectrum was converted into absorbance mode and baseline corrected to ensure consistency and enable accurate comparison between samples. All spectra were processed with Spectragryph software (v. 1.2.16.1).

3.3.4 Gas-Chromatography and mass spectrometry following thermochemiolysis (Pyr/GC-MS)

Green compost underwent a preliminary thermochemiolysis aimed to simplify the complex organic matrix of the samples through the depolymerization of natural biopolymers (specifically inducing the cleavage of ether and ester bonds) and methylating terminal hydroxyl and carboxyl groups. Thermochemiolysis coupled with GC-MS analysis was carried out as described by **Vinci et al. (2019)**. Briefly, 500 mg of sample was derivatized with 1 mL of a 25% tetramethylammonium hydroxide (TMAH) methanolic solution and placed in a quartz vessel. After 2 hours, the quartz vessel was introduced into a Pyrex tubular reactor (50 cm $\times$ 3.5 cm i.d.) and heated at 400 $^{\circ}\text{C}$ for 30 minutes using a Barnstead Thermolyne 21,100 tubular furnace. The depolymerized and derivatized volatile compounds developed

during the pyrolysis were transported by a helium stream (20 mL min^{-1}) into two dichloromethane solutions, arranged in series and kept in an ice bath. The two solutions were then combined, concentrated by rotary evaporation, and the final residue was redissolved in 1 mL of dichloromethane prior to GC–MS analysis.

Analyses were performed on a Perkin Elmer Autosystem XL gas chromatograph equipped with an RTX-5MS WCOT capillary column ($30 \text{ m} \times 0.25 \text{ mm}$; film thickness $0.25 \text{ }\mu\text{m}$) and coupled to a PE Turbomass-Gold quadrupole mass spectrometer via a heated transfer line at $250 \text{ }^{\circ}\text{C}$. The oven temperature program consisted of an initial hold at $60 \text{ }^{\circ}\text{C}$ for 1 minute, a ramp of $7 \text{ }^{\circ}\text{C min}^{-1}$ up to $320 \text{ }^{\circ}\text{C}$, and a final hold at $320 \text{ }^{\circ}\text{C}$ for 10 minutes. Helium was used as carrier gas (1.90 mL min^{-1}), with the injector set at $250 \text{ }^{\circ}\text{C}$ and operating in split mode (30 mL min^{-1} split flow). Mass spectra were acquired in electron impact mode (70 eV), scanning from m/z 45 to 650 with a cycle time of 0.2 s. Compound identification was achieved by both comparing spectra to those of authentic standards and the NIST 05 mass spectral library, and with the support of the appropriate scientific literature (**Gitonga et al., 2024; Wu et al., 2024**). The areas of the peaks in the pyrograms were integrated, grouped by compound class, and expressed both in absolute terms and as percentages of the total chromatogram area. The compound classes included: nitrogen-containing compounds, fatty acid methyl esters (FAMES), branched FAMES and hydroxyacids, unsaturated and branched FAMES, aromatic compounds, nitrogen-containing aromatics (including indole-related structures), and lignin-derived moieties (classified into p-hydroxyphenyl [P], guaiacyl [G], and syringyl [S] units).

3.3.5 *Microbiological analyses*

Microbiological analyses involved the determination of the following microbial groups: total bacteria, siderophore-producing bacteria, spore-forming bacteria, lactic and acetic acid bacteria, mold and yeast, *Escherichia coli*, fecal streptococci, total and fecal coliforms.

The extraction of microorganisms from Green Compost was performed by shaking for 30' at 120 rpm in a 100 ml flask, the equivalent of 5 g of dried and 2-mm sieved fertilizer, with an equivalent of 45 ml of 0.85% NaCl solution. After the shaking, the suspension was left to decant for 5 min. Then, 1 ml of the suspension was sampled and subjected to serial dilutions with water, up to dilute the sample 1:100'000'000 times. For each dilution and for each microbial group, three aliquots of 10 µL were plated on the proper substrate, which varied as a function of the examined microbial group. In detail, total bacteria were counted on soil extract agar (**Subba Rao, 1977**); siderophores forming-bacteria were counted on a selective agar medium without iron (**Scher and Baker, 1982**); spore-forming bacteria were counted by plating, on Nutrient Agar, ten-fold dilutions of a suspension previously heated at 90 °C for 10 min (**Sadfi et al., 2001**); lactic bacteria were counted on MRS Agar (**de Man et al., 1960**); acetic acid bacteria were counted on WL Agar (**Green and Gray, 1950**); mold and yeast were counted on Rose-Bengal Agar (**Jarvis, 1973**); *Escherichia coli* was counted on Tryptone Bile X-Gluc Agar (**Delisle and Ley, 1989**).

For the determination of fecal streptococci and total and fecal coliforms, serial decimal dilutions of each sample were inoculated into tubes containing the specific growth substrate and it was applied the multiple tube method (MPN = most probable number) (**Bonadonna et al., 2002**).

All agar plates or tubes were incubated for 2-5 days at 44 °C for *Escherichia coli* and fecal coliforms; at 37 °C for fecal streptococci and total coliforms; at 25 °C for molds and yeast; at 28 °C for all the other microbial groups. Results were reported as CFU (colony-forming units) or MPN (most probable number) per gram of dried fertilizer, where the MPN was determined via Mc Crady tables.

3.3.6 *Pot trials experiment in greenhouse*

The pot experiment was conducted in a controlled greenhouse environment at the University of Salerno. Climatic conditions were continuously monitored using a portable meteorological station (PCE Instruments PCE-FWS 20N) positioned 130 cm above the soil surface. The air temperature was maintained between 25 and 28 °C using an air conditioning system, while relative humidity ranged from 37% to 50%. Plants were exposed to a 16-hour photoperiod under artificial lighting provided by Indium art 1158 Sap-T 400 Cnr-L Graphite Disano lamps (250 W, 230 V, 50 Hz), followed by 8 hours of darkness. Green compost and pellets were incorporated into a neutral, unfertilized peat substrate (Mannaflor Tray 80/20 0.4) prior to sowing. Three biological replicates were established for each treatment. Each 500 mL pot (10 cm diameter) was sown with 15 lettuce seeds (*Lactuca sativa* L. genotype Eden, Maraldi S.R.L., Italy) and 1 radish seed (*Raphanus sativus* L. genotype Olter, small round, white-tipped type). Pots were irrigated three times per week with 125 mL of water (pH 7.34; EC 518.4 $\mu\text{S}/\text{cm}$). Fertilization with synthetic NH_4NO_3 was applied only to treatment T2, at a total dose of 0.26 g per pot (0.13 g at sowing and 0.13 g ten days later). Treatments were designed to evaluate the effects of green compost-derived pellets, green compost, and synthetic fertilizer, applied alone or in combination, as follows: T1 (CTRL): unfertilized as control; T2 (MIN): synthetic fertilizer as control (0.26 g NH_4NO_3 per pot); T3 (PEL): pellets at equivalent N dose to synthetic fertilizer (3.36 g per pot); T4 (PEL2): green compost pellets at double N

dose (6.72 g per pot); T5 (PEL/MIN): 50% pellets + 50% synthetic fertilizer; T6 (COM): green compost at equivalent N dose to pellets (3.4 g per pot); T7 (COM/MIN): 50% compost + 50% synthetic fertilizer; T8 (COM/PEL): 50% pellets + 50% compost. All amendments were thoroughly mixed into the substrate at sowing. Harvest was carried out approximately 30 days after sowing.

3.3.7 Agronomic Surveys

During the crop cycle, plant height was measured with a standard ruler, and soil moisture was monitored using a Fieldscout TDR (Time Domain Reflectometry) sensor. At harvest, for lettuce, aboveground and belowground fresh biomass (g per pot) were determined using an electronic balance, while for radish, fresh and dry weight of leaves, edible roots, and whole roots were recorded separately. Root length and leaf number per plant were also measured, and collar diameter was assessed with a digital caliper. All samples were subsequently oven-dried at 65°C for 48 hours to determine dry biomass.

3.3.8 Physiological Surveys

Physiological surveys were performed throughout the crop cycle and at harvest. Specifically, key leaf pigments, including chlorophyll, flavonoids, anthocyanins, and the NFI index (chlorophyll/flavonoid ratio), which are indicative of primary and secondary plant metabolism, were measured using the MPM100 instrument (Opti-Sciences). Canopy temperature was monitored with a FLIR Ex-Series thermal camera, and vegetation indices, including the Normalized Difference Vegetation Index (NDVI) and the Photochemical Reflectance Index (PRI), were recorded using Decagon sensors.

3.3.9 *Qualitative Surveys*

At harvest, a series of qualitative analyses were performed. Leaf area was measured using a portable Li-Cor LI-3000 leaf area meter. Leaf colour was assessed using a CR-210 Chroma Meter (Minolta Corp., Osaka, Japan) equipped with a standard D65 illuminant. The resulting colorimetric data were expressed in the CIELAB colour space as L*, a*, and b* values. Nitrate concentration (NO_3^-) was determined using a Horiba LAQUAtwin device.

3.3.10 *Statistical Analysis*

The experimental data were subjected to one-way ANOVA, and mean comparisons were performed using Duncan's multiple range test. Differences were considered statistically significant at $p < 0.05$. Pearson's correlation and principal component analysis of the evaluated attributes were conducted by GraphPad Prism V8 software (GraphPad Co., San Diego, CA, USA) and XLSTAT Addinsoft version 2014.5.03 (New York, NY, USA), respectively.

3.4 RESULTS

3.4.1 *Chemical analyses of green compost and pellet*

The investigated green compost, derived from spinach, red chicory, escarole, and olive pruning residues, together with its pelletized form, was chemically characterized. The material exhibited a markedly alkaline pH (8.65 ± 0.26 ; **Table 1**), likely influenced by both the composition of the feedstock and the ammonification of organic nitrogen occurring during composting, which releases ammonia capable of generating hydroxide ions upon pH-dependent protonation. This alkalinity, typical of plant-based composts, may exert a buffering effect once applied to soil, counteracting acidity and improving the chemical environment for microbial activity and plant growth (Maffia et al., 2024a). However, in very alkaline soils, such composts should be applied with caution, as excessive pH increases can impair plant and

microbial metabolism and decrease the solubility of micronutrients such as Fe, Cu, Mn, and Zn (**Pizzeghello et al., 2021**). The electrical conductivity (EC) was relatively high (**Table 1**), indicating an appreciable concentration of soluble salts and mineral nutrients. This suggests that the compost can provide readily available nutrients, although application rates should be carefully adjusted for salt-sensitive crops and soil salinity should be periodically monitored. Elemental analysis revealed total carbon and nitrogen contents of 28.19% and 2.66% (dry weight basis), respectively, corresponding to a C/N ratio of approximately 12 (**Table 1**). Such a low ratio, together with the relatively high nitrogen content, indicates a compost still undergoing partial mineralization, with a substantial fraction of labile organic carbon rather than fully stabilized humic material (**Maffia et al., 2024b**). The ash content reached 34.34% (dry weight), consistent with the nutrient-rich nature of both olive pruning residues and leafy vegetable wastes. This composition supports the idea of a material rich in mineral components but only moderately stabilized from a biochemical standpoint. ICP-MS measurements revealed a nutrient-rich composition, where potassium (61754 mg/kg) and calcium (33638 mg/kg) were the predominant nutrients, thus representing the most impactful elements for the high salinity. Then, a relatively high concentration was also detected for magnesium (6040 mg/kg), phosphorus (8653 mg/kg) and iron (7745 mg/kg). Other essential micronutrients, including manganese (421 mg/kg), copper (112 mg/kg), zinc (81.7 mg/kg), and boron (49.3 mg/kg), were also present at levels suitable to support plant and microbial metabolism. Although sodium content was relatively high for a compost, with an average value of 6162 mg/kg, it was much lower than potassium and calcium. Therefore, it is not enough to affect the soil structure e promote soil erosion in case of soil application. The pellets maintained chemical characteristics largely consistent with the original material, exhibiting slightly elevated carbon (33.59%) and hydrogen (3.89%) contents while nitrogen remained essentially

unchanged (2.68%), indicating that the pelleting process did not substantially alter nutrient composition. Most macro- and micronutrient concentrations in pellets were comparable with those observed in compost, excepted for some elements whose content during the process of pelletization changed, such as for magnesium, potassium, manganese and iron (**D. Ronga et al., 2020; Pizzeghello et al., 2021**). From a chemical standpoint, the mechanical compression applied during pelletization may slightly influence the distribution of organic fractions, but without inducing relevant transformations in the bulk composition. The resulting pellets can thus be regarded as nutrient-rich, stable organic amendments capable of improving soil fertility by supplying essential macro- and micronutrients while contributing to the long-term accumulation of organic matter. In addition, their compact form offers improved handling, storage, and application efficiency compared with the loose compost.

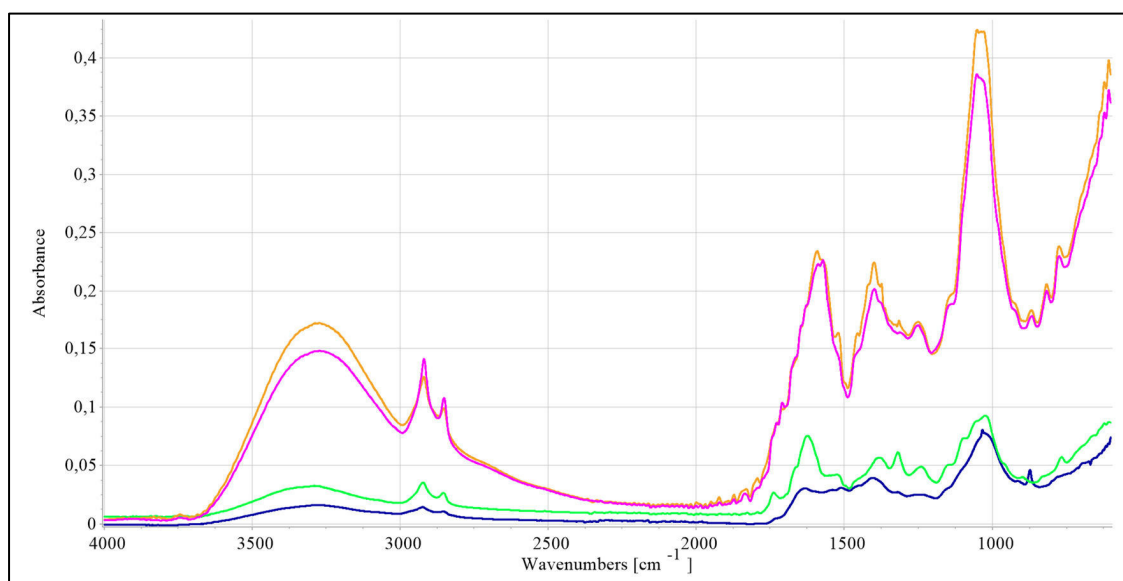
Table 1. Chemical analyses were performed on green compost and pellets. pH, electrical conductivity (mS/cm), and ash content (% w/w) were determined on the compost, while elemental composition (C, H, N, S) and nutrient contents were analyzed on both compost and pellets using ICP-MS.

GREEN COMPOST			PELLETS
pH		8.65 ± 0.26	
CE	mS/cm	29.86 ± 0.54	
Ashes	% (w/w)	34.34	
C	% (w/w)	28.19	33.59
N	% (w/w)	2.66	2.68
H	% (w/w)	3.22	3.89
S	% (w/w)	<1%	<1%
Mg	mg/kg	6040 ± 1266	5009 ± 749
P	mg/kg	8653 ± 189	7978 ± 407
K	mg/kg	61754 ± 6130	40517 ± 250
Ca	mg/kg	33638 ± 131	34296 ± 2064
B	mg/kg	49.3 ± 3.0	49.3 ± 1.4
Mn	mg/kg	421 ± 37	352.2 ± 29.9
Fe	mg/kg	7745 ± 597	9170 ± 1461
Cu	mg/kg	112 ± 14	113.15 ± 2.68
Zn	mg/kg	81.7 ± 7.4	79.1 ± 1.2
Na	mg/kg	6162 ± 481	3659 ± 34

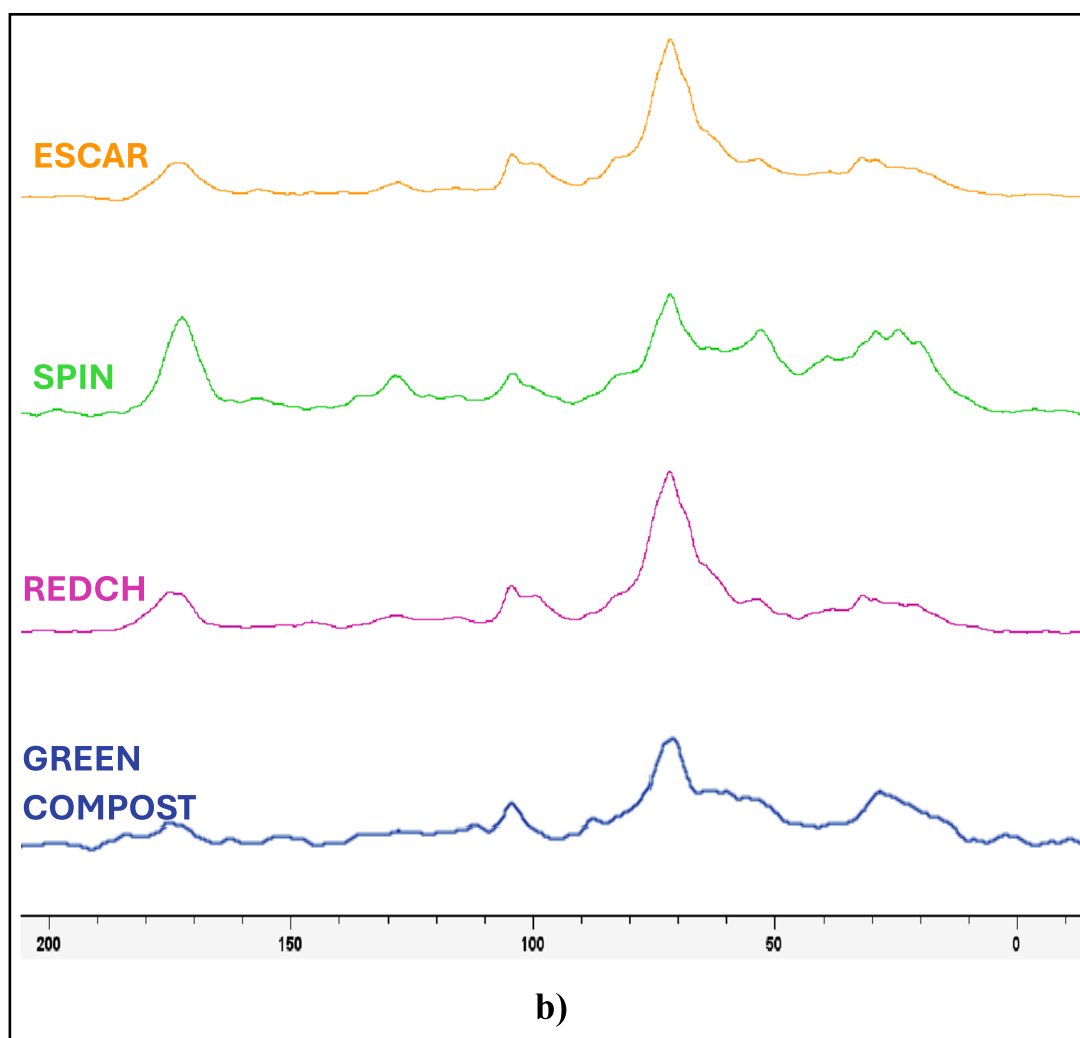
3.4.2 Compositional Characterization of starting biomasses and derived Green Compost

The chemical composition of the starting biomasses plays a crucial role in determining the transformations occurring during composting and, consequently, the molecular properties of the final product. To track these changes, ATR FT-IR and solid-state ^{13}C CPMAS NMR analyses were performed on the feedstocks (REDCH, SPIN, ESCAR), revealing marked compositional differences that influenced their evolution during composting. The compost obtained from the mixture of REDCH, ESCAR, SPIN, and olive pruning residues (35:35:10:20 w/w) was then analyzed by ATR FT-IR and ^{13}C CPMAS NMR to assess the degree of organic matter transformation and stabilization, and by Pyr-GC/MS to investigate the molecular nature of the residual macromolecular structures.

The ATR FT-IR spectra of the examined samples are shown in **Figure 1a**, while the solid-state ^{13}C CPMAS NMR spectra of the starting biomasses (REDCH, ESCAR, and SPIN) and the resulting Green Compost provide a detailed overview of carbon distribution, with the NMR spectrum shown in **Figure 1b** and the corresponding absolute and normalized integrations reported in the relative table in **Figure 1c**.



a)



b)

Spectral range (ppm)	REDCH	SPIN	ESCAR	GREEN COMPOST
230-190 (Carbonyl-C)	13.35×10 ⁶	43.19×10 ⁶	1.55×10 ⁶	6.49×10 ⁶
190-160 (Carboxyl-C)	231.39×10 ⁶	507.68×10 ⁶	208.43×10 ⁶	32.44×10 ⁶
160-145 (O-Aryl-C)	82.12×10 ⁶	151.79×10 ⁶	72.11×10 ⁶	17.23×10 ⁶
145-110 (Aryl-C)	176.70×10 ⁶	340.55×10 ⁶	140.64×10 ⁶	42.49×10 ⁶
110-60 (O-Alkyl-C)	1625.90×10 ⁶	1902.90×10 ⁶	1900.28×10 ⁶	416.02×10 ⁶
60-45 (CH₃O/C-N)	169.66×10 ⁶	494.37×10 ⁶	254.53×10 ⁶	50.78×10 ⁶
45-0 (Alkyl-C)	406.23×10 ⁶	1135.37×10 ⁶	531.34×10 ⁶	117.71×10 ⁶
HI	0.49	1.08	0.54	0.502
Alk/O-Alk	0.28	0.81	0.32	0.283
LR	2.07	3.26	3.53	2.946

c)

Figure 1. Solid-state ATR FT-IR (a) and ¹³C CPMAS NMR (b) spectra of REDCH, SPIN, ESCAR and Green compost; the box (c) reports the NMR integrations (normalized to the total) the most relevant NMR-based indexes.

At the macroscopic level, both spectroscopic techniques revealed marked compositional differences between the initial biomasses. In particular, REDCH and ESCAR displayed distinct spectral features, as compared to SPIN. In the ATR FT-IR spectra, the broad absorption band in the 3200–3400 cm⁻¹ region is attributed to O–H stretching vibrations of hydroxyl groups, commonly arising from hydrogen-bonded alcohols, phenols, and carboxylic acids. The absorption bands at 2920 and 2850 cm⁻¹ were attributed to alkyl C–H stretching vibrations, indicating the presence of lipidic and aliphatic structures. The weaker absorption between 2850 and 2750 cm⁻¹ was ascribed to C–H stretching of methoxyl (–OCH–) groups, mainly arising from lignin-derived compounds. The prominent band at ~1720 cm⁻¹ corresponded to C=O stretching vibrations of carboxylic acids, esters, and ketones, acting as indirect indicators of the degree of organic matter oxidation. The band near 1620 cm⁻¹ was associated with aromatic C=C skeletal vibrations. The strong absorption in the 1030–1080 cm⁻¹ range was attributed to C–O stretching of polysaccharides, while the bands in the 700–

900 cm^{-1} region were assigned to the out-of-plane C–H bending vibrations of aromatic rings (Curcio et al., 2025; Machado et al., 2020).

Based on these assignments, the spectra of REDCH and ESCAR were characterized by stronger absorptions across the main regions (O–H, aliphatic C–H, and polysaccharide-related C–O), reflecting their carbohydrate-rich composition, including cellulose, hemicelluloses, and pectins (Lang et al., 2025; Zhu et al., 2025). Instead, SPIN showed weaker signals overall, indicating fewer carbohydrate structures and a higher proportion of proteins, organic acids, and phenolic compounds.

ATR FT-IR spectra of the green compost displayed a broad O–H band (3200–3400 cm^{-1}) and aliphatic C–H absorptions (2920 and 2850 cm^{-1}), but with a relative decrease in polysaccharide-related C–O absorption (1030–1080 cm^{-1}) and an increased intensity of carbonyl (1720 cm^{-1}) and aromatic (1620 cm^{-1}) signals, indicative of oxidation and enrichment in recalcitrant fractions. Signals in the 2850–2750 cm^{-1} range, attributable to methoxyl groups, along with aromatic C–H bands at 700–900 cm^{-1} , reflected the persistence of lignin-derived moieties, mainly from the woody fraction of olive pruning residues. The presence of residual labile carbon was suggested by the remaining O–H and C–O absorptions, in agreement with the relatively low C:N ratio observed in elemental analyses. Altogether, these spectral features revealed a balanced coexistence between recalcitrant aromatic domains and residual labile polysaccharides, consistent with an advanced but incomplete humification process (Curcio et al., 2025; Verrillo et al., 2021; Bento et al., 2021).

The solid-state ^{13}C CPMAS NMR spectra of the starting biomasses (REDCH, ESCAR, and SPIN) and the resulting Green Compost provide a detailed overview of carbon distribution, with the spectrum shown in **Figure 1b** and the corresponding absolute and normalized integrations reported in the relative table in **Figure 1c**. The region between 0–45 ppm was

attributed to (CH₂)_n and CH₃ alkyl carbons, mainly deriving from aliphatic chains in lipids, cuticular waxes, cutin, and fatty acids of both plant and microbial origin. The 45–60 ppm interval was assigned to methoxyl carbons, associated with lignin-derived aromatic methoxyls, and C–N carbons in oligo- and polypeptides. The 60–110 ppm range corresponds to O-alkyl carbons, primarily reflecting structural polysaccharides such as cellulose, hemicellulose, and pectins, as well as soluble carbohydrates. Within this interval, the most intense resonance around 70 ppm is associated with the overlapping C2, C3, and C5 carbons of pyranosidic rings, while signals near 65 and 80 ppm correspond to C6 and C4 carbons, respectively; anomeric (C1) carbons resonate between 100 and 110 ppm. Signals in the 110–145 ppm region arise from unsubstituted aromatic carbons mainly derived from lignin, phenolic compounds, or polycondensed aromatics, whereas the 145–160 ppm region corresponds to quaternary aromatic carbons bonded to hydroxyl or methoxyl groups. Finally, the 160–190 ppm region is attributed to carbonyl carbons of carboxylic acids, esters, and esterified biopolymers, representing polar and reactive groups that contribute to nutrient retention, cation exchange, and interactions with soil components (Verrillo et al., 2021; de Aquino et al., 2019).

Among the fresh biomasses, REDCH and ESCAR showed the highest contribution of O-alkyl-C, confirming their richness in structural carbohydrates. In contrast, SPIN exhibited almost half the carbohydrate-derived carbon content and displayed intense signals in both methoxyl-C/peptidic C–N and aromatic-C regions, indicating a higher proportion of proteins and lignin (Waseem et al., 2021; Cerulli et al., 2024). The relatively higher lignin content in spinach was expected, being a horticultural product with typical lignin concentrations ranging between 4–5%. FT-IR spectra confirmed this trend, highlighting an enrichment of phenolic components in SPIN and a predominance of carbohydrate-derived carbon in REDCH and

ESCAR.

These findings were further corroborated by the NMR-derived indexes (Table 2), commonly used to assess the biochemical stability of organic substrates (**Bento et al., 2020b; de Aquino et al., 2019; Monda et al., 2017; Pane et al., 2016**). The HI and Alk/O-Alk ratios were very similar for REDCH and ESCAR and significantly lower than those observed for SPIN, whose higher values (two to three times greater) reflected its higher lipidic and aromatic content. For all biomass types, the LR ratio exceeded 1, suggesting a relevant presence of amino acids and proteinaceous material, with REDCH showing the lowest value. After composting, the ^{13}C CPMAS NMR spectrum of the Green Compost revealed well-defined signals across all carbon regions, indicating the formation of a chemically complex and stabilized organic structure following composting. The coexistence of Alkyl-C and aromatic-C peaks indicated the formation of recalcitrant, hydrophobic moieties responsible for the structural stability of the compost, whereas the persistence of intense O-alkyl-C signals suggested the presence of residual polysaccharides, although partially degraded, and other labile compounds. Such signals demonstrate that, despite ongoing humification, the compost retains microbially available carbon, thus contributing to soil nutrient cycling and biological activity. In fact, an advanced humification state should consist of an extensive microbial and enzymatic degradation of carbohydrates, determining a decrease in the intensity of these peaks accompanied by a selective preservation of peaks related to more recalcitrant moieties. Also NMR indexes confirmed these observations. In fact, the hydrophobic index and the Alk/O-Alk ratio resulted of 0.502 and 0.283, respectively, with values relatively low for a typical compost. Also the LR ratio (2.946), being significantly higher than 1, revealed that an important contribution for the CH₃O/C-N signal was provided by amino acids and proteins, thus confirming a residual non-negligible presence of labile carbon and justifying the

relatively high nitrogen content in the compost (**Table 1**). Altogether, the ^{13}C CPMAS NMR results highlight the transition from carbohydrate-rich and proteinaceous biomasses to a more chemically stabilized product, where long-term structural stability (due to hydrophobic and aromatic fractions) coexists with short-term functional activity (provided by residual carbohydrates and reactive carboxylic groups). This dual molecular character makes the Green Compost a valuable organic amendment for improving soil quality and supporting both microbial and plant activity (**Curcio et al., 2025**). The Pyr-GC/MS pyrogram of green compost is shown in **Figure 2**.

Figure 2. GC chromatograms of Green compost obtained by thermochemiolysis conducted at 400 °C with the tetramethylammonium hydroxide.

Most of the peaks were identified through comparison with the NIST mass spectral library, supported by the interpretation of characteristic fragmentation patterns and, where available, with authentic standards. The complete list of identified compounds, along with their chemical class, molecular weight, and diagnostic ions, is reported in **Supporting Table S1**. All signals detected in the pyrograms were integrated and classified into specific compound classes, as summarized in **Table 2**.

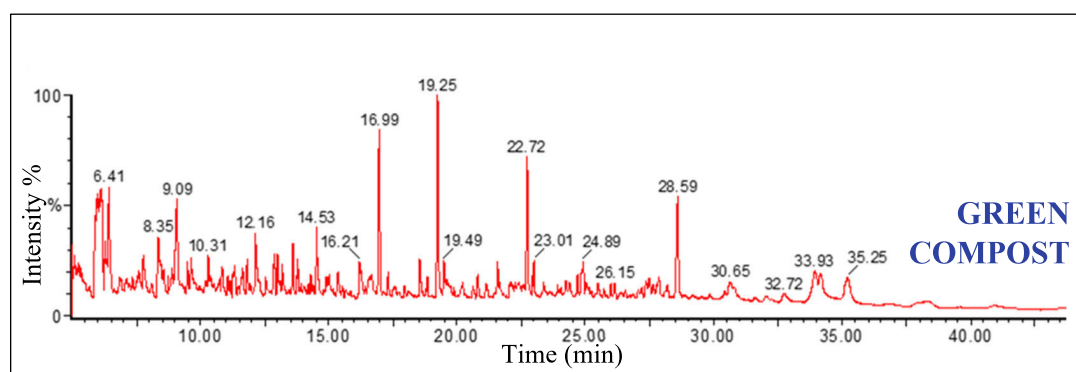


Table 2. Integration (normalized values, %) of GC-MS pyrograms of Green compost. The labels FA, FAME, P, G, and S correspond to fatty

acids, fatty acid methyl esters, p-hydroxyphenyl, guaiacyl and syringyl monomeric lignin units, respectively.

% Compounds class	GREEN COMPOST
Branched FA and hydroxyacids	10.63 ± 0.02
Dioic acids	0.00 ± 0.00
FA mono- and di-unsaturated	9.52 ± 0.02
FAME	1.39 ± 0.00
Lignin - P	0.67 ± 0.02
Lignin - G	23.09 ± 0.00
Lignin - S	13.01 ± 0.01

The pyrolysis products of compost were dominated by lignin-derived compounds, which represented about 36.77% of the total peak area. Among these, guaiacyl (G) units were the most abundant (23.09%), followed by syringyl (S) units (13.01%), while p-hydroxyphenyl (P) units were only detected in minor amounts (0.67%). The prevalence of G- and S-type structures is consistent with the woody fraction of the starting feedstock (olive pruning residues), whose lignin-rich composition strongly influenced the chemical profile of the final material. The low content in lignin P-units, which are typically high in herbacees, are ascribable mostly to the leafy inputs (spinach, radish, and escarole).

A significant contribution in the composition was provided by branched fatty acids and hydroxy acids (10.6%), which are recognized as molecular markers of microbial biomass. This suggests that the composting process promoted intense microbial activity and turnover, likely stimulated by the presence of nitrogen-rich vegetable wastes (**Pizzanelli et al., 2023b; Maffia et al 2024b**). A further fraction of mono- and di-unsaturated fatty acids (9.5%) was also detected, together with a minor content of saturated FAMES (1.4%), reflecting the persistence of plant- and microbial-derived lipidic structures after composting.

Interestingly, the content in dicarboxylic acids was below the detection limit, possibly due to their extensive degradation during the thermophilic stages of composting or to their limited

abundance in the initial feedstock. Specifically, the main signals detected in compost were assigned to benzoic acid, 3,4-dimethoxy-, methyl ester (Lignin-G unit) at RT 19.248 min, to benzoic acid, 3,4,5-trimethoxy-, methyl ester (Lignin-S unit) at RT 22.72 min, to 2-propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester (Lignin-G unit) at RT 24.378 min, and to tetradecanoic acid, 10,13-dimethyl-, methyl ester (branched FA) at RT 28.587 min.

The integrated spectroscopic evidence demonstrates that the labile carbohydrate fractions of REDCH and ESCAR underwent extensive oxidative depolymerization, while the more recalcitrant proteinaceous, lipidic, and lignin-derived components (particularly abundant in SPIN and the olive pruning fraction) persisted or were selectively enriched. The simultaneous increase in aromatic and carbonyl functionalities, combined with the presence of microbial lipid markers, indicates that composting promoted both mineralization and humification processes. The final compost thus integrates recalcitrant structures that guarantee long-term carbon stability with functional moieties (carboxylic, hydroxyl, amminic) that sustain cation exchange, microbial colonization, and nutrient release (**Pizzanelli et al., 2023**). In conclusion, the compositional trajectory from carbohydrate-dominated feedstocks (REDCH and ESCAR) and protein/lignin-rich SPIN to a chemically complex compost enriched in aromatic, lipidic, and microbially derived compounds highlights a dual functionality. The resulting material acts simultaneously as a stable carbon reservoir and an active biofertilizing agent, enhancing both soil organic matter persistence and short-term biological fertility through its heterogeneous molecular architecture. (**Pizzanelli et al., 2023c; Maffia et al., 2024c**).

3.4.3 Microbial characterization of Green Compost

In **Table 3** are shown the results of the microbiological analyses of the green compost (C). The examined groups included total bacteria, filamentous fungi (molds), spore-forming and siderophore-producing bacteria, lactic acid and acetic acid bacteria, and yeasts, all known for

their beneficial roles in organic matter degradation, plant protection, and plant growth stimulation. The compost showed high concentrations of beneficial microorganisms, with total bacteria, siderophore-producing bacteria, and spore-forming bacteria ranging from 6.35×10^6 to 9.32×10^6 CFU g⁻¹, indicating an active and diversified microbial community. Yeasts and filamentous fungi were detected at 1.1×10^5 CFU g⁻¹, while lactic acid and acetic acid bacteria reached 8.4×10^5 and 4.66×10^5 CFU g⁻¹, respectively. These data suggest that the green compost hosts a rich and functionally diverse microbial population, potentially beneficial for soil biological activity and plant development. Regarding the presence of undesirable microorganisms from a sanitary and hygienic standpoint (*Escherichia coli*, fecal streptococci, total and fecal coliforms), the compost was found to be microbiologically safe. In fact, *E. coli*, fecal streptococci, and fecal coliforms were completely absent, while total coliforms were detected at only 2.5×10^1 MPN g⁻¹. The very low or absent counts of these microorganisms confirm the effective hygienization achieved during the composting process, which produces a microbiologically stable and safe soil improver suitable for agricultural applications.

Table 3. Microbiological analyses of anaerobic Green Compost. The results are expressed as CFU (colony-forming units) or MPN (Most probable number) per g of dried material.

	Total bacteria (CFU/g)	Siderophore producing bacteria CFU/g)	Spore- forming Bacteria (CFU/g)	Yeast (CFU/g)	Mold (CFU/g)	Lactic Acid Bacteria (CFU/g)	Acetic Bacteria (CFU/g)
Green Compost	6.36x10 ⁶	9.32x10 ⁶	6.35x10 ⁶	1.1x10 ⁵	1.1x10 ⁵	8.4x10 ⁵	4.66x10 ⁵
	Escherichia coli (CFU/g)	Fecal Streptococci (MPN/g)	Total Coliform (MPN/g)	Fecal Coliform (MPN/g)			
Green Compost	absent	absent	2.5x10 ¹	absent			

3.4.4 Results of agronomic and non-destructive physiological surveys on lettuce during the crop cycle

Table 4 shows the results of the physiological and agronomic parameters measured on lettuce plants across the different treatments. The data indicate that green compost pellets, alone or combined with synthetic fertilizer or compost, positively influenced the physiological and agronomic traits of lettuce. Specifically, treatments with double N-dose pellets (T4) or combinations of pellets and compost (T5, T8) increased plant height, collar diameter, and NDVI and PRI indices. Soil moisture varied among treatments, with higher values observed in the pellet + synthetic fertilizer or pellet + compost combinations. Moreover, T4 exhibited the highest chlorophyll content and NFI index. Overall, green compost pellets contributed to improved growth and physiological status of lettuce compared to the controls.

Table 4. Results of agronomic and non-destructive physiological surveys on lettuce during the crop cycle

Treatments	T1 (CTRL)	T2 (MIN)	T3 (PEL)	T4(PEL2)	T5 (PEL/MIN)	T6 (COM)	T7 (COM/MIN)	T8 (COM/PEL)
Plant Height (cm)	12.25 bc	14.50 a	11.50 cd	10.00 e	10.33 de	11.00 cde	11.83 bc	12.83 b
Collar diameter (mm)	2.53 ab	2.18 b	1.45 c	2.68 a	2.32 ab	2.04 b	2.15 b	2.36 ab
TDR (% soil water content)	8.20 d	11.35 d	17.85 c	17.65 c	28.85 a	23.50 b	26.70 ab	24.30 ab
ChlM	0.14	0.16	0.17	0.18	0.16	0.16	0.15	0.17
FlvM	0.07 b	0.03 cd	0.03 cde	0.10 a	0.01 de	0.06 b	0.01 e	0.03 c
AnthM	0.16 abc	0.17 ab	0.16 abc	0.19 a	0.13 c	0.15 bc	0.17 ab	0.18 ab
NFI	1.85 de	4.48 cde	6.40 c	1.53 e	17.76 a	3.77 cde	10.37 b	5.49 cd
Canopy Temp. (°C)	22.43 b	23.70 ab	24.90 a	23.67 ab	23.80 a	24.37 a	24.23 a	23.60 ab
NDVI	0.58 ab	0.59 ab	0.39 c	0.55 abc	0.67 a	0.47 bc	0.61 ab	0.71 a
PRI	-0.76 bcd	-0.72 abc	-0.80 cd	-0.65 a	-0.76 bcd	-0.70 ab	-0.85 d	-0.70 ab

T1 (CTRL): unfertilizer control; T2 (MIN): synthetic fertilizer control (0.26 g NH_4NO_3 per pot); T3 (PEL): green compost pellets at equivalent N dose to synthetic fertilizer (3.36 g per pot); T4 (PEL2): green compost pellets at double N dose (6.72 g per pot); T5 (PEL/MIN): 50% pellets + 50% synthetic fertilizer; T6 (COM): green compost at equivalent N dose to pellets (3.4 g per pot); T7 (COM/MIN): 50% compost + 50% synthetic fertilizer; T8 (COM/PEL): 50% pellets + 50% compost. TDR = Time Domain Reflectometry; ChlM = leaf chlorophyll content index; FlvM = leaf flavonol contents index; AnthM = leaf anthocyanin contents index; NFI = nitrogen-

flavonol index (CHL/FLAV ratio); NDVI = normalized difference vegetation index; PRI = photochemical reflectance index; Different letters indicate significant differences according to Duncan's test ($p \leq 0.05$).

3.4.5 Results of agronomic and physiological not destructive surveys on lettuce during the harvest

Table 5 summarizes the physiological and agronomic parameters measured on lettuce plants across the different experimental treatments. Overall, treatments containing pelleted green compost (T3, T4, T5, and T8) and their combinations with compost or synthetic fertilizer generally exhibited intermediate to higher leaf chlorophyll content compared to the controls (T1 unfertilized control and T2 synthetic fertilizer). Flavonol content was the highest in the compost treatment (T6), while anthocyanin content and the NFI index varied among treatments, with T3 and T8 showing significantly positive values. Leaf temperature was the highest in treatments with higher pellet rate (T8), whereas NDVI and PRI values indicated greater photosynthetic efficiency in T3, T4, and T6. From an agronomic perspective, plants treated with pellets and their combinations (T3, T4, T5, T6, T7, T8) exhibited increased plant height and larger collar diameter compared to the unfertilized control. Soil moisture (TDR) varied among treatments, being highest in the unfertilized control (T1), while substrates amended with pellets and compost exhibited higher soil moisture.

Table 5. Results of agronomic and physiological not destructive surveys on lettuce at harvest

Treatments	T1 (CTRL)	T2 (MIN)	T3 (PEL)	T4(PEL2)	T5 (PEL/MIN)	T6 (COM)	T7 (COM/MIN)	T8 (COM/PEL)
Plant Height (cm)	10.33 f	15.67 ab	12.33 de	11.33 def	14.33 bc	10.67 ef	13.00 cd	16.50 a
Collar diameter (mm)	1.60 b	2.43 a	2.72 a	2.72 a	1.62 b	2.60 a	2.46 a	2.90 a
TDR (% soil water content)	30.60 a	26.15 b	18.85 c	23.00 b	16.90 c	18.53 c	16.23 c	17.60 c
ChlM	0.20 bc	0.19 bc	0.18 c	0.17 c	0.21 b	0.12 d	0.26 a	0.24 a
FlvM	0.20 b	0.04 f	0.09 d	0.16 c	0.05 ef	0.24 a	0.07 de	0.03 f

AnthM	0.15 ab	0.14 ab	0.16 a	0.15 ab	0.13 bc	0.15 ab	0.10 c	0.13 ab
NFI	1.10 c	3.62 b	1.41 c	1.00 c	3.05 b	0.74 c	3.36 b	6.31 a
Canopy Temp. (°C)	24.77 c	25.43 abc	24.80 bc	26.03 a	25.00 bc	25.37 abc	25.73 ab	24.80 bc
NDVI	0.78 ab	0.80 ab	0.82 a	0.80 ab	0.69 b	0.84 a	0.75 ab	0.77 ab
PRI	-0.73	-0.69	-0.74	-0.76	-0.70	-0.77	-0.72	-0.73

T1 (CTRL): unfertilizer control; T2 (MIN): synthetic fertilizer control (0.26 g NH₄NO₃ per pot); T3 (PEL): green compost pellets at equivalent N dose to synthetic fertilizer (3.36 g per pot); T4 (PEL2): green compost pellets at double N dose (6.72 g per pot); T5 (PEL/MIN): 50% pellets + 50% synthetic fertilizer; T6 (COM): green compost at equivalent N dose to pellets (3.4 g per pot); T7 (COM/MIN): 50% compost + 50% synthetic fertilizer; T8 (COM/PEL): 50% pellets + 50% compost. TDR = Time Domain Reflectometry; ChlM = leaf chlorophyll content index; FlvM = leaf flavonol contents index; AnthM = leaf anthocyanin contents index; NFI = nitrogen-flavonol index (CHL/FLAV ratio); NDVI = normalized difference vegetation index; PRI = photochemical reflectance index; Different letters indicate significant differences according to Duncan's test ($p \leq 0.05$).

3.4.6 Results of lettuce production and quality surveys at harvest

Data on leaf number, root length, shoot and root biomass, leaf area, color indices, and nitrate content are summarized in **Table 6**. In particular, the highest leaf number was observed in the synthetic fertilizer control (T2) and in treatments with green compost pellets or their combinations (T3, T5). Root length varied among treatments, with the longest roots recorded in T3 and T8. Fresh and dry biomass of aboveground and belowground were generally higher in T3, T4, T5, and T8 compared to the unfertilized control (T1). Fresh and dry biomass of both shoots and roots were generally higher in T3, T4, T5, and T8 compared to the unfertilized control (T1). Specifically, shoot fresh weight increased by 102% in T3, 50% in T4, 97% in T5, and 123% in T8 compared to T1, and by 27–44% compared to the synthetic fertilizer treatment (T2). Root fresh weight showed similar trends, with T4 (+187% vs. T1, +42% vs. T2) and T8 (+123% vs. T1, +11% vs. T2) being the most responsive. Dry biomass of shoots and roots also reflected these patterns, highlighting the beneficial effect of pelletized compost and combined treatments on both above- and belowground growth. Leaf area was greater in combined treatments and in pellet applications (T7, T8). Brightness (L) and color indices (a

and b) differed across treatments, with T7 and T8 showing the higher luminosity values. Nitrate accumulation was higher in synthetic fertilizer treatments (T2, T4), while pelletized compost and compost combinations (T3, T6, T8) showed intermediate values.

Table 6. Results of lettuce production and quality surveys at harvest

Treatments	T1 (CTRL)	T2 (MIN)	T3 (PEL)	T4(PEL2)	T5 (PEL/MIN)	T6 (COM)	T7 (COM/MIN)	T8 (COM/PEL)
TN° leaves (n° pot ⁻¹)	28.00 c	38.67 a	37.67 ab	35.33 ab	39.00 a	35.33 ab	36.67 ab	32.67 bc
Roots Length (cm pot ⁻¹)	14.00	16.33	16.67	14.33	15.00	15.00	13.67	15.67
Aboveground Fresh Weight (g pot ⁻¹)	12.67 c	27.67 a	28.50 a	19.00 b	25.00 a	14.33 bc	17.67 bc	28.33 a
Aboveground Dry Weight (g pot ⁻¹)	0.77 c	1.23 ab	1.40 a	1.35 a	1.43 a	1.13 ab	1.00 bc	1.43 a
Belowground Fresh Weight (g pot ⁻¹)	8.00 cd	11.00 abc	11.50 ab	11.50 ab	8.50 bcd	7.00 d	12.50 a	13.33 a
Belowground Dry Weight, g pot ⁻¹)	0.90 ce	1.33 bc	1.33 bc	2.20 a	1.33 bcd	0.75 e	1.45 b	1.77 b
Leaf Area (cm ² pot ⁻¹)	2174.00 ab	2128.00 ab	1212.00 c	1912.00 b	1229.00 c	2709.00 a	2114.00 ab	836.00 c
L	58.56 a	53.94 b	49.90 b	52.96 b	52.81 b	51.13 b	44.84 c	51.27 b
a	-17.07	-18.09	-16.64	-17.47	-17.15	-17.02	-15.77	18.37
b	32.85 a	29.09 bc	26.85 cd	26.30 cd	31.68 ab	25.00 d	24.56 d	32.24 ab
NO ₃ ⁻ (ppm)	2550.00 c	2783.00 a	2567.00 c	2740.00 a	2343.00 d	2520.00 c	2700.00 ab	2617.00 bc

T1 (CTRL): unfertilizer control; T2 (MIN): synthetic fertilizer control (0.26 g NH₄NO₃ per pot); T3 (PEL): green compost pellets at equivalent N dose to synthetic fertilizer (3.36 g per pot); T4 (PEL2): green compost pellets at double N dose (6.72 g per pot); T5 (PEL/MIN): 50% pellets + 50% synthetic fertilizer; T6 (COM): green compost at equivalent N dose to pellets (3.4 g per pot); T7 (COM/MIN): 50% compost + 50% synthetic fertilizer; T8 (COM/PEL): 50% pellets + 50% compost. L: brightness index; a: green index; b: yellow index; NO₃⁻: nitrates. Different letters indicate significant differences according to Duncan's test ($p \leq 0.05$).

3.4.7 Results of agronomic and non-destructive physiological surveys on radish during the crop cycle

Table 7 reports the physiological and morphological parameters of radish under different treatments. The chlorophyll index was highest in the unfertilized control (T1), while pellet- and compost-based treatments showed reduced values. Flavonol content recorded the highest value

in T4, whereas the NFI index was markedly higher in fertilized treatments. Soil water content decreased under fertilization, with the lowest value in T8. Leaf temperature was reduced in fertilized plants. Morphologically, plant height and collar diameter were enhanced, particularly in T8, confirming the positive effect of the pellet + compost combination. Spectral indices (NDVI and PRI) were also higher in fertilized treatments compared to the unfertilized control, consistent with greater canopy development and higher photosynthetic efficiency.

Table 7. Results of agronomic and non-destructive physiological surveys on radish during the crop cycle

Treatments	T1 (CTRL)	T2 (MIN)	T3 (PEL)	T4(PEL2)	T5 (PEL/MIN)	T6 (COM)	T7 (COM/MIN)	T8 (COM/PEL)
Plant Height (cm)	12.2 ₅ bc	14.5 ₀ a	11.5 cd	10 e	10.3 ₃ de	11.0 ₀ cd e	11.83 bc	12.83 b
Collar diameter (mm)	2.53 ab	2.18 b	1.45 c	2.68 a	2.32 ab	2.04 b	2.15 b	2.36 ab
TDR (% soil water content)	8.20 d	11.3 ₅ d	17.85 c	17.6 ₅ c	28.8 ₅ a	23.5 b	26.7 ab	24.30 ab
ChlM	0.14	0.16	0.17	0.18	0.16	0.16	0.15	0.17
FlvM	0.07 b	0.03 cd	0.03 cde	0.10 a	0.01 de	0.06 b	0.01 e	0.03 c
AnthM	0.16 abc	0.17 ab	0.16 abc	0.19 a	0.13 c	0.15 bc	0.17 ab	0.18 ab
NFI	1.85 de	4.48 _e cd	6.40 c	1.53 e	17.7 ₆ a	3.77 _e cd	10.37 b	5.49 cd
Canopy Temperature (°C)	22.4 ₃ b	23.7 ₀ ab	24.9 a	23.6 ₇ ab	23.8 ₀ a	24.3 ₇ a	24.23 a	23.60 ab
NDVI	0.58 ab	0.59 ab	0.39 c	0.55 _c ab	0.67 a	0.47 bc	0.61 ab	0.71 a
PRI	-0.76 bcd	-0.72 _c ab	-0.80 cd	-0.65 _a	-0.76 bcd	-0.70 ab	-0.85 d	-0.70 ab

T1 (CTRL): unfertilizer control; T2 (MIN): synthetic fertilizer control (0.26 g NH₄NO₃ per pot); T3 (PEL): green compost pellets at equivalent N dose to synthetic fertilizer (3.36 g per pot); T4 (PEL2): green compost pellets at double N dose (6.72 g per pot); T5 (PEL/MIN): 50% pellets + 50% synthetic fertilizer; T6 (COM): green compost at equivalent N dose to pellets (3.4 g per pot); T7 (COM/MIN): 50% compost + 50% synthetic fertilizer; T8 (COM/PEL): 50% pellets + 50% compost. TDR = Time Domain Reflectometry; ChlM = leaf chlorophyll content index; FlvM = leaf flavonol contents index; AnthM = leaf anthocyanin contents index; NFI = nitrogen-flavonol index (CHL/FLAV ratio); NDVI = normalized difference vegetation index; PRI = photochemical reflectance index; Different letters indicate significant differences according to Duncan's test ($p \leq 0.05$).

3.4.8 Results of agronomic and physiological not destructive surveys on radish at harvest

Table 8 reports the physiological and agronomic parameters of radish under the different treatments at harvest. The chlorophyll index was highest in combined applications, with T8 showing the greatest increase, whereas the lowest values were observed in the unfertilized control (T1) and in compost alone (T6). Flavonol content reached its maximum in T5, while anthocyanin levels remained generally low, with minimum values also in T5. The NFI index revealed marked differences among treatments, with a sharp increase in synthetic fertilization. Spectral indices further reflected treatment effects: NDVI reached the highest value in T5, while PRI showed smaller variations, though remaining higher in combined treatments. Morphological traits confirmed these trends: plant height was significantly greater in T8 and T5, whereas collar diameter was larger in T6 and T8. Finally, soil moisture was highest in the unfertilized control, whereas fertilized treatments showed lower values, particularly under synthetic fertilization.

Table 8. Results of agronomic and physiological not destructive surveys on radish during the harvest

Treatments	T1 (CTRL)	T2 (MIN)	T3 (PEL)	T4(PEL2)	T5 (PEL/MIN)	T6 (COM)	T7 (COM/MIN)	T8 (COM/PEL)
Plant Height (cm)	14.00 b	21.67 a	16.67 b	15.00 b	22.33 a	17.00 b	20.33 a	23.67 a
Collar diameter (mm)	5.94 bc	6.32 bc	5.39 c	5.91 bc	5.80 bc	7.65 a	6.44 b	7.59 a
TDR (% soil water content)	22.73 c	16.73 d	23.90 bc	26.50 a	27.17 a	25.45 ab	24.00 bc	24.20 bc
ChlM	0.35 d	0.38 cd	0.37 cd	0.37 cd	0.44 bc	0.36 d	0.47 ab	0.52 a
FlvM	0.16 bc	0.10 d	0.12 cd	0.10 d	0.23 a	0.10 d	0.17 b	0.13 bcd
AnthM	0.04 a	0.03 ab	0.03 ab	0.03 ab	0.01 c	0.04 a	0.02 bc	0.02 bc
NFI	1.33 c	4.80 a	2.69 bc	3.20 b	2.63 bc	2.55 bc	2.82 bc	3.71 ab
Canopy Temperature (°C)	32.55 ab	30.67 e	31.05 de	31.53 cd	32.27 abc	32.87 a	31.97 bc	32.85 a
NDVI	0.46 e	0.59 d	0.48 e	0.74 ab	0.77 a	0.63 cd	0.69 bc	0.73 ab
PRI	-0.75 abc	-0.85 c	-0.84 bc	-0.82 abc	-0.74 abc	-0.71 a	-0.73 ab	-0.79 abc

T1 (CTRL): unfertilizer control; T2 (MIN): synthetic fertilizer control (0.26 g NH₄NO₃ per pot); T3 (PEL): green compost pellets at equivalent N dose to synthetic fertilizer (3.36 g per pot); T4 (PEL2): green compost pellets at double N dose (6.72 g per pot); T5 (PEL/MIN): 50% pellets + 50% synthetic fertilizer; T6 (COM): green compost at equivalent N dose to pellets (3.4 g per

pot); T7 (COM/MIN): 50% compost + 50% synthetic fertilizer; T8 (COM/PEL): 50% pellets + 50% compost. TDR = Time Domain Reflectometry; ChlM = leaf chlorophyll content index; FlvM = leaf flavonol contents index; AnthM = leaf anthocyanin contents index; NFI = nitrogen-flavonol index (CHL/FLAV ratio); NDVI = normalized difference vegetation index; PRI = photochemical reflectance index; Different letters indicate significant differences according to Duncan's test ($p \leq 0.05$).

3.4.9 Results of radish production and quality surveys at harvest

The production and quality of radish were evaluated at harvest by measuring leaf and root traits, including fresh and dry weight of leaves, edible roots, and roots, as well as leaf area, colorimetric parameters of the edible part, and nitrate content. As shown in **Table 9**, Leaf number per pot varied among treatments, with T2 (synthetic fertilizer) showing the highest count compared to the unfertilized control. Root length was significantly increased by T5 and T8 compared to the unfertilized control. Fresh leaf weight ranged from 2.75 g in the unfertilized control to 7.35 g in T8, corresponding to an increase of 167% relative to the unfertilized control and 28% relative to the synthetic fertilizer treatment (T2). Dry leaf weight showed a similar trend, with T5 achieving 0.75 g, +36% over T2 and +36% over the unfertilized control. Fresh edible root weight was markedly enhanced in T8, representing a 553% increase over the unfertilized control and 206% increase over T2, while dry edible root weight peaked in T3 and T5, showing substantial improvement compared to the unfertilized control. Fresh and dry root weights followed a comparable pattern, with T8 and T7 producing the highest values. Leaf area was greatest in T8, approximately 48% higher than the unfertilized control and 57% higher than T2. Colorimetric analysis of the edible part revealed that L* values ranged from 37.94 to 52.74, a* from 10.47 to 23.54, and b* from 5.61 to 10.31, reflecting variations in brightness and chromaticity induced by the different fertilization treatments. Nitrate content was also influenced by treatments, with the highest concentrations observed in T7 and T5, and the lowest in the unfertilized control (350.0 ppm).

Table 9. Results of radish production and quality surveys at harvest.

Treatments	T1 (CTRL)	T2 (MIN)	T3 (PEL)	T4(PEL2)	T5 (PEL/MIN)	T6 (COM)	T7 (COM/MI N)	T8 (COM/PEL)
Number of leaves (n° pot-1)	4.67 ^b _c	6.67 ^a	4.33 ^c	3.50 ^b _c	5.00 ^{bc}	4.67 ^{bc}	5.67 ^{ab}	5.67 ^{ab}
Roots Length (cm pot-1)	12.50 ^d	24.83 ^a _b	21.00 ^c	23.00 ^b	25.17 ^a	23.00 ^b	24.50 ^{ab}	25.67 ^a
Fresh weight of leaves (g pot-1)	2.75 ^c	5.75 ^a _b	4.15 ^{bc}	3.40 ^c	5.85 ^{ab}	5.85 ^{ab}	3.50 ^c	7.35 ^a
Dry weight of leaves (g pot-1)	0.55 ^a _b	0.70 ^a	0.20 ^c	0.23 ^c	0.75 ^a	0.70 ^a	0.60 ^{ab}	0.45 ^b
Fresh weight of edible roots (g pot-1)	1.50 ^b	3.20 ^b	2.40 ^b	4.29 ^b	4.80 ^b	1.75 ^b	0.90 ^b	9.80 ^a
Dry weight of edible roots (g pot-1)	0.27 ^c _d	0.37 ^b _c	0.60 ^a	0.10 ^d	0.65 ^a	0.13 ^d	0.50 ^{ab}	0.10 ^d
Fresh weight of roots (g pot-1)	2.50 ^c	2.90 ^c	4.70 ^b	4.05 ^b _c	6.77 ^a	6.45 ^a	7.15 ^a	7.63 ^a
Dry weight of roots (g pot-1)	0.30 ^d	0.37 ^d	0.75 ^{bc} _d	0.50 ^c _d	0.97 ^{ab} _c	1.25 ^a	1.43 ^a	1.10 ^{ab}
Leaf Area (cm ² pot-1)	231.20 ^b	218.20 ^b _c	119.40 ^c	172.50 ^b _c	273.50 ^{ab}	200.30 ^{bc}	194.20 ^{bc}	343.30 ^a
L	39.81 ^b	43.63 ^b	39.11 ^b	52.74 ^a	37.94 ^b	40.58 ^b	40.39 ^b	44.03 ^b
a	10.47 ^d	23.26 ^a _b	23.54 ^a	12.15 ^c _d	21.34 ^{ab}	16.83 ^{bc} _d	18.90 ^{ab}	17.68 ^{abc}
b	7.72 ^c _d	8.82 ^b _c	9.66 ^{ab}	10.31 ^a	6.79 ^d	7.79 ^{cd}	5.61 ^e	7.69 ^{cd}
NO ₃ - (ppm)	350.00 ^c	543.30 ^a	416.70 ^b	460.00 ^b	593.30 ^a	543.30 ^a	606.70 ^a	563.30 ^a

T1 (CTRL): unfertilizer control; T2 (MIN): synthetic fertilizer control (0.26 g NH₄NO₃ per pot); T3 (PEL): green compost pellets at equivalent N dose to synthetic fertilizer (3.36 g per pot); T4 (PEL2): green compost pellets at double N dose (6.72 g per pot); T5 (PEL/MIN): 50% pellets + 50% synthetic fertilizer; T6 (COM): green compost at equivalent N dose to pellets (3.4 g per pot); T7 (COM/MIN): 50% compost + 50% synthetic fertilizer; T8 (COM/PEL): 50% pellets + 50% compost. L: brightness index; a: green index; b: yellow index; NO₃-: nitrates. Different letters indicate significant differences according to Duncan's test ($p \leq 0.05$).

3.4.10 Agronomic evaluation of treatments

The present study demonstrated that the application of pelletized compost, either alone or in combination with synthetic fertilizer or raw compost, significantly influenced physiological, agronomic, and productive parameters of both baby leaf lettuce (*Lactuca sativa* L.) and radish (*Raphanus sativus* L.) throughout the crop cycle and at harvest. However, the responses varied between the two species, highlighting the crop-specific physiological and morphological adaptations to nutrient availability and soil conditions. During the crop cycle, lettuce plants treated with compost pellets (T3, T4) or combinations of pellets with compost/synthetic fertilizer (T5, T8) exhibited greater plant height and collar diameter compared to the unfertilized controls. These treatments were also associated with higher NDVI and PRI values, reflecting enhanced vegetative vigor and improved nitrogen use efficiency. Such results are consistent with previous evidence showing that pelletized organic amendments not only supply readily available nitrogen but also improve substrate structure and water availability (**Diacono & Montemurro, 2011**). Chlorophyll content (ChlM) and the Nitrogen Fluorescence Index (NFI) were also higher in pellet-based treatments, indicating improved nutritional status and photosynthetic activity. It is well documented that organic amendments stimulate photosynthesis by providing a slow-release nitrogen source and enhancing rhizosphere microbial activity (**Ayangbenro et al., 2022**). Conversely, treatments with raw compost (T6) favored flavonol accumulation, reflecting enhanced secondary metabolism likely induced by mild nutrient limitation, as reported for leafy vegetables under moderate stress (**Machado et al., 2025**). At harvest, the positive impact of pelletized compost was confirmed by yield data. Shoot fresh biomass increased by 102–123% compared with the unfertilized control and by 27–44% compared with synthetic fertilization, with the highest values in T3 and T8. Root biomass followed a similar trend, with increases of up to +187% (T4 vs. unfertilized control).

These results are in agreement with **Hernández et al. 2015**, who found that green compost significantly enhanced lettuce biomass compared to synthetic fertilization alone. Nitrate accumulation was markedly higher in synthetic fertilizer treatments (T2, T4), while pelletized compost and combined applications (T3, T5, T8) resulted in intermediate values, suggesting more gradual nitrogen release. The observed reduction in nitrate accumulation in lettuce treated with compost and pelletized amendments may be attributed to enhanced nitrate retention and gradual release in the soil. Organic matter and colloidal components of the amendments likely increase nutrient adsorption and promote microbial immobilization, thereby limiting excessive nitrate uptake by plant tissues and contributing to safer and more balanced nitrogen availability (**Verrillo et al., 2021**). This aspect is of particular importance, as lettuce is among the vegetable crops most prone to nitrate accumulation, with direct implications for food safety (**Santamaria, 2006**). The integration of organic and synthetic fertilization therefore appears to be an effective strategy to sustain yields while reducing nitrate accumulation in leaves. The chemical and molecular characterization of the compost and pelletized form supports these agronomic outcomes. The presence of residual labile carbohydrates and oxygen-containing functional groups, as revealed by ^{13}C CPMAS NMR and ATR FT-IR analyses, provides microbially available substrates that can sustain nutrient cycling in the soil. Moreover, lignin- and aromatic-rich components ensure long-term structural stability of the soil organic matter, potentially enhancing water retention and cation exchange capacity. The slightly higher stability of the pellet compared to the bulk compost may explain the more sustained release of nutrients and the consistent improvements in plant growth observed in T3 and T8 (**Maffia et al 2024b**).

In radish, the impact of fertilization was equally evident but more complex, as the edible organ is the storage root. During the crop cycle, combined applications of pelletized compost with

synthetic fertilizer or raw compost (T5, T8) promoted higher plant height and collar diameter compared to the controls. NDVI and PRI indices confirmed improved canopy development and higher photosynthetic efficiency in fertilized treatments (**Hnizil et al., 2024**). At harvest, the response in root yield was particularly striking. Fresh edible root weight in T8 was 553% higher than in the unfertilized control and 206% higher than in synthetic fertilization (T2). Leaf biomass also increased substantially, with T8 showing a 167% increase compared with the unfertilized control. These results support those of **Almaramah et al. (2024)** who reported significant yield improvements in radish following compost amendment. The structural composition of the compost, combining labile carbohydrate fractions with stable lignin and alkyl residues, likely contributed to the enhanced root development in radish. Such a dual role, providing both immediate nutrient availability and long-term organic matter stabilization, can explain the substantial yield increases observed in T8, which received the combined application of pellets with synthetic or raw compost (**Pizzanelli, S., et al., 2023c**).

Quality traits were also affected. Colorimetric parameters (L^* , a^* , b^*) of the edible root varied across treatments, with pellet and combined applications enhancing brightness and chromatic uniformity, traits that improve marketability. Nitrate content was highest in synthetic and combined treatments (T5, T7), though still lower than the levels observed in lettuce. This indicates that radish accumulates nitrates less readily, yet fertilization practices should still be optimized to avoid excessive accumulation. Comparing the two crops, lettuce appeared more sensitive to nitrogen form and more prone to nitrate accumulation, whereas radish benefited more strongly in terms of yield from pelletized compost, especially when combined with synthetic or raw compost. Nevertheless, in both crops, pelletized compost proved to be a valuable tool to enhance growth, improve yield, and limit nitrate accumulation compared to synthetic fertilization alone. Importantly, although nitrate concentrations were higher in some

treatments, particularly in lettuce, the values observed in this study remained within the legal thresholds established by European Commission Regulation (EC) No 1258/2011 for leafy vegetables, thus ensuring product safety and compliance with food quality standards (**Di Serio et al., 2025**).

Overall, these findings highlight the potential of pelletized compost as an effective agronomic strategy to reconcile productivity with sustainability. The observed increases in yield and vegetative vigor in both crops, together with the more balanced nitrate content, demonstrate the ability of pelletized compost to enhance nutrient use efficiency and product quality while reducing reliance on synthetic fertilizers. The integration of stabilized and easily manageable organic amendments can therefore contribute to the transition toward more circular and resilient agricultural systems, capable of valorizing organic residues and preserving long-term soil fertility.

3.5 CONCLUSIONS

The use of green compost pellets, either alone or in combination with synthetic fertilizers or raw compost, significantly improved plant growth, physiological performance, and yield in both lettuce and radish. Lettuce exhibited a higher sensitivity to nitrogen form and a greater tendency to accumulate nitrates, although concentrations remained within legal safety limits, while radish showed a stronger response in terms of edible root development.

From a compositional and molecular perspective, both the compost and its pelletized form displayed a balanced nutrient profile, rich in essential macro- and micronutrients, and characterized by the coexistence of labile and recalcitrant organic fractions. **The analysis of the initial feedstocks (escarole, red chicory, and spinach residues) revealed high contents of O-alkyl and carboxylic functional groups, which were progressively transformed**

during composting into more stable aromatic and carboxylated structures. Spectroscopic evidence confirmed this molecular evolution, highlighting a compost matrix that integrates bioavailable aliphatic moieties with persistent carbon-rich domains, features that underpin its dual agronomic function of immediate nutrient supply and gradual carbon release. The combined use of compost and its pelletized counterpart proved particularly effective, as it integrated the biological activity and nutrient richness of compost with the higher stability, handling efficiency, and controlled-release properties of pellets. This synergy maximized nutrient-use efficiency and enhanced both short-term productivity and long-term soil quality. **Overall, linking the molecular characteristics of the original plant residues to those of the mature compost provided mechanistic insight into the material's functionality.** Green compost pellets thus represent an innovative and sustainable agronomic strategy capable of enhancing vegetable production, improving product quality, and reducing environmental impacts associated with conventional mineral fertilization.

Data availability

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request.

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Supporting Information

Supporting Table

Supporting Table S1. List of compounds identified in the GC-MS pyrograms of Green compost, including compound class compound name, molecular weight (MW), the most intense and diagnostic signals and the retention time. The labels FA, FAME, P, G, and S correspond to fatty acid, fatty acid methyl ester, *p*-hydroxyphenyl, guaiacyl and syringyl monomeric lignin units, respectively, whereas the symbol >> specifies that the specific mass signal is predominant in the mass spectrum).

Compounds identified in GC-MS pyrograms of Green compost	Compound name	MW	Intense and diagnostic signals			
				RT	ABS	
BRANCHED FA	METHYL 5,9 DI METHYL DECANOATO	214	214, 87, 74, 171	17.314	0.0076	
BRANCHED FA	TETRADECANOIC ACID, 10,13-DIMETHYL-, METHYL ESTER	222	74, 87, 270,227, 143, 129	27.49	0.0091	
BRANCHED FA	TETRADECANOIC ACID, 10,13-DIMETHYL-, METHYL ESTER	222	74, 87, 129, 143, 270	28.587	0.0583	
BRANCHED FA	HEXADECANOIC ACID, 14-METHYL-, METHYL ESTER	284	74, 87, 143, 241, 284	30.44	0.0063	
BRANCHED FA	HEXADECANOIC ACID, 14-METHYL-, METHYL ESTER	284	74, 87, 143, 241, 284	30.638	0.0198	
BRANCHED FA	HEXADECANOIC ACID, 15-METHYL-, METHYL ESTER	284	74, 87, 143, 241, 284	31.6	0.0029	
BRANCHED FA	HEPTADECANOIC ACID, 16-METHYL-, METHYL ESTER	298	74, 87 255, 298, 143		0.0000	
BRANCHED FA MONO	11-HEXADECENOIC ACID, 15-METHYL-, METHYL ESTER	282	69, 97, 110, 152, 208, 225, 250, 130.961		0.0024	
TOT			BRANCHED FA		0.1063	± 0.01916
DIOIC	HEXADECANEDIOIC ACID, DIMETHYL ESTER			-	-	
DIOIC	HEXADECANEDIOIC ACID, DIMETHYL ESTER	314	74, 84, 98, 241, 283, 314	-	-	
TOT			DIOIC		-	
FA DI-UNS.	9,11-OCTADECADIENOIC ACID, METHYL ESTER, (E,E)-	294	67, 81, 95, 294, 263, 150		0	
FA DI-UNS.	10,13-EICOSADIENOIC ACID, METHYL ESTER	322	67, 81, 95, 291, 322	35.24	0.041084606	
FA MONO	8-OCTADECENOIC ACID, METHYL ESTER, (E)-			32.734	0.011747044	
FA MONO	8-OCTADECENOIC ACID, METHYL ESTER, (E)-	296	55, 74, 97, 111, 180, 222, 364, 296		0	
FA MONO	9-OCTADECENOIC ACID, METHYL ESTER, (E)-	296	55, 74, 97, 111, 180, 222, 364, 296		0	
FA MONO	9-OCTADECENOIC ACID, METHYL ESTER, (E)-	296	55, 74, 97, 111, 180, 222, 364, 253.941		0.042389069	
TOT			FA MONO E DI-UNS.		0.095220719	± 0.02055
FAME	METHYL TETRADECANOATE	242	74, 87, 242, 211	22.932	0.005887843	
FAME	PENTADECANOIC ACID, METHYL ESTER	256	74, 87, 256, 213	24.683	0.007975925	
TOT			FAME		0.013863768	± 0.00148
LIGNIN - G	PHENOL, 3,4-DIMETHOXY-	154	154, 139, 111, 93	12.155	0.034068809	
LIGNIN - G	PHENOL, 2,6-DIMETHOXY-	154	154, 139, 111, 93	12.529	0.008332545	
LIGNIN - G	BENZENE, 4-ETHENYL-1,2-DIMETHOXY-	164	164,149, 121, 103, 77	12.9	0.020440965	
LIGNIN - G	1,2,4-TRIMETHOXYBENZENE	168	168, 153, 125	15.16	0.003304691	
LIGNIN - G	PHENOL, 2-METHOXY-4-(1-PROPENYL)-, (E)-	164	164,149, 131, 77	15.359	0.013519847	
LIGNIN - G	BENZALDEHYDE, 3,4-DIMETHOXY- + ALCANO	166	166, 95, 77	16.203	0.02359893	
LIGNIN - G	BENZENE, 1,2-DIMETHOXY-4-(1-PROPENYL)-	178	178, 163, 107, 91, 77		0	
LIGNIN - G	BENZOIC ACID, 4-HYDROXY-3-METHOXY-, METHYL ESTER	182	182, 151		0	
LIGNIN - G	HOMOVANILLYL ALCOHOL	168	137>> 168,94,77		0	
LIGNIN - G	ETHANONE, 1-(3,4-DIMETHOXYPHENYL)-	180	165, 180, 122, 77	18.54	0.015436869	
LIGNIN - G	BENZENE, 4-BUTYL-1,2-DIMETHOXY-	194	151>>, 194, 77		0	
LIGNIN - G	BENZOIC ACID, 3,4-DIMETHOXY-, METHYL ESTER	196	196, 165, 79	19.248	0.075944928	
LIGNIN - G	PHthalide, 4,6-DIMETHOXY-	194	165, 194, 77	21.138	0.008470851	
LIGNIN - G	2-PROPENOIC ACID, 3-(2,3-DIMETHOXYPHENYL)-, (E)-	208	208, 177, 77	23.013	0.01211674	
LIGNIN - G	2-PROPENOIC ACID, 3-(3,4-DIMETHOXYPHENYL)-, METHYL ESTER	222	222, 191, 151, 119, 91, 77	24.378	0.007404531	
LIGNIN - G	2-PROPENOIC ACID, 3-(3,4-DIMETHOXYPHENYL)-, METHYL ESTER	222	222, 191, 207, 77, 109	27.369	0.008248639	
LIGNIN - G	BENZENE, 4-BUTYL-1,2-DIMETHOXY-	194	151>>, 194, 107, 77		0	
TOT			LIGNIN - G		0.230888345	± 0.01873
LIGNIN - P	1,4-BENZENEDICARBOXYLIC ACID, DIMETHYL ESTER	194	163, 194, 135, 103		0	
LIGNIN - P	BENZENEPROPANOIC ACID, 4-METHOXY-, METHYL ESTER	194	121>>,194, 134,77		0	
LIGNIN - P	2-PROPENOIC ACID, 3-(4-METHOXYPHENYL)-, METHYL ESTER	192	161, 192, 133, 118, 89, 77		0	
LIGNIN - P	BENZOIC ACID, 4 (TRIMETHYLSILYL)OXY, METHYL ESTER	224	224, 209, 181, 165	24.253	0.006701213	
TOT			LIGNIN - P		0.006701213	± 0.00335
LIGNIN - S	2',3',4' TRIMETHOXYACETOPHENONE	210	195, 210, 152, 77	21.567	0.009028656	
LIGNIN - S	BENZOIC ACID, 3,4,5-TRIMETHOXY-, METHYL ESTER	226	226, 211, 195, 183, 155	22.72	0.047874543	
LIGNIN - S	2,3,4,6-TETRAMETHOXYSTYRENE	224	224, 209, 151	24.837	0.009089547	
LIGNIN - S	1,2,3 trimethoxybenzene	168	168, 153,110	11.33	0.019455243	
LIGNIN - S	1,2,4-TRIMETHOXYBENZENE	168	168, 153, 125, 110	13.014	0.014176678	
LIGNIN - S	BENZENE, 1,2,3-TRIMETHOXY-5-METHYL-	182	182, 167, 139, 107	13.81	0.018708157	
LIGNIN - S	PHENOL, 2,6-DIMETHOXY-4-(2-PROPENYL)-	194	194, 179, 151, 119, 77		0	
LIGNIN - S	BENZENE, 1,2,3-TRIMETHOXY-5-(2-PROPENYL)-	208	208, 193, 133, 105, 79	20.789	0.011767659	
TOT			LIGNIN - S		0.130100482	± 0.01418

Chapter IV

Original research paper

“Sustainable valorization of waste horticultural biomasses to develop a novel hydrochar to improve soil health and modulate the lettuce metabolism”

Rossella Curcio^{1,2}, Annamaria Di Serio³, Andrea Sica², Silvana Cangemi⁴, Riccardo Spaccini⁴, Diana Agrelli⁴, Patrizia Iannece³, Domenico Ronga², Pierluigi Mazzei²

¹*DAFE - Department of Agricultural, Forest, Food, and Environmental Sciences, University of Basilicata, 10, Viale dell'Ateneo Lucano - Potenza (PZ)*

²*Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA Department of Chemistry and Biology*

³*Department of Chemistry and Biology, University of Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Italy*

⁴*Department of Agriculture, University of Naples Federico II, Via università, 100, 80055, Portici, Italy*

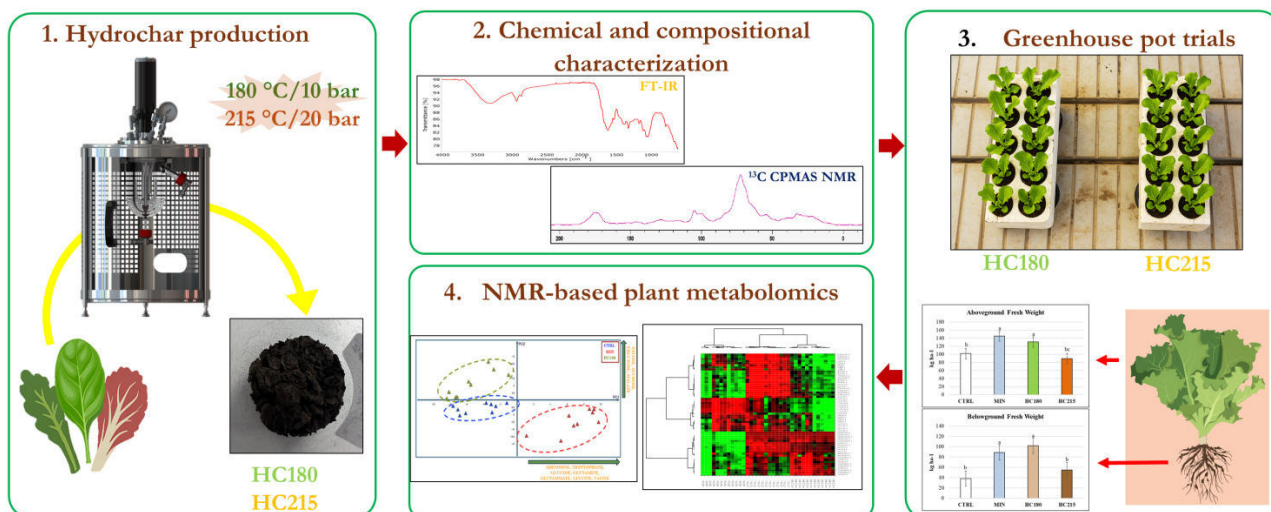
*** Corresponding author:** Pierluigi Mazzei, orcid.org/0000-0002-5312-4969, pmazzei@unisa.it

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4.1 ABSTRACT

Hydrothermal carbonization (HTC) represents an innovative and sustainable approach exploiting sub-critical water and useful to convert waste biomass into a novel solid material with a huge potential for soil and plant health. Two hydrochar types were produced, under mild (180 °C, 10 Bar) and severe (215 °C, 20 Bar) HTC conditions (HC180 and HC215, respectively), by processing spinach, red chicory, and escarole wastes resulting from industrial horticultural production. These hydrochars were characterized for elemental composition, mineral content, and molecular composition via advanced techniques (¹³C CPMAS NMR and FT-ATIR) and compared with initial biomass types. HTC progressively converted labile, carbohydrate-rich residues into carbon-dense, aromatic materials, with the greater severity reducing nutrient availability. Solid hydrochars retained up to 2.82 g of water per g of material, thus representing a tool to make soil more resilient against the drought effects related to climatic changes. Greenhouse pot experiments on baby-leaf lettuce revealed that HC180 strongly promoted plant shoot and root growth, matching conventional mineral fertilization, while HC215 showing only limited short-term effects. ¹H NMR metabolomics indicated that the most performing hydrochar stimulated the lettuce primary metabolism by increasing sugars and amino acids linked to energy production and stress responses, highlighting a biostimulant effect mediated by enhanced nutrient availability and soil-microbiome interactions. These findings demonstrate that mild HTC conditions produce hydrochars with rapid biostimulant activity (characterized by a balanced composition of labile carbon and nitrogen), partially preserve active functional groups, and supply a spectrum of plant-available nutrients, offering a tunable strategy for sustainable management of horticultural residue.

Keywords: *biomass recycle; sub-critical water; Hydrothermal carbonization; plant biostimulants; NMR-based metabolomics; degraded soils restoration.*



Graphical Abstract

4.2 INTRODUCTION

Nowadays, we are forced to face urgent environmental challenges, including the degradation and overexploitation of fertile soils, the water scarcity, the disposal of waste biomass and the costs for soil fertilization to produce food. About the soils, one of the most critical issues is represented by the widespread decline in the soil organic matter (SOM), undermining ecosystem services, plant productivity and microbial vitality (Ye et al., 2023). In Mediterranean regions, long-standing land use and intensive cropping practices have accelerated SOM mineralization, leaving many soils with less than 2% organic carbon in the topsoil, with adverse effects such as vulnerability to erosion, nutrient leaching, and structural collapse. The problem is especially severe in horticultural production districts which are involved in the continuous growth of short-cycle crops and provide only minimal compensation of organic residues. For example, the Sele Plain in the Southern Italy hosts the second european productive site for the production of baby-leaf vegetables for the fresh-cut market, where horticultural products are cultivated continuously under green-house with the production generating large amounts of waste biomass (Zhang et al., 2023). These residues

are highly perishable, developing a large microbial load and posing considerable logistical and environmental challenges for their storage, management, and disposal. Only few of these productive sites are equipped with an in-site composting plant developing solid compost for soil, while most of the other ones leave the vegetable wastes stored in special sites to rot (**Zhang, et al., 2023**). However, the composting process has important drawbacks such as relatively long maturation times (4-6 months), relevant emission of carbon dioxide and other volatiles, risk of pathogen development, and significant carbon loss through microbial respiration (**Li, et al., 2020; Ayilara et al., 2020; Ye et al., 2023**). These limitations highlight the need to invest on more efficient and sustainable processes and technologies capable of convert organic residues into useful products for soil health and agriculture.

Hydrothermal carbonization (HTC) represents a promising strategy for converting fresh, high-moisture biomass into hydrochar (HC), a carbon-rich material with a high environmental, energetic and agronomic potential (**Bento et al., 2021; Islam et al., 2021**). The process takes place in a closed reactor where the biomass is put in contact with water and heated at temperatures higher than 160 °C, leading to the generation of autogenous pressure, due to water vaporization, and determining the conditions for the subcritical water. In this state, the physicochemical properties of water (e.g., dielectric constant, ionic product, and solvent polarity) change significantly, enhancing its ability to act simultaneously as reactant, solvent, and catalyst. This unique environment promotes hydrolysis, dehydration, decarboxylation, and condensation reactions that progressively transform the organic matrix into a more stabilized and carbon-rich material detaining new physicochemical properties (**Yu et al., 2022; Pauline et al., 2020, Knappe, 2018**). HTC can be very advantageous because allows the direct processing of wet biomass without the need for energy-intensive drying, employs water as the reaction medium rather than organic solvents, operates at relatively mild

temperatures (180-250 °C), and ensures high carbon recovery, making it an energy-efficient and environmentally sustainable valorization route (Xu et al., 2021; Kumar et al., 2020). The agronomic effectiveness of solid HC derives from factors such as a large number of terminal oxygenated functional groups, high organic matter content, chemical stability, which, collectively, exert a determinant role in improving soil structure, increasing soil water and nutrients retention, releasing nutrients through a slow release, promoting plant and soil microbial activity (Zhang et al., 2020; Bento et al., 2021). According to Shanmugam and co-workers (2025), the extent and nature of HC agronomic benefits are strongly determined by the operational parameters applied during the HTC process (e.g., reaction temperature, system pressure, residence time, biomass-to-water ratio), which ultimately define both the yield and the chemical and physical characteristics of the final product. Moreover, the intrinsic composition of the feedstock (e.g., lignin, cellulose, protein content) plays a key role in governing the structure, stability, and reactivity of the resulting HC (Khoo et al., 2020; Afolabi et al., 2020; Islam et al., 2021). Therefore, a comprehensive characterization of feedstock and resulting HC is essential to delve into the reaction dynamics and the structural changes occurring during HTC. Advanced spectroscopic techniques, such as solid-state CPMAS-NMR and ATR-FT-IR, have proven particularly effective in unraveling the effects of HTC on biomass composition, identifying the molecular structure and finding useful correlations with its abilities as soil improver. In this context, Bento and co-workers (2021) have exploited CPMAS NMR to inform on structural changes induced in several HC lines, which were produced via different HTC conditions and were proved to exert a role as plant biostimulant and fertilizer. Similarly, Lin et al. (2023) have employed FT-IR spectroscopy to demonstrate that glucose-derived HC is enriched in terminal oxidated functional groups and aromatic structures, suggesting high polarity and chemical reactivity, which make it suitable

for further activation and functionalization. At the soil–plant system level, HC has been shown to enhance nutrient uptake, stimulate root development, and modulate physiological and metabolic processes through the gradual release of bioactive molecules (**Fregolente et al., 2021; Bargmann et al., 2014; de Jager et al., 2021**). Cow manure- and reed straw-derived HCs applied to acidified soils have improved lettuce biomass up to reach an increase of 18.7% with an extent depending on feedstock characteristics and soil pH (**Yin et al., 2022**). In another study, spent grain-based HC composites improved substrate quality and stimulated the germination and growth of *Brassica rapa*, further supporting the potential of hydrochar as a multifunctional amendment for leafy vegetable cultivation (**Miao et al., 2024**). Moreover, recent studies suggest that HC can also modulate primary metabolic processes in plants. In **Lang et al. (2023)** the authors have shown that HC amendments enhance soil microbial metabolism, including carbohydrate and amino acid metabolism, while **Lima** and coworkers (**2024**) have tested liquid and solid hydrochar from aboveground corn biomass on *Lactuca sativa* seeds, proving that molecules such as linear and aromatic carboxylic acids and amino acids were implicated in auxin-like bioactivity.

Therefore, in the present study, two HCs were developed from ready-to-eat fresh-cut vegetable residues (spinach, escarole, red chicory) under two different HTC conditions and comprehensively characterized in terms of yield, elemental composition (CHNS analysis and ICP–MS), and structural features (FT–IR spectroscopy and solid-state ^{13}C CPMAS NMR). The same analytical approach was applied to characterize also the feedstock biomasses used for HC production, enabling a direct comparison between the raw materials and the corresponding HCs. The hydrochars potential as soil fertilizer and as solution to restore degraded soil was then assessed in greenhouse experiments with polystyrene seed trays, by

using baby lettuce as model plant. Finally, the HC effects on the primary metabolism were investigated using ^1H NMR-based metabolomics.

4.3 MATERIALS AND METHODS

4.3.1 *Hydrochar production*

Solid hydrochar (HC) was developed via HTC by processing waste biomass (red chicory, spinach, and escarole) deriving from the production of ready-to-eat fresh-cut vegetables and supplied by the Agricultural Producers Organization "TERRAMORE" (Eboli, Italy) sited in the Sele Plain. HTC was performed by treating fresh and solid biomass with subcritical water by means of a system based on a 1-liter hydrothermal reactor (Buchi-Glass; **Supporting Figure S1**). The latter one was equipped with a cyclone (Buchi-Glass), to maintain suspended solid material during the HTC, and coupled to an external digital unit (HUBER CC-315B), determining a time-dependent modulation of the temperature gradients and guaranteeing thermal stability during the reaction. For the HTC, 50 g of each of the 3 studied biomass types were reduced to fragments smaller than 2 mm and then loaded into the reactor with 300 mL of water (1:2 m:V ratio). Two HTC reactions were performed (in duplicate) at the conditions of either 180 °C/10 bar or 215 °C/20 bar. In detail, the temperature program consisted of 3 min at 25°C, 80 min to rise the temperature from 25 to 180 or 215 °C (depending on the reaction set up), 60 min at the stable set temperature (180 or 215 °C), followed by 80 min to allow the decrease of the temperature at 25°C. The solid phases obtained after the reactions carried out at either 180 (HC180) or 215 (HC215) °C was separated by the liquid fraction and washed with water (300 mL of water) for three times. Details on the methods adopted to evaluate reaction yields, water retention and elemental content in solid HC are reported in **Supporting Paragraphs S1 and S2**.

4.3.2 Solid-state CPMAS NMR and FT-IR spectroscopies

The molecular composition of spinach, red chicory, escarole biomass types as well as those of HC180 and HC215 samples was characterized in the solid state via the Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (CPMAS NMR) and the Attenuated Total Reflectance Fourier Transform Infrared (FT-IR) spectroscopies. ^{13}C CPMAS NMR spectra were acquired with a Bruker Avance 300 MHz wide-bore magnet (Bruker Biospin, Rheinstetten, Germany), equipped with a CPMAS probe, operating at 25 °C, with ^{13}C and ^1H frequencies of 75.47 and 300.13 MHz, respectively. Finely powdered solid samples (60–80 mg), previously sieved to 0.5 mm, were placed into 4 mm zirconia rotors, sealed with Kel-F caps, and spun at $10,000 \pm 1$ Hz. The ^{13}C NMR spectra were obtained through the CPMAS experiment, with a setup including 1 ms of contact time, 1814 time domain points, a spectral width of 300 ppm (22,727.3 Hz), a recycle delay of 5 s, 8 dummy scans, and 8000 scans. Proton decoupling was performed using the TPPM15 (time proportional phase modulation) sequence. NMR free induction decays (FIDs) were processed with Bruker Tospin (v 4.0.1) software, Fourier transformed using 2-fold zero-filling and an exponential filter function of 250 Hz, followed by phase- and baseline-correction. Each ^{13}C CPMAS NMR spectrum was divided into specific resonance regions, whose areas were integrated and expressed both in absolute values (normalized per 100 mg of sample) and as percent of the total spectral range: 0–45 ppm (alkyl-C), 45–60 ppm (methoxyl-C and N-alkyl-C), 60–110 ppm (O-alkyl-C), 110–145 ppm (aryl-C), 145–160 ppm (phenol-C), 160–190 ppm (carboxyl-C), and 190–230 ppm (Carbonyl-C). Important indexes, such as the hydrophobicity index (1), the lignin ratio (2) and the Alk/O-Alk ratio (3) were calculated based on specific ^{13}C NMR intervals, according to the following equations (Monda et al., 2017; de Aquino et al., 2019):

$$HB = \frac{[(0 - 45) + (45 - 60) + (110 - 145) + (145 - 160)]}{[(45 - 60) + (61 - 90) + (90 - 110) + (160 - 190)]}$$

$$LR = \frac{(45 - 60)}{(145 - 160)}$$

$$Alk/O-Alk\ Ratio = \frac{(0 - 45)}{[(61 - 90) + (90 - 110)]}$$

ATR FT-IR analyses were carried out examining dried and sieved samples (≤ 0.5 mm). Analyses were performed in duplicate using a PERKIN ELMER Frontier spectrometer, with 96 scans per sample, a spectral range included within 4000 and 600 cm^{-1} , and a resolution of 16 cm^{-1} . For each acquisition set, a background spectrum was recorded and subtracted. Each spectrum was converted into absorbance mode and baseline corrected to ensure consistency and enable an accurate comparison among samples. All IR spectra were processed with *Spectragryph* software (v. 1.2.16.1).

4.3.3 NMR-based metabolomics

The bioactivity of solid hydrochars on lettuce plant was assessed via green-house pot experiments with lettuce. Details on the method are reported in **Supporting Paragraph S3**. For NMR-based metabolomics, fresh lettuce samples were reduced to fine powder with mortar and pestle under liquid nitrogen. The extraction was conducted by putting in contact 150 mg of powdered material with 1.6 mL of a solution composed by ultrapure water and methanol (50% V:V). The suspension was then ultrasonicated (10 min at 22 °C) and stirred on a linear shaker (10 min at 350 rpm). The supernatant was collected, after centrifugation (10 min, 13'000 rpm at 4°C), and evaporated to dryness using a rotary evaporator at 50 °C and at 130 rpm. The solid residue was solubilized again with 760 μL of an aqueous buffer prepared with deuterated water (78% $\text{D}_2\text{O}:\text{H}_2\text{O}$, V:V, Eurisotop, 99% D_2O), brought at pH 6 (0.05 M phosphate buffer) and

added with the internal standard 3-Trimethylsilylpropionic Acid (TMSPA) up to attain a final concentration of 0.3 mg/mL. The sample was then transferred into 5 mm high-precision NMR tubes, degassed and finally loaded into the NMR magnet. 10 extractions and acquisitions were performed per each treatment. The identification of the primary metabolome was carried out by assigning most of the detected proton signals through the use of a composite approach, based on (i) the interpretation of 1D and homo- and hetero-correlated 2D NMR spectra of representative lettuce extracts, (ii) the spectral examination of the detected proton signals, (iii) the evaluation of inherent literature (**Lanzotti et al., 2022; Chadwick et al., 2024; Sobolev et al., 2005; Feizi et al., 2020**) and (iv) the comparison of the signals detected in the extracts spectra with those of certified standards. Two-dimensional homo- and hetero-nuclear NMR spectra were acquired using a 500 MHz Bruker spectrometer (11.75 T, Bruker Oxford/Spectrospin Cryomagnet) equipped with a BBO probe. The 2D experiments included ^1H - ^1H COSY, TOCSY (mixing time: 0.08 s), and NOESY (mixing time: 0.8 s), as well as ^1H - ^{13}C HSQC (optimized for $J_{\text{CH}} = 145$ Hz) and HMBC (long-range $J_{\text{CH}} = 8$ Hz). Acquisition parameters for all 2D spectra included: 16 dummy scans, 48 scans, spectral widths of 16 ppm (^1H , 6410.3 Hz) and 300 ppm (^{13}C , 30186.8 Hz), with 2048 points in F2 and 256 increments in F1. Details on the method adopted to integrate ^1H 1D NMR spectra and exploit the data for chemometrics (PCA and Heatmap) are reported in the **Supporting Paragraph S4**.

4.4 RESULTS AND DISCUSSION

4.4.1 Solid yield and water retention

The extent of solid yield resulting from the HTC reaction depends on a number of factors, including biomass type and amount, HTC conditions, and reaction residence time. In particular, higher HTC severity is expected to decrease the solid yield, due to a partial translocation of carbon, nitrogen and other components into the liquid and gaseous phases (**Ekpo et al., 2016**).

The yields of HC180 and HC215 solid hydrochars are shown in **Supporting Fig. S2a**. The HC formation was associated to a substantial decrease in mass as compared to the initial dry biomass, with a loss even higher than the 75%. This resulted from the alternation of processes, including the partial decomposition of organic matter followed by its migration into the liquid phase, the release of volatiles, the partial extraction of soluble organic and inorganic components. The reaction conducted for 1 hour at 180 °C was accompanied by an autogenic pressure of 10 Bar and retained the largest amount of biomass, which corresponded to 24.7 +/- 0.78%. Conversely, the HTC performed for 1 hour at 215°C and 20 Bar led to a much lower yield (17.25 +/- 0.23 %) (**Supporting Fig. S2a**). Therefore, the combination of higher temperatures and pressures determined a more severe alteration and degradation of the feedstock. At the same time, the extraction extent was fostered by the more pronounced sub-critical character reached by the water (**Wang et al., 2018; Knappe et al., 2018; Cao et al., 2019**). It is important to underline that the examined HTC conditions provided a yield which was never lower than 17%, thus suggesting this as a relatively efficient process to develop a satisfying amount of solid material.

The water retention test (**Supporting Fig. S2b**) revealed that HC180 and HC215 adsorbed 2.82 and 2.43 grams of water per gram of hydrochar, respectively. They represents relevant amounts of water and suggest that by supplying soil with these materials the amount of retained and bioavailable water can be improved, thereby making the soil more resilient to drought effects due to climate change. Such a strategy may be particularly efficient for degraded sandy soils undergoing desertification and characterized by a typical poor organic matter content.

4.4.2 *Elemental analyses*

CHNS elemental analysis and ICP-MS techniques were applied to elucidate the content in nutrients and several elements in both the HCs and their corresponding feedstocks (**Supporting Table 1**). Both HCs exhibited a higher carbon concentration than the initial biomasses. In fact, while the three initial biomass types contained an average carbon content of 37.67%, the solid hydrochars reached values of 45.76% (HC215) and 46.66% (HC180). Such an enrichment in carbon reflects the preferential degradation of oxygen-rich compounds, mostly represented by polysaccharides and alcohols, in part condensed in aromatic structures. This finding well agrees with the variation in the H/C ratio (**Supporting Table 1**), whose relative decrease in HCs than the initial biomasses indicated the partial conversion of aliphatic polar components into aromatic structures. About the nutrients, the spinach was the feedstock with the highest amount of nitrogen, with a value even 2.5 times higher than escarole and red chicory. In case of N, the content in both HCs was comparable to the average amount in all the three biomass types. Interestingly, it slightly decreased at higher HTC severity (3.95 and 3.42% in HC180 and HC215, respectively) indicating a partial nitrogen loss, likely due to the release of ammonium and volatile nitrogen-based organic compounds (**Leng et al., 2021**). No information was obtained about the sulphur, since its content was below the detection limit in all cases (0.5%). The contents in the macronutrients phosphorous and potassium was relatively high in all cases, where the escarole showing the lowest values (4,89 and 38,52 g/kg, respectively). It is interesting to note that the phosphorous content in both HCs was lower than in the initial biomass, with HC215 showing an even lower content. This proves that HTC promotes the hydrolysis of P-based organic compounds, including phospholipids, nucleic acids and nucleotides, thus releasing phosphate ions in the liquid phase, with an extent directly depending on temperature severity. The K content in both HCs decreased as compared to the initial

biomass, with HC180 retaining the higher amount. Since the potassium is expected to be adsorbed by solid HCs through ionic interactions driven by terminal charged sites developed during HTC (mostly represented by deprotonated carboxyl groups), the fact that the K was lower in HC215 can reflect a weaker retention due to a lower number of charged sites and resulting from more exacerbated decarboxylation and condensation.

Polivalent mesonutrients, such as Ca^{2+} and Mg^{2+} , were more abundant in both HCs than in the initial materials, again due to the ionic adsorptions which were more efficient in hydrochars due to a larger amount of HTC-induced terminal deprotonated carboxyl groups.

In terms of micronutrients, the levels in all the original biomass types were relatively satisfying, with the escarole containing the largest amount of Fe, B and Mo, while the spinach exhibited the largest concentration of Mn, Zn and Co as well as of Cu, along with values similar to those detected for the red chicory (**Supporting Table 1**). Again, most of these elements, which are cations, resulted more abundant in both hydrochars than in the initial biomass. It is plausible that these elements, initially solubilized into the aqueous phase during the early stages of HTC, subsequently were adsorbed onto the solid matrix at elevated temperatures. This process likely occurs via cation exchange or coordination with terminal nitrogen- and oxygen-containing functional groups (e.g., $-\text{NH}_2$, $-\text{COOH}$, phenolic $-\text{OH}$) formed in the HC, resulting in the apparent enrichment of these elements in the solid fraction (**Zhong et al., 2023**). The only exception was observed for boron, whose content was similar or lower than the initial biomass. This can be explained by the acidic environment determined by HTC which retained this element in neutral boric acid form, thus making it not eligible for ionic interactions. The sodium content, which was relatively high in spinach (16.5 g/kg) and can be one of the main driver of salinity, resulted acceptable in both HCs. Finally, potentially toxic elements (Al, Be) and heavy metals (Cd, Cr, Ni, Pb, As, Hg, Tl, V) were present in relatively low concentrations, which,

even though slightly higher than in the initial biomasses, were below the legal thresholds. These findings suggested that the HTC of red chicory, escarole and spinach produced nutrient-rich solid materials, potentially efficient when applied in a soil as a source of beneficial elements both as a bioavailable form for plants and through a slow-release mediated by microbial mineralization (**Funke et al., 2010**). The application of hydrochars may represent an innovative, sustainable and cost-effective way to replace mineral and commercial fertilizers in the management and requalification of unfertile and degraded soils.

4.4.3 Solid-state CPMAS NMR and ATR FT-IR spectroscopies

The compositional profiles of processed biomass types and both the HCs were examined via ATR FT-IR and ^{13}C CPMAS NMR spectroscopies in the solid-state (**Fig.1**). Such an analytical approach aimed not only to highlight the main structural alterations induced by the HTC process, but also to pave the way to deciphering the correlations between HCs composition and their potential action in soil. About the ATR FT-IR spectra (**Fig. 1a**), the broad absorption band in the $3200\text{--}3400\text{ cm}^{-1}$ region was attributed to O–H stretching vibrations of hydroxyl groups, commonly arising from water, alcohols, phenols and carboxylic acids. The bands at 2920 and 2850 cm^{-1} were ascribed to alkyl C–H stretching vibrations, indicating the presence of lipidic and aliphatic structures. The weaker absorption at 2780 cm^{-1} was assigned to C–H stretching of methoxyl groups, mainly due to lignin-derived compounds. The prominent band ranging within 1720 and 1670 cm^{-1} corresponded to C=O stretching vibrations of carboxylic acids and esters, potentially useful to reveal the degree of organic matter oxidation. The weak adsorption in the region included between 1670 and 1500 cm^{-1} was attributed to the N–H bending in secondary amines arising from proteins and amino acids. The region included within 1500 and 1430 cm^{-1} was assigned to C–H bending in lipidic alkanes.

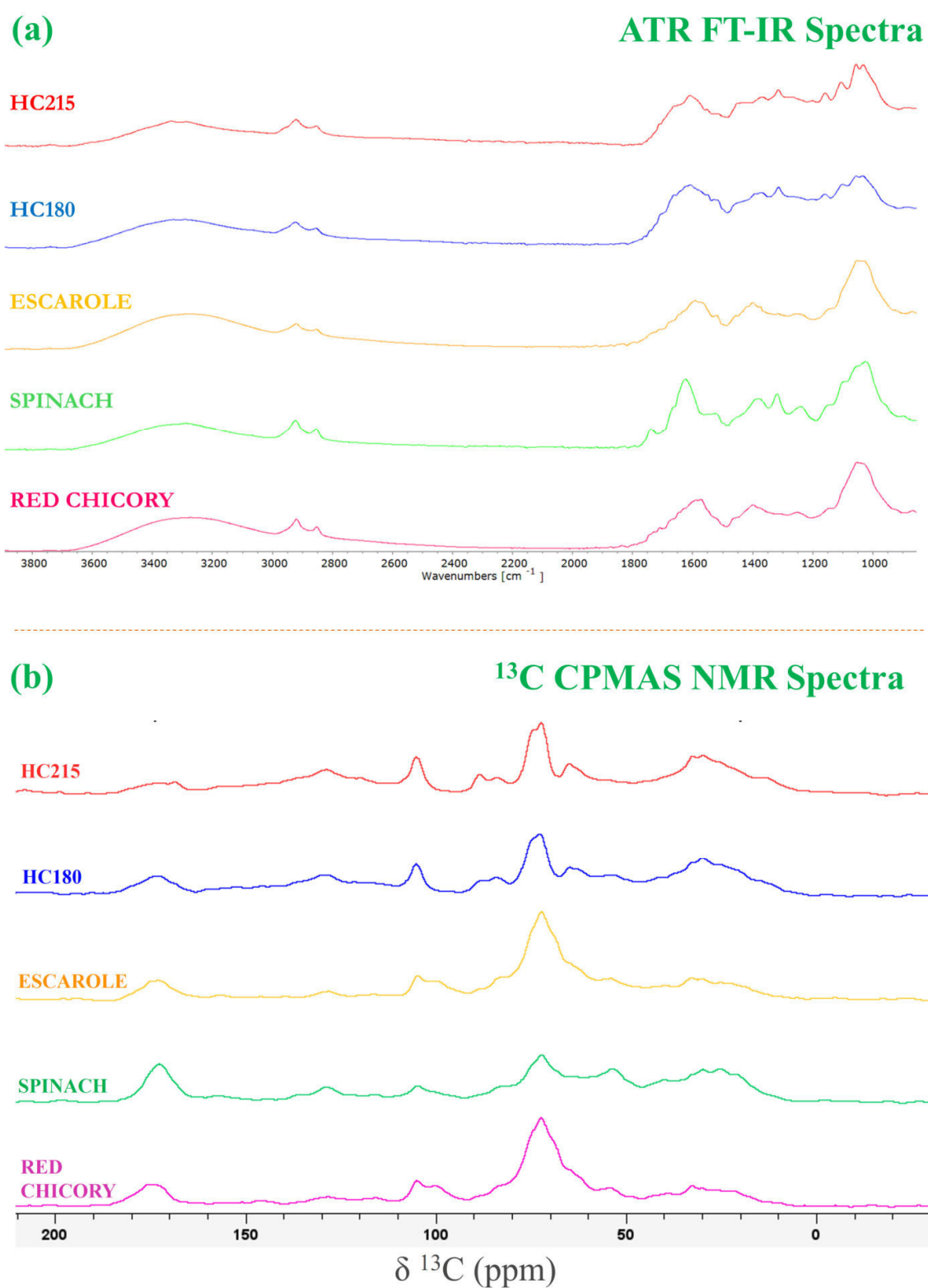


Fig. 1. Solid-state ATR FT-IR (a) and ^{13}C CPMAS NMR (b) spectra of red chicory (pink), spinach (green), escarole (orange) feedstock biomasses and the resulting solid hydrochars, obtained by an HTC conducted at 180 (HC180, blue) and 215 (HC215, red) $^{\circ}\text{C}$.

The strong absorption in the 1200–1000 cm^{-1} range was ascribed to C–O stretching of polysaccharides, while the bands in the 700–900 cm^{-1} region were attributed to the C–H bending in alkene and aromatic rings (Curcio et al., 2025; Machado et al., 2020). The spectra of red chicory and escarole were characterized by intense absorptions in the regions of O–H, aliphatic C–H, and C–O, thus suggesting a certain richness in carbohydrates, typically represented by cellulose, hemicellulose and oligosaccharides (Lang et al., 2025; Zhu et al., 2025). Although the spinach IR spectrum showed relatively weaker absorptions, in these regions, especially the signals of proteins and organic acids resulted relatively high (Kaliyaperumal et al., 2020; Vargas et al., 2022). IR spectra of HC180 and HC215 indicated that the HTC induced a pronounced alteration in the profile of the initial biomasses (Sailer et al., 2022). In particular, most of carbohydrates signals underwent a drastic reduction in intensity, as a consequence of their dehydration, decarboxylation and decomposition. Also the signal attributed to peptidic moieties decreased in intensity, becoming even weaker with increasing HTC severity. At the same time, the broad aromatic peak at around 1550 cm^{-1} appeared relatively more intense in both the HCs, as compared to the average response detected in all the biomass types. This proved a partial enrichment in condensed polycyclic aromatic structures, which are characteristic of carbonaceous materials formed under HTC conditions (Sailer et al., 2022), along with a decrease in labile saccharidic and peptidic material.

The ^{13}C CPMAS NMR spectra of initial biomasses (Fig. 1b) were dominated by O-alkyl-C resonances (60–110 ppm), deriving from the predominance of structural polysaccharides (cellulose, hemicelluloses, pectins) and soluble carbohydrates in plant residues (Bento et al., 2021; de Aquino et al., 2019) whose global amounts rounded up to the 42, 60 and 61% of total area for spinach, red chicory and escarole, respectively (Supporting Table 2). The second more abundant fraction was represented by the apolar aliphatic components, marked by the

peak in alkyl-C region (0-45 ppm), which include lipids, cuticular waxes, and protein side chains. The evident signal at 45–60 ppm, arises from the superimposition of lignin methoxyl and C–N functional groups in peptides and amino acids. The aromatic components in the 110-145 ppm interval include both un-substituted and C-substituted rings followed by the O-bearing aryl compounds pertaining to phenolic and lignin derivatives displayed beneath the 145-160 ppm band (**Fig. 1b**). The peaks resonating between 160 and 230 ppm are indicative of carboxyl/carbonyl functionalities pertaining to acids, amides and ester bonds. NMR spectra were very consistent with the relevant molecular traits revealed by FT-IR spectra. In fact, also NMR spectra confirmed the carbohydrates dominance in red chicory and escarole. The hydrochar materials showed a relatively lower intensity of O-alkyl-C signals than those observed in the initial biomasses. Conversely, both HCs exhibited a markedly higher content in aromatic components and lipids, deriving from the thermal transformation of plant components. Part of these aromatic structures are expected to derived from the partial carbohydrates carbonization which, after a thermal decomposition, dehydration and ring breaking, were converted into active intermediates (i.e radicals, aldehydes and ketones) capable to trigger subsequent condensation. The comparison between HC180 and HC215 unveiled that more severe HTC conditions promoted a larger abundance of aromatic singals and lipids in HC215, along with a decrease in the peaks of carbohydrates and carboxyls. This aspect is relevant because delineates a more hydrophobic nature of HC215 as well as suggests a lower number of charged sites, which well agrees with the above discussed lower water retention (**Supporting Fig. S2b**) and the lower content in adsorbed cationic nutrients.

NMR indexes, such as HB, lignin and A/OA ratios (**Supporting Table 2**), are very useful in summarizing and highlighting relevant molecular traits of studied organic materials. In fact, they are commonly exploited to assess structural properties, including the composition and the

organic dynamics, as well as to provide elements to elucidate the biochemical processes involved in its formation and the correlation with the beneficial action exerted on both soil and plants (**Bento et al., 2020b; de Aquino et al., 2019; Monda et al., 2017; Pane et al., 2016**). The higher values of the HB index and A/OA ratio found in HCs samples, as compared with fresh plant residues (**Supporting Table 2**), indicated an increase in the content of apolar, recalcitrant aromatic and aliphatic compounds, which are often associated with SOC accumulation processes (**de Aquino et al., 2019**). This resulted from the drastic decrease in labile carbon of carbohydrates and peptides, determining a more hydrophobic and recalcitrant character in both HCs and a more biochemically stable material. In this respect, the LR value of initial biomasses suggested that the peak risen in the 45-60 ppm interval gained of a contribution of organic N in amino acids and proteinaceous material (**Bento et al., 2021**). Conversely, the marked decrease of LR ratio in both HCs samples confirmed the removal of thermolabile material and the formation and preservation of aromatic lignin derivatives. Moreover, while part of proteins was retained at 180 °C, the more severe hydrothermal conditions reached at 215 °C led to a marked reduction of proteinaceous material. **Santoro et al 2025** proposed a molecular-level explanation of the mechanisms occurring during the HTC process, according to which the fibrous network of cellulose is fragmented into nano- to micro-sized cellulose particles, forming spherical fragments with a minimal interface in contact with the reaction water. In this way, cellulose undergoes hydrolysis, dehydration and decarboxylation, with extents depending on the combined effects of temperature, pressure, and the subcritical conditions of water, resulting in the formation of a material enriched in condensed aromatic rings (**Bento et al., 2021; Falco et al., 2011; Wörner et al., 2024; Wu et al., 2017**). Both IR and NMR data highlighted a consistent altered composition of HTC into more hydrophobic and biochemically stable materials. Although these resamples most of the

typical properties of common organic fertilizers, such as the green compost, the investigated hydrochars have been produced only in few hours, through a cost-effective and ecofriendly approach. Moreover, this proves that solid hydrochar gains the potential to promote long-term carbon stabilization in soil and enhance soil organic matter persistence.

4.4.4 Greenhouse pot trials with *Lactuca sativa*

Greenhouse pot trials were carried out to verify a potential role as fertilizer of investigated hydrochars, by using *Lactuca sativa* seedlings as model plant. As shown in **Fig. 2a**, HC180 treatment induced a significant increase in the aboveground fresh biomass (30% more than CTRL), which was even comparable to that induced by the mineral fertilizer, whereas HC215 neither produced a significant increase than the CTRL nor affected the plant through a phytotoxic action. The fact that HC180 induced a clear increase in the lettuce aboveground biomass represents a finding of paramount importance. In fact, the epigeal part of *Lactuca sativa* consists in the most valuable tissue, being it the edible and marketable part. Therefore, HC180 may help producers to gain a significant increase in lettuce production without resorting in any fertilizer addition. A similar trend was observed also for the belowground phytomass (**Fig. 2b**), where HC180 significantly enhanced root development (168% more than the CTRL), eliciting a response similar to that induced by MIN. Although HC215 treatment fostered a better average response in roots development than the control, the enhanced response variability did not permit to consider these results significant. Even though the lettuce roots are not edible part of the plant, the fact that HC180 induced a drastic root increase might be an interesting aspect. In fact, this means that the root apparatus benefited by HC180 through both a biostimulation and a supply of nutrients. The largest root volume translates in a more efficient soil exploration of treated plants, thereby increasing the probabilities to find nutrients and water and favoring plant resilience even in adverse environmental conditions. In fact, the better response in the

aboveground part of plants treated with HC180 (**Fig. 2a**) may also be derived from the enhanced root apparatus.

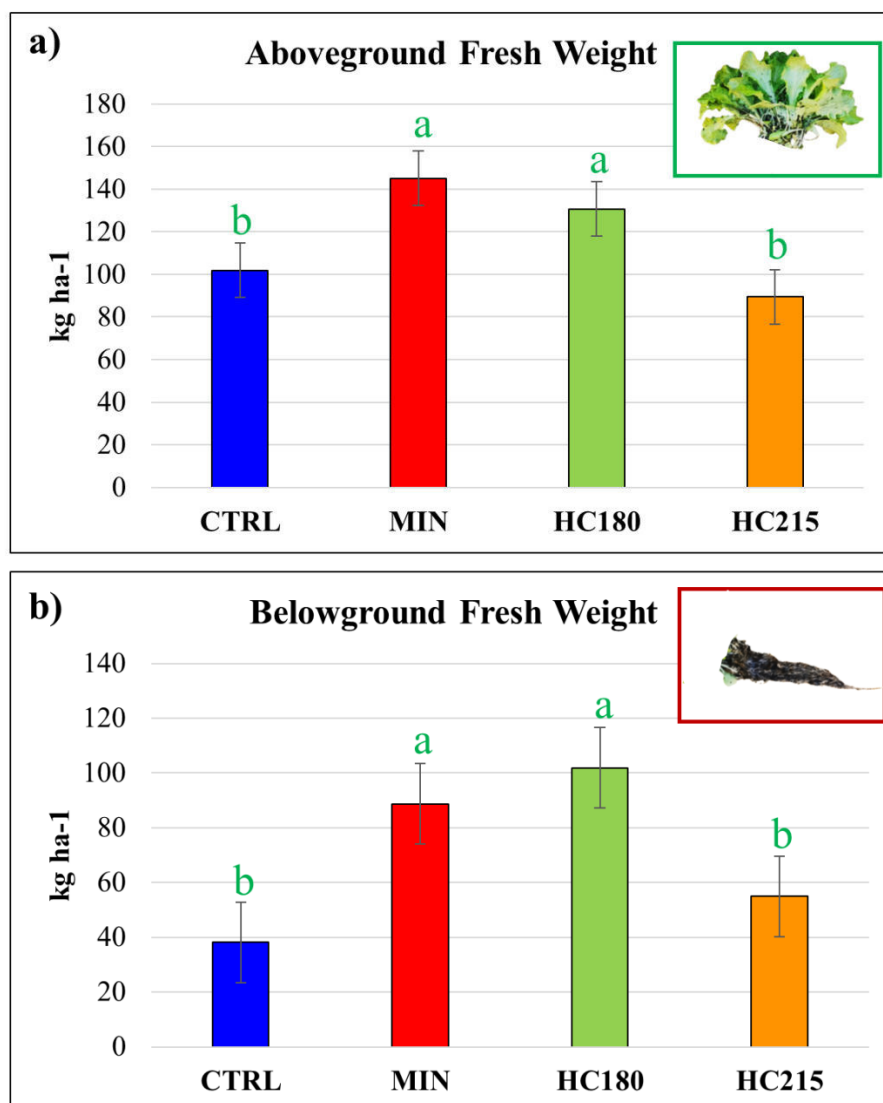


Fig. 2. Production of aboveground (a) and belowground (b) fresh biomass (kg ha⁻¹) of *Lactuca sativa* seedlings resulting from greenhouse pot-trials and grown in peat, without (CTRL) or with the supply a mineral fertilizer (MIN), the hydrochar fertilizers obtained at 180 (HC180) and 215 (HC215) °C. Different letters above the bars indicate statistically significant differences among treatments according to one-way ANOVA ($p < 0.05$).

The agronomic results highlighted the biostimulant potential of HC180, which effectively promoted both shoot and root growth, up to approach the performance of conventional mineral fertilization. From a functional perspective, HC180 can be considered as a rapid-acting

amendment exhibiting a great chemical reactivity, the increased cation exchange capacity, and the release of labile organic compounds (**Canencio et al., 2025**). In contrast, HC215, being more carbonized and recalcitrant, exhibits a limited short-term effect on plant growth. However, it may indeed contribute to long-term soil carbon stabilization and slow nutrient release (**Shen et al., 2024; Wang et al., 2025; Canencio et al., 2025; Skrzypczak et al., 2023**).

4.4.5 *Hydrochar-Induced Modulation of Lettuce primary metabolome*

The identification of the modulation exerted by HC180 on the primary metabolome of treated lettuce plants is of pivotal importance to shed light on the biochemical mechanisms activated by such an innovative soil and plant improver. Therefore, a set of experiments was conducted by paying attention only for the HC180 product, being it the only one which induced a significant improvement in the lettuce production. In particular, the primary metabolome of HC180-treated plants was identified by aqueous extract of lettuce leaves through high-resolution liquid-state NMR spectroscopy. In order to minimize possible degradative and oxidative phenomena the analyses were performed immediately after each sample extraction and preparation. The assignment of the most intense proton peaks was carried out with a composite approach, based on (i) the interpretation of the 1D and 2D homo- and hetero-correlated NMR spectra of a representative lettuce extract, (ii) the analysis of the diagnostic elements in proton multiplets, (iii) the evaluation of inherent literature (**Lanzotti et al., 2022; Chadwick et al., 2024; Sobolev et al., 2005; Feizi et al., 2020**) and (iv) the comparison of the extract's spectra with those of certified standards. The most relevant proton regions of a representative lettuce extract with related peaks assignment are shown in **Supporting Fig. S3**. NMR revealed a lettuce primary metabolome composed of 23 compounds, including amino

acids, organic acids, carbohydrates, alcohols, and nucleosides. Most of the peaks detected in the alkyl and hydroxyalkyl region (ranging between 0.65 and 3.1 ppm; **Supporting Fig. S3a**) were attributed to GABA, aspartate, glutamine, malate, glutamate, acetate, threonine, citrate, leucine, valine and isoleucine; the signals observed in the hydroxyalkyl region, included between 3.05 and 4.45 ppm (**Supporting Figure S3b**), were assigned to glucose (G), fructose (F), sucrose (S), methanol and choline; the signals appearing in the range 5-9 ppm (**Supporting Figure S3c**), which are mostly represented by hydrogen involved in double bonds and aromatic compounds, included not only the anomeric signals of S, G and xylose (Xyl), but also the signals of cytidine, formate, adenosine, fumarate, tryptophan, phenylalanine and tyrosine. From a general point of view, the most intense and predominant proton signals were represented by the multiplets of carbohydrates. This was expected, since the carbohydrates represent the most abundant compounds in lettuce tissue, with an average content ranging within 2.5 and 3% and with the fibers representing about the 50%. The cytidine is a nucleotide consisting of cytosine and ribose which is relatively abundant in the polar fraction of the lettuce leaves. It is noteworthy that the obtained aqueous extract was also rich in malate, which is the most abundant organic acid in lettuce, as well as contained a relevant content in both citrate and fumarate.

Leaves extractions were conducted on HC180-treated plants as well as on both control plants and plants supplied with mineral fertilizer (serving as a positive control). ^1H NMR spectra were acquired, integrated, segmented into defined spectral buckets, and compiled into a data matrix, which was subsequently processed via the Principal Component Analysis (PCA). The score plot obtained by combining PC1 and PC2 (accounting for 50.12% of the total variance; **Fig. 3a**) revealed a clear grouping for each of the examined plant-type. In particular, the HC180 and CTRL groups were respectively collocated in a region characterized by negative PC1 values,

while the MIN group was positioned at positive PC1 values. On the other hand, HC180 and CTRL samples were discriminated against each other along the PC2. A contextual analysis of the loading plot enabled the identification of the most representative loading vectors associated with the two axes, which were then subjected to ANOVA test (Tukey and Benjamini-Hochberg tests; p -value < 0.05) in order to select the metabolites significantly discriminating among the examined groups. The separation occurring along the PC1 axis was due to a larger amount of adenosine, tryptophan, glucose, glutamine, glutamate, leucine, and valine, in MIN as compared to both HC180 and CTRL leaves, in which these compounds exhibited an almost similar concentration. Conversely, the discrimination along the PC2 was determined by a larger amount of xylose, sucrose, fructose and malate in HC180-treated plants, as compared to both MIN and CTRL samples. In **Fig. 3b** is shown the PCA score plot obtained by combining PC2 and PC3 (explaining 40.69% of total variance). Interestingly, the PC3 retained variables which further denoted interesting differences among the primary metabolomes of studied samples. In particular, most of MIN and HC180 observations were associated to positive PC3 values, while most of CTRL ones were associated to negative values. Such a collocation was due to the higher levels of GABA, aspartate, alanine and methanol in HC180 and MIN.

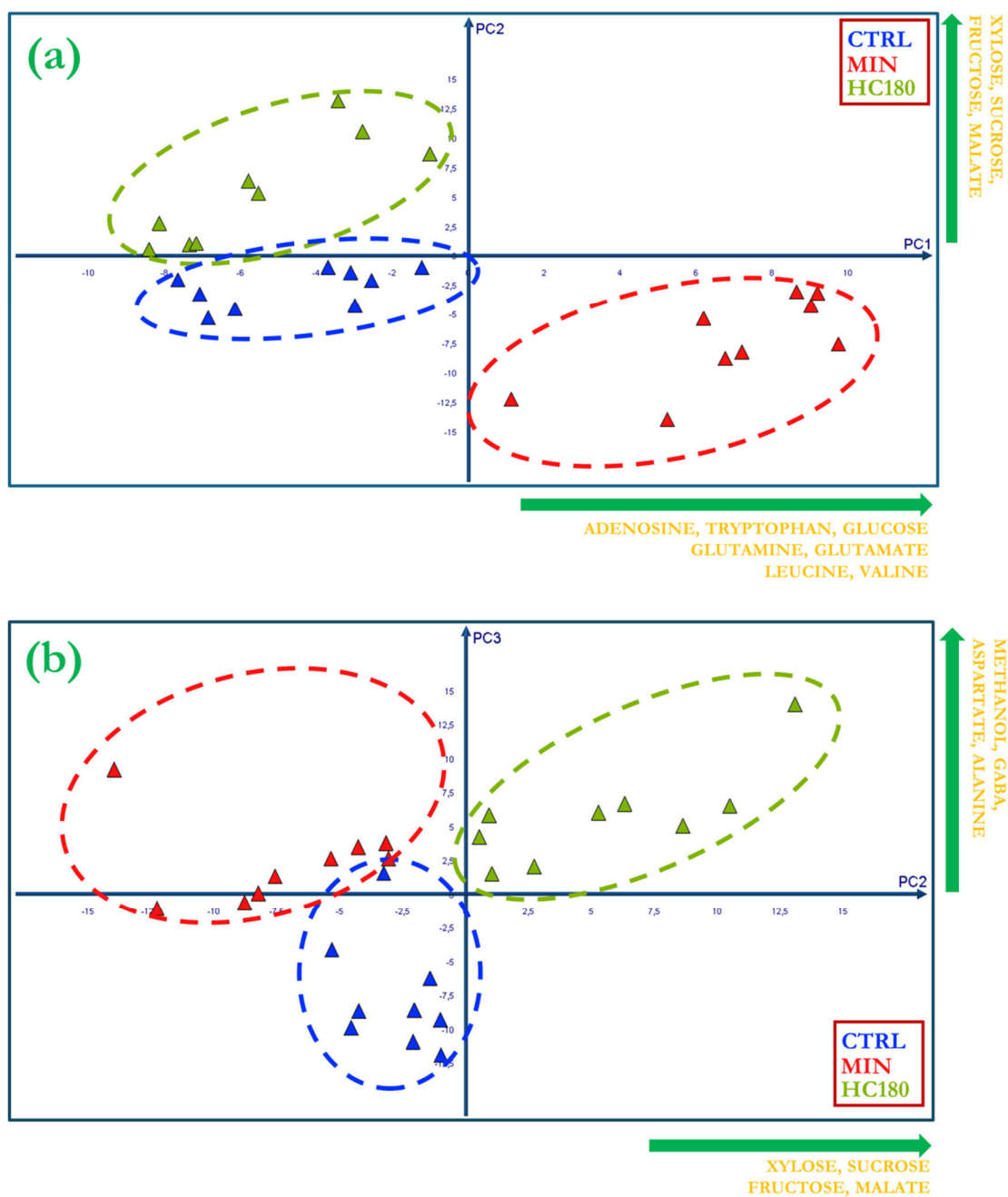


Fig. 3. PCA score plots (PC1 vs PC2, a; PC2 vs PC3, b) based on ^1H NMR spectra of extracts from lettuce plants resulting from the following treatments: control (CTRL samples; blue triangles), addition of either a mineral fertilizer (MIN, red triangles) or hydrochar obtained at 180 °C (HC180, green triangles). The names and the orthogonal directions of the loading vectors discriminating significantly the investigated sample types are reported in yellow along the edges of each score plot.

Further details regarding the role played by the variables discriminating significantly each treatment are displayed in **Supporting Table S3**.

These results proved that the root and shoot growth biostimulation induced by HC180 treatment (**Fig. 2**) was accompanied by a clear change in the primary metabolome of lettuce, as reflected in the overexpression of carbohydrates, such as fructose, xylose and sucrose. Such an outcome is very relevant since suggests the role of HC180 in improving the plant vitality, making lettuce more efficient in performing photosynthesis and biosynthesizing carbohydrates. Plants use to accumulate sucrose in leaves as temporary energy storage, especially when the glucose production exceeds the rapidity with which it is decomposed via glycolysis. Moreover, sucrose may serve as both a form of carbohydrates transport and precursor of essential compounds, such as cellulose, starch and proteins. Accordingly, the PC1 indicated that also the glucose content in HC180 plants was higher than CTRL, although it was lower than in MIN (**Supporting Table S3**). It is also important to underline that several factors induced by the supply of HC180 concurred to improve the overall quality of the growth substrate, which was enabled to provide more nutrients, increased the number of exchangeable sites retaining water and essential cations, and supplied labile organic matter, useful to support the soil microorganisms. Concomitantly, the higher content in malate indicated a more pronounced Krebs cycle in HC180-treated plants which well agrees with the larger energy consumption occurred to support the vital functions and a more enhanced lettuce growth and development. Similar observations were reported by **Bhattarai H.R. et al. 2025**, demonstrating that hydrochar acts as an organic amendment with significant biostimulant effects on plants. Conversely, the accumulation of amino acids, such as aspartate, GABA and alanine may represent a form of storage of nitrogen-based compounds in the lettuce leaves, resulting by a satisfying nitrogen availability. However, the over-expression of GABA and aspartate sometimes occurs as a plant response to external

biochemical stimuli and specific growth conditions. The MIN plants exhibited a profile which reflected the large abundance of bioavailable nitrogen provided by the $\text{Ca}(\text{NO}_3)_2$ fertilizer. In fact, MIN lettuces showed the highest levels of amino acids such as leucine, valine, glutamine, glutamate, tryptophan, and isoleucine, as well as the highest concentration in the nucleoside adenosine, thus suggesting an enhanced amino acid biosynthesis pathways (Zhou et al., 2021).

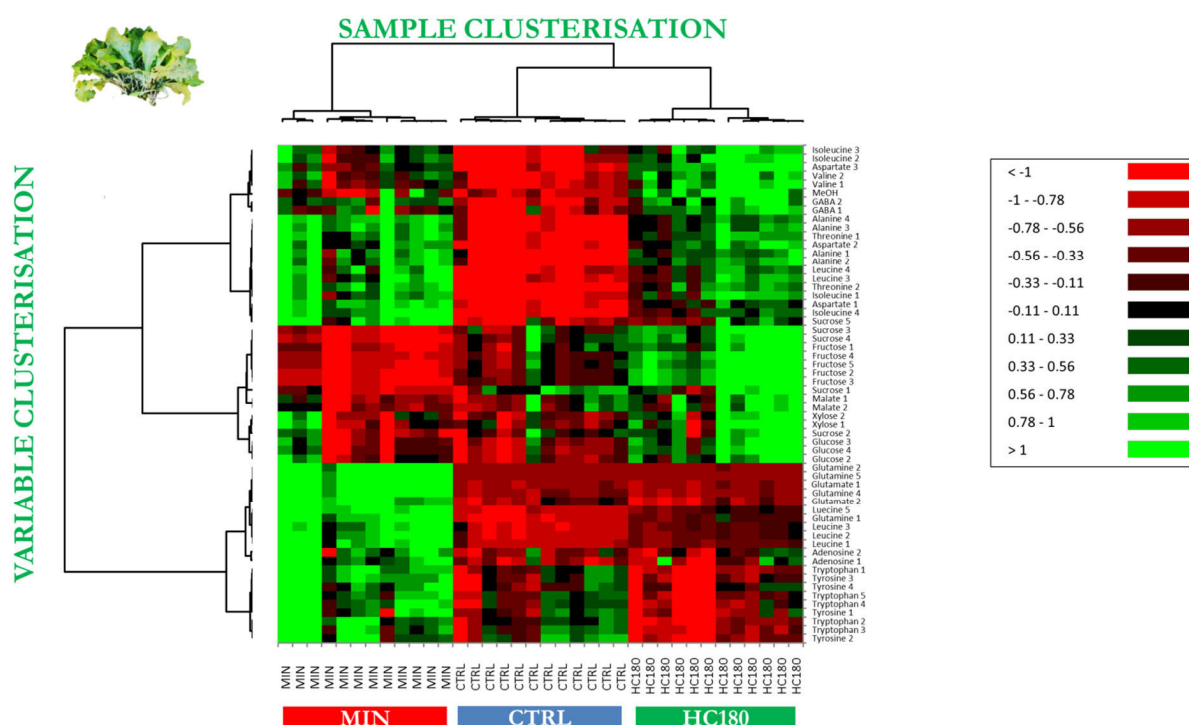


Fig. 4. Clustered heatmap showing the relative abundance of the most significant metabolites in lettuce leaves subjected to control (CTRL), mineral fertilizer (MIN), and hydrochar 180 °C (HC180) treatments. The color scale ranges from red (lowest concentrations) to green (highest concentrations), with black indicating intermediate values. Both samples (top dendrogram) and metabolites (left dendrogram) were clustered according to their similarity. The numbers following the metabolites names specify different NMR peaks belonging to the same compound.

each sample type was clustered in distinct groups. CTRL were generally characterized by the lowest relative concentrations for most metabolites, particularly glucose, valine, alanine, and aspartate, indicating a reduced metabolic turnover and limited nitrogen assimilation. In contrast, the HC180 treatment induced a peculiar response in the primary metabolome, with a pronounced accumulation of carbohydrates and nitrogen-containing metabolites (**Lanzotti et al., 2016**). The heatmap not only confirmed and reinforced the results emerged by PCA analysis, but also provided new insight among variables. In particular, the variables clusterization highlighted the strong correlation among the abundant carbohydrates and malate detected in HC180. Moreover, it also underlined a direct correlation among GABA and aspartate in HC180 samples, being them linked to redox homeostasis and nitrogen remobilization as a further tool supporting a biostimulant-like response (**Chen et al., 2020**). Interestingly, the variables clusterisation also correlated the amino acids which were overexpressed in MIN, revealing a tight linkage among the aromatic amino acids tyrosine and tryptophan. Concluding, the HC180 promoted a broad metabolic reprogramming in lettuce, reinforcing the hypothesis of its biostimulant activity through the stimulation of primary metabolism, nitrogen assimilation, and energy-related pathways (**Bhattarai H.R. et al., 2025**).

4.5 CONCLUSIONS

This study demonstrates that HTC is an effective strategy for the sustainable valorization of horticultural residues, enabling the production of HC with tailored physicochemical and agronomic properties. The comparative analysis of the initial feedstocks with the HCs revealed significant compositional differences, especially in terms of carbohydrate and protein content as well as in the hydrophobicity and biochemical stability. The combined variation of temperature and autogenous pressure significantly influenced solid yield, water retention

capacity, elemental composition, and molecular structure, resulting in HCs with distinct functionalities. HC180 exhibited a pronounced biostimulant effect on lettuce, stimulating both shoot and root growth to levels comparable with those elicited by mineral fertilization, whereas HC215 showed only a limited agronomic impact on the examined model plant. This notwithstanding, HC215 retained a role as improver of soil chemical properties and a valid strategy for carbon sequestration. The biostimulant properties of HC180 were also reflected in the metabolomic profile, with increased levels of simple and intermediate sugars, GABA, aspartate, and alanine, indicating enhanced energy metabolism, as proved and confirmed by heatmap clusterization.

Concluding we have proved that the solid hydrochar produced by a virtuous and innovative process of valorization of red chicory, spinach and escarole waste biomass represents an unprecedented, sustainable and ecofriendly solution to improve soil health and provide more resilience to adverse environmental conditions in soils, thus candidating as a way to help repairing degraded soils. In particular, solid HC represents a source of labile organic matter and nutrients, thus compensating and replacing the costs of mineral fertilizers. Moreover, it not only can increase the water retention capacity, thus favoring soil to better contrast drought periods, but also represents an efficient strategy for carbon sequestration, improving soil structure, soil resistance to erosion and, indirectly, promoting soil microbiota and biodiversity. The biostimulant role of HC180 line candidate it as an effective natural promoter of plant growth, obtained in full respect of the circular economy principles and according to the 3 R's paradigm.

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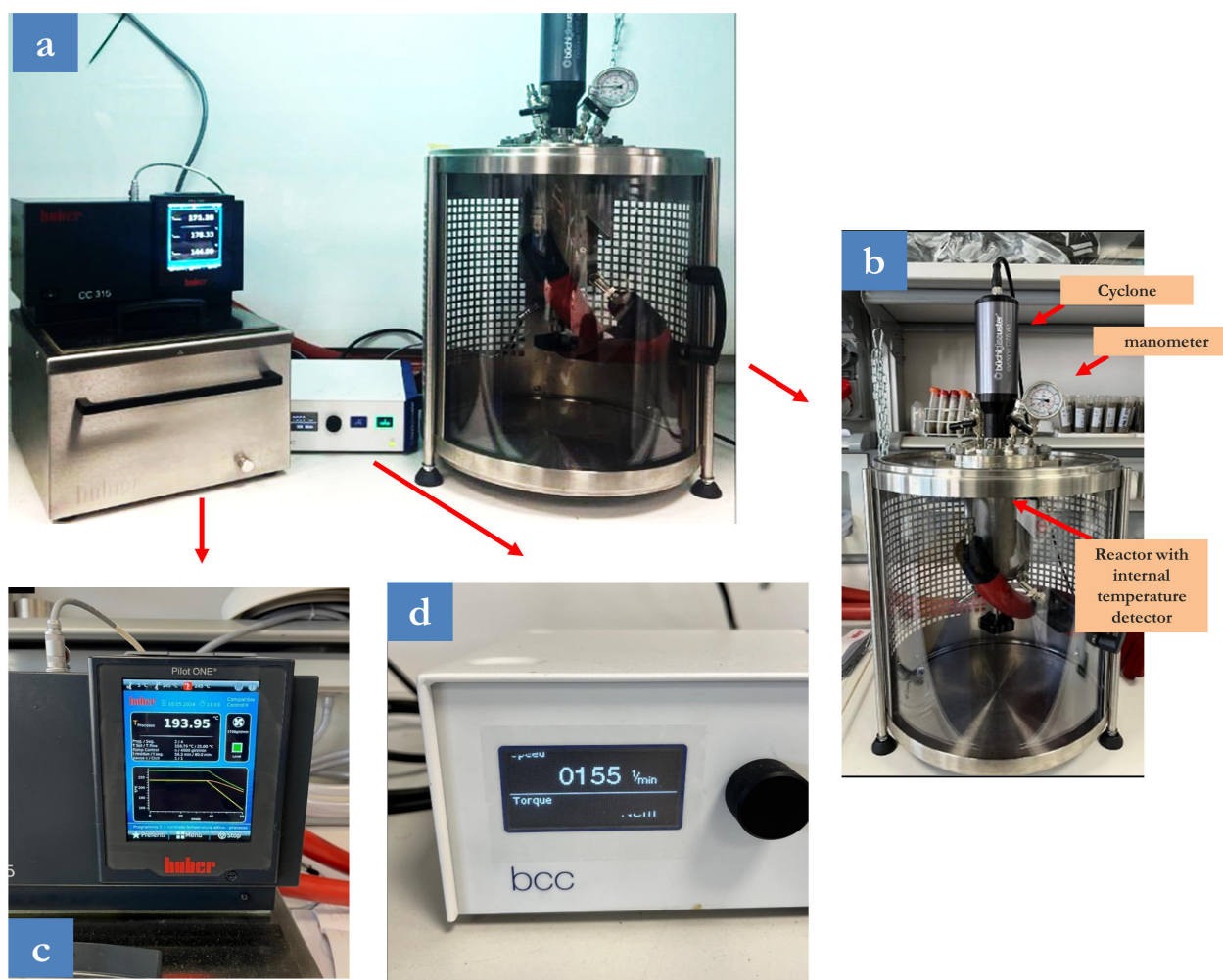
Supporting Information

Supporting Figures: 3

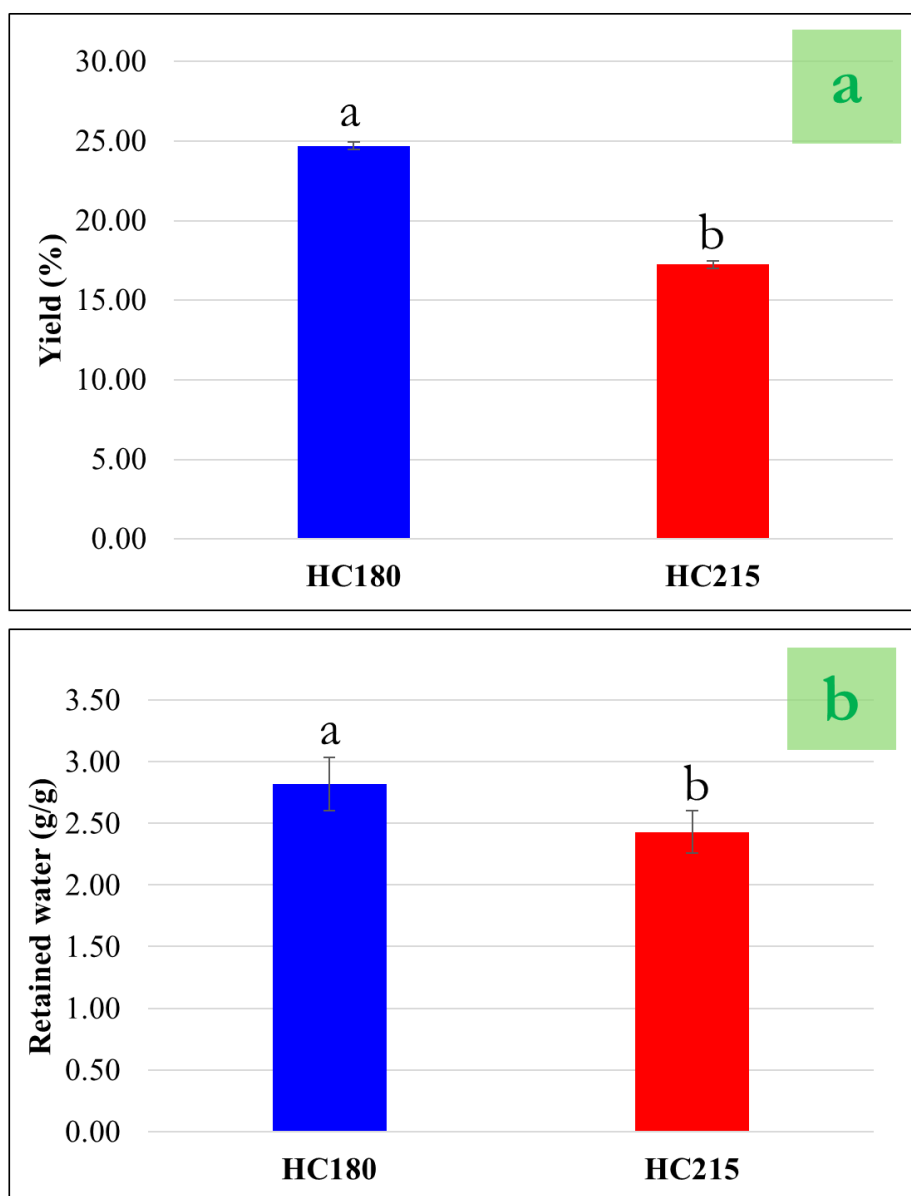
Supporting Table: 3

Supporting Paragraphs: 4

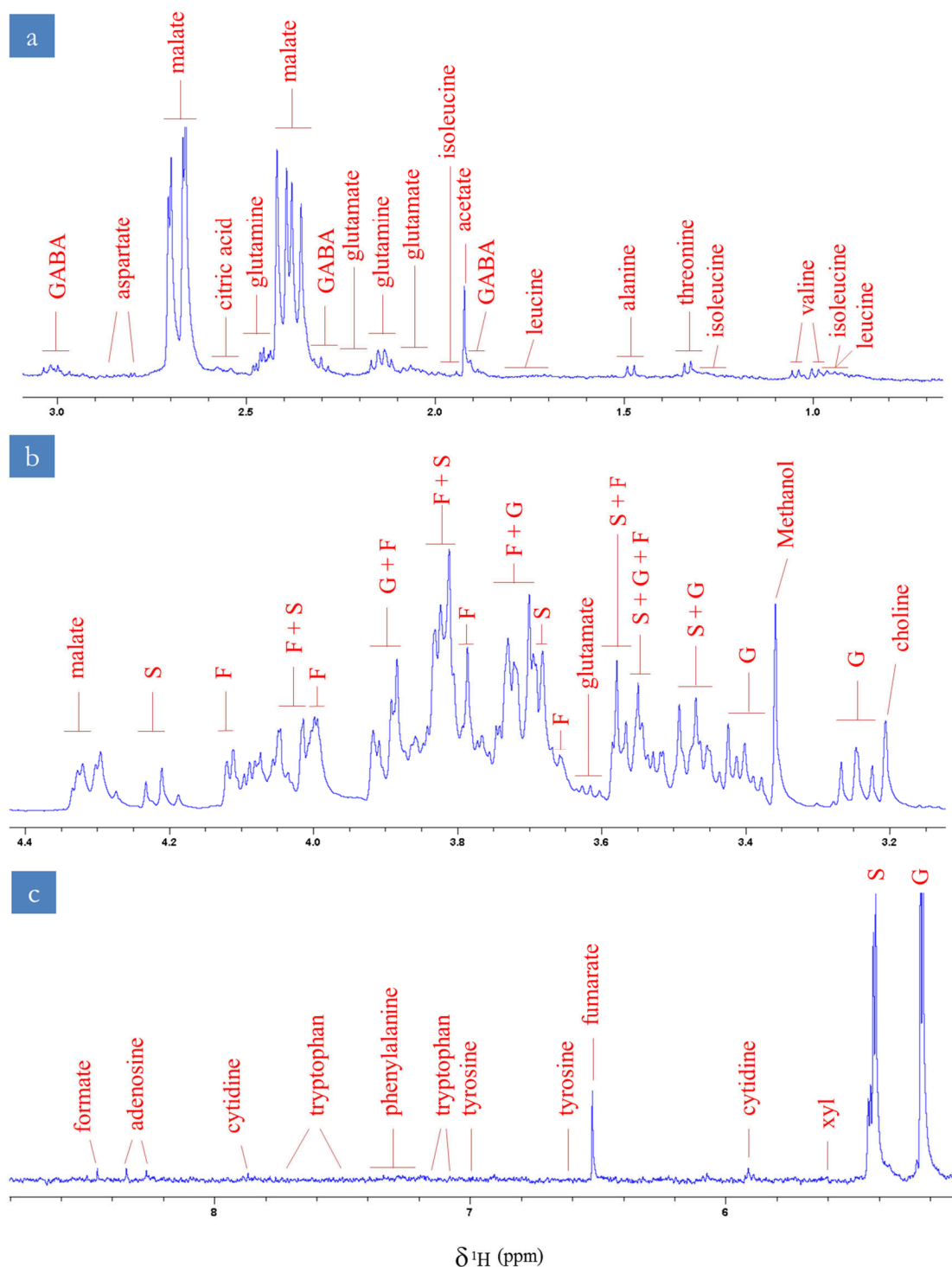
Supporting Figures



Supporting Fig. S1. Complete system to perform the hydrothermal carbonization (HTC) of fresh and solid waste biomass with sub-critical water (a). The system is composed by the core reactor which is connected to a manometer and an internal temperature detector (b), and associated to two external units to modulate and monitor the temperature, during the HTC process (c), and to control the cyclone (d), respectively.



Supporting Fig. S2. Solid yield (%) a) and retained water (g/g; b) of hydrochar resulting from the hydrothermal carbonization of red chicory, spinach and escarole waste biomass obtained at 180 (HC180, blue) and 215 (HC215, red) °C. Histograms represent mean values $\pm$ standard deviations while the different letters above the bars indicate statistically significant differences among treatments according to one-way ANOVA ($p < 0.05$).



Supporting Fig. S3. Spectral regions of high-resolution ^1H NMR spectrum of a representative lettuce extract: (a) alkyl and hydroxyalkyl (0.65–3.1 ppm), (b) hydroxyalkyl (3.05–4.45 ppm) and (c) double bonds, aromatic compounds and amides (5–9 ppm) regions. The peaks assignments are specified in the figures, where G, F, S, and xyl abbreviations stand for glucose, fructose, sucrose, and xylose, respectively.

Supporting Table

Supporting Table 1

Elemental composition of both waste biomass types (red chicory, spinach, escarole) and the resulting hydrochars obtained at 180 and 215 °C (HC180 and HC215, respectively) as detected by the elemental analyzer (C, N, H, S; % w:w) and the ICP-MS (mg kg⁻¹).

	Red chicory			Spinach			Escarole			HC180			HC215		
C	38.90	±	0.22	37.88	±	0.47	36.25	±	0.45	46.66	±	2.56	45.76	±	0.26
N	2.54	±	0.22	6.09	±	0.01	2.08	±	0.12	3.95	±	0.16	3.42	±	0.03
C/N	17.9			7.3			20.3			13.8			15.6		
H	5.41	±	0.04	4.97	±	0.04	5.57	±	0.16	5.51	±	0.31	4.96	±	0.02
H/C	1.67			1.57			1.84			1.42			1.30		
S	< 0.5			< 0.5			< 0.5			< 0.5			< 0.5		
P	7121.24	±	159.35	5779.96	±	59.22	4891.09	±	1.77	3853.45	±	522.40	3295.90	±	128.93
K	57170.68	±	466.26	63016.29	±	1090.00	38522.83	±	714.68	29229.99	±	3470.78	17816.84	±	438.73
Ca	5692.16	±	266.31	12158.59	±	98.37	12348.26	±	163.59	16346.08	±	580.34	16719.99	±	662.14
Mg	2028.83	±	95.58	6807.02	±	437.43	1860.22	±	167.98	3838.48	±	414.90	2774.00	±	26.49
Fe	92.87	±	26.63	89.11	±	10.02	681.96	±	74.00	384.95	±	26.18	641.59	±	25.44
Mn	35.73	±	1.71	143.63	±	7.23	39.28	±	2.40	77.27	±	2.34	95.96	±	1.69
Zn	23.01	±	0.33	42.94	±	0.31	31.10	±	3.29	80.92	±	18.28	83.04	±	1.17
Cu	15.22	±	0.72	14.64	±	0.25	13.25	±	0.05	26.29	±	1.52	31.13	±	0.38
B	17.48	±	0.90	28.11	±	0.57	30.50	±	0.89	28.72	±	1.75	16.48	±	0.46
Mo	0.49	±	0.02	1.01	±	0.00	1.65	±	0.05	3.01	±	0.36	3.18	±	0.17
Co	0.10	±	0.01	0.44	±	0.00	0.30	±	0.07	0.49	±	0.05	0.66	±	0.01
Na	391.75	±	7.38	16498.65	±	182.73	4925.59	±	131.05	4528.45	±	696.76	2686.96	±	103.90
Ni	1.04	±	0.00	0.34	±	0.07	1.17	±	0.27	3.60	±	0.56	2.92	±	0.25
Se	0.04	±	0.01	0.03	±	0.00	0.25	±	0.05	0.13	±	0.00	0.11	±	0.02
Al	405.50	±	93.42	237.97	±	22.33	1085.56	±	523.66	908.17	±	16.94	1144.71	±	83.77
As	0.04	±	0.01	<0,025			0.20	±	0.02	0.19	±	0.02	0.21	±	0.06
Be	<0,05			<0,05			0.08	±	0.02	<0,05			0.07	±	0.00
Cd	0.07	±	0.00	0.65	±	0.01	0.21	±	0.00	0.53	±	0.02	0.64	±	0.01
Cr	0.16	±	0.00	0.26	±	0.02	1.50	±	0.56	6.98	±	0.44	4.87	±	0.40
Hg	<0,5			<0,5			<0,5			<0,5			<0,5		
Pb	0.10	±	0.04	0.09	±	0.00	0.67	±	0.13	0.56	±	0.03	0.89	±	0.02
Tl	<0,025			0.11	±	0.00	0.06	±	0.01	0.05	±	0.00	0.04	±	0.00
V	<0,25			<0,25			2.10	±	0.86	1.20	±	0.10	2.01	±	0.13

Supporting Table 2

Integrations of the ^{13}C CPMAS NMR spectra (reported as absolute values (a.v.) and as percentages (%) of both waste biomass types (red chicory, spinach, and escarole) and the resulting hydrochars obtained at 180 and 215 °C (HC180 and HC215, respectively). The table also reports NMR-derived indexes, including the hydrophobic index (HI), the alkyl/hydroxy-alkyl ratio (Alk/O-Alk), and the lignin ratio (LR).

Spectral range (ppm)	Red chicory		Spinach		Escarole		HC 180		HC 215	
	a.v.	(%)	a.v.	(%)	a.v.	(%)	a.v.	(%)	a.v.	(%)
230-190 (C=O-C)	13351916.25	0.53	43194919.18	1.06	1551344.20	0.05	69722631.66	1.71	51245982.92	1.34
190-160 (COOH-C)	231394360.09	9.13	507676432.97	12.44	208426425.13	7.30	341886125.55	8.38	195019795.11	5.10
160-145 (O-Aryl-C)	82124273.14	3.24	151791041.03	3.72	72113562.86	2.53	236074037.11	5.78	226977886.34	5.93
145-110 (Aryl-C)	176700781.08	6.97	340550264.26	8.34	140640674.30	4.93	554829651.70	13.59	606734157.71	15.86
110-60 (O-Alkyl-C)	1456242996.77	57.43	1408534089.01	34.51	1645750652.67	57.66	1451774577.15	35.57	1416703512.51	37.03
60-45 (CH ₃ O/C-N)	169658551.25	6.69	494367677.67	12.11	254533593.27	8.92	322700451.96	7.91	179310175.88	4.69
45-0 (Alkyl-C)	406232555.29	16.02	1135372249.93	27.82	531340567.60	18.62	1104711834.86	27.06	1149895697.05	30.06
HI	0.49		1.08		0.54		1.19		1.30	
Alk/O-Alk	0.28		0.81		0.32		0.76		0.81	
LR	2.07		3.26		3.53		1.37		0.79	

Supporting Table S3

Variables associated to the axes PC1, PC2 and PC3 of the PCA score-plot shown in **Figure 3** and involved in the discrimination among the investigated lettuce leaves subjected to control (CTRL), mineral fertilizer (MIN), and hydrochar 180 °C (HC180) treatments, as revealed by ANOVA test (Tukey and Benjamini-Hochberg tests, with a p -value < 0.05). Different letters (A-C) indicate the metabolites whose concentrations are statistically different.

	Significant metabolites	CTRL	MIN	HC180
PC1	Adenosine	B	A	B
	Tryptophan	B	A	C
	Glucose	C	A	B
	Glutamine	B	A	B
	Glutamate	B	A	C
	Leucine	B	A	B
	Valine	C	A	B
PC2	Xylose	A	A	A
	Sucrose	A	B	A
	Fructose	B	C	A
	Malate	A	B	A
PC3	Methanol	B	B	A
	GABA	B	A	A
	Aspartate	C	A	B
	Alanine	C	A	B

Supporting Paragraphs

Supporting Paragraph SP1

Solid hydrochar yield and HC water retention:

The moisture content in both the initial biomass and the resulting HC types was determined gravimetrically. In detail, the initial biomass and the HC were dried in an oven at 70 °C until the constant weight. The biomass dry weight was used to calculate the initial moisture content, while the HC yield was determined on the basis of the following formula (1) and expressed as a relative value (%).

$$Yield (\%) = \frac{\text{hydrochar dry weight (g)}}{\text{initial biomass dry weight (g)}} \times 100 \quad (1)$$

Dried biomass, HC180 and HC215 were then stored under dry conditions.

The water holding capacity of the hydrochar was determined gravimetrically. Briefly, a funnel was collocated on a glass cylinder. Then, a filter was put into the funnel and wet with water. Subsequently, 0.5 grams of dried hydrochar were placed on the moistened filter and added gradually of distilled water until percolation was observed from the bottom of the funnel, indicative of the material water saturation. The amount of retained water was expressed as gram of water retained per gram of hydrochar (3 replicates per HC type). Hydrochar yield and water retention results were processed by ANOVA test (p -value < 0.05; Tukey), where different letters indicated statistically significant differences among compared treatments.

Supporting Paragraph SP2

Analysis of Elemental Composition (C, H, N, S) and Nutrient Content via ICP-MS

The organic elemental composition was determined using a CHNS-O analyser (Flash 1112 EA, Thermo). Samples (5 mg) were weighed in tin capsules and introduced into the combustion reactor at 980 °C, using pure oxygen as the combustion gas and pure helium as the carrier gas. After combustion, the resulting gases were carried by the helium flow to a gas chromatography (GC) column for separation. Finally, the gases were detected by a Thermal Conductivity Detector (TCD). The relative weight percentages of carbon (C), hydrogen (H), nitrogen (N), and sulphur (S) were measured directly, using 2,5-bis(5-tert-butyl-benzoxazol-2-yl)thiophene (BBOT) as a calibration standard. Oxygen content was calculated by difference. The essential nutrients and other elements, including metals and other non-essential nutrients, were examined via Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Briefly, about 0.1 g of samples were mixed with 3 mL HNO₃ 67 %, 1 mL HCl 37 % and 1 mL HF 47 %, in a Teflon vessel, and digested into a microwave system (Ultrawave, Milestone), according to the following program: increase for 25 min up to achieve 240 °C followed by 10 min of steady state at 240 °C. After cooling, the digested solutions were diluted up to achieve a volume of 10 mL (depending on the nutrient concentration, the solution was further diluted up to 200 times) for the ICP-MS analyses. The following isotopes were monitored in KED (Kinetic Energy Discrimination) mode and used as on-line internal standards: ²⁷Al, ⁷⁵As, ¹¹B, ⁹Be, ⁴⁴Ca, ¹¹¹Cd, ⁵⁹Co, ⁵²Cr, ⁶⁵Cu, ⁵⁷Fe, ²⁰²Hg, ³⁹K, ⁷Li, ²⁴Mg, ⁵⁵Mn, ⁹⁵Mo, ²³Na, ⁶⁰Ni, ³¹P, (²⁰⁶+²⁰⁷+²⁰⁸)Pb, ¹²¹Sb, ⁷⁸Se, ⁸⁸Sr, ²⁰⁵Tl, ⁵¹V, ⁶⁶Zn.

Supporting Paragraph SP3

Greenhouse Trials experiment

The potential of HC180 and HC215 as soil fertilizers was evaluated in peat-based pot trials conducted on baby-leaf lettuce (*Lactuca sativa* L. var. *Eden*) plants grown under controlled greenhouse conditions (Venlo-type glasshouse, University of Salerno, Fisciano, Italy). Throughout the experiment, light was supplied using 250 W lamps (Indio 1158 Sap-T 400 Cnr-L Grafite Disano), set to a photoperiod of 16 h light/8 h dark, while greenhouse temperature and relative humidity were maintained in the ranges of 25–28 °C and 37–50%, respectively. Polystyrene seed trays (cell diameter 40 mm, height 55 mm; volume 40 mL) were used for the assay. For each treatment, peat was manually mixed with either HC180 or HC215, and each plug was filled with the corresponding mixture, resulting in average HC doses of 0.47 g plug⁻¹ (HC180) and 0.30 g plug⁻¹ (HC215). The HC doses applied in the peat-based trials were selected based on the nutrient content of each hydrochar type, aiming to provide a comparable contribution of mineralizable nitrogen while avoiding excessive alteration of substrate structure. Specifically, the higher carbon content and lower nitrogen availability of HC215, as compared to HC180, justified the use of a slightly lower dose, ensuring balanced nutrient supply and optimal plant growth. The experimental design included a peat-only control (CTRL) and a pot added with mineral fertilizer (MIN) consisting in a calcium nitrate [Ca(NO₃)₂] application at 0.21 g plug⁻¹ at sowing and repeated 10 days later. Seven plugs (replicates) were prepared for each treatment, and each plug was sown with two seeds. Irrigation was applied three times per week, with 125 mL of water per plug. Lettuce plants were harvested 38 days after sowing, and fresh biomass was immediately determined by measuring separately the aboveground (shoot) and belowground (root) weights to minimize water loss and ensure accuracy. The results

were processed by ANOVA test at a p -value < 0.05 (Tukey), where different letters indicated statistically significant differences among treatments.

Supporting Paragraph SP3

Multivariate analysis of NMR data

The spectral range included within 10 and 0.7 ppm of 1D ^1H NMR spectra of lettuce extracts, was divided into n -parcels (buckets) of equal size (0.04 ppm, each corresponding to about 8 Hz), thus generating a matrix composed by 484 buckets per spectrum/replicate. 1D proton spectral bucketing was conducted using MestReC software (v. 4.9.9.9). Each bucket was then integrated and the resulting values were divided by the area of the internal standard TMSPA (semi-quantitative standardisation). Principal Component Analysis (PCA) was used to evaluate data, identify similarities and dissimilarities among samples and find variables (loading vectors) discriminating the different types of lettuce. One-Way ANOVA (ANalysis Of VAriance) test was used to examine and select the most important and significant loading vectors associated with the explored principal components of PCA. In all cases, they were considered only the loading vectors capable to discriminate significantly (p value at least < 0.05 for both Tukey and Benjamini-Hochberg tests; α confidence level of 0.05) the different types of investigated lettuce samples. The same variables were then used to develop the clustered heatmap. The latter one was carried out by centring and reducing the values and displaying them according to a colour scale which ranged from green (relatively higher/more concentrated) to dark red (relatively lower/less concentrated) through black (intermediate values). Samples and variables were clustered using Euclidean's distance and the Ward's aggregation method. All the statistical applications reported in this study were executed through the XLStat software (v. 2016, Addinsoft, Paris, France).

Chapter V

Original research paper

“Deciphering the mechanism of action elicited by innovative liquid hydrochars resulting from hydrothermal carbonization of horticultural biomass”

Rossella Curcio^{1,2,*}, Maite Olaetxea^{3,4}, Álvaro Rubio^{3,4}, Angel María Zamarreño^{3,4}, José María García-Mina^{3,4}, Pierluigi Mazzei^{2,*}

¹*DAFE - Department of Agricultural, Forest, Food, and Environmental Sciences, University of Basilicata, 10, Viale dell'Ateneo Lucano - Potenza (PZ)*

²*Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA Department of Chemistry and Biology*

³*Universidad de Navarra, Instituto de Biodiversidad y Medioambiente BIOMA, Irunlarrea 1, 31008, Pamplona, España.*

⁴*Universidad de Navarra, Facultad de Ciencias, Departamento de Biología Ambiental, Grupo Biología y Química Agrícola, Irunlarrea 1, 31008, Pamplona, España.*

*** Corresponding authors:** Pierluigi Mazzei and Maite Olaetxea

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5.1 ABSTRACT

The valorization of horticultural biomass through sustainable technologies is a key challenge in circular agriculture. In the present work, liquid hydrochars (LHCs) were produced via hydrothermal carbonization (HTC) of leafy horticultural residues (spinach, red chicory, endive) from the Sele Plain (Italy), under two conditions: 180 °C/10 bar (LHC180) and 215 °C/20 bar (LHC215). Chemical characterization revealed that LHC180 contained higher levels of labile organic carbon, soluble nutrients, and bioactive molecules, including auxins, cytokinins, jasmonates, and salicylic acid, while LHC215 exhibited a more condensed structure with reduced phytohormone content.

The mechanism of action of LHCs in plant promotion was assessed using a previously defined functional framework of sedimentary humic acids (SHA) in plant development. Bioassays on *Lepidium sativum* and hydroponic experiments on *Arabidopsis thaliana* showed that LHC180 strongly promoted seed germination, root development and morphology through IAA-ABA modulation, as well as shoot biomass, whereas LHC215 induced more limited growth responses. Both LHCs activated cytokinin (Ck) levels in shoots and upregulated the expression of genes encoding nutrient transporters (COPT2), iron homeostasis regulators (FRO2), and plasma membrane H⁺-ATPases (AHA1, AHA7) in roots. They also enhanced the nutrient use efficiency (NUE) of major macro- and micronutrients in both roots and shoots.

These results highlight hydrochar-derived LHCs, particularly LHC180, as promising bioactive amendments for sustainable fertigation, growth stimulation, and nutrient management, offering a practical strategy for valorizing vegetable residues within a circular bioeconomy framework.

Keywords: *Biostimulant activity, Phytohormones, Nutrient use efficiency, Gene expression, Circular agriculture.*

5.2 INTRODUCTION

Every year, large amounts of organic biomass is generated by agricultural farms and, in particular, by the industry of horticultural products. This is the case of the production site of the Sele Plain in Campania (Southern Italy) which represents the second European site for the production and export of high-quality leafy vegetables for the fresh-cut (fourth range) industry, with more than 50'000 tons of vegetable waste per year (FAO, 2019; Magama et al. 2022). These organic residues not only represent a disposal challenge but also an underexploited source of bioactive compounds, such as polyphenols, organic acids, and other secondary metabolites (Alhnidi et al., 2020; Gimenez et al., 2020). Leafy vegetable wastes are particularly rich in indolic precursors and phenolic acids linked to auxins and salicylic acid (Zhang et al., 2025; Tang et al., 2022), which serve as antioxidants and signaling molecules influencing plant growth, stress responses, and nutrient efficiency. Traditionally, practices such as composting or vermicomposting have been employed to recycle and valorize these organic residues. However, these approaches are time-consuming, space-demanding, and may lead to greenhouse gas emissions or nutrient losses during the process (Li, Y. et al., 2020; Ayilara, M. S. et al., 2020). Therefore, there is a growing need for innovative and more efficient strategies to recover and valorize these biomasses, turning them from waste into resources that can create added value for farmers and contribute to the sustainability of the agri-food chain.

Among the emerging approaches, hydrothermal carbonization (HTC) has recently attracted increasing attention as a promising technology for the valorization of agro-industrial residues. HTC converts high-moisture biomass into a carbon-rich solid hydrochar (HC) by processing it with water under subcritical conditions (180–250 °C; autogenous pressure ranging within 4 and 20 Bars), promoting hydrolysis, dehydration, decarboxylation, and condensation

reactions that stabilize the organic matrix and generate products with distinct physical and chemical properties (**Bento et al., 2021; Islam et al., 2021; Yu et al., 2022**). Importantly, HTC allows the direct processing of wet biomass without energy-intensive drying steps, enhancing the overall efficiency and sustainability of the process (**Xu et al., 2021; Kumar et al., 2020**). HTC produces a liquid phase, referred to as liquid hydrochar (LHC), which can serve as soil fertilizer being it relatively rich in organic matter and labile carbon and inorganic components, including important nutrients (**Mutsven et al., 2024; Chen et al., 2025**). HTC temperature governs the reaction and final LHC composition, where moderate HTC conditions ($\leq 160\text{--}180\text{ }^{\circ}\text{C}$) favor the generation of bioactive molecules, whereas harsher settings reduce their availability and promotes a solid fraction carbonization (**Tang et al., 2022; Lu et al., 2021**). Under moderate conditions, partial hydrolysis of proteins, amino acids, and phenolic constituents yields low-molecular-weight metabolites that may act as auxins, cytokinins, or stress-related signaling compounds (e.g., salicylic and jasmonic acids). At the same time, the mildly acidic environment established during HTC promotes the release and solubilization of conjugated phytohormones, enhancing their availability in the liquid phase (**Tang et al., 2022**).

Consequently, LHC emerges as a complex aqueous mixture of soluble organic compounds, nitrogenous molecules, and low-molecular-weight metabolites, with a composition strongly influenced by feedstock and process severity (**Pauline et al., 2020; González-Arias et al., 2022**). Although often discarded as process water, LHC retains bioactive compounds such as polyphenols, amino acids, and phytohormone-like metabolites, along with dissolved organic carbon and essential macro- and micronutrients that can support both plant growth and soil functionality (**Huang et al., 2021; Sun et al., 2014**). Its direct exploitation for agricultural or industrial applications could therefore enhance nutrient recovery, provide biostimulant and

antioxidant functions, and contribute to the overall efficiency and sustainability of the HTC process (Chen et al., 2025; Tang et al., 2022).

Therefore, in this study we aimed to valorize horticultural biomass (red chicory, spinach, and escarole waste leaves) from the Sele Plain production site through HTC. In particular, we produced two types of hydrochars by using, during the HTC process, a temperature of either 180 °C (pressure of 10 Bars) or 215 °C (pressure of 20 Bars). The chemical and biological characterization of LHC was performed, focusing on its content of bioactive molecules (e.g., polyphenols, nitrogen-containing compounds) (Gimenez, M., et al., 2020; Li, H., et al., 2023), essential nutrients, and plant hormones (Lu et al., 2021; Chen et al., 2025).

The effects of LHC application on *Arabidopsis thaliana* were investigated to assess its influence on vegetative growth, root development, and secondary metabolism, while also evaluating the agronomic effectiveness of these compounds for potential agricultural use. Therefore, the mechanism of action of LHCs in promoting plant growth was explored in relation to previous findings on the functional framework of sedimentary humic acids (SHA) in plant development (Aguirre et al., 2009; Mora et al., 2010; Mora et al., 2014; Olaetxea et al., 2018). SHA represent fractions of organic matter with well-documented bioactivity and multiple applications in agriculture (Chen et al., 2004; Rose et al., 2014). Beyond assessing growth and nutritional responses, the nutrient use efficiency of key macro- and micronutrients was determined. A targeted molecular analysis was then conducted to evaluate the expression of genes involved in nutrient transport, and plasma membrane ATPase activity, while LHC-induced changes in endogenous hormone levels were also examined. This integrative approach provides insight into how LHC composition (modulated by hydrothermal processing conditions, affects plant physiology and metabolism) supporting its potential as a bioactive amendment for sustainable horticultural practices within circular economy frameworks.

5.3 MATERIAL AND METHODS

5.3.1 *Liquid Hydrochar (LHC)*

Hydrochar was produced via hydrothermal carbonization (HTC) by processing the organic by-products of ready-to-eat fresh-cut vegetables (spinach, red chicory, and escarole) supplied by the Agricultural Producers Organization "TERRAMORE" (Eboli, Italy). HTC was performed by treating the solid biomass with subcritical water in a 1-L Büchi-Glass reactor equipped with a cyclone to maintain the solid material in suspension during the process and connected to an external digital unit (HUBER CC-315B) that controlled the time-dependent modulation of temperature gradients, ensuring thermal stability throughout the reaction (**Supporting Figure 1** illustrates the reactor and its main components). For the HTC, 150 g of fresh biomass (comprising 50 g of each of the three vegetable types, previously reduced to fragments smaller than 2 mm) were processed with 300 mL of water for 1 h at either (I) 180 °C/10 bar or (II) 215 °C/20 bar, referred to as LHC180 and LHC215, respectively. The programmed heating profile included 3 min at 25 °C, 80 min to reach the target temperature (180 or 215 °C), 60 min of reaction, and 80 min for cooling back to 25 °C.

After the reaction (one replicate per treatment), the liquid phase (LHC) was separated from the solid residue, vacuum-filtered (Whatman 41 cellulose acetate filters, 20 µm pore size), and stored at –80 °C until subsequent analyses.

5.3.2 *Chemical analyses on LHCs*

5.3.2.1 *Determination of pH, electrical conductivity and terminal carboxyl- and amine-sites*

The pH and electrical conductivity of each LHC were measured with HANNA HI5222 dual-channel instrument associated with a glass single ceramic double junction pH electrode and a potentiometric probe, respectively. The measurements (n=2) were performed on the samples

without relying on dilution. The determination of terminal carboxyl- and amine-sites was carried out via the Bradford. Briefly, the latter one exploits the Bradford reagent which contains the dye Coomassie Brilliant Blue G-250 and provides a brownish color to the solution until the dye is present in the free form. When it forms a complex with terminal carboxylic and amino groups, it assumes a blue colour, with an intensity depending on the amount of these sites. In detail, the reaction was launched by putting in contact 600 μ L of LHC with 600 μ L of Bradford reagent (diluted 1:2 with water), in absence of light. After 10 min, the reaction mixture was analyzed using a spectrophotometer (Thermo Spectronic 20 Genesys) at 595 nm. The absorbance values were converted into quantitative data by comparing them with a calibration curve, built up by inducing the reaction with increasing concentrations of the standard BSA (Bovine Serum Albumin; 0.01–0.075 mg/mL; $r^2 = 0.96$). For each set of reactions, they were launched two replicates per sample and a blank (the sample volume was replaced by 600 μ L of water). The results were expressed as mg/mL of BSA equivalents.

5.3.2.2 Determination of total polyphenol content via Folin Ciocalteu assay

The total phenol content in LHCs was determined via the Folin-Ciocalteu assay. The reaction was conducted by combining 0.25 mL (diluted 1:2 with distilled water) of LHC (diluted 1:2 with water) with 3.5 mL of Folin-Ciocalteu reagent (diluted 1:3 with water) and 0.2 mL of 10% (w/v) Na_2CO_3 . The reaction mixture was left to react for 4 minutes in the dark. Then, 3.2 mL of distilled water were added, and the reaction was allowed to proceed for 2 hours and 30 minutes in the dark. Absorbance of each reaction mixture was measured with a spectrophotometer (Thermo Spectronic 20 Genesys) at 765 nm. The absorbance values were converted into quantitative data through a comparison with a calibration curve, built up by inducing the reaction with increasing concentrations of the standard gallic acid (0.01–0.50

mg/mL; $r^2 = 0.99$) in ethanol at 70%. For each set of reactions, they were launched four replicates per sample and a blank (the sample volume was replaced by 0.25 mL of water). The results were expressed as mg/mL of gallic acid equivalents.

5.3.2.3 Nutrients content

The nutrient content was measured in both LHCs using inductively coupled plasma optical emission spectroscopy (ICP-OES, iCAP PRO X, Thermo Scientific). The samples were diluted 1:10 with distilled water prior to analysis.

5.3.2.4 Total Organic Carbon and Total Organic Nitrogen determinations.

The Total Organic Carbon (TOC) and Total Organic Nitrogen (TON) contents of both LHC180 and LHC215 samples were determined using a TOC-L CPH Total Organic Carbon Analyzer (Shimadzu). Prior to injection, samples were diluted 1:500 with distilled water. The total organic carbon (TOC) and total organic nitrogen (TON) concentrations of SHA were previously determined by **Aguirre et al. (2009)**.

5.3.2.5 UV-VIS and Fluorescence spectroscopies

Optical characterization of LHCs was performed by UV-Vis and fluorescence spectroscopy according to **Fuentes et al. (2006)**.

For UV-Vis analysis, samples were analyzed using a Cary 60 UV-Vis spectrophotometer (Agilent) with a 1 cm path length cuvette. Solutions containing 100 mg L⁻¹ of TOC were prepared in 0.1 M NaOH, with a final pH of 12. The spectroscopic indexes E_4/E_6 ratio, ϵ_{600} , ϵ_{280} and E_{ET}/E_{BZ} ratio were calculated.

For fluorescence analysis, solutions containing 50 mg L⁻¹ of OC in 0.1 M NaOH (pH 12) were used. Spectra were acquired with a Shimadzu RF-6000 spectrofluorophotometer. The Milori index (A_{440}) and Kalbitz index (I_{350}/I_{480}) were determined. Synchronous-scan excitation

spectra were recorded in the range of 300–520 nm, with simultaneous scanning of excitation and emission wavelengths at a constant offset of 55 nm.

5.3.2.6 *Hormone concentration*

The extraction and quantification of the acidic phytohormones indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and jasmonoyl isoleucine (JA-Ile) were carried out on the initial LHCs, following a modified protocol based on **Bosch et al., 2024**. For this purpose, 600 μ L of each sample was mixed with 1 mL of a solvent mixture composed of methanol, water, and formic acid (90:9:1, v/v/v), supplemented with 2.5 mM sodium diethyldithiocarbamate and 10 μ L of a methanolic stock solution containing deuterium-labeled internal standards (reported in **Supporting Table S1**). Extraction was conducted through a two-step process involving agitation and centrifugation. Combined supernatants (1 mL) were evaporated to dryness at 40 °C, and the residues were reconstituted in 0.25 mL of a mixture consisting of methanol and an aqueous acetic acid solution (0.133% v/v) in a 40:60 (v/v) ratio. Samples were centrifuged at 20,000 rcf prior to UHPLC injection. Hormone separation was performed using a Dionex Ultimate 3000 UHPLC system with a reverse-phase column. Detection and quantification were achieved via an Exploris 120 high-resolution mass spectrometer (Thermo Fisher Scientific), equipped with an OptaMax NG ion source, a C-Trap, an Ion-Routing Multipole, and a High Field Orbitrap analyzer with quadrupole filtering. Analyses employed a product ion scan in negative ion mode, with quantification based on multilevel calibration curves using internal standards. For each hormone, three fragment ions were monitored: the most abundant fragment (fragment 1) was used for quantitation, while fragments 2 and 3 served for identity confirmation.

Cytokinins were extracted from both the initial LHC samples, following a modified protocol described in Olaetxea et al., 2024. The total content of cytokinins affected by SHA (namely trans-zeatin (tZ), trans-zeatin riboside (tZR), cis-zeatin riboside (cZR), dihydrozeatin riboside (DHZR), isopentenyladenosine (iP), and isopentenyladenosine riboside (iPR)) was considered for quantification. Each sample (600 μ L) was mixed with 1.75 mL of cold (-20°C) extraction solvent (methanol:water:formic acid, 15:4:1, v/v/v) and supplemented with 20 μ L of a 100 ng/mL stock solution of deuterium-labeled internal standards (reported in **Supporting Table S1**) to allow accurate quantification. The extraction was performed overnight at -20°C , followed by centrifugation to remove any precipitated material. A second extraction was then carried out using 1.25 mL of the same extraction solvent, and the resulting supernatants were combined for subsequent analysis. The pooled extracts were purified through Sep-Pak C18 cartridges (Waters Co., Milford, MA), conditioned with methanol and extraction solvent. An additional volume of extraction solvent was passed through the cartridge to ensure complete elution. The eluate was concentrated to near dryness and reconstituted in 1.5 mL of 1 M formic acid. Samples were then loaded onto an Oasis MCX column (Waters Co.) preconditioned with methanol and 1 M formic acid. The column was sequentially washed with 1 M formic acid, methanol, and 0.35 M ammonium hydroxide solution. Final elution was performed with 0.35 M ammonium hydroxide in 60% methanol. The eluate was dried, re-dissolved in 200 μ L methanol and 200 μ L of 0.04% formic acid in water, and centrifuged before UHPLC injection. Cytokinins were separated using the same UHPLC system and reverse-phase column, and quantified using the Exploris 120 mass spectrometer operated in positive ion mode with a product ion scan. Multilevel calibration curves with internal standards were used for quantification, and data acquisition and processing were performed with TraceFinder 5.1 EFS

software. As with acidic hormones, three fragment ions were monitored per cytokinin, with the most intense used for quantification and the others for identity confirmation.

5.3.3 *Phytotoxicity and Biostimulant assay of LHCs*

A preliminary bioassay was carried out to evaluate the potential phytotoxic or biostimulant effects of the developed LHCs on cress seeds (*Lepidium sativum* L.). The latter ones were treated with ultrapure water (CTRL, control), SHA (biostimulant control), LHC180 and LHC215 tested at the concentrations of 50 and 100 mg L⁻¹ of organic carbon (**Mora et al., 2010; Olaetxea et al., 2015**). For each treatment, three Petri dishes (9 cm diameter) were prepared, each containing 10 seeds placed on filter paper moistened with 5 mL of the respective solution. The dishes were sealed with Parafilm® to maintain humidity and incubated in the dark at 25 °C for five days under controlled conditions (25 °C, 75% RH).

At the end of the incubation period, germinated seeds were analysed using ImageJ® software (NIH, USA). Root lengths were measured, and germination rates were recorded. From these data, the following indices were calculated, using the ultrapure water treatment as the reference (100%) (**Sobarzo-Bernal, O., et al., 2021; Benech Arnold, R. L., et al., 1991**):

$$SG\% = \frac{Gs}{Gc} \% \quad (a)$$

$$RGR\% = \frac{Rs}{Rc} \% \quad (b)$$

$$GI\% = (SG\% \times RGR\%) \% \quad (c)$$

The formula (a) permitted to calculate the seeds germination (SG %), indicating the percentage of seeds that successfully germinated in the treatment as compared to the control, where G_s and G_c are the average numbers of germinated seeds in the treated and control groups, respectively. The formula (b) enabled the identification of the Relative Growth of Root (RGR%), where R_s and R_c are the average root lengths of the treated and control seedlings, respectively. The formula (c) permitted to calculate the germination index (GI%) which accounts for both the germination rate and root growth.

5.3.4 Evaluation of phytohormones-like behaviour of LHC180

Following the quantification of phytohormones in the LHCs, the bioassay was designed to assess whether the observed biostimulant activity could be attributed only to the hormonal component or to the overall molecular pool of the LHC. Based on its higher phytohormone content, LHC180 was selected for this test. Cress seeds (*Lepidium sativum* L.) were treated with four different solutions: ultrapure water (CTRL, negative control), SHA, (100 mg OC L⁻¹; biostimulant control), LHC180 (100 mg OC L⁻¹), and a synthetic mixture reproducing the concentrations of the phytohormones detected in LHC180 at the 100 mg OC L⁻¹ dilution (MIX-P). The experiment settings are the same as those used in the above-mentioned experiment (Sobarzo-Bernal et al., 2021; Benech Arnold et al., 1991).

5.3.5 Hydroponic experiment with *Arabidopsis thaliana*

The experiment conducted in this study was carried out under hydroponic conditions, using *A. thaliana* (Col-0) wild-type plants. Four treatment groups were established: (1) untreated wild-type plants (CTRL, negative control); (2) wild-type plants treated with sedimentary humic acid (SHA, biostimulant control); (3) wild-type plants treated with LHC180; and (4) wild-type plants treated with LHC215. Five biological replicates (containers) were used for

the CTRL, SHA, and LHC180 treatments, while three biological replicates were used for the LHC215 treatment due to the limited availability of the LHC215 sample. Preliminary assays (data not shown), performed with various concentrations of organic extracts based on literature-reported organic C application rates (Mora et al., 2010; Olaetxea et al., 2015), identified 100 mg L⁻¹ organic C as the optimal concentration for promoting Arabidopsis growth. This concentration was thus adopted for evaluating the effects of LHC180 and LHC215 under elevated carbon availability. The general methodology applied in all the experiments is outlined as follows: the seeds were surfaced-sterilized with diluted bleach for 1 min (1 % bleach/water of commercial bleach) rinsed to remove bleach residues and stored at low temperature overnight. The cultivation process was set in 1.4 L containers with 34 growth cones per container. Each of the seeds was placed in a growth cone previously charged with bacteriological agar [8% agar in water (*p/p*)]. During the first 10 days, the containers were filled with water and afterward, the water was changed by a nutrient solution composed by the following salts: 0.375 mM MgSO₄·7H₂O, 0.25 mM KH₂PO₄, 0.375 mM KNO₃, 0.500 mM Ca(NO₃)₂·4H₂O, 4.44 μM Fe from Fe-HBED, 0.0375 mM KCl, 2.00 μM MnSO₄·H₂O, 0.75 μM CuSO₄·5H₂O, 1.25 μM ZnSO₄·7H₂O, 0.05 mM H₃BO₃, and 0.0375 μM (NH₄)₆Mo₇O₂₄. The pH of the nutrient solution was maintained at 6. The growth chamber conditions were set at 21 °C day/18 °C night, 75% humidity, and with a photoperiod of 10/14 h day/night fixing the irradiance at 175 μmol m⁻² s⁻¹.

Seven days after the initial nutrient solution application, containers were homogenized by reducing plants to 18 per container, removing individuals with growth markedly above or below the average. The nutrient solution was then replaced as previously described. After seven days, a second replacement was carried out, redistributing plants among containers to balance size classes without discarding any. Three days later, when plants reached optimal

size, treatments were applied by adding 100 mg OC L⁻¹ of SHA (biostimulant control), LHC180 or LHC215 to the respective containers, while five tuppens received only nutrient solution (negative control). Harvest was conducted 7 days after treatment (DAT), with roots and shoots immediately frozen separately at -80 °C for subsequent analysis. Based on this procedure, the performed analysis are reported in the next paragraph.

5.3.6 Plant fresh weight, root morphology, hormone and nutrients concentration

At harvest, fresh weight of both roots and shoots was determined gravimetrically for each replicate. In addition, roots from 12 individual plants (three per each of the four treatment groups) were carefully washed and scanned at 600 dpi using an Epson Perfection V700 Photo scanner. The scanned images were analyzed with the WinRHIZO Arabidopsis scanner-based image analysis system software (Regent Instruments, Montreal, QC, Canada, 2012b) set at 600 dpi. The software allowed detailed quantification of root morphological parameters, including total root length (mm), number of tips, bifurcations (forks), and crossings. Outputs included numerical data of root length distribution, enabling comprehensive assessment of root system architecture and morphology.

Root samples were flash-frozen in liquid nitrogen and ground to a fine powder using a pre-chilled ceramic mortar. The resulting homogenates were stored at -80 °C until subsequent analysis. Hormone concentration in plant tissues was measured as explained above. Hormones were extracted from 0.1 g aliquots of frozen ground plant material.

For nutrients concentration analysis, plant samples were harvested 7 DAT, oven-dried at 60 °C for 48 h and subsequently ground to a fine powder. An aliquot of 100 mg of dried material was digested for 20 min at 200 °C with 6 mL of 65% HNO₃ and 2 mL of 33% H₂O₂ using a microwave digestion system (ETHOS UP, Milestone). After digestion, the resulting

supernatant was recovered, diluted 1:10 with distilled water, and analyzed by ICP-OES. Nutrient use efficiency (NUE) was calculated based on the obtained data, following the approach described by **Urrutia et al. (2014)** as the ratio between shoot dry weight (μg) and nutrient concentration in the shoot ($\mu\text{g}\cdot\text{g}^{-1}$).

5.3.7 Gene expression by qRT-PCR

Genes related to nutrient uptake and transport along with two H^+ -plasma membrane ATPases were analyzed. In particular, the focus was on *IRT1* (iron-regulated transporter 1), *FIT* (FER-like iron deficiency-induced transcription factor), *FRO2* (a low-iron-inducible root ferric chelate reductase), *NRT2.1* (a high affinity nitrate transporter), *COPT2* (copper transporter protein 2), and the two H^+ -plasma membrane ATPases, *AHA1* and *AHA7*. *EF1 α* (Elongation factor 1-alpha) and *UBQ10* (polyubiquitin-10) were used as reference genes.

Total RNA was isolated from 100 mg of frozen and ground roots collected 7 DAT using the NucleoSpin RNA Plant kit (Macherey-Nagel). RNA integrity and concentration were analyzed by fluorescence spectroscopy using a Qubit Fluorometer (Thermo Fisher Scientific). To determine the RNA concentration a Qubit RNA XR assay kit was applied. To evaluate the RNA quality, a Qubit RNA IQ assay kit was used. The Qubit assays utilize fluorescence dyes that selectively bind to RNA, allowing for accurate quantification and quality analysis. Reverse transcription was developed by applying iScript cDNA synthesis kit (Bio-Rad) and using aliquots containing 2 μg of total RNA. RT-qPCR reactions were prepared with 1 μL of cDNA template, 5 μL of SYBR Green Supermix (Bio-Rad), 1 μL of DNase-free deionized water, and 1.5 μL of the primer mix with 2 μM forward and reverse primers. The analysis and high-throughput data acquisition was developed on a CFX384 Touch Real-Time PCR System (Bio-Rad). Samples were mixed and placed into a thermocycler with a configuration

consisting in 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Gene expression analysis was performed using five independent root RNA samples per treatment, with each sample analyzed in duplicate RT-qPCR reactions.

Gene IDs and primer sequences are summarized in **Supporting Table S2**.

5.3.8 *Statistics*

Data statistics were developed using STATISTICA 10. Significant differences ($p \leq 0.05$) among treatments were evaluated by one-way analysis of variance followed by the Fisher LSD post hoc test. The normal distribution of data was analyzed by applying Shapiro–Wilk's test and the homogeneity of variances by Levene's test.

5.4 RESULTS AND DISCUSSION

5.4.1 *Chemical characterization of LHC180 and LHC215*

The main chemical characteristics of examined LHCs, including pH, EC and nutrient composition, are listed in **Table 1**. Both LHCs were acid, with LHC180 showing a lower pH (pH 4.52 vs 5.21). This reflects a more enhanced decomposition of organic acids under stronger HTC conditions (Malhotra et al., 2020; Falco et al., 2011; Fregolente et al., 2021). Moreover, the higher pH of LHC215 may be also attributed to the decarboxylation and/or the condensation of the terminal acid sites in the solid phase thus reducing the overall acidity of the resulting LHC (Czerwińska, K., et al., 2024). EC followed the same trend (7.07 vs. 5.46 mS cm⁻¹), indicating a higher concentration of soluble ions in LHC180. This can be probably associated to a structural alteration of the solid hydrochar, promoting the development of new sites with pH-dependent charges capable to retain on the solid phase multivalent cations. This

could have affected the concentration of ions in the LHC thus explaining the observed decrease in electrical conductivity (Niinipuu, M., et al., 2020). The TOC decreased from 9705 mg L⁻¹ in LHC180 to 4792 mg L⁻¹ in LHC215, thus indicating that milder HTC favors the solubilization of relatively labile organic compounds, whereas harsher conditions promote carbon loss via decarboxylation and condensation into more recalcitrant structures (Malhotra et al., 2020; Spitzer et al., 2018). TOC in SHA was around 400000 mg L⁻¹ (Aguirre et al. 2009). TON also decreased from 3.92 to 2.72 mg L⁻¹. This, along with the simultaneous decrease previously observed in the solid fraction (Curcio et al., 2025; not yet published, reported in Chapter V), indicates that part of the nitrogen may be lost via HTC-induced volatilization, in addition to incorporation into recalcitrant N-containing structures. High temperatures likely promote the thermal degradation of labile organic N compounds, consistent with previous reports of minor but significant nitrogen losses under severe HTC conditions (Huang et al., 2016; Zhang et al., 2023). The concentration of the organic nitrogen in SHA was around 1000 mg L⁻¹ (Aguirre et al., 2009). Macronutrient analysis confirmed that HTC severity strongly affects element solubilization. LHC180 was enriched in soluble K (1346 mg L⁻¹), Mg (135 mg L⁻¹), Na (211 mg L⁻¹), and P (141 mg L⁻¹), whereas all these elements decreased substantially in LHC215. Sulfur and several micronutrients, such as Mn, Zn and B, followed the same trend, highlighting stronger leaching under milder conditions. These data are very important since prove that the processing of horticultural biomass with the adopted HTC conditions enables the extraction of a significant amount of macro-, meso- and micronutrients, bioavailable for plants and soil microorganisms. Moreover, these results indicate a trade-off between nutrient solubilization and carbon stabilization: mild HTC promotes the release of soluble nutrients into the liquid phase, while harsher conditions reduce

nutrient availability through carbon condensation, nitrogen transformation, and partial volatilization.

Table 1. Chemical characterization of liquid hydrochars produced at 180 °C (LHC180) and 215 °C (LHC215). Parameters include pH, electrical conductivity (EC), elemental composition (C, N, S), and nutrient concentrations determined by Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES).

		LHC180	LHC215
pH		4.52	5.21
CE	mS/cm	7.07	5.46
TOC	mg/L	9705.00	4792.00
TON	mg/L	3.92	2.72
S	mg/L	66.58	28.41
P	mg/L	141.00	48.35
Ca	mg/L	0.00	0.32
Mg	mg/L	135.36	65.12
K	mg/L	1346.55	603.01
Na	mg/L	211.43	89.00
Si	mg/L	1.00	0.55
Sr	mg/L	0.14	0.03
Ba	mg/L	0.11	0.01
Al	mg/L	3.47	1.84
B	mg/L	0.70	0.33
Mn	mg/L	0.87	0.16
Zn	mg/L	0.45	0.00
Mo	mg/L	0.00	0.00

5.4.2 Total polyphenol content and terminal carboxyl- and amine-sites in LHCs

The determination of total polyphenol contents in LHC180 and LHC215 were 0.5 and 0.4 mg GAE mL⁻¹, respectively (**Figure 1a**). The fact that the TPC contents were relatively high can be due to the capacity of sub-critical water to extract polyphenols from solid vegetables. Moreover, studies on olive mill residues have demonstrated that HTC can also promote the hydrolysis of complex polyphenolic structures, leading to the release of simpler bioactive compounds such as hydroxytyrosol and tyrosol (**Gimenez, M., et al., 2020; Tirado, D. F., et al., 2019**). Interestingly, the fact that LHC215 showed a slightly lower TPC content indicates

that by increasing the HTC severity the pool of soluble polyphenols is reduced, likely due to their condensation into the solid hydrochar matrix or their thermal degradation in the aqueous phase. This means that HTC conditions influence both the abundance and the nature of phenolic compounds in liquid hydrochars, with potential consequences for their antioxidant properties and biostimulant activity.

The Bradford assay performed on LHCs to quantify their content in terminal carboxyl- and amine-sites. The values resulted almost similar with 0.098 and 0.09 mg BSA mL⁻¹ for LHC180 and LHC215, respectively (**Figure 1b**). Although relatively small, such a difference between LHC180 and LHC215 was statistically significant, indicating that intensification of the hydrothermal process parameters and consequent reaction severity promotes enhanced degradation of compounds with sites characterized by pH-dependent charges. This is perfectly in line with the results on pH, salinity and the nutrients content in previous literature (**Li, H., et al., 2023; Sun et al., 2020**). Part of detected sites can be ascribed to proteins and amino acids whose content has diminished as a function of the reaction temperature. In fact, the HTC may have promoted their hydrolysis and deamination, producing ammonia with a slightly alkalization of LHC (**Alhndi et al., 2020; Sun et al., 2020**). In addition, some of the released nitrogen may be incorporated into heterocyclic compounds via Maillard-type reactions with reducing sugars, while a portion of NH₃ can be lost through volatilization under elevated temperature and alkaline conditions (**Wang, T., et al., 2018**).

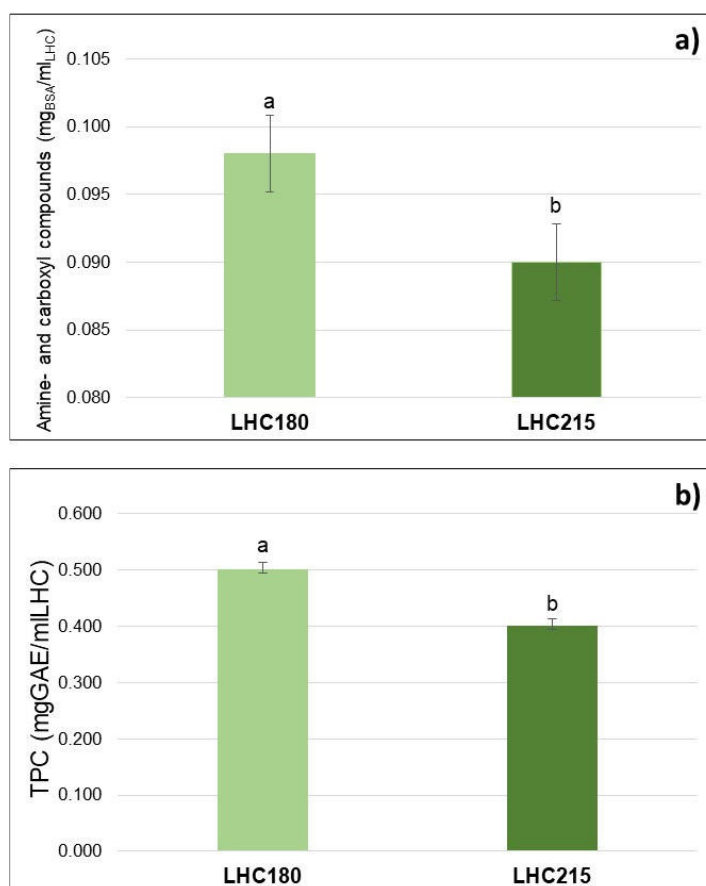


Figure 1. a) Content in terminal amine- and carboxyl-groups as detected via Bradford assay in LHC180 and LHC215. Values are expressed as mg of bovine serum albumin equivalents (BSA) /mL of LHC; b) Total polyphenol content (TPC) detected in LHC180 and LHC215. Values are expressed as mg of gallic acid equivalents (GAE)/ mL of LHC. In both cases, different letters above the bars indicate statistically significant differences (one-way ANOVA, $p < 0.05$).

5.4.3 *Phytohormonal content*

Liquid fraction obtained from hydrothermally carbonized plant biomass, such as LHCs, have been reported to contain a notable fraction of bioactive compounds, including phytohormones and hormone-like metabolites, which can act as biostimulants (Chen, S., et al., 2025; Lu et al., 2021). However, the concentration of the different plant regulators analysed in SHA was below the detection limits (Aguirre et al., 2009). In addition, it has been proven that the feedstocks used in this study (red chicory, spinach and escarole) are naturally rich in bioactive

constituents (Imani Asl et al., 2024; Leibar-Porcel et al., 2020). These intrinsic hormonal pools and their metabolic precursors, such as amino acids and phenolic compounds, represent valuable substrates for transformation during HTC. In this context, under milder reaction conditions, the HTC process promotes the release, alteration and, in some cases, *de novo* formation of such a type of compounds (Y. Tang et al. (2022)). Partial hydrolysis of plant macromolecules (including proteins, amino acids, and phenolic constituents) generates low-molecular-weight metabolites that can function as auxins, cytokinins, or stress-related signaling molecules (e.g., salicylic acid and jasmonic acid). Moreover, the acid environment, which typically characterizes the HTC process, favors the liberation of conjugated phytohormones, enhancing their solubility in the aqueous fraction (Y. Tang., et al., 2022).

The exam of studied LHCs indicated the content of phytohormones as well as a clear effect of processing severity on their composition (Table 2). A substantial amount of acid phytohormones were detected in LHC180, including salicylic acid, indole-3-acetic acid, and jasmonic acid. They were revealed also several cytokinins, such as cis-zeatin, trans-zeatin, isopentenyl adenine, along with their riboside derivatives (cis-zeatin riboside, trans-zeatin riboside, isopentenyl adenine riboside). They were identified also abscisic acid and the jasmonate conjugate although at lower concentrations. In LHC215 the total amount of phytohormones was markedly lower. SA, IAA, and JA were less abundant than in LHC180, while ABA resulted even below the detection limit. Similarly, cytokinins were strongly diminished, with only trace levels of cZ and tZ still detectable, whereas cZR, tZR, iP, and iPR were no longer quantifiable (Table 2).

Each of these phytohormones plays a distinct role in plant physiology. SA is a phenolic signaling molecule involved in photosynthesis, ion transport, and defense responses against biotic and abiotic stress (Leibar-Porcel et al., 2020). The high abundance of SA in LHCs can

be attributed to both the phenolic richness of the initial feedstock and the transformations promoted by HTC (in case of LHC180), where mild to moderate reaction conditions allow partial hydrolysis and oxidation of phenolic precursors without extensive degradation (Sambyal & Singh, 2021). Under harsher conditions, SA content decreased sharply, reflecting the thermal sensitivity of phenolic compounds and their condensation into recalcitrant fractions. IAA, which represents one of the principal auxins, regulates cell elongation, root branching, vascular patterning, and organ initiation in plants (Imani Asl et al., 2024). Its high abundance in LHC180 can be related to the hydrothermal release of tryptophan from protein hydrolysis, which is a key precursor for indolic auxins, and to indole derivatives formed via Maillard-type reactions (Chen et al., 2025; Cai et al., 2023). At higher severity, IAA levels decreased due to oxidation into inactive metabolites such as OxIAA and to thermal degradation of the indolic ring (Nissen et al., 1990; Chen et al., 2025). JA functions as a stress-related hormone, mediating responses to biotic and abiotic stressors such as pathogen attack, drought, and salinity. Its presence in LHC180 likely stems from the hydrolysis of membrane lipids and subsequent β -oxidation of polyunsaturated fatty acids, which are precursors of the oxylipin pathway (Eng et al., 2021). However, being a thermolabile oxidized fatty acid derivative, JA undergoes rapid degradation under harsher HTC conditions, explaining its decline in LHC215. Cytokinins, including zeatin derivatives and isopentenyladenine, regulate cell division, leaf senescence, nitrogen-responsive growth, and grain number. CK content was significantly higher under mild HTC conditions and was largely reduced in LHC215, with several CKs dropping below detection limits. This trend reflects the thermal sensitivity of these adenine derivatives, where higher temperatures and pressures promote degradation via decarboxylation, oxidation, or dehydration and milder conditions

favor CK biosynthesis by preserving nucleoside precursors released from solubilized proteins and nucleic acids (Matusková et al., 2023).

These results highlighted the determinant role played by HTC temperature and pressure in modulating the profile of soluble bioactive phytohormones in LHCs. Milder conditions favor the accumulation of SA, IAA, JA, and CKs, enhancing their potential as biostimulants, whereas excessive severity leads to their degradation.

Table 2. Acid phytohormone and cytokinin contents (pmol mL⁻¹) detected in LHC180 and LHC215. The abbreviations SA, IAA, JA, ABA, JA-Ile, cZ, tZ, cZR, iP, tZR, and iPR correspond to salicylic acid, indole-3-acetic acid, jasmonic acid, abscisic acid, jasmonate conjugate, cis-zeatin, trans-zeatin, cis-zeatin riboside, isopentenyl adenine, trans-zeatin riboside, and isopentenyl adenine riboside, respectively.

		LHC180	LHC215
Acid phytohormones	IAA	91.27	32.66
	ABA	1.33	< 0.5
	SA	1222.56	243.50
	JA	40.06	20.27
	JA-Ile	0.57	0.54
Cytokinins	tZ	6.27	1.58
	cZ	11.87	1.91
	tZR	0.25	<0.06
	cZR	3.73	<0.06
	iP	1.69	<0.1
	iPR	0.58	<0.06

5.4.4 Humification degree of LHCs assessed by Spectroscopic Indexes and Synchronous Fluorescence Using SHA and SRFA as References

In order to evaluate the humification degree of developed LHCs, they were examined and compared to two model and commercial humic substances. In particular, we considered the

sedimentary origin humic acid (SHA) and Suwannee River fulvic acid (SRFA) which have been already characterized previously (Aguirre et al., 2009; Thorn, 1987).

Fuentes et al. (2006) suggested that the complementary consideration of the molar absorptivity coefficients at 600 and 280 nm, the absorbance ratios at 253 and 220 nm (E_{Et}/E_{bz}) and the fluorescence index (A_{440} ; Milori et al. 2002) allows a determination of the humification degree of investigated organic material. In Table 3 are reported the UV-VIS and Fluorescence indices of LHC180 and LHC215. The results report that the molar absorptivity coefficients at 600 nm and 280 nm indicated that the degree of humification of LHCs was lower than that of the sedimentary humic acids (SHA) and the IHSS fulvic acid standard (SRFA). However, the E_{Et}/E_{bz} index, which indicates that the humification could be associated with the presence of more substituted aromatic rings in the molecules, was similar for all the studied materials. Similarly, no clear relationship was found between humification and E_4/E_6 values. Chen et al. (1977) suggested that these results may not be related to the aromaticity of the samples, but rather to differences in molecular weight, noting an inverse relationship between E_4/E_6 and molecular weight. In this context, the molecular weight of LHCs was more similar to that of SRFA, and, as expected, lower than that of SHA.

Figure 2 displays the comparison among the synchronous fluorescence spectra of SHA, SRFA, and the two developed LHCs. Materials characterized by a pronounced humification degree typically exhibit intense peaks at wavelengths longer than 450 nm, with an increase in humification causing a shift of peaks toward higher wavelengths and vice versa (Senesi et al., 1991). In this study, SRFA showed its highest intensity at an average wavelength of around 425 nm, whereas SHA exhibited prominent signals at wavelengths even beyond 520 nm, reflecting its higher humification state. Consistent with the UV-VIS and fluorescence indexes

reported in **Table 3**, SHA demonstrated a considerably more advanced humification than SRFA.

Both examined LHCs displayed similar profiles, characterized by an intense peak at 345 nm accompanied by a weaker shoulder at 360 nm. This pattern, together with A440 and I350/I480 values, indicates a low degree of humification and the predominance of labile, chemically simple organic matter rather than highly condensed or aromatic structures. Such labile organic fractions are potentially available as a source of carbon and energy for soil microorganisms, which may enhance nutrient mineralization and overall soil biological activity.

Fluorescence indexes further support these observations: the A440 values of both LHCs were comparable to those of SRFA but substantially lower than those of the humate system, which generally exhibited higher A440 values due to its more advanced humification (**Fuentes et al., 2006**). Higher fluorescence intensity at shorter wavelengths has been associated with simpler chemical structures, such as less condensed aromatic rings or reduced conjugation in aliphatic chains (Senesi et al., 1991), consistent with the I350/I480 results showing a greater proportion of simple structures in LHCs compared to more humified materials.

Finally, the profile of synchronous fluorescence spectra shifts toward longer wavelengths as humification increases; these results confirm that both LHCs possess a lower humification degree than SHA and SRFA, and that harsher hydrothermal carbonization treatment did not substantially alter the humified conformation of the products. Overall, despite the low humification degree, the presence of labile organic matter supports the potential application of LHCs as bioactive soil amendments capable of stimulating microbial activity and potentially improving nutrient availability for plants, highlighting their promise in sustainable horticultural practices and circular economy strategies.

Table 3. UV–VIS and fluorescence spectroscopic parameters of LHC180 and LHC215, SHA and a Suwannee River fulvic acid (SRFA). UV–VIS data include molar absorptivity coefficients at 280 and 600 nm, EEt/Ebz ratio, and E4/E6 values, while fluorescence indices comprise A₄₄₀ and I₃₅₀/I₄₈₀ ratios, used to assess the humification degree and molecular complexity of the samples.

^aRatio of absorbances at 465 and 665 nm (E4/E6); ^bmolar absorptivity at 600 nm (L·cm⁻¹·mol of OC⁻¹); ^cmolar absorptivity at 280 nm (L·cm⁻¹·mol of OC⁻¹); ^dratio of absorbances at 253 and 220 nm (EEt/Ebz); ^earea under fluorescence emission spectra with λ_{exc} = 440 nm (A₄₄₀); ^fratio of fluorescence intensities at 350 and 480 nm in synchronous-scan excitation spectra (I₃₅₀/I₄₈₀).

Sample	UV-VIS indexes				Fluorescence indexes	
	E ₄ /E ₆ ^a	ε ₆₀₀ ^b	ε ₂₈₀ ^c	E _{Et} /E _{Bz} ^d	A ₄₄₀ ^e	I ₃₅₀ /I ₄₈₀ ^f
SHA	5.1	30.3	68	1.2	1200319	0.1
SRFA	14.8	2.3	73	2.6	402372	0.4
LHC180	12.1	0.4	22	1.8	136481	15.7
LHC215	10.5	0.6	19	1.4	228370	14.0

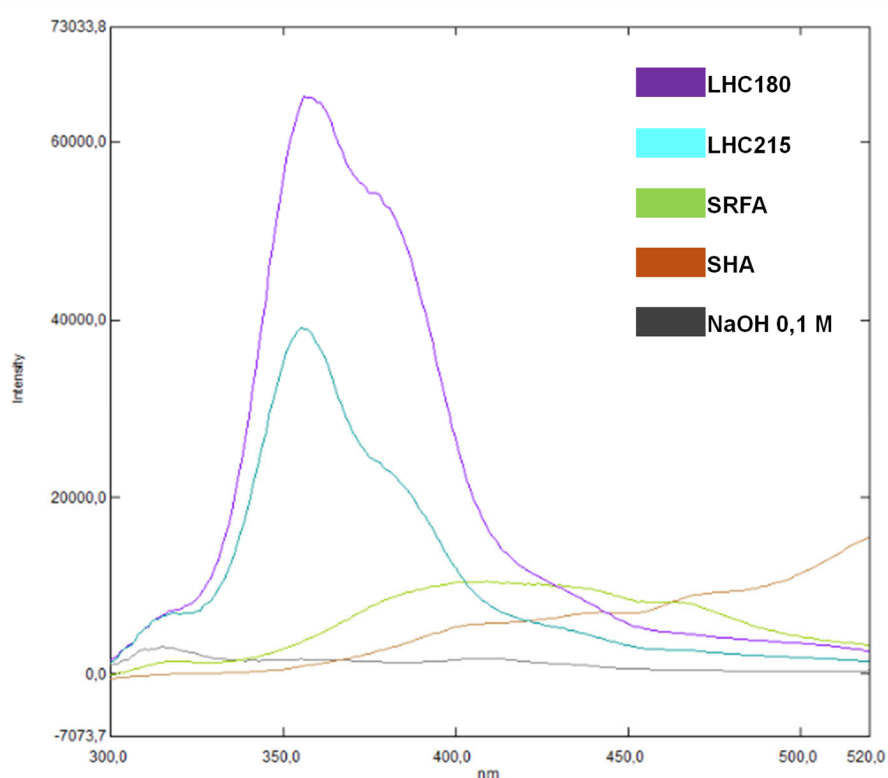


Figure 2. Synchronous fluorescence spectra of LHC obtained at 180 (LHC180, purple) and 215 (LHC215, light blue) °C, sedimentary humic acids (SHA, brown) and Suwannee river fulvic acids (SRFA, green), showing the spectral shifts associated with humification degree and compared with a control (NaOH 0.1M, grey).

5.4.5 Phytotoxicity and biostimulant assay with LHCs

The phytotoxicity assay was launched to evaluate the potential effects of LHCs on the modulation of both seed germination and early development of cress seeds (**Vitti et al., 2024; Bento et al., 2020; Fregolente et al., 2020**). Seeds treated with undiluted LHCs did not germinate at all, indicating a strong phytotoxic effect of the raw samples (**Q. Lang, et al., 2025**). At a concentration of 50 mg L⁻¹ of organic carbon, no significant differences were observed than the CTRL (data not shown). The application of LHCs at a concentration of 100 mg L⁻¹ of organic carbon elicited a significant positive effect on root development (**Fig. 3a**). In particular, seeds exposed to LHC180 showed the highest root elongation, which was significantly greater than the CTRL and comparable to the positive effects induced by SHA (**Mora et al., 2010; Olaetxea et al., 2015**). Similarly, LHC215 promoted a certain root elongation, although to a slightly lower extent than LHC180. By contrast, no significant effects were detected on epicotyl growth (data not shown). The analysis of germination indexes provided further insights into the biostimulant activity of LHCs (**Sobarzo-Bernal, O., et al., 2021; Benech Arnold, R. L., et al., 1991**) (**Fig. 3b**). Seed germination percentage (SG%) remained unaffected by the LHC180, whereas it slightly decreased in the case of LHC215 and SHA (90%). In contrast, the root growth rate (RGR%) was consistently stimulated by the LHC treatments (180: 116.1%; 215: 112.7%) as compared to the CTRL, with results similar to those obtained with SHA (111.4%). Consequently, the germination index (GI%), which integrates both germination and root growth responses, showed the most pronounced increase for the LHC treatments, with the most significant one observed for LHC180, which reached 116.1%, whereas LHC215 did not show any relevant improvement (101.4%). Overall, the stimulatory impact was more pronounced on root growth than on germination. Although these are only preliminary results, we can conclude that LHC180,

when applied at a concentration of 100 mg L^{-1} of organic carbon, may represent an effective and innovative biostimulant for plants.

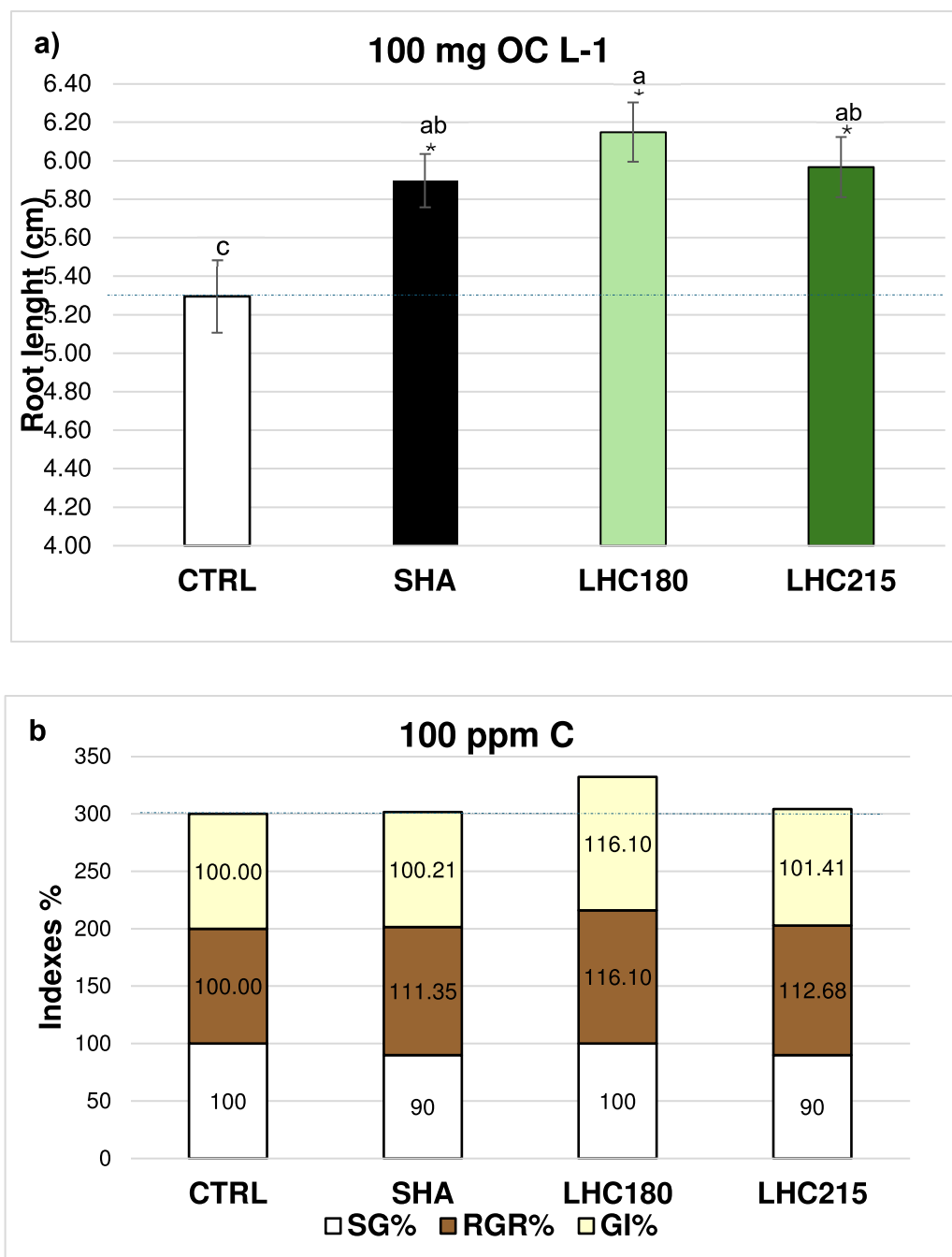


Figure 3: Effects of 100 mg OC L^{-1} of LHC180 and LHC215 on cress seed germination and early seedling development. (a) Root elongation 5 days after the treatments ($n=3$; Different letters above bars indicate statistically significant differences among treatments via one-way ANOVA at α, p value < 0.05); (b) Germination-related indexes: germination indexes (GI%), seed germination % (SG%), relative growth root (RGR). CTRL and SHA labels indicate seeds treated with water and sedimentary humic acids, respectively.

5.4.6 Identification of phytohormone-like response in LHC180

The cress seed bioassay highlighted distinct differences among the treatments (**Fig. 4a**). Root length was again significantly enhanced by LHC180, as compared to both the untreated control and SHA ($p < 0.05$), with LHC180 eliciting the most enhanced roots development. While SHA increased root length, as compared to CTRL, although with an effect less pronounced than that induced by LHC180. In contrast, the MIX-P did not significantly differ from the CTRL and resulted in shorter roots than both LHC180 and SHA.

These patterns were further corroborated by relative indices (**Fig. 4b**). SG% remained fairly consistent across all treatments, whereas RGR% was highest in seedlings treated with LHC180, followed by SHA, reflecting the greater biostimulant power of the innovative product. The GI% peaked in LHC180-treated seeds, reinforcing the conclusion that its stimulatory effect cannot be solely attributed to its phytohormonal content. Instead, the results suggest a synergistic action of the multiple bioactive components present in LHC180, which exceeds the effect of SHA (Massaya et al., 2022; Olaetxe et al., 2024).

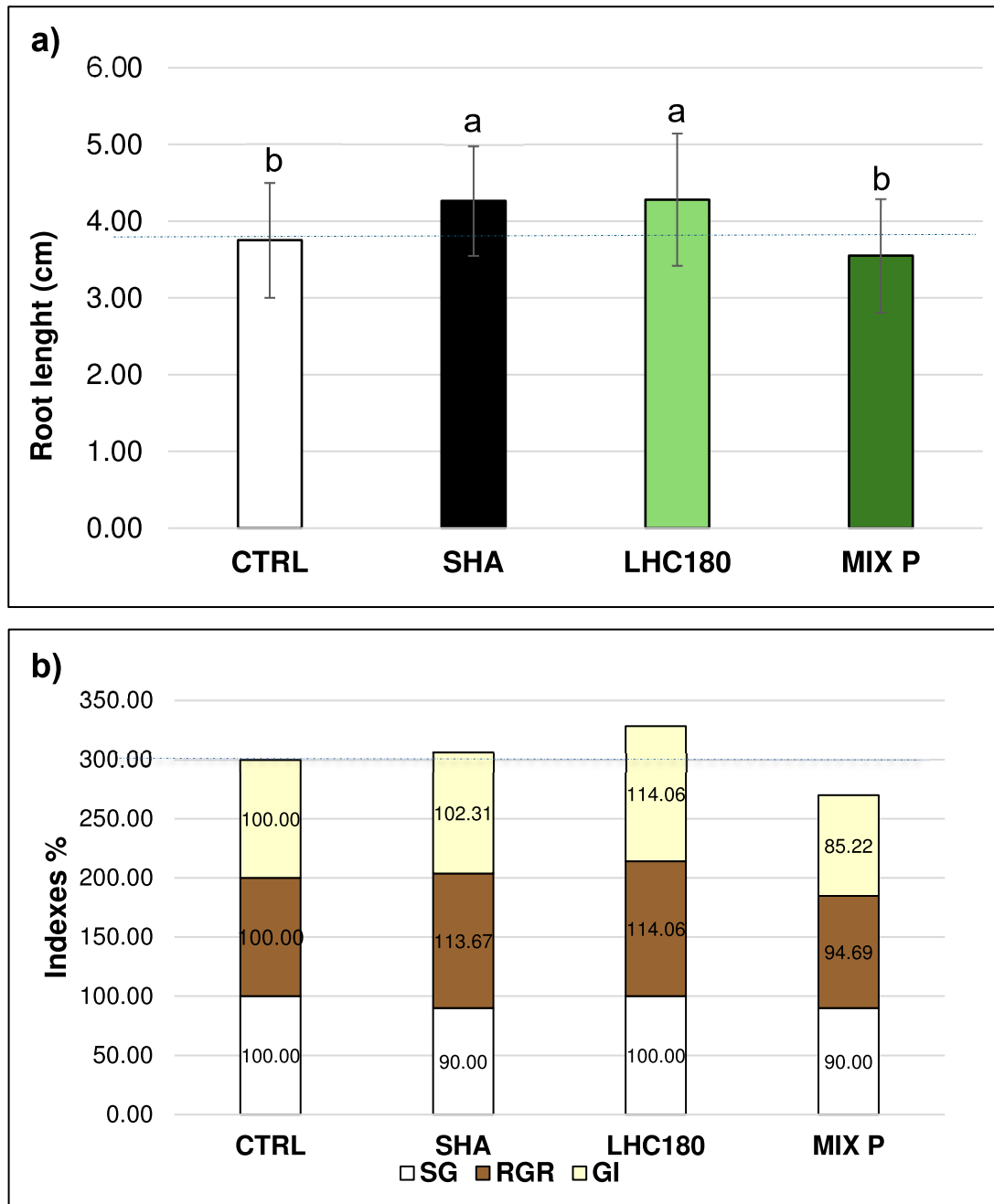


Figure 4. Effects of induced by LHC180 and the solution of phytohormones (MIX-P) on cress seed germination and early seedling development at the concentration of 100 mg OC L^{-1} : (a) Root length (cm) measured 5 days after treatments ($n=3$; Different letters above bars indicate statistically significant differences among treatments via one-way ANOVA at $a, p \text{ value} < 0.05$); (b) Germination-related indices, including seed germination (SG%), relative root growth (RGR%), and germination index (GI%). CTRL and SHA labels indicate seeds treated with water and sedimentary humic acids, respectively.

5.4.7 Study of the mechanism of action of LHC180 and LHC215 using an established functional humic acid plant stimulation framework.

5.4.7.1 The LHCs promoted shoot and root fresh weight in *A. thaliana*.

The two LHCs were applied together with SHA at a concentration of 100 mg OC L⁻¹ to evaluate their potential effects on plant development based on a functional plant stimulation framework (Aguirre et al., 2009; Jannin et al., 2012; Mora et al., 2010; Olaetxea et al., 2018). The application of both LHC180 and LHC215 at this concentration significantly promoted shoot growth in Arabidopsis (Fig. 5a; Supporting Figures S2).

Regarding roots, two main aspects were considered: overall root growth and modifications in root morphology. Root growth was also enhanced by both LHCs; however, the expected promotion under SHA application was not consistently observed (Fig. 5b). Morphological analysis using WinRHIZO software indicated that both SHA and LHCs increased root length and the number of tips (Fig. 6a, 6b; Supporting Figures S4), whereas no significant increases were observed for root forks or crossings (Supporting Figure S4).

Relating plant development to the physicochemical characteristics of the compounds, neither the degree of humification nor the hormone content alone fully explains the ability to promote shoot and root growth. Both products, despite their substantially lower degree of humification compared to SHA, were able to stimulate plant development. This suggests that, rather than humification degree itself, the molecular conformation in solution may play a more critical role. Indeed, the conformation of humic acids has been shown to be crucial for promoting root and shoot growth (Olaetxea et al., 2021; Aranaz et al., 2023).

Similarly, LHCs contained measurable concentrations of key phytohormones (Table 3). However, a reconstructed phytohormone mixture based on LHC180 did not induce root

stimulation in the comparative bioassay previously reported indicating that the observed bioactivity likely arises from synergistic effects of multiple molecular components within the LHCs, rather than the action of individual hormones alone (**Fig. 4**). These observations suggest that the plant growth-promoting effect of LHCs does not derive solely from the hormonal concentration in solution, although a complementary role of phytohormones in growth stimulation cannot be excluded, pointing to a complex, multifactorial mechanism of action that involves both molecular conformation and chemical composition.

Overall, further studies are required to elucidate the relationship between the structural features of LHCs in solution and their plant growth-promoting effects.

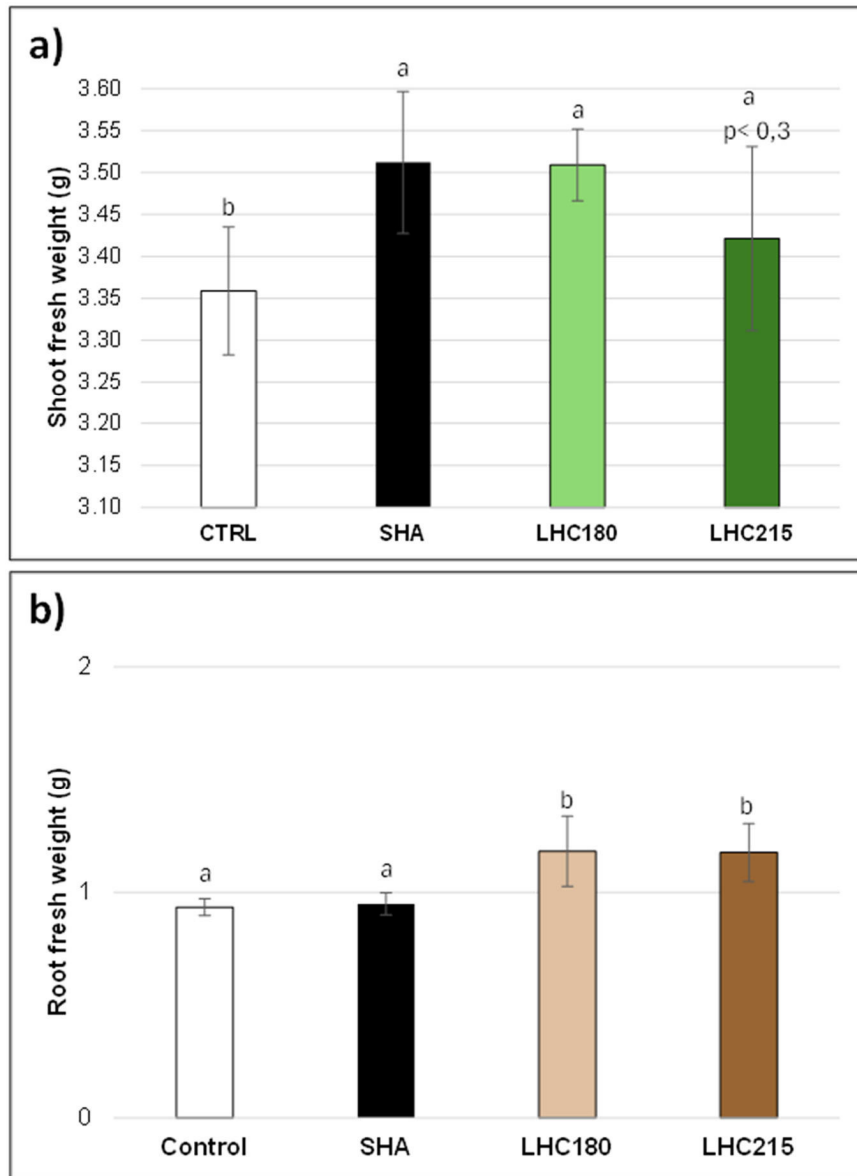


Figure 5. (a) Shoot fresh weight and (b) root fresh weight of *Arabidopsis thaliana* plants treated with LHC180, LHC215, and sedimentary humic acids (100 mg OC L⁻¹). The figures show average values $\pm$ SD. CTRL indicates seeds treated with water. Different letters above bars indicate statistically significant differences among treatments determined by one-way ANOVA ($p < 0.3$).

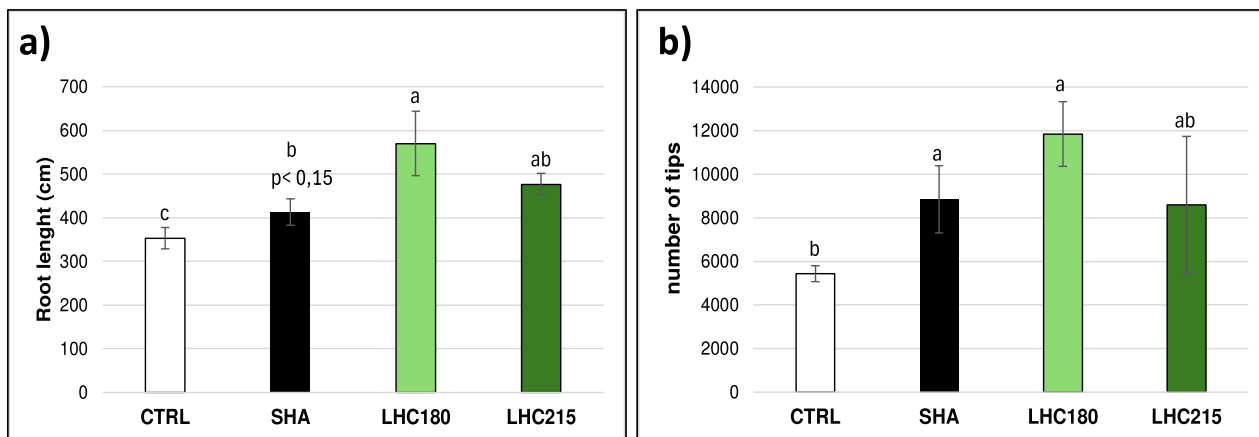


Figure 6. Morphological traits of *Arabidopsis thaliana* roots treated with SHA and LHC180 (100 mg OC L⁻¹). (a) Root length and (b) number of root tips. Data represent average values $\pm$ SD. CTRL indicates seeds treated with water. Different letters above bars indicate statistically significant differences among treatments determined by one-way ANOVA ($p < 0.05$).

5.4.7.2 Hormonal changes

Previous studies demonstrated that SHA application altered principally IAA, ABA, cytokinin and JA/JA-Ile hormonal responses, leading to shoot and root growth stimulation (Mora et al., 2010; Olaetxea et al., 2018).

In this study, the cytokinin-enhancing effect of SHA was also evident for both LHC180 and LHC215 in shoots (Figure 7b). Similarly, the typical SHA-driven modulation of the IAA–ABA balance in roots was reproduced, although the two LHCs behaved differently. LHC180 promoted the accumulation of both IAA and ABA, whereas LHC215 failed to induce significant variations in either hormone (Olaetxea et al., 2019) (Figure 7c, d). This divergent behavior suggests that LHC180 retains a higher fraction of bioactive compounds capable of triggering the auxin–ABA signaling cascade involved in root development. (Sharp et al., 2022).

No significant increases were observed in JA/JA-Ile and salicylic acid (SA) levels compared to the control (Supporting Figure S4).

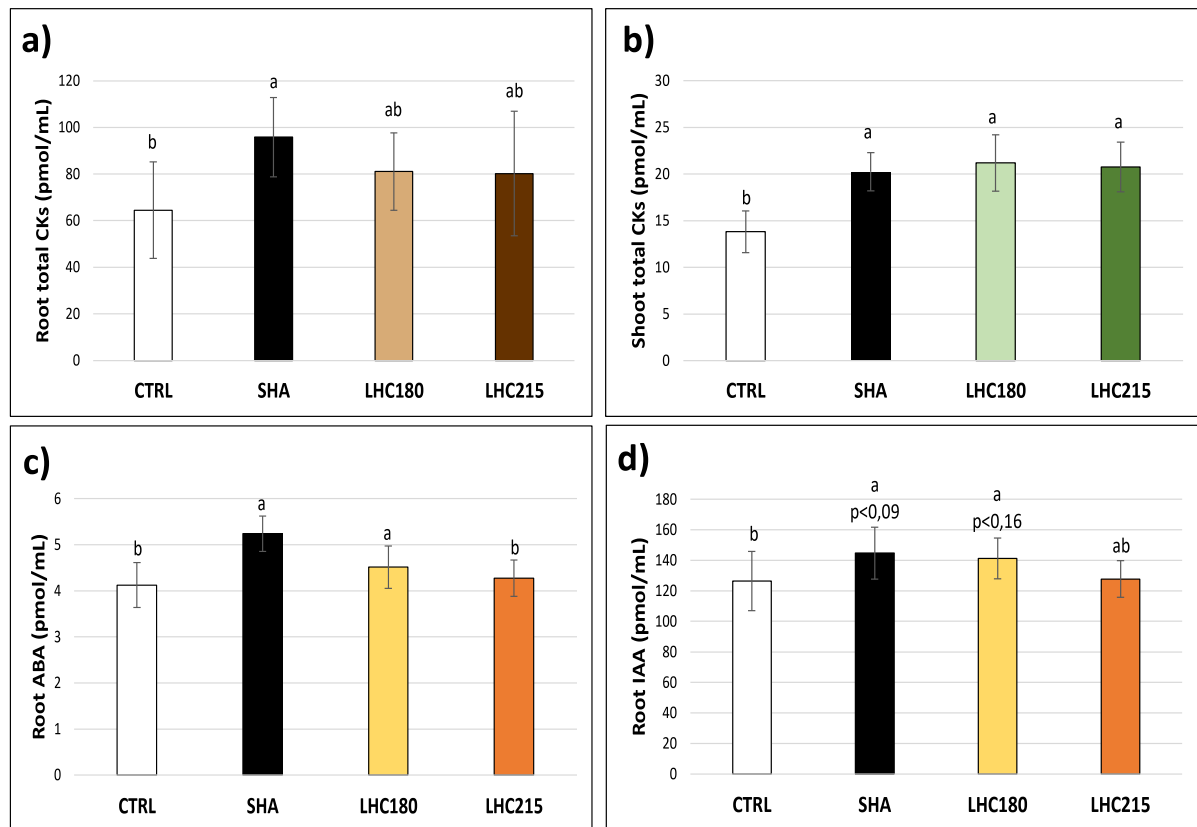


Figure 7. Hormone contents in *Arabidopsis thaliana* treated with LHC80, LHC215, and sedimentary humic acids (SHA) at 100 mg OC L⁻¹. (a, b) Cytokinin levels in shoots and roots, respectively; (c, d) indole-3-acetic acid (IAA) and abscisic acid (ABA) levels in roots. CTRL and SHA indicate seeds treated with water and humic acids, respectively. Data represent average values $\pm$ SD. Different letters above bars indicate statistically significant differences among treatments (one-way ANOVA, $p \leq 0.05$).

5.4.7.3 Nutrient transporters and H⁺-plasma membrane ATPase.

The ability of SHA to induce the upregulation of nutrient-related genes and enhance shoot nutrient use efficiency for key macro- and micronutrients is well documented (Aguirre et al., 2009; Jannin et al., 2012; Olaetxea et al., 2018). Accordingly, the expression of the iron uptake-related genes FIT and FRO2, as well as the copper transporter COPT2, was activated in roots treated with SHA (Fig. 8). Regarding the LHC treatments, the results showed that LHC215 significantly increased the expression of FRO2 and COPT2 (Fig. 8b, c), indicating

that the more condensed LHC can still trigger nutrient transporter activation, particularly for Fe and Cu acquisition.

Consistent with previous studies (Aguirre et al., 2009; Olaetxea et al., 2019), SHA also upregulated both plasma membrane H⁺-ATPase homologues (AHA1 and AHA7). Similarly, both LHC180 and LHC215 significantly enhanced the expression of these H⁺-ATPase genes (Fig. 8d, e), confirming that root proton pump activation represents a conserved response mechanism. These findings are in line with previous works demonstrating a close relationship between H⁺-ATPase regulation and the enhancement of nutrient uptake at the root level (Mora et al., 2010).

Expression patterns in all treatments were associated with improved nutrient use efficiency in both roots and shoots for several essential macro- and micronutrients (Fig. 9, Supporting Figure S5). This coordinated transcriptional response is known to be governed by upstream IAA/NO stimulation, which contributes to shoot and root growth as well as to root morphological remodeling (Mora et al., 2014). In these experiments, in addition to the well-established IAA/NO–root ATPase–nutrient uptake pathway induced by SHA (Zandonadi et al., 2010; Olaetxea et al., 2018), both LHC180 and LHC215 were also (at least partially) able to activate this signaling cascade. Interestingly, root morphological traits such as total root length and number of tips were less stimulated under LHC215 treatment (Fig. 6). Consistently, root IAA levels were not enhanced by LHC215 (Fig. 7), which may explain the limited morphological response. Likewise, root ATPase activation is typically accompanied by cytokinin accumulation in the shoot, explaining the observed shoot growth promotion (Mora et al., 2010; Olaetxea et al., 2018). Under both LHC treatments, root ATPase upregulation occurred in parallel with increased shoot cytokinin levels (Figs. 7b and 8d, e).

The present results confirm and extend the well-established capacity of humic substances to enhance plant nutrient acquisition and growth. As previously reported, SHA treatment upregulated key nutrient-related genes (FIT, FRO2, and COPT2) and plasma membrane H⁺-ATPase homologues (AHA1 and AHA7), resulting in improved nutrient use efficiency in both roots and shoots (**Figs. 8 and 9; Aguirre et al., 2009; Jannin et al., 2012; Olaetxea et al., 2018**). Importantly, both hydrochar-derived products, LHC180 and LHC215, were also able to trigger these responses, albeit with distinct profiles. LHC215 significantly stimulated FRO2 and COPT2 expression (Fig. 8b, c), highlighting its potential to enhance Fe and Cu acquisition even though its effects on root morphology and IAA/ABA levels were limited (**Figs. 6 and 7**). In contrast, LHC180 exerted a broader activity, activating the IAA–ABA hormonal axis, promoting root elongation and tip formation. Both LHC treatments increased shoot cytokinin levels and root H⁺-ATPase expression (**Figs. 5b, 6, 7, 8d, e**).

These patterns suggest that LHC180 operates through a dual mode of action, simultaneously modulating hormonal signaling and nutrient uptake pathways, while LHC215 primarily targets nutrient transporter activation and proton pump regulation. The observed coordination between root H⁺-ATPase upregulation and cytokinin accumulation in the shoot aligns with previous models linking auxin–ABA–cytokinin crosstalk to root-driven nutrient uptake and shoot growth promotion (**Mora et al., 2010; Olaetxea et al., 2018; Zandonadi et al., 2010**). Unlike SHA, which has been reported to modulate the JA/JA-Ile signaling pathway (Olaetxea et al., 2019), no significant variation in jasmonate-related responses was detected under our experimental conditions. This may be related to the sampling time or to the relatively high basal JA levels observed in control plants, potentially masking treatment effects.

Taken together, these findings indicate that hydrochar-derived LHCs mimic key functional traits of humic acids, enhancing root and shoot growth predominantly through hormonal

crosstalk and proton pump activation. The differential effects observed between LHC180 and LHC215 likely reflect differences in their composition and chemical complexity, which in turn may be influenced by HC production parameters such as temperature and pressure. Overall, the study provides a coherent mechanistic framework linking LHC bioactivity to improved root morphology, nutrient uptake, and hormonal regulation, offering a promising basis for their application as biostimulants in sustainable agriculture.

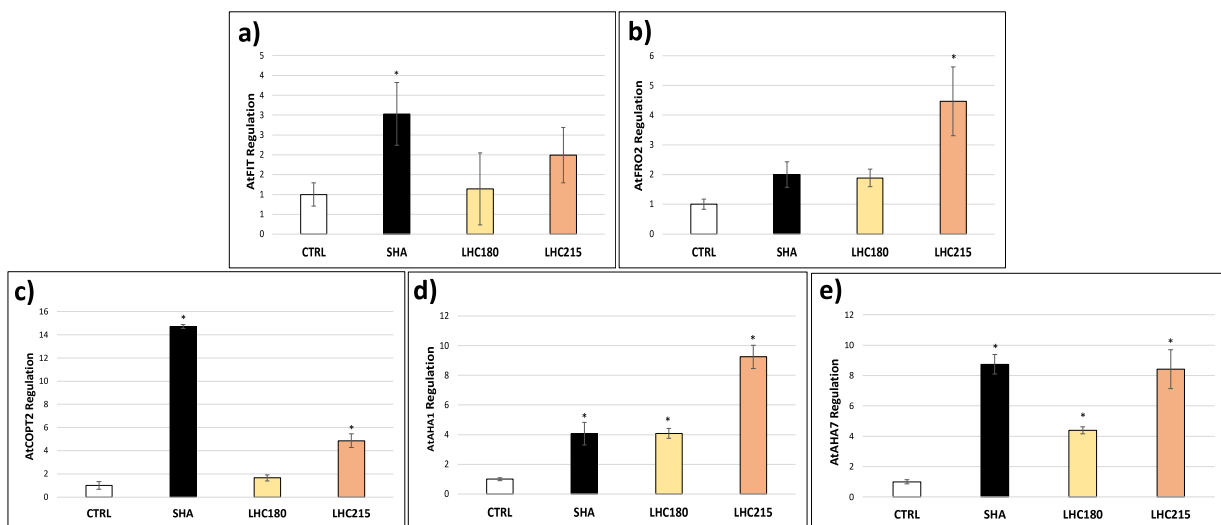


Figure 8. Relative expression of nutrient transporter genes, *FIT* (a), *FRO2* (b), *COPT2* (c), and plasma membrane H⁺-ATPase isoforms *AHA1* (d) and *AHA7* (e), in roots of *Arabidopsis thaliana* treated with LHC AGR180, LHC AGR215, and sedimentary humic acids (SHA) at a concentration of 100 mg OC L⁻¹. Different letters indicate statistically significant differences among treatments (one-way ANOVA, p ≤ 0.05).

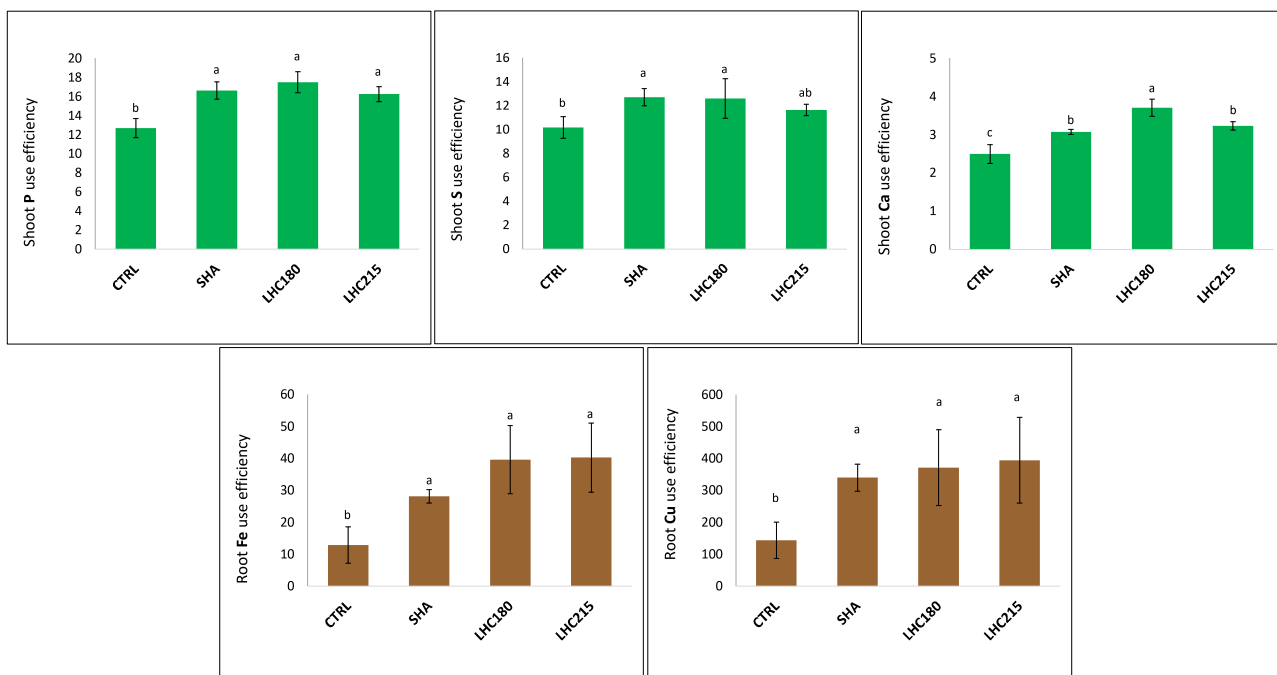


Figure 9. Nutrient use efficiency (NUE) in roots (brown) and shoots (green) of *Arabidopsis thaliana* after treatment with LHC180, LHC215, and SHA at 100 mg OC L⁻¹. Different letters indicate significant differences among treatments (one-way ANOVA, $p \leq 0.05$).

5.5 CONCLUSIONS

In conclusion, hydrochars derived from horticultural biomass can be effectively transformed into liquid products exhibiting strong biostimulant activity, capable of enhancing shoot growth, root development, and nutrient use efficiency. Among the two LHCs studied, LHC180 displayed a broader and more pronounced bioactive profile, simultaneously modulating hormonal signaling pathways (IAA–ABA–CK), promoting root morphogenesis and enhancing root ATPase–nutrient uptake pathway, whereas LHC215 primarily enhanced nutrient transport and proton pump activity, likely reflecting differences in hydrochar composition and quality influenced by production parameters such as temperature and pressure.

The plant-derived and liquid nature of these products makes them particularly suitable for integration into sustainable fertigation systems, where they could improve nutrient efficiency, crop performance, and resilience while valorizing vegetable processing residues. Moreover, their targeted effects on root morphology, hormone regulation, and nutrient acquisition suggest that tailored hydrochar-based biostimulants could be designed for specific crop requirements or growth stages.

Future research should focus on **field-scale evaluations** to validate their practical agronomic benefits under real cultivation scenarios. In addition, investigating the long-term impacts on soil health and microbiome interactions would provide further insights into the broader sustainability potential of these materials. Overall, these findings position hydrochar-derived liquid products as a promising and versatile tool for advancing circular bioeconomy strategies in agriculture.

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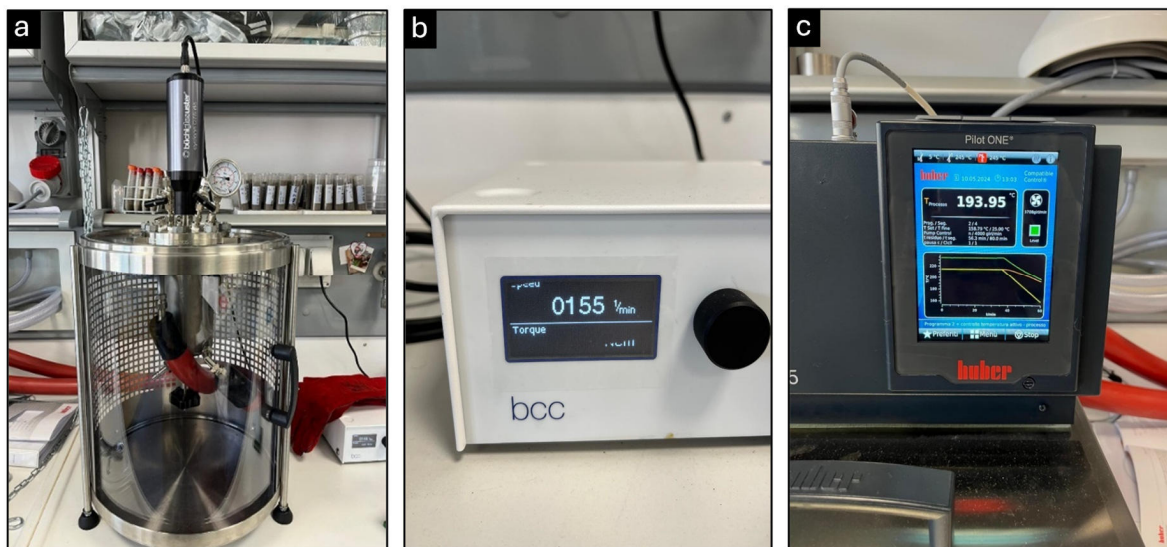
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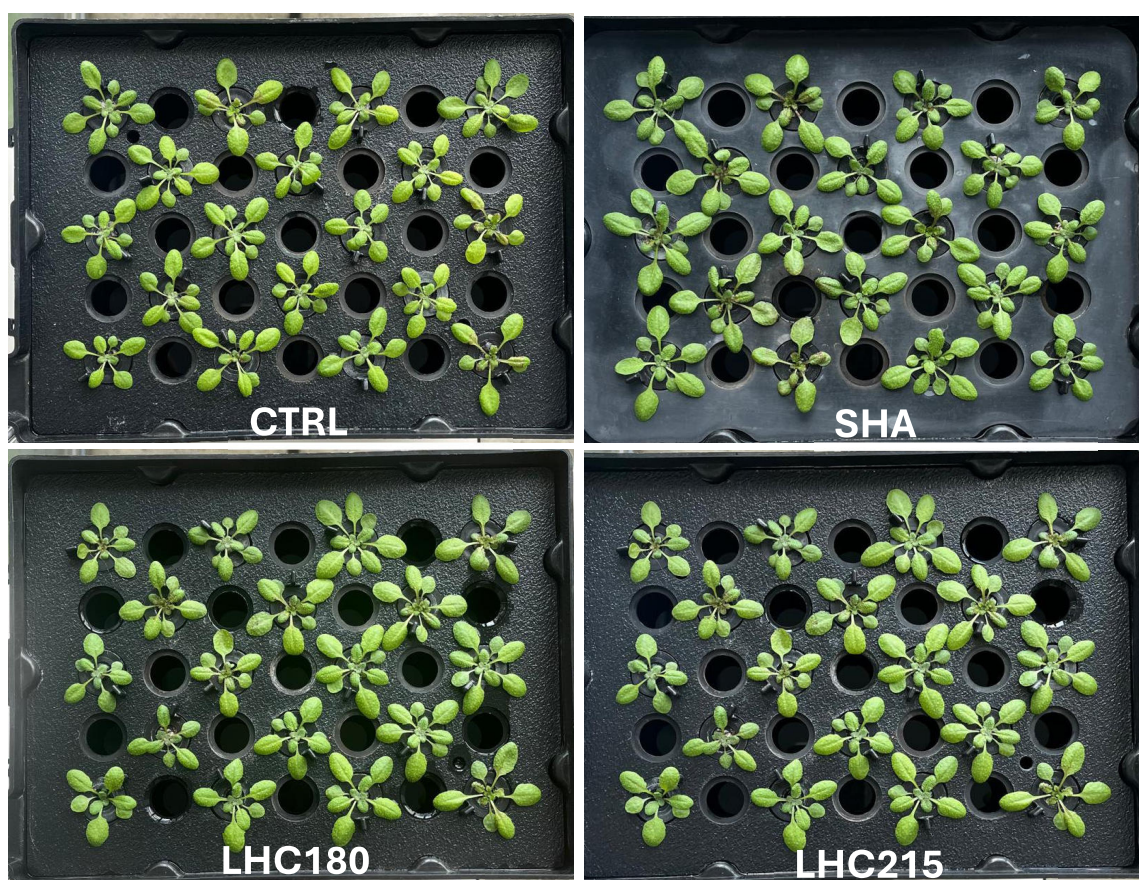
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Supporting Information

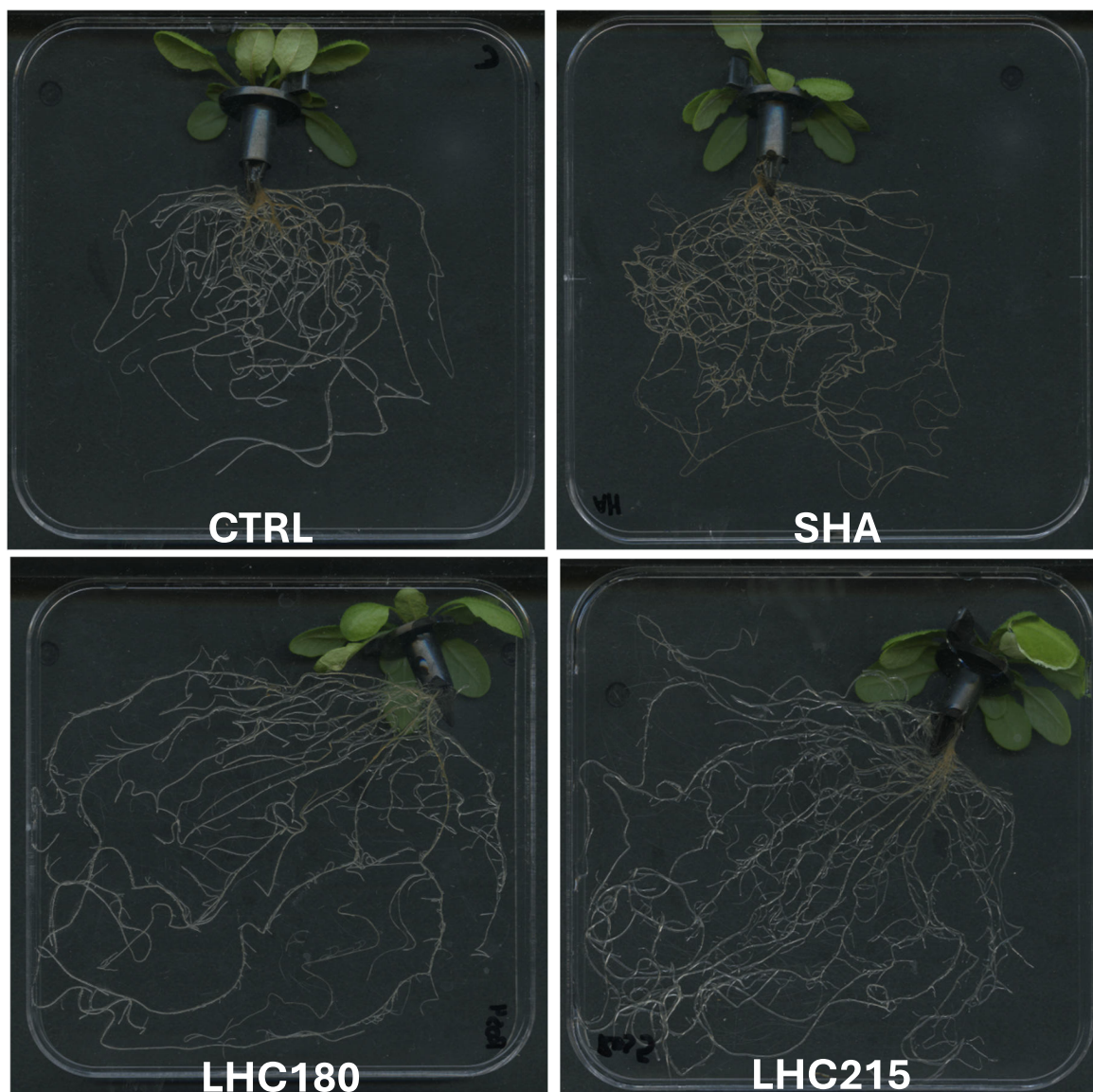
Supporting Figures



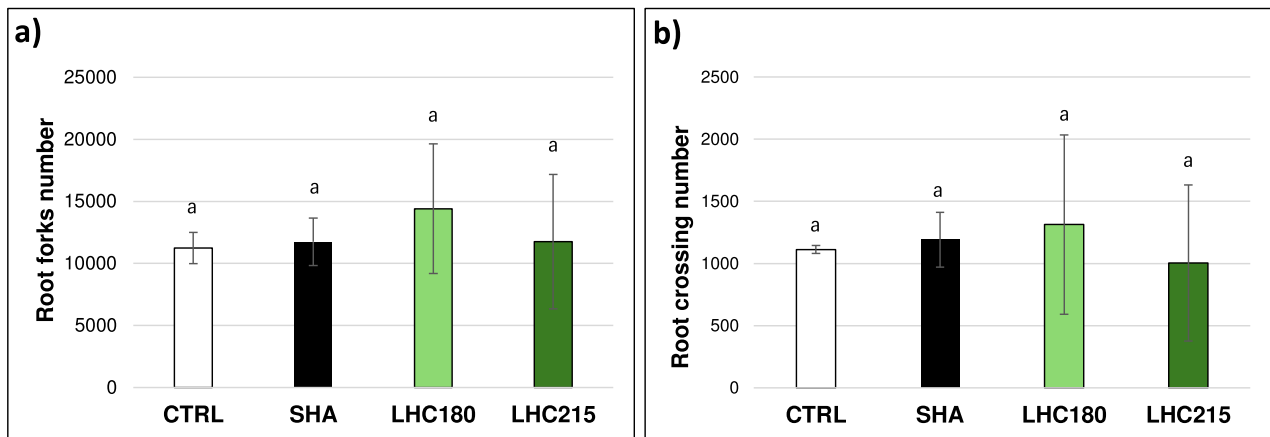
Supporting Figure S1. Lab-scale 1-L Buchi-Glass reactor used for hydrothermal carbonization (HTC) of biomass. (a) Hydrothermal reactor; (b) agitation system control (cyclone); (c) External controller used to modulate temperature and supply heat during the HTC process.



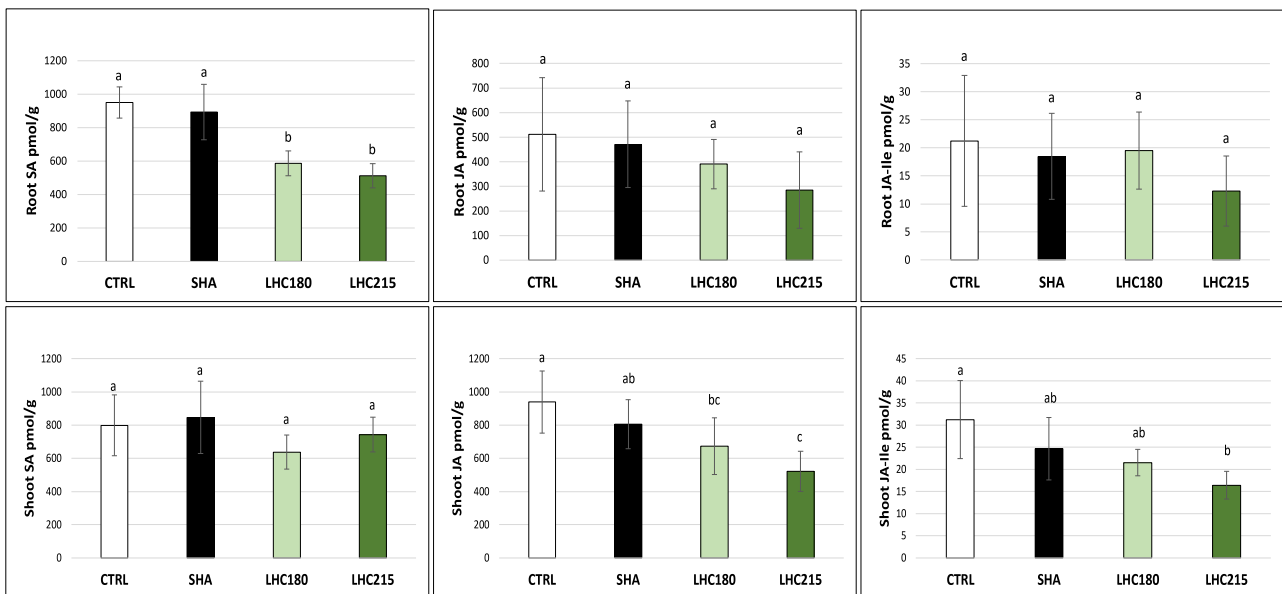
Supporting Figure S1. Hydroponic experiment with *Arabidopsis thaliana* at harvest day. Plants were grown under four treatment conditions: control (CTRL), sedimentary humic acid (SHA), LHCs obtained at 180 °C (LHC180), and LHC obtained at 215 °C (LHC215). The image was acquired under standardized conditions, with the camera positioned at a fixed height and angle to ensure impartial visual comparison across treatments.



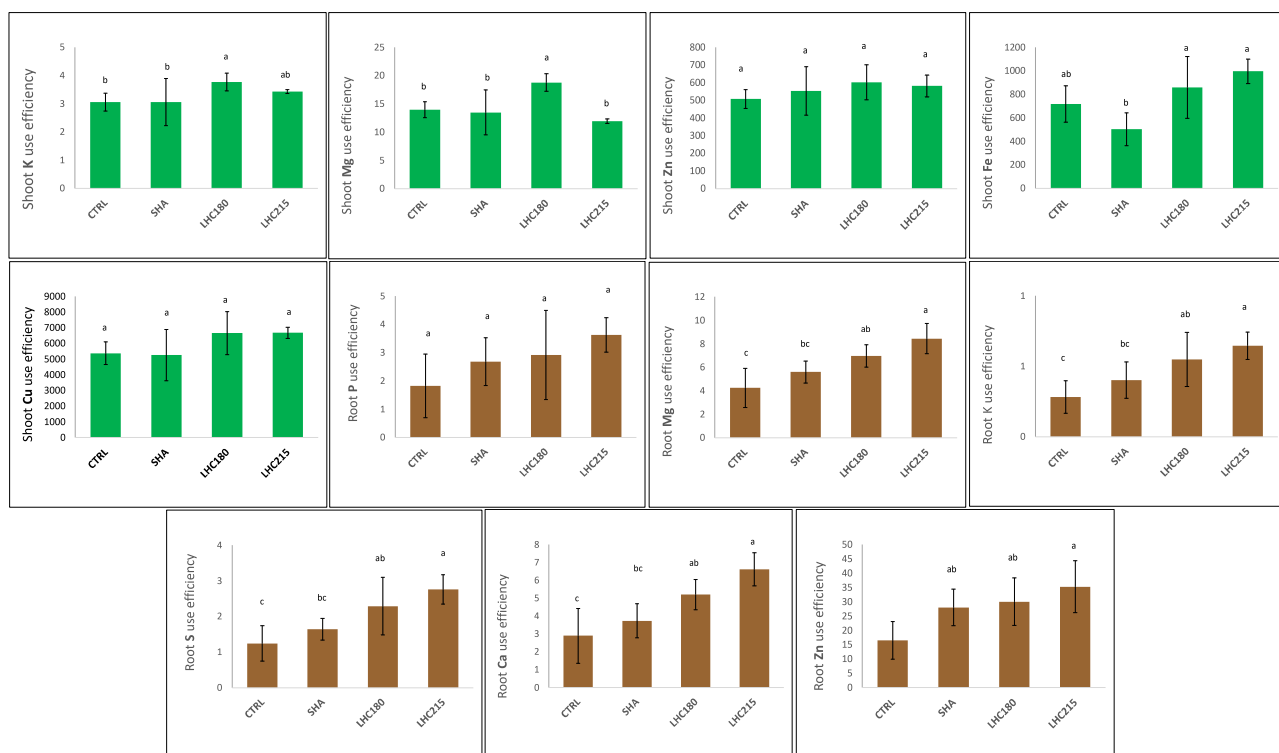
Supporting Figure S2. Root system architecture of *Arabidopsis thaliana* plants at harvest, following hydroponic cultivation under four treatment conditions: control (CTRL), sedimentary humic acid (SHA), LHCs obtained at 180 °C (LHC180), and at 215 °C (LHC215). Root samples were scanned using the WinRHIZO image analysis system under standardized acquisition settings, maintaining fixed scanner resolution, orientation, and sample positioning to ensure unbiased comparison. The image illustrates treatment-dependent differences in total root length and morphological traits.



Supporting Figure S3. Root morphological parameters, including the number of forks (a) and crossings (b), in *Arabidopsis thaliana* analyzed using winRHIZO software after treatment with LHC180, LHC215, and SHA at 100 mg OC L⁻¹. No significant differences were observed among treatments (one-way ANOVA, $p > 0.05$).



Supporting Figure S4. JA/JA-Ile and SA content in shoots and roots of *Arabidopsis thaliana* after treatment with LHC180, LHC215, and SHA at 100 mg OC L⁻¹. (one-way ANOVA, Fisher's LSD post hoc test, $p \leq 0.05$).



Supporting Figure S5. Supplementary data on nutrient use efficiency (NUE) parameters in roots and shoots of *Arabidopsis thaliana* after treatment with LHC180, LHC215, and SHA at 100 mg OC L⁻¹. Different letters indicate significant differences among treatments (one-way ANOVA, $p \leq 0.05$).

Supporting Tables

Supporting Table S1. Internal standards (deuterium-labeled phytohormones) used for UHPLC-MS/MS analysis, including their parent ions (m/z), main fragment ions (m/z), and collision energies (CE, V) applied for fragmentation. The standards were obtained from the following suppliers: D-IAA, D-ABA, D-JA, D-JA-Ile, D-tZ, D-tZR, D-iP, and D-iPR from OlChemim Ltd; D-SA from Sigma-Aldrich.

Abbreviations used: D indicates deuterium-labeled compounds. D-IAA (indole-3-acetic acid), D-ABA (abscisic acid), D-SA (salicylic acid), D-JA (jasmonic acid), D-JA-Ile (jasmonoyl-isoleucine), D-tZ (trans-zeatin), D-tZR (trans-zeatin riboside), D-iP (isopentenyladenosine), and D-iPR (isopentenyladenosine riboside).

D-standard	parent ion m/z	CE (V)	m/z fragment 1
D-IAA	179.08744	8	135.0976
D-ABA	269.16654	8	159.1297
D-SA	141.04952	18	97.0598
D-JA	214.1497	10	62.0327
D-JA-Ile	332.26515	18	140.1502
D-tZ	225.15067	15	137.0681
D-tZR	357.19293	18	225.1507
D-iP	210.16203	14	137.0681
D-iPR	342.20429	18	210.162

Supporting Table S2. Gene IDs and primer sequences.

Gene	TAIR ID	Sense	Primer sequence
<i>FRO2</i>	AT1G01580	Forward	CGTATCAAGTTTGGAACATCCACTT
		Reverse	CCATCATTGGGAACATATACATGAA
<i>IRT1</i>	AT4G19690	Forward	TGCAATCTCTCCAGCAACTTCAACTG
		Reverse	TCGCAAGAGCTGTGCATTTGACGAA
<i>FIT</i>	AT2G28160	Forward	GGTTAGGCAAGTTTAAGCTCTG
		Reverse	GGAGAAGGTGTTTGTCCATCTC
<i>COPT2</i>	AT3G46900	Forward	TGCACATGACCTTCTTTTGG
		Reverse	GTCATCGGAGGGTTTGTGTA
<i>NRT2.1</i>	AT1G08090	Forward	CCGACAAGACGGCCAAGTTCGACCT
		Reverse	TCCGTAGAGAAGAACGAAGATCCAAG
<i>sAHA7</i>	AT3G60330	Forward	AGAAATAGCGCAACGGAAC
		Reverse	TTGCAGCTGATTCAACCTTC
<i>AHA1</i>	AT2G18960	Forward	TTGAAGTTTGCCATTCGGTA
		Reverse	GCCCATTGAGCTTCTCTTTC
<i>EF1</i> □	AT5G60390	Forward	AGATCAACGAGCCCAAGA
		Reverse	CCGTTCCAATACCACCAAT
<i>UBQ10</i>	AT4G05320	Forward	AACGGGAAAGACGATTAC
		Reverse	ACAAGATGAAGGGTGGAC

Chapter VI

Original research paper

“Innovative alginate-based systems to increase soil water retention and enable a slow-release of humic acids, calcium and iron in sustainable agriculture”

Pellegrino Alfieri^{1,‡} & Rossella Curcio^{1,2,‡}, Falcone Giovanni¹, Andrea Sica¹, Patrizia Iannece³, Nicola Funicello⁴, Salvatore De Pasquale⁴, Russo Paola¹, Pierluigi Mazzei^{1,*}

¹*Department of Pharmacy, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano, SA, Italy*

²*DAFE - Department of Agricultural, Forest, Food, and Environmental Sciences, University of Basilicata, 10, Viale dell'Ateneo Lucano - Potenza (PZ)*

³*Department of Chemistry and Biology (DCB), University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano, SA, Italy*

⁴*Department of Physics, University of Salerno, University of Salerno, Via Giovanni Paolo II, 132, 84084 Fisciano, SA, Italy*

**Corresponding Author: Pierluigi Mazzei and Rossella Curcio*

[‡]Pellegrino Alfieri and Rossella Curcio contributed equally to this work.

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6.1 ABSTRACT

Formulations derived from alginate ionotropic gelation can retain water and plant nutrients and ensure their slow release. In this work, novel alginate-based systems were developed and their potential applications as tools to enhance soil properties and fertility were explored. The investigated systems consisted of hydrogel layers (H) and beads (B) produced using either calcium or iron (III) cations as crosslinkers. The resulting products were characterized in terms of degradability, water and cation release rates, and structural rigidity, considering the ratio between alginate and crosslinker concentrations. The calcium and iron content incorporated into beads was quantified via optical ICP, corresponding to 800 µg and 125.12 µg of nutrient per single bead, respectively. The degradation extent of calcium and iron beads was further assessed in the presence of the chelating agents EDTA and citric acid, respectively. Hydrogels and beads were also incubated in sandy soil to monitor degradation under realistic conditions.

An optimal and stable formulation was identified for developing alginate-based systems loaded with bioactive, water-soluble humic materials isolated from compost (HSCOM), vermicompost (HSVC), and solid anaerobic digestate (HSDIG). These humic materials were characterized using advanced spectroscopic techniques, including ¹H-NMR, DOSY 2D NMR, and FT-IR. Magnetic resonance imaging (MRI) and rheology confirmed that the incorporation of humic substances preserves the gel network and viscoelastic properties. Seed coating experiments with alginate, either alone or loaded with humates, were evaluated via scanning electron microscopy (SEM). Bioassays with coated seeds demonstrated that HSDIG- and HSVC-enriched hydrogels exerted biostimulant effects on watercress germination. These alginate-based systems represent a promising multifunctional tool, providing a slow-release source of bioactive humates, water, calcium, and iron. Their efficiency can be further

optimized by tailoring the formulation according to soil characteristics and the intended application.

Keywords: *Humic substances, NMR, Rheology, climate change, nutrients, biomass recycle, alginates, hydrogels, beads*

6.2 INTRODUCTION

The growing global population and the consequent need to ensure food security are driving an ever-increasing demand for fertile soils and effective strategies to preserve and improve their quality (Godfray et al., 2010; Ali et al., 2024). This challenge is further aggravated by widespread and uncontrolled land degradation, the adverse effects of climate change, and the rising costs of fertilizers and biostimulants (Yang et al., 2024). Within this framework, the development of innovative, efficient, and sustainable approaches to enhance plant growth, soil fertility, and ecosystem resilience has become a key priority for modern agriculture (Hossain et al., 2020; Wijerathna-Yapa et al., 2022). Among the most promising materials proposed for this purpose, hydrogels derived from natural polysaccharides have attracted increasing attention. These materials, typically produced through ionotropic gelation, form three-dimensional networks stabilized by hydrophilic interactions and ionic bonds with multivalent cations, which allow them to retain up to 99% of water within their structure (Kaur et al., 2023; Oladosu et al., 2022; Wang et al., 2023; Abgna et al., 2025). Hydrogels have long been exploited in medical, pharmaceutical, and cosmetic applications, whereas their use in agriculture has gained attention only in recent years, mainly due to their potential to enhance the availability of water and nutrients (Zagórska-Dziok et al., 2023). Their capacity for gradual water release improves water use efficiency, mitigates drought stress, and reduces irrigation frequency (Abobatta et al., 2018; Neethu et al., 2018). Furthermore, hydrogels can

promote plant growth through the controlled and continuous release of essential elements (**Fu et al., 2024**)

Among the various biopolymers used in hydrogel production, alginates are particularly valued for their non-toxic, biocompatible, and renewable nature (**Bahram et al., 2016**). These polysaccharides, commonly extracted from brown algae such as *Ascophyllum nodosum* and *Laminaria hyperborea*, are linear copolymers composed of β -D-mannuronic acid (M) and α -L-guluronic acid (G) units linked by 1,4-glycosidic bonds. The monomers are arranged in blocks of M, G, or alternating MG sequences, whose distribution strongly influences the structural and mechanical characteristics of the resulting hydrogels (**Hecht et al., 2016; Cao et al., 2020; Savić Gajić et al., 2023; Savarese et al., 2025**). The performance of alginate hydrogels is affected by several factors, including the alginate-to-crosslinker ratio, gelation method, type of crosslinking ion, and the presence of additives.

Alginate-based systems can be applied to soils either as hydrogel layers or as spherical beads a few millimeters in diameter, both designed to improve soil fertility and resilience. Their beneficial effects derive from enhanced physical properties—such as soil structure and macroporosity—as well as from the gradual release of water, nutrients, and bioactive compounds that sustain plant and microbial activity (**Chatzoudis & Rigas, 1998; Banedjschafie & Durner, 2015; Tariq et al., 2023**). Beads are particularly suitable for delivering controlled doses of nutrients and bioactive molecules, whereas hydrogel layers are more effective in water retention and in improving soil physicochemical properties. Recent studies have shown that alginate systems can provide a sustained release of nutrients and bioactive compounds, enhancing seedling vigor, growth, and productivity (**Gholamian et al., 2021; Liu et al., 2024**). A promising strategy involves the use of alternative crosslinkers to calcium—such as iron—which serves not only as a structural ion but also as an essential plant

micronutrient. This approach is particularly advantageous in alkaline soils, where iron bioavailability is often limited due to pH-dependent precipitation phenomena (**Oades, 1963; Kratochvílová et al., 2023; Kumar et al., 2024**). Another relevant strategy consists in the encapsulation of organic materials within alginate matrices to enhance their biostimulant potential. In this regard, humic substances (HS), lignohumates, and lignosulfonates have received increasing attention for their ability to stimulate soil microbial activity and plant metabolism (**Muscolo et al., 2013; Hu et al., 2022; Wang et al., 2023; Kaur et al., 2023**). For instance, **Kratochvílová et al. (2023)** developed poly(acrylic acid–acrylamide) hydrogels loaded with lignohumates, observing improved water retention and soil porosity, whereas **Song et al. (2020) and Antunes et al. (2024)** reported enhanced soil hydraulic and nutrient-holding properties using green alginate hydrogels enriched with lignosulfonates and konjac flour.

Based on these considerations, this study focused on the development and characterization of innovative calcium- and iron-based alginate systems, formulated both as hydrogels and beads, with or without the inclusion of humic substances (HS) isolated from recycled biomasses such as compost, vermicompost, and solid anaerobic digestate. The systems were characterized with respect to their degradability, water and cation release dynamics, and structural rigidity, with particular attention to the effects of different alginate-to-crosslinker ratios. Rheological analysis were performed to assess the viscoelastic behavior and mechanical stability of the hydrogels, providing insights into their potential performance under soil conditions. Finally, alginate-based coatings were applied to seeds to investigate possible biostimulant effects on germination and early plant development.

6.3 MATERIALS AND METHODS

6.3.1 *Alginate-based formulations: optimization of calcium and iron hydrogel and beads*

Sodium alginate used in this study was supplied by Thermo Fisher Scientific (1% at 20°C, viscosity 350–550 mPa·s, pH 5.5–8). Hydrogels (H) and beads (B) were produced via ionotropic gelation using Ca^{2+} (calcium chloride, anhydrous, Titolchimica) and Fe^{3+} (ferric chloride hexahydrate, BeanTown Chemical, >97%) as crosslinkers.

For H preparations, increasing amounts of alginate powder were hydrated in ultrapure water (Milli-Q, Sartorius Ariun Pro) at 20°C under continuous stirring to achieve final concentrations of 1, 1.5, 2, and 3% (w/v). Calcium- (HCa) and iron-based (HFe) hydrogels were then obtained by adding 25 mM calcium chloride or ferric chloride solutions, respectively, to the hydrated alginate in a 1:1 (v/v) ratio. The mixtures were stirred until gelation was complete. Hydrogels were stored at 4°C for 17 hours to allow slow but complete gelation. Samples were subsequently centrifuged at 6000 rpm for 10 minutes to remove micro- and macro-sized air bubbles. In the case of HFe, centrifugation resulted in the formation of a more rigid phase at the bottom of the tube and a viscous gel-like phase. The rigid phase was assumed to consist of precipitated iron not involved in alginate gelation; only the viscous phase was used for further testing and analysis. Each hydrogel preparation was performed in duplicate.

Beads were produced using the drop-weight method. Briefly, hydrated alginate solution was released into a solution containing a high concentration of crosslinker cations. Dripping was controlled through a glass funnel equipped with a stopcock to modulate the flow rate (**Supporting Figure S1**). The process was conducted slowly and steadily, with an average release of one droplet per second, maintaining a distance of 17 cm (optimized, **Supporting**

Figure S2) between the funnel tip and the surface of the crosslinker solution. Multiple bead series were prepared using different concentrations of hydrated alginate (1, 1.2, and 1.5% w/v) and crosslinker solutions (50, 70, 125, and 250 mM). Beads were left in the crosslinker solution at 4°C for 17 hours to ensure complete gelation, particularly in the core. Each preparation was performed in duplicate and consisted of forty beads per set.

6.3.2 *Isolation and characterization of humates*

Aiming to incorporate compounds with a bioactive nature into the alginate-based systems under investigation, humified water-soluble compounds were isolated by soil organic amendment. The latter ones consisted in green compost (COM), solid anaerobic digestate (DIG), and vermicompost (VC) deriving from DIG rielaboration. In detail, COM was produced by the compost plant of the Terramore Producers Organization (Eboli, Italy), through a 3-month composting process (with active aeration) by utilising as feed production by-products, which included curly endive, spinach, red chicory, and olive pruning residues. DIG was produced by the C&F Energy biogas plant (Capaccio, Italy) via the anaerobic digestion of livestock buffalo manure. In the same plant, part of DIG material was subsequently converted into VC through the action of earthworms, in controlled conditions, which converted DIG into an even more stabilized and humified material.

The extraction of humates from green compost (HSCOM), digestate (HSDIG) and vermicompost (HSVC) was performed by mixing 30 g of dried and sieved (2 mm) organic material with 1 L of a KOH alkaline extracting solution [1M]. The suspension was agitated for 16 hours at 150 rpm on a rocking platform. Then, the supernatant was separated from the pellet via centrifugation at 6500 rpm and at 25 °C for 20 minutes (BECKMAN J-25). Subsequently, a second rapid extraction cycle was repeated by putting in contact the residual

pellet with the extraction solution for 2 hours. The supernatants obtained from the two extraction cycles were combined and then filtered through sterile, coarse mesh gauze. The supernatant pH was corrected to 7, through an HCl solution [1M], and then dialyzed (membranes with a cut-off of 3500 Da) against distilled water to remove excess salts. The extracts were lyophilised using an Edwards MODULYO 5289 freeze dryer and stored in dry environments at room temperature and away from light until further use. The average extraction yield was 10% by weight (approximately 3 grams from 30 grams). Alginate systems were loaded with the humates by replacing water with humic solutions in the phase of alginates hydration. Due a different solubility of humates, the humic solutions were prepared with ultrapure water and resolubilised up to achieve the concentrations of 100 mg/L, for vermicompost and compost extracts, and 25 mg/L for digestate extracts.

6.3.3 Evaluation of hydrogels and beads dehydration extent

Two experiments were set up to study how the cation concentration in the crosslinker solutions for beads preparation impacts on both the total amount of absorbed water and their dehydration trend. The beads were prepared using a 1.2% (w/v) sodium alginate solution, crosslinked by dripping into aqueous solutions of calcium chloride (CaCl_2) and ferric chloride (FeCl_3) at different concentrations. For Ca^{2+} crosslinking, the following concentrations were tested: 250, 200, 125, 100, 75, and 50 mM, whereas for Fe^{3+} , considering its trivalent nature, proportionally charged concentrations were selected: 166.7, 133, 83.3, 66.6, 50, and 33.3 mM. For each concentration, 25 beads were formed and subsequently placed in pre-weighed aluminum trays, which were kept in a climate-controlled chamber. Bead weight was monitored every 45 minutes over a total period of 4.5 hours, with net mass calculated by subtracting the weight of the empty tray.

In a second phase, the experiment was repeated using a formulation enriched with humic substances (HSCOM, HSVC and HSDIG), compared with a control sample (CTRL) to evaluate their effect on water retention. In all enriched formulations, the alginate concentration was maintained at 1.2%, and a constant CaCl_2 concentration of 50 mM was used for crosslinking. Similarly, 25 beads per formulation were prepared, placed in aluminum trays, and monitored for 4.5 hours with weight measurements every 45 minutes. To ensure data reliability, all experiments were performed in duplicate (experimental replicates), maintaining constant environmental conditions throughout the duration of the tests. Also for hydrogels the dehydration extent was studied as a function of crosslinker solution. In the first experiment, 3 grams of HCa, prepared with different alginate concentrations (1, 1.5, 2 and 3% w/v), were put homogeneously into plastic vessels and placed into a growth chamber (temperature of 22.4°C and 53% of humidity). Aiming to quantitatively estimate the water loss, over the time, the residual H weight was progressively measured at time intervals ranging from 0.5 to 44.30 hours after the begin of the experiment. All tests were performed in duplicate.

The second experiment simulated a more realistic scenario by placing HCa, prepared with different concentrations of alginates (1, 1.5, 2 and 3%), in contact with a model soil, previously sieved at 2 mm. The latter one was sampled from a periurban site in Fisciano (Salerno, Italy) and was characterized by a sandy texture, a pH of 6.8, and an electrical conductivity of 158.40 $\mu\text{S}/\text{cm}$ (non saline). For such an experiment, it was designed an artisanal system, conceived to confine the hydrogels between two layers of soil and, at the same time, permit a periodical and non-destructive visual monitoring of hydrogels. As shown in **Supporting Figure S3**, the system consisted of three cylinders, where one (a, the outer) had a diameter of 28.48 mm, while the other two (b and c, to be inserted into a) had a diameter of 26.16 mm. Two overlapped layers of a hydrophilic nylon filter (100 micron mesh) were placed on one of the margins of

both cylinders b and c where, in the first case, served to create an interface between the upper layer of soil and hydrogels while, in the second case, served to contain the lower layer of soil. Such a system was conceived to permit to periodically remove the cylinder b in order to visualize the hydrogel, in a non-destructive way and without incurring in particles waste from the upper soil layer. The lower layer of soil (6 g) was loaded into the system and kept 3 cm above the bottom by the nylon filter in cylinder c to guarantee the water drainage and avoid waterlogging. Then, 6 mL of HCa were placed above the soil. Finally, the upper layer of soil (6 g), contained into cylinder b by the filter, were put in direct contact with the top surface of the hydrogel. Periodically and up to 14 days following the begin of the experiment, cylinder b was gently removed by the system to inspect the hydrogels evolution, assess their condition and photograph the state. A control test were carried out by excluding the H (**Supporting Figure S3**). The water content was periodically measured using gravimetric methods, weighing the entire system and subtracting the weight of cylinders, filters and dry soil. The experiment was conducted in controlled conditions, by keeping the cylinders into a growth chamber (temperature of 22.4°C and 53% of humidity). All experiments were conducted in duplicate.

6.3.4 Evaluation of Ca and Fe beads content and release test using optical-ICP

The average amount of either calcium or iron entrapped into each single bead was estimated by comparing the electrical conductivity of the crosslinker solution before and after the formation and the removal of beads, and assuming that the difference in conductivity was due to the sequestration of cations. Electrical conductivity was measured via benchtop conductivity-meter (HANNA Instruments, HI5522), calibrated with a certified reference standard of 1.413 dS/m. The quantitative estimation was conducted against a calibration

curves built up by measuring increasing concentration of either Ca^{2+} or Fe^{3+} (range 10–100 mM), with R^2 values of 0.9956 and 0.9848, respectively. The electrical conductivity of ultrapure water was negligible.

Aiming to assess the crosslinker release in water, 40 beads (prepared with 50 mM calcium or iron solutions) were placed in contact with 80 mL of ultrapure water for 3 days at 20 °C. Then, 10 mL of the solution kept in contact with beads were collected from each set (in duplicate) and acidified with HNO_3 up to achieve a 3% nitric acid solution (v/v). The cations were then analyzed using ICP-OES (Perkin Elmer Optima 7000 Dual View). Ca^{2+} and Fe^{3+} concentrations were measured in triplicate for each sampling set and the quantitative estimation was conducted against calibration curves (range 0.01–50 mg/L, $r^2 = 0.999$).

6.3.5 *Beads degradation test with chelating agents*

Aiming to evaluate the degradation extent of studied beads, it was set up an experiment in which beads were put in contact with chelating agents for a defined time interval. In detail, they were prepared calcium and iron beads by using a 1.2% (w/v) solution of hydrated alginates. Calcium beads were prepared by using either 25 or 50 mM crosslinker solutions, while they were adopted the concentrations of 6.6 and 30 mM for iron beads. The concentrations of iron solutions were relatively lower than for calcium ones due to the trivalent nature of this ion.

The test was conducted by putting in contact 40 beads, for each bead line, with 40 ml of a solution containing the proper chelating agent. In particular, they were used ethylenediaminetetraacetic acid (EDTA, $\geq 99\%$, Sigma-Aldrich, Germany) and citric acid (anhydrous, Ward's Science) for calcium and iron, respectively. The test was performed in controlled conditions, in the dark, at a temperature of 23°C and with a humidity of 53%.

Concomitantly, a control test was launched in absence of chelating agents. The degradation extent of beads was visually monitored and photographed after 0, 4 and 9 days from the begin of the experiment. All tests were carried out in duplicate

6.3.6 *Beads degradation test in contact with a real soil*

Representative beads were put in direct contact with a model soil to evaluate their degradation extent in a more realistic scenario. Also in this case, it was adopted the same system with cylinders and the model soil described above. All the beads were prepared by using an hydration alginate solution brought at a concentration of 1.2 % w/v. The concentrations for the crosslinker solutions were 25 and 50 mM, for calcium beads, and 6.6 and 33 mM, for iron beads. The humidity of soil into the cylinders has been maintained at the field capacity for the entire duration of the experiment. The experiment lasted 20 days and was conducted in controlled conditions, by keeping the cylinders into a growth chamber (temperature of 22.4°C and 53% of humidity). All experiments were conducted in duplicate.

6.3.7 *Compositional characterization of humates*

Humates were characterized by high-resolution liquid-state Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared (FTIR) spectroscopies.

NMR analysis were carried out on samples obtained by dissolving 2 mg of humates into 1 ml of 0.05 M sodium hydroxide solution prepared using deuterated water (99.8% D₂O/H₂O, Eurisotop). Each sample was vortexed and ultrasonicated, up to attain its total dissolution. Then, each sample was transferred into borosilicate NMR tubes, degassed, debubbled and finally loaded into a 500 MHz magnet (11.75 Tesla, Bruker Oxford/spectrospin cryomagnet) equipped with a BBO (Broad-Band Observe) probe. ¹H NMR spectra were acquired through an on-resonance presaturation technique (at a power of 55 ± 5 dB) to suppress the residual signal of the aqueous solvent, by setting 8 dummy scans, 128 effective scans, a 90° spin torsion

pulse ranging within 8.5 and 10.5 μ s with a power of -10 dB; spectral window of 16 ppm (6410.256 Hz), 16384 acquisition points and a total recycling time of 5 s. For the processing of 1D ^1H NMR spectra it was performed the Fourier Transform by applying an apodization filter of 1 Hz without “zero filling”. The analysis was conducted in duplicate.

In order to identify the most relevant signals and compounds and confirm their structural pathways, they were acquired ^1H - ^1H homo-correlated 2D spectra such as COSY (COrrelation SpectroscopY), TOCSY (TOtal Correlation SpectroscopY) and NOESY (Nuclear Overhauser Effect SpectroscopY) and hetero-related 2D spectra ^1H - ^{13}C such as HSQC (Heteronuclear Single-Quantum Coherence) and HMBC (Heteronuclear Multiple-Bond Coherence). All 2D NMR spectra were set up with 16 dummy scans, 48 scans, and a spectral window of 16 (6410.3 Hz) and 300 (30186.8 Hz) ppm for the ^1H and ^{13}C nuclei, respectively. In addition, 2048 points and 256 experiments were set as size for F2 and F1 dimensions, respectively. In detail, TOCSY and NOESY were optimized by using mixing times of 0.08 and 0.8 s, respectively, while HSQC and HMBC were optimized according to a scalar J_{CH} coupling of 145 Hz and a “long-range” 8 Hz J_{CH} connectivity. All spectra were processed using Bruker Topspin software (v. 4.1.4, Bruker Biospin, Rheinstetten, Germany). 2D DOSY NMR (Diffusion Ordered Spectroscopy) were conducted to estimate the self-diffusion coefficients of humates and were acquired by a stimulated echo sequence with bipolar gradients, including two spoil gradients and a supplementary delay to suppress eddy currents. This sequence was preferred to the traditional sequence to minimize signal losses due to the short relaxation times typical of the materials under study. The acquisition was carried out by setting gradients (δ) of 1700 μ s, which evolved from 0.674 to 32.030 G cm^{-1} in 64 increments and selecting a 0.15s long diffusion delay (Δ) between the encoding and decoding gradients. A total of 32 scans were acquired for each increment.

FTIR analysis were carried out via ATR (Attenuated Total Reflection) technique on solid and dried samples, sieved at 0.5 mm. The analysis were conducted in duplicate on the PERKIN ELMER Frontier instrument, by setting 64 scans, a wavelength range included within 4000 and 600 cm^{-1} , with a resolution of 2 cm^{-1} . For each acquisitions set, it was examined a background spectrum. A baseline correction was conducted for each spectrum in order to make them suitable for direct and reliable comparison. All spectra were processed using PerkinElmer Spectrum IR software (v. 10.6.2).

6.3.8 Assessment of alginate hydrodynamic radius via 2D DOSY NMR

Hydrogels (H) and beads (B) were developed through ionotropic gelation by using the sodium alginate as gelling agent (Thermo Fisher Scientific; 1% at 20°C, with a viscosity of 350–550 mPas and a pH ranging between 5.5 and 8). The alginate average hydrodynamic radius was extrapolated based on the hydrogen self-diffusion coefficients, as detected via 2D DOSY NMR (Diffusion Ordered Spectroscopy). The alginate was dissolved (0.3% w/v) into a water solution ($\text{H}_2\text{O}:\text{D}_2\text{O}$ 10:90, v:v; where deuterated water was 99.8% $\text{D}_2\text{O}/\text{H}_2\text{O}$, supplied by Eurisotop) containing sodium (0.02 M). Then, the sample was transferred in a borosilicate NMR tube, loaded into a 500 MHz magnet (11.75 Tesla, Bruker Oxford/spectrospin cryomagnet) equipped with a BBO (Broad-Band Observe) probe. The self-diffusion coefficients of alginate hydrogens were acquired by a stimulated echo sequence with bipolar gradients, including two spoil gradients and a supplementary delay to suppress possible eddy currents. The acquisition was carried out by setting gradients (δ) of 2000 μs , which evolved from 0.674 to 32.030 G cm^{-1} in 32 increments, and selecting a 0.4 s long diffusion delay (Δ) between the encoding and decoding gradients. A total of 24 scans were acquired for each increment. In order to build up a calibration curve, the same acquisition parameters were applied to launch the DOSY NMR experiment on a set of dextran standards, characterized by

a certified molecular weight (5, 25, 50, 150, 270 and 410 KDa, Analytical standard for GPC, Supelco, Merck), dissolved in the same solution (H₂O:D₂O 10:90, v:v; NaCl 0.02 M, 0.3% w/v).

In all cases, 2D DOSY NMR spectra underwent a Fourier Transform performed by applying an apodization of 5 Hz and a two-fold zero-filling. The self-diffusion values were calculated for the most intense signals detected in alginate and dextran DOSY and averaged.

The same DOSY NMR approach was also applied to the calcium hydrogels (HCa) loaded with humic substances (HSCOM, HSDIG, and HSVC), in order to evaluate possible changes in the diffusion behaviour and molecular aggregation patterns resulting from the interaction between alginate and humic molecules.

6.3.9 Alginate-based systems characterisation via MRI

HCa (1, 2 and 3% w/v) and HFe (2, 2.5 and 3% w/v) as well as hydrogels incorporating humic extracts were analysed by using Magnetic Resonance Imaging (MRI) technique. These materials were loaded into borosilicate glass tubes (NORELL, diameter of 1 cm) and, due to their viscoelastic behaviour, were kept steady and in vertical position for 8 hours, in order to promote both the slow settling on the tube bottom and the evacuation of air macrobubbles. Then, tubes were loaded into the Bruker Avance 300 MHz wide-bore magnet (Bruker Isospin, Rheinstetten, Germany) equipped with a μ -imaging MICRO 5 probe, operating at a temperature of 298 ± 1 K.

¹H MRI experiments consisted in multi-slice multi-echo (MSME) and Diffusion pulsed field gradient (PFG), allowing to measure the T₂ spin-spin relaxation times and the self-diffusivity of water molecules contained into the hydrogels, respectively. In detail, MSME consisted of 12 scans, a recycle time of 3 seconds, 30 experiments acquired with an increasing number of

spin echoes (ranging from 11 to 330 ms). Spin-density images were obtained by extracting the experiment with the shortest spin-echo time (11 ms). PFG was performed by adopting a pulsed field gradient integrated with stimulated echoes (with a little and big delta of 1.7 and 20 ms, respectively, in 11 experiments). The PFG experiments were set with a recycle delay of 4 seconds, 6 scans, and increasing gradient intensities (0, 4.5, 17.9, 40.3, 71.7, 161.3, 286.8, 448.1, 645.3, 878.3, 1147.1 s/mm²) across 11 experiments.

In all cases, it was used an axial geometry by selecting 8 slices with a width of 1.35 mm. For each sample, T₂ and diffusivity values were calculated by averaging the values detected for the 3 central slices, evaluating 3 regions of interest (ROIs) per slice (**Supporting Figure S4**).

MSME data were fitted to a single exponential decay, according to the equation (Eq. 1):

$$M_t = M_0 \exp(-t/T_2) \quad (\text{Eq. 1})$$

where, M_0 and M_t represent the magnetization intensity resulting at the minimum spin-echo delay and after the i -th spin-echo interval, respectively.

PFG data were analyzed by nonlinear least-squares regression of the equation (Eq 2):

$$M_t = M_0 \exp [-\gamma^2 G^2 \delta^2 (\Delta - \delta/3) D] \quad (\text{Eq 2})$$

where M_0 and M_t represent the magnetization intensity resulting after the application of gradients at the minimum and at the i -th strength, respectively, γ is the proton magnetogyric ratio, G is the pulsed field gradient intensity, δ is the duration of the field gradient pulse, and Δ is the time interval between the leading edges of the field gradient pulses. The Bruker ParaVision 5.1 software was employed for MRI data processing.

6.3.10 Hydrogels rheological investigation

All produced hydrogels or hydrogels that showed the most interesting properties were subjected to rheological analysis aimed to highlight to physico-chemical properties of hydrogels change in function of different concentrations of crosslinking agent, as well as the incorporation of humic substances. MCR 102 rheometer (Anton Paar, Graz, Austria) was used to perform all analysis choosing the following setup: instrument configuration the plate-plate (PP25, plate diameter 24.985 mm), measuring gap at 1mm, and measuring temperature 25 ± 1 °C. To carefully evaluate all rheological properties of hydrogels (i.e., flowability, viscoelasticity, and thixotropy) three different test were carried out:

- Flow sweep test (rotational measurement): evaluation of viscosity in function of shear rate (from 0.1 to 1000 s⁻¹);
- Amplitude sweep test (oscillatory measurement): evaluation of both storage and loss moduli in function of shear strain (from 0.1 to 100 %, with constant angular frequency 1 s⁻¹), to identify the Linear ViscoElastic Region (LVER) of each hydrogel, the maximum of deformation was settled at 5 % drop of storage modulus value). Data were reported in function of the Loss factor, the ratio of the viscous and the elastic portion of the viscoelastic deformation behaviour (Mezger, T. (2020). The rheology handbook: for users of rotational and oscillatory rheometers. European Coatings.) ;
- Shaer test (transient measurement): a five-step cycle to evaluate viscosity over time when subjected to different shear rate (step 1: 75 s at 0.1 s⁻¹ shear rate, step 2: 75 s at 50 s⁻¹ shear rate, step 3: 75 s at 0.1 s⁻¹ shear rate, step 4: 75 s at 100 s⁻¹ shear rate, and step 5: 75 s at 0.1 s⁻¹ shear rate).

The software used to set all tests parameters and to analyse the data obtained was RheoCompass™ Software (Anton Paar, Graz, Austria). All tests were performed in triplicate and the results were reported as mean value and standard deviations. Results were analyzed by GraphPad Prism 10.1.0 software. In detail, results of Flow sweep test (in the medium range from 0.1 to 100 s⁻¹) were exploited as dataset for a regression study according to the Power-Law model

$$\eta = c \cdot \dot{\gamma}^p$$

where η is the viscosity, c is the consistency index (viscosity value at 1.0 s⁻¹), and p is the flow index ($p < 1$ means shear thinning behaviour).

6.3.11 Effect of alginate treatments on seed germination

Calcium and iron alginates were prepared with 1 and 2.5 % w/v, respectively, and using a 25 mM crosslinker concentration. These alginate formulations were put in contact with cress seeds in order to evaluate their effect on the germination. Seeds were immersed, for 30 minutes, into alginate calcium hydrogels, either with or without the enrichment with humic materials. In addition, seeds were also immersed into alginate a representative iron hydrogel and water (serving as test control). In case of hydrogels loaded with humates, the materials were prepared by hydrating the alginates with the solutions of humates, obtained by using a concentration of 100 mg/l, for VC and COM, and 25 mg/l, for DIG.

After the treatment, the seeds underwent a test to evaluate the phototoxicity and possible biostimulant effects (**Roca-Pérez, L. et al., 2011**). The test was conducted in Petri dishes (Ø of 9 cm) and consisted in loading 20 seeds per dish which were put on two layers of filter paper, which were moistened with 5.5 ml of ultrapure water. Each dish was sealed with Parafilm and each treatment was replicated (two dishes per treatment). All materials were

sterilized via autoclaving and the dishes were prepared under a laminar flow hood (following a preliminary 15'-long UV treatment). For each dish, germinated seeds were numbered daily, while radicle and shoot lengths in germinated seeds were measured six days after the initiation of the test by ImageJ software (v1.53k, NIH, USA). The results were used to calculate the indices shown in **Supporting Table S1**.

6.3.12 Scanning Electron Microscopy (SEM) analysis of coated *Lepidium sativum* seeds

The morphology of cress seeds (*Lepidium sativum*), either uncoated or coated with alginate-based hydrogels, was investigated by scanning electron microscopy (SEM), using a Tescan Solaris instrument (Tescan Orsay Holding, Brno, Czech Republic). Prior to observation, the samples were mounted on aluminum stubs (Agar Scientific, Stansted, UK) and coated with a thick gold layer (200–400 Å) (LEICA EMSCD005 metallizator), according to the standard ISO 13322-1. SEM imaging was conducted at 55° tilt using a 5 keV electron beam.

In particular, a comparative analysis was performed between control seeds, which were immersed in distilled water for 30 minutes, and treated seeds, which were immersed for 30 minutes in a 2% (w/v) calcium alginate hydrogel (HCA) to allow coating formation.

The analysis aimed to verify the effective formation and adhesion of the hydrogel coating layer and to assess its surface uniformity and continuity. Mean coating thickness was measured directly from SEM images using ImageJ (National Institutes of Health, Bethesda, MD, USA), a Java-based open-source image analysis software.

6.4 RESULTS AND DISCUSSION

6.4.1 Alginate Beads

6.4.1.1 Optimization of production parameters

The optimal distance between the funnel tip and the surface of the crosslinker solution during bead production was first optimized to identify the setup that maximized the formation of uniformly sized, pseudo-spherical beads. Beads were produced with the funnel tip positioned at distances of 4, 10, 14, 17, 20, and 25 cm from the surface of the crosslinker solution and subsequently compared. Only at distances greater than 20 cm did the resulting beads appear irregular and elongated, exhibiting a non-spherical and heterogeneous morphology. Such irregular shapes are expected to compromise reproducibility in terms of release kinetics and decomposition rate per bead. In contrast, beads produced at shorter distances displayed a more uniform and spherical morphology (**Supporting Figure S2**), with a distance of 17 cm providing the optimal outcome. A similar response was observed for iron-based beads, and consequently, a funnel-to-solution distance of 17 cm was systematically adopted in all experiments. Interestingly, iron beads acquired a characteristic red color, attributable to the presence of oxidized iron.

Subsequently, the influence of the crosslinker-to-alginate ratio on bead quality was investigated. Initially, several hydrated alginate solutions with concentrations ranging from 1 to 1.5% w/v were tested. From both a macroscopic and practical perspective, an alginate concentration of 1.2% w/v was found to be optimal, providing smooth extrusion from the funnel while avoiding issues associated with excessively high viscosity. Using these parameters, calcium- and iron-based beads were prepared with increasing crosslinker concentrations (5–500 mM). As the concentration of Ca^{2+} or Fe^{3+} increased, bead formation accelerated, reflecting the higher availability of cations to participate in alginate network

crosslinking. This trend continued up to 250 mM, beyond which no significant changes in formation rate were observed. Conversely, at concentrations below 50 mM, beads were irregular or failed to form entirely. These findings are critical for establishing a reproducible bead production protocol, as the rate of bead formation can influence both the encapsulation efficiency of bioactive compounds and the resistance of the material to natural degradation. Selecting an appropriate crosslinker concentration is therefore essential to achieve a consistent, efficient, and tailored product.

6.4.1.2 *Effect of Crosslinking Ions on Water Retention of Alginate Beads*

To evaluate the influence of crosslinking ions and humic substances on the stability and release behavior of alginate-based systems, a series of experiments was conducted using beads (B) prepared with increasing concentrations of either Ca^{2+} or Fe^{3+} ions, with or without the addition of three selected humic extracts. Monitoring mass variations over time allowed assessment of both structural integrity and water retention capacity under controlled conditions. Alginate beads crosslinked with Ca^{2+} (**Figure 1a**) exhibited a pronounced decrease in residual mass within the first 45 minutes. Interestingly, beads prepared with the lowest calcium concentration retained the largest amount of water, whereas the fastest water release was observed at the highest concentration. Beads crosslinked with Fe^{3+} (**Figure 1b**) displayed superior structural stability compared to calcium-based ones, with residual water exceeding 80% even at the highest tested concentration (33.3 mM) after 45 minutes. The higher valence of Fe^{3+} allows the formation of stronger and more numerous crosslinks along the alginate backbone, thereby enhancing resistance to degradation and mass loss over time. Beads enriched with humic substances and crosslinked with CaCl_2 at a fixed concentration of 50 mM (**Figure 1c**) showed an intermediate release profile. Despite the presence of the organic matrix, variations in residual mass across different formulations were limited, ranging between 35% and 37% after 45 minutes. This suggests that humic substances can modulate water retention and the structural organization of the alginate matrix, without markedly affecting short-term release kinetics. Comparative analysis of all bead formulations highlights that Ca^{2+} crosslinking promotes faster water release and lower structural

stability, whereas Fe^{3+} crosslinking substantially enhances matrix integrity and enables a more controlled release. Beads containing humic substances exhibit an intermediate capacity to retain mass over time, reflecting a moderate modulatory effect associated with the organic matrix. These findings provide valuable guidance for optimizing controlled-release systems, particularly for applications where water retention is critical, and support the rational selection of crosslinking ions and matrix compositions according to specific functional requirements.

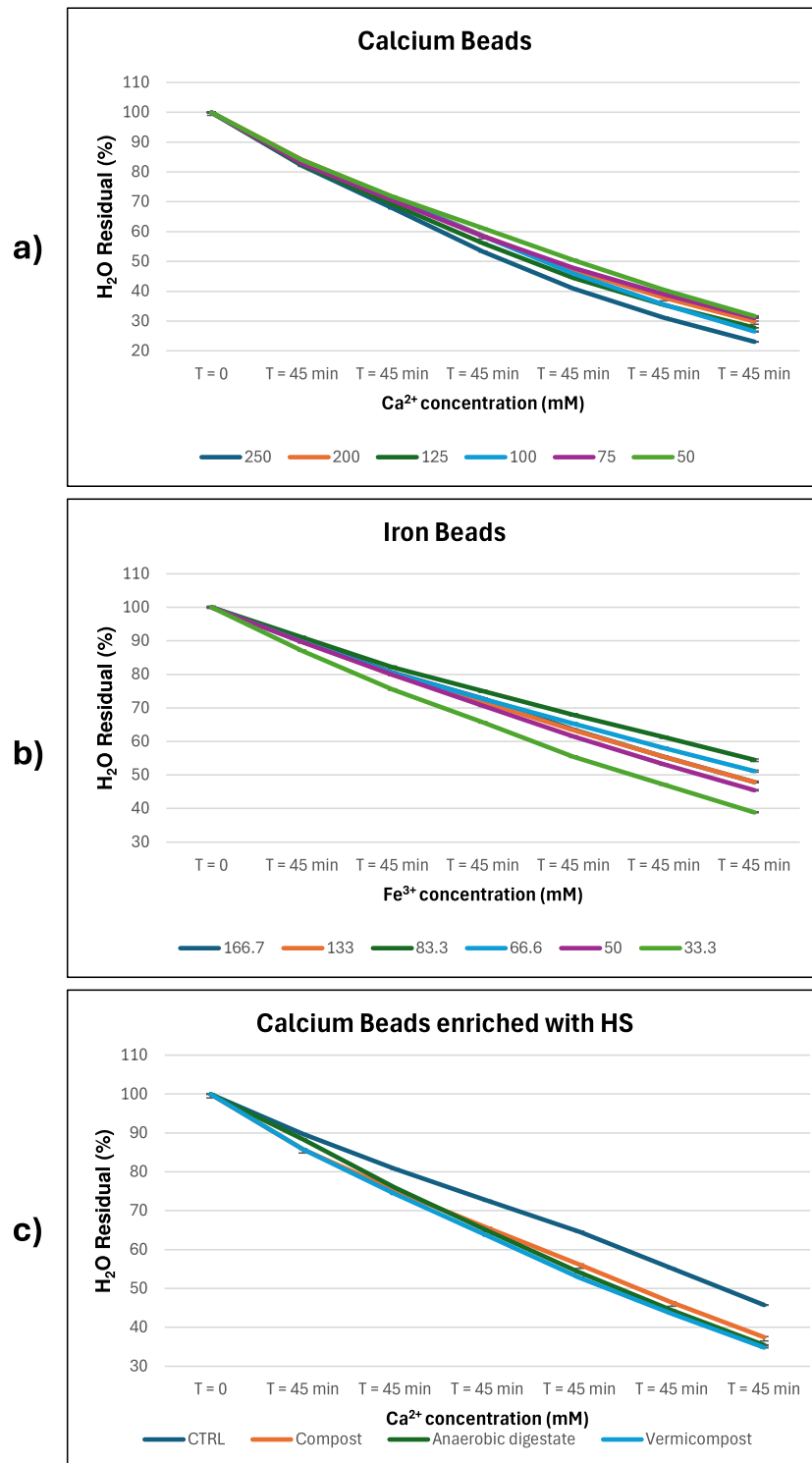


Figure 1. Water retention profiles of alginate beads under controlled conditions. (a) Calcium-crosslinked beads (50–250 mM Ca^{2+}). (b) Iron-crosslinked beads (33.3–166.7 mM Fe^{3+}). (c) Calcium-crosslinked beads enriched with humic substances (50 mM CaCl_2). Beads were monitored for 4.5 hours to assess residual water content. Error bars represent standard deviations from duplicate experimental runs.

6.4.1.3 Beads crosslinkers content and release

The amount of crosslinkers entrapped in the bead matrix was estimated based on the residual electrical conductivity detected in the crosslinker solution after bead formation and removal. Iron and calcium beads were prepared using a 50 mM crosslinker solution combined with 1.2% alginate. This combination provided an optimal balance, ensuring a regular spherical shape without creating an overly rigid structure. For beads formed with ferric chloride, the average Fe^{3+} content was 125.12 μg per bead, whereas for calcium chloride beads, the average Ca^{2+} content was 800 μg per bead (**Table 1**). The significantly higher calcium content per bead compared to iron is attributed to the trivalent nature of iron, which promotes rapid formation of a larger number of ionic interactions with the alginate anionic sites. This results in a more rigid structure, reducing the penetration of free iron cations into the bead core. Assessment of the average cation content is crucial, as it allows prediction of the maximum amount of these nutrients potentially releasable into the soil and enables dose adjustment. To estimate the release of cations in the absence of microbial decomposition, a set of experiments was conducted in which 40 beads were placed in 80 mL of water for 3 days under controlled conditions. The resulting solutions were analyzed using ICP-OES. The data (**Table 1**) indicated that cation release occurred in both cases, with 24.32 μg of calcium and 50.3 μg of iron released per bead. This shows that calcium is released at a considerably slower rate than iron.

The slower release of calcium can be explained by multiple factors: a larger proportion of calcium acts as a crosslinker, smaller pore size, and a more stable bead structure. Part of the cations contributes to ionic bonding within the alginate matrix, while another fraction remains in the bead pores as free or weakly retained ions in the bead water. These results provide critical

information regarding the amount of cations potentially released through simple water contact, supporting dose regulation to meet expected nutrient requirements. Once introduced into soil, however, this release pattern may be strongly influenced by microbial activity, which disrupts the bead structure and promotes cation liberation via multiple mechanisms, including cation exchange.

Table 1. Quantitative estimation (μg per bead; mg in 40 beads) of cations (calcium or iron) used as crosslinkers and entrapped into the beads networks. Assessment of the average cations release occurred by putting 40 beads in contact with 80 ml of water for 3-days and based on ICP-OES analysis.

	Cations content ($\mu\text{g}/\text{bead}$)	Total cations content in 40 beads (mg)	Concentration of cations in the release solution (mg/l)	Amount of released cations in 80 ml (mg)	Residual cation content (total mg/ μg per bead)	Amount of released cation (%/ $\mu\text{g}/\text{bead}$)
Calcium	800 ± 2	$32 \pm 0,080$	12.15 ± 0.07	0.972	$31.028 \pm ,68$	3.04 ± 24.32
Iron (III)	$125,12 \pm 2$	$5.005 \pm 0,055$	25.15 ± 0.49	2.012	$2.993 \pm 74,82$	40.2 ± 50.3

6.4.1.4 Beads decomposition mediated by chelating agents and when put in contact with soil

The resistance of beads to soil decomposition plays a critical role in either limiting or promoting the release of nutrients and/or incorporated bioactive compounds. Soil properties, microclimatic conditions, and the activity of both microorganisms and edaphic fauna contribute to accelerating bead degradation. To simulate the action of root exudates in soil, calcium beads (prepared with 25 and 50 mM Ca^{2+} solutions) and iron beads (prepared with 6.6 and 33 mM Fe^{3+} solutions) were exposed to appropriate chelating organic acids. BCa and BFe were placed in contact with 150 mM EDTA and 99 mM citric acid, respectively, while control tests were conducted in the absence of chelating agents. Bead degradation was visually

monitored and photographed at 0, 4, and 9 days under controlled conditions (22.3 °C) (**Figure 2a–d**). After 9 days, calcium beads prepared with 25 mM calcium exhibited a noticeably lighter color than those prepared with 50 mM calcium, reflecting a lower crosslinker density and, consequently, a less stable structure. Nonetheless, both 25 and 50 mM BCa remained largely unchanged in the absence of chelating agents or biotic activity. Exposure to EDTA, however, induced progressive decomposition in all beads over time. The organic acid promoted calcium chelation, weakening the alginate network. Complete dissolution of 25 mM beads occurred after 4 days of EDTA exposure, whereas 50 mM BCa remained visible, albeit with a lighter color due to partial crosslinker removal. This demonstrates that beads with lower crosslinker content possess reduced structural stability and resistance.

Iron beads exhibited a similar trend. The 6.6 mM BFe displayed a lighter color and less perfect spherical shape compared to 33 mM BFe, reflecting the lower crosslinker density. In the absence of citric acid, no significant modification was observed. Addition of citric acid induced time-dependent decomposition in all beads, with a more pronounced effect for 6.6 mM BFe. During the initial hours, decoloration was limited to the bead surface, indicating that cation release initially affected only the external layer, while the core remained darker (**Supporting Figure S5**). After one day, gradual structural breakdown allowed citric acid to penetrate the core, and after 4 days, the red core was no longer visible in both 6.6 and 33 mM BFe (**Figure 2c,d**). A similar behavior is expected for calcium beads, although the white color prevented qualitative observation. In conclusion, calcium and iron beads prepared with lower crosslinker concentrations (25 and 6.6 mM, respectively) exhibited lower resistance than beads with higher crosslinker content. Under identical chelating agent concentrations, beads with higher crosslinker density contained more cations both within the network and in the pores, limiting the availability of free chelating agents and slowing degradation. These results

indicate that bead resistance and release rates can be tailored through formulation, allowing optimization of their performance for specific applications.

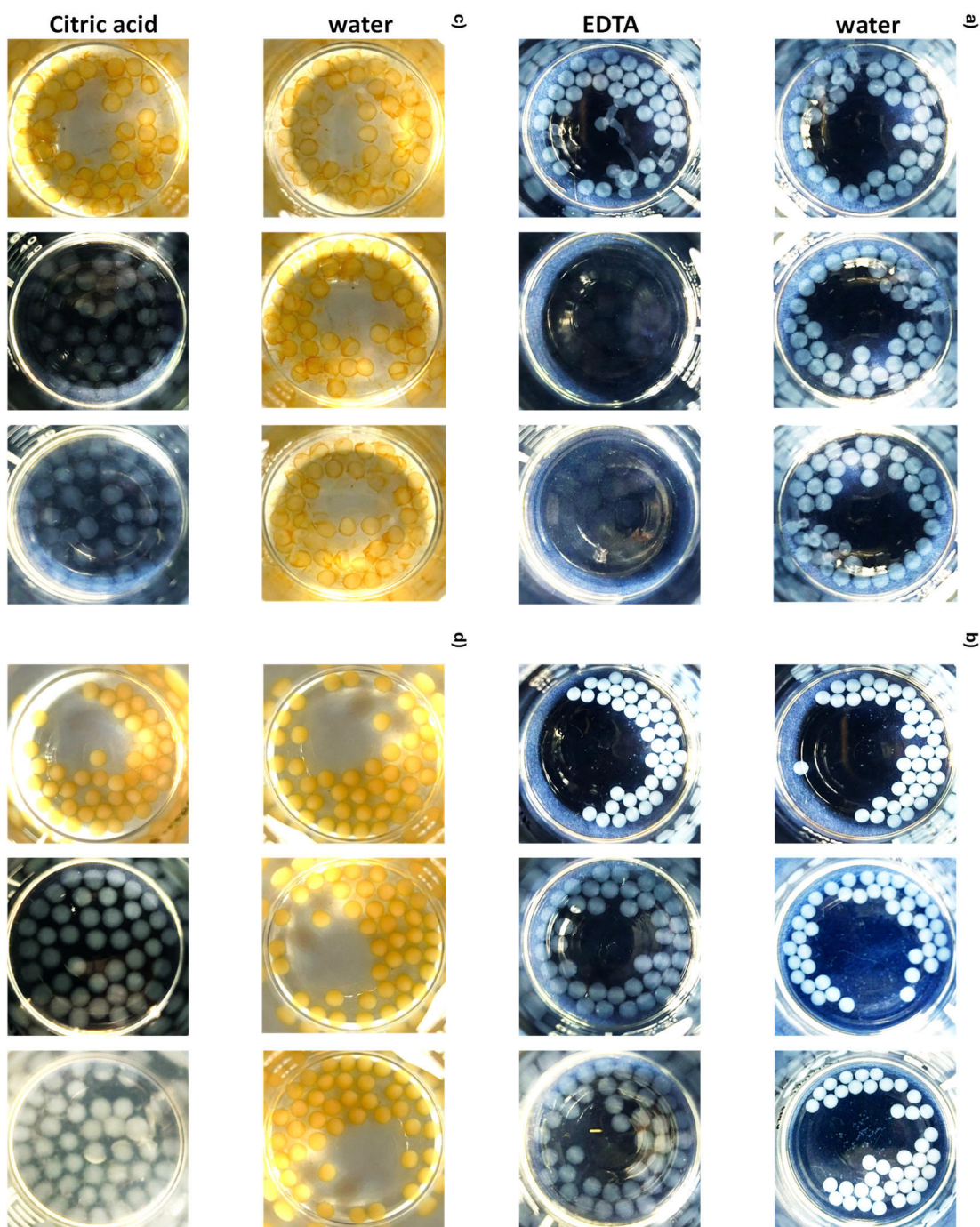


Figure 2. Calcium (plot a, 25 mM; plot b, 50 mM) and iron (plot c, 6.6 mM; plot d, 33 mM) beads prepared with alginate (1.2 % w/v) and placed in contact with either water or a chelating agent (EDTA and Citric acid for calcium and Iron, respectively). In each plot are exhibited the images of beads after 0 (left-side), 4 (center) and 9 (right-side) after the begin of the experiment, launched with water (upper-side) or the chelating agent (bottom-side).

An additional experiment was conducted to evaluate the behavior of the beads when placed in contact with real soil. BCa and BFe were positioned within a cylindrical system, confined between two soil layers, and maintained for 20 days under controlled temperature, air humidity, and soil moisture, which was kept at field capacity. The qualitative evolution of the beads was continuously monitored. Notable differences were observed at the end of the experiment for both BCa and BFe (**Supporting Figure S6**). Despite soil inhumidification, all beads released most of their water, resulting in a significant reduction in size.

For calcium beads, 25 mM BCa were almost completely decomposed, whereas 50 mM BCa retained a pseudo-spherical shape (**Supporting Figure S6**). A similar trend was observed for iron beads, although decomposition was more pronounced even for 33 mM BFe. These observations are consistent with the results obtained from the chelating agent tests, confirming the strong correlation between crosslinker type and concentration and the rate of bead decomposition. In soil, additional factors, such as root and microbial activity, as well as the pressure exerted by the overlying soil layer, accelerate decomposition and induce physical deformation.

The observed BFe decomposition highlights its potential role as a tool for releasing oxidized iron in a bioavailable form. Part of the Fe^{3+} released during BFe breakdown is expected to remain bound to alginate fragments, which, particularly in neutral and alkaline soils, helps prevent precipitation of this micronutrient.

6.4.1.5 Humates characterization and loading in beads

Given the beneficial role of humic substances for rhizosphere activity, plant roots, and soil microbiota, a procedure was designed to enhance the potential of the examined alginate systems by incorporating these materials. The aim was to develop a reliable system ensuring gradual and controlled release in soil. Specifically, the focus was on the water-soluble fraction

of organic matter extracted from green compost (HSCOM), vermicompost (HSVC), and solid digestate (HSDIG). These materials are effective when applied as exogenous, biochemically stable organic matter, and part of their beneficial effects is attributed to the hydrosoluble components. The type and extent of benefits depend strongly on the composition of the humic material, which, in turn, reflects the quality of the source biomass. Consequently, the composition of the studied humates was examined using high-resolution liquid-state NMR and FT-IR.

1D NMR proton spectra of HSCOM, HSDIG and HSVC are showed in the **Figure 3a**. The relatively intense peaks resonating in the two spectral ranges 0.5-0.88 and 0.93-1.33 ppm were attributed to lipid compounds and, in particular, to methyl and methylene chains in fatty acids, respectively. The signals resonating in the region 1.8–2.9 ppm were ascribed to functional groups either bonded to weakly electron-withdrawing groups, such as the nitrogen, or vicinally bonded to aromatic or carboxyl groups. Within such a spectral range, it was detected an intense singlet at 1.804 ppm which was assigned to the methyl hydrogens in acetic acid, whose highest content was detected in HSDIG. The family of overlapped signals resonating in the range 2.87-4.55 ppm was attributed to the signals of hydrogens bound to strongly electron-withdrawing groups, such as the oxygen, and mostly representing carbohydrates. Finally, the very broad signals observed in the 6.5–7.8 ppm region were attributed to aromatic hydrogens, mostly arising from phenolic and lignin-derived compounds in a polymeric and/or aggregated state. Within this envelope, three distinct resonances at 6.51, 6.82 (adjacent to hydroxyl groups), and 7.35 ppm (adjacent to quaternary carbons) were detected and ascribed to relatively mobile oligomeric or monomeric lignin units. The most deshielded signal (7.35 ppm) was assigned to aromatic protons vicinal to hydroxyl groups.

It is noteworthy that in all the extracts it was appreciated a relevant content in lipid and phenolic compounds. In particular, HSCOM contained, in absolute, the lowest amount of these two classes of compounds. This notwithstanding, it is also interesting the fact that HSCOM and HSVC profiles resulted relatively similar, between each other, despite they were extracted by organic materials deriving from different biomass and processes (**Figure 3a**). In fact, the former was obtained by a composting process conducted on fresh vegetables wastes, while the latter was attained by the earthworm valorisation of solid digestate deriving from liquid livestock wastes. Conversely, HSDIG appeared much more different than the other two humates. In fact, while HSCOM and HSVC exhibited a similar profile for the carbohydrates region, which was characterized by few relatively broadened peaks, HSDIG spectrum showed a significantly higher content in carbohydrates. In addition, this region in HSDIG was populated by a larger number of peaks, mostly characterized by a relatively sharp shape, as well as the spectrum also included three pronounced peaks in the range 5.02-5.34 ppm attributable to anomeric functional groups in carbohydrates. These elements denoted the presence, in HSDIG, of relatively small molecules with a fast tumbling rate thus revealing a significant abundance of residual mono- and oligo-saccharides. Importantly, this finding indicated that the processed organic material reached a less advanced humification stadium in DIG, as compared to those reached with vermicomposting and composting. In fact, it exhibited a lower hydrophobic index, accompanied by a relative abundance of labile carbon.

Conversely, the aromatic profile was very similar for HSDIG and HSVC, while HSCOM indicated a significantly lower content in this class of molecules. The similarity between HSVC and HSDIG may be explained by a higher content in phenolic compounds in liquid livestock wastes which was then slowly and only partially decomposed during both anaerobic digestion and vermicomposting. The presence of phenols in these extracts is not obvious since,

being most of them associated with biostimulant and hormone-like properties, could concur to explain their potential bioactivity (**Canellas et al., 2015; Muscolo et al., 2020**). Moreover, only in case of HSGID it was detected a intense singlet at 8.3 ppm, ascribable to the formic acid, which is a typical product developed during acidogenesis in anaerobic digestion (**Wang et al, 2021**).

The FT-IR spectra of HSCOM, HSDIG, and HSVC are shown in **Figure 3b** while the assignments of the most intense absorption bands are summarised in **Supporting Table S2**. The band at 3400 cm^{-1} was attributed to O–H stretching, N–H stretching, hydrogen-bonded O–H. The adsorption bands at $2935\text{--}2925$ and 2850 cm^{-1} were assigned to alkyl C–H stretching, while the band at $2925\text{--}2850\text{ cm}^{-1}$ to methoxyl C–H stretching. The band at $1725\text{--}1710\text{ cm}^{-1}$ corresponded to the C=O stretching in carboxyls, whereas the one at $1640\text{--}1600\text{ cm}^{-1}$ to aromatic C=C skeletal vibrations. The adsorption at $1512\text{--}1506\text{ cm}^{-1}$ was ascribed to N–H deformation and C=N stretching, while the band at $1419\text{--}1416\text{ cm}^{-1}$ to O–H deformation and C–O stretching of phenolic OH. The adsorptions at $1460\text{--}1450\text{ cm}^{-1}$ and at $1269\text{--}1261\text{ cm}^{-1}$ were attributed to C–H asymmetric bending of CH_3 groups and C=O stretching of aryl esters, respectively. The spectral range included within 1220 and 1000 cm^{-1} refers mostly to C–O stretchings, where the band at 1219 cm^{-1} was ascribed to aryl ethers and phenols, that at 1184 cm^{-1} to secondary alcohols and ethers and that at $1130\text{--}1000\text{ cm}^{-1}$ to polysaccharides and alcohols (**Enev et al., 2014; Machado et al., 2020; Li et al, 2024**).

The IR spectra (**Figure 3b**) confirmed a marked difference subsisting among the examined humates as well as revealed that the peaks in HSCOM spectrum were slightly more broadened than for HSDIG and HSVC. This may be indicative of a more heterogeneous composition in this extract resulting from more advanced humification state. In particular, IR confirmed most of the information unveiled by NMR spectroscopy. In fact, HSDIG exhibited very intense adsorptions in all the bands attributed to carbohydrates, especially for the bands corresponding to the ranges $1184\text{--}1110$ and $3400\text{--}3200\text{ cm}^{-1}$. The peaks assigned to aromatic and phenolic moieties ($1640\text{--}1600$ and $1470\text{--}1415\text{ cm}^{-1}$) were very pronounced in all cases, with HSCOM and HSDIG showing the weakest and strongest adsorptions. It is interesting to note a certain

adsorption at around 2880 and accompanied by a peak at 1254 cm^{-1} , mostly ascribable to methoxyls in guaiacyl and syringyl lignin units. Finally, the regions attributed to aliphatic CH (2935-2900; 1460; 1400-1350 cm^{-1}) as well as carboxyl C=O stretching peak (1720 cm^{-1}), prevalently belonging to lipidic structures, appeared relatively more pronounced in HSDIG and HSVC.

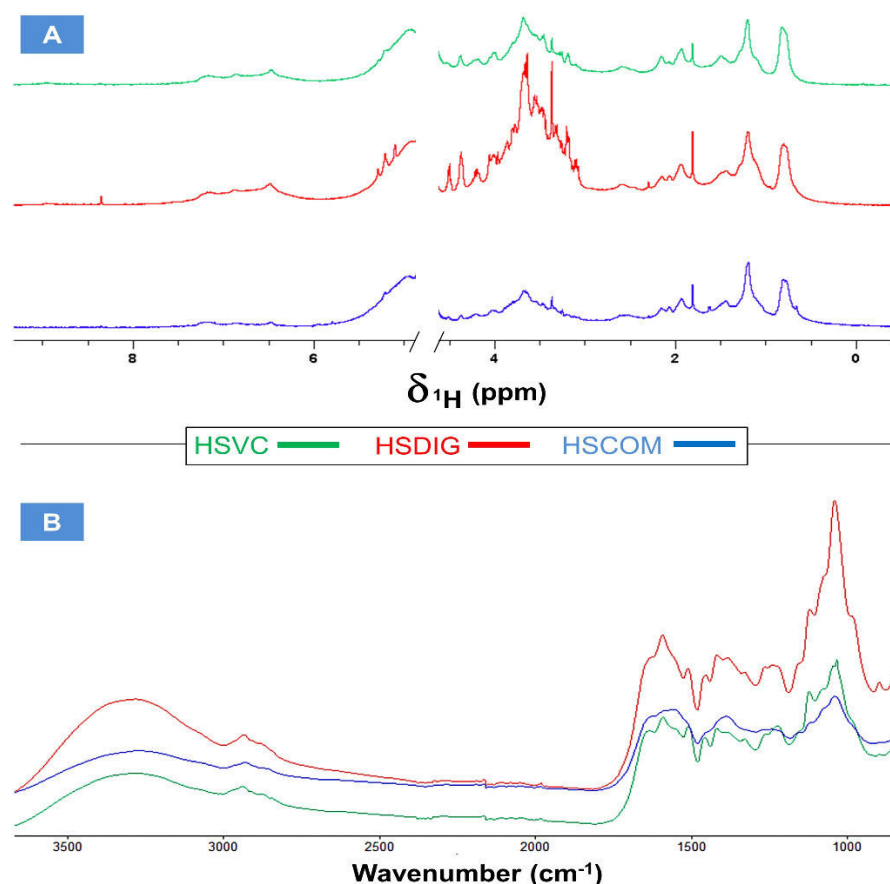


Figure 3: (a) Liquid-state high resolution ^1H NMR and (b) solid-state Fourier transform infrared (FT-IR) spectra of humates for green compost (HSCOM, blue), solid anaerobic digesatte (HSDIG, red) and Vermicompost (HSVVC, green). The distorted portion of spectra involving the water suppression (4.7-4.9 ppm) was digitally removed.

The most relevant IR information well agree with NMR results and confirm that are HSDIG contain the highest carbohydrate content, while HSCOM contains the lowest level in phenols and lipids.

The DOSY-NMR technique (**Wimmer et al., 2022**) was employed to investigate the diffusive behavior of the molecules composing the examined humates. Because molecular diffusivity is inversely proportional to hydrodynamic radius (**Chapman et al., 1980**), this approach allows discrimination between monomeric and polymeric forms and provides insights into possible aggregation states of complexed compounds. DOSY 2D NMR spectra are obtained via an inverse Laplace transform and project proton signals along the y-axis according to their self-diffusion coefficients. Specifically, proton signals from molecules with small hydrodynamic radii appear lower on the scale (higher logD values), whereas signals from larger molecules or aggregates are projected upward. **Figure 4** displays the superimposition of the 2D DOSY NMR spectra of HSCOM, HSDIG and HSVC. From a macroscopic perspective, the fact that the projections of most compounds of HSVC and HSCOM resulted aligned in DOSY spectra not only proved that the components, despite their molecular heterogeneity, aggregated to form a typical supramolecular complex (**Piccolo & Drosos, 2024**), but also indicated that the average hydrodynamic radius of the humic aggregates did not change relevantly according to the humic type. Conversely, in case of HSDIG, the diffusive response showed a certain variability as a function of the class compounds. In particular, most of carbohydrates signals in HSDIG showed a greater mobility as compared to both the HSDIG aromatic and aliphatic domains and the other humates. The first evidence confirmed a relevant presence of residual monosaccharides (small and relatively mobile) in digestate humate. Despite the ^1H NMR spectra did not show any marked difference among the alkyl signals, DOSY unveiled a different aggregation state of these components, resulting from a humate-specific hydrophobic aggregation of lipidic moieties. In particular, the largest complex was observed for HSDIG, while the smallest one was appreciated for HSCOM. Such a response in HSDIG may be explained by a more enhanced segregation promoted by the

relatively hydrophilic nature of the extract. The fact that singlet at 1.804 ppm exhibited in all cases a fast diffusivity further supported its assignment as acetic acid. Finally, the DOSY projections in the aromatic region only indicated a slightly larger aggregation for HSDIG components, which well correlated with the behaviour of the lipidic components, and may be again explained a more enhanced segregation state of hydrophobic moieties. Concluding, this results indicated that all the extracts exhibited a singular molecular profile, depending on the source organic biomass, the applied valorization process and the resulting humification state, which can be determinant for the extract bioactivity.

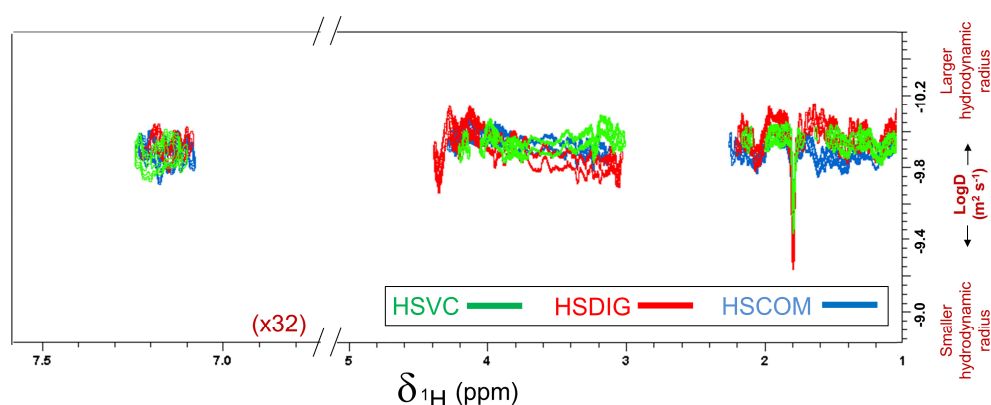


Figure 4: Overlapping of ^1H DOSY NMR spectra of humates from compost (blue), anaerobic digestate (red) and vermicompost from digestate (green). The negative logarithm of the diffusivity coefficients is shown along the vertical axis. The figure depicts the spectral regions included between 1 and 5.2 ppm (on the right) and 6.2 and 7.35 ppm (on the left, x 32).

6.4.2 Hydrogels

6.4.2.1 *Hydrogels layers characterization and water retention capacity*

Hydrogel alginate layers (H) represent another promising delivery strategy for soil. As compared to the alginate beads, the H are mostly oriented towards applications as soil structure ameliorative and supplementary reservoirs of water and bioactive agents, such as humates, with a relatively less pronounced role as cations source. They may act as a soil improver and plant growth promoter, especially when applied in the proximity of the rhizosphere.

Firstly, diffusion NMR spectra were employed to determine the average molecular size of the sodium alginate used in this work. Such an evaluation was carried out by comparing the diffusion coefficient of studied sodium alginate with a calibration curve built up with the diffusion values of standard dextrans (25, 150, and 410 kDa). The average molecular size was estimated to be approximately 551 kDa (**Supporting Figures S7**).

Subsequently, calcium alginate hydrogels (HCa) were developed and characterized through different formulations. Two complementary experiments were carried out to investigate their dehydration behaviour, both under free-drying conditions and in contact with a model soil (**Figure 5 a, b, c**).

In the first test (**Figure 5a**), the dynamics of water loss from HCa prepared with different alginate concentrations (1, 1.5, 2, and 3% w/v) were monitored under controlled humidity (53%) and temperature (22.4 °C). A very rapid water release was observed for the 1.5% formulation, which lost about 32% of its initial water content after only 4.5 hours, whereas all the other hydrogels showed lower losses (around 21%). After 22 hours, clear differences among samples were evident: the 1.5% HCa maintained the highest water loss rate, while 1%, 3%, and 2% HCa dehydrated progressively more slowly. Complete dehydration was achieved

after 44.3 hours for all formulations. Overall, the 1.5% HCa exhibited the weakest water-holding ability, while the 2% formulation showed the slowest and most gradual water release. The dehydration curves displayed a biphasic trend, with a rapid initial phase dominated by the evaporation of loosely bound water (mainly located in the outer and more porous regions of the hydrogel) followed by a slower phase involving water molecules more strongly retained through ion–dipole and hydrogen-bond interactions. These results highlight that increasing alginate concentration enhances hydrogel rigidity and internal crosslinking, thereby slowing down dehydration. Consequently, the 2% HCa formulation appears more suitable for soils requiring a gradual and sustained water release, such as those with low water potential or high microbial activity, whereas the 1.5% HCa could be preferred in conditions where a faster water delivery is desired.

In the second experiment (**Figure 5b**), the same HCa formulations were placed in contact with a sandy soil to simulate more realistic conditions. Gravimetric monitoring showed that all H-treated systems experienced a slower water loss compared to the soil-only control, confirming that the presence of hydrogels effectively delayed evaporation. Among the formulations, the 1.5% and 2% HCa displayed the lowest cumulative water loss after 14 days, indicating their superior capacity to moderate soil dehydration. As illustrated in **Figure 5c**, morphological differences were also detected after 14 days of soil contact. Hydrogels with 1% and 1.5% alginate showed marked contraction and partial structural collapse during dehydration, while the 2% and 3% formulations maintained a more stable morphology. This greater structural integrity suggests that denser HCa formulations can better withstand drying–rewetting cycles, potentially restoring their original volume and functionality upon rehydration. In practical soil applications, this resilience would enable multiple hydration cycles and long-term moisture regulation.

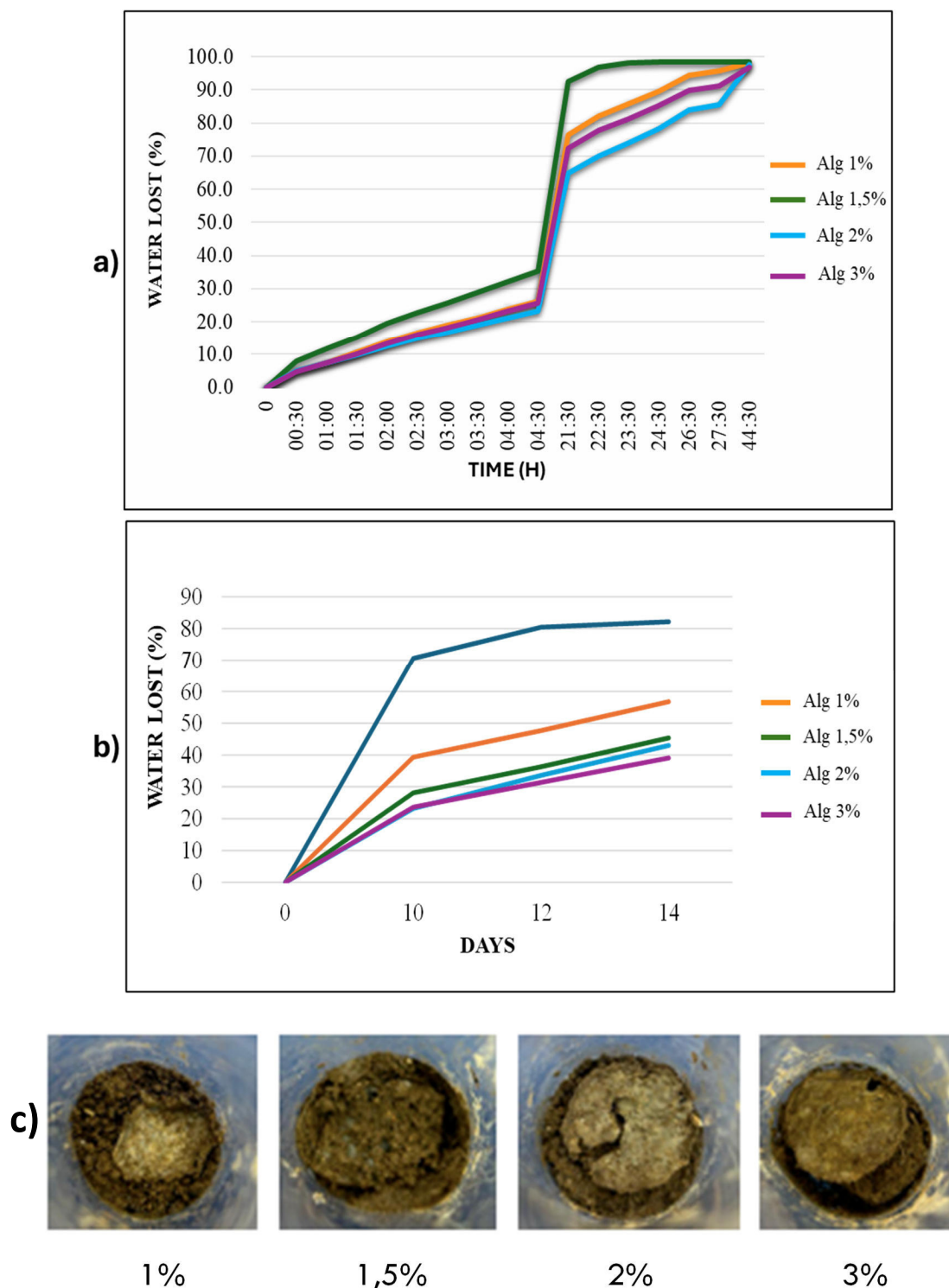


Figure 5: (a) Dehydration kinetics of calcium alginate hydrogels prepared with different alginate concentrations (1, 1.5, 2, and 3% w/v) and monitored for 46 hours under controlled conditions (T 22.4 °C; RH 53%). (b) Cumulative water loss of HCa formulations (1, 1.5, 2, and 3% w/v) in contact with a sandy soil under controlled environmental conditions, recorded up to 14 days. (c) Morphological appearance of the same HCa formulations after 14 days of contact with soil, showing the progressive structural changes related to alginate concentration.

3.2.2 Hydrogel rheological characterization

Understanding the rheological properties of hydrogels provides insight into the connection between their chemical structure and macroscopic behaviour (**Falcone et al., 2024**). This knowledge is also essential for the optimization of hydrogels with tailored properties suited to specific applications (**Stojkov et al., 2021**). For this reason, after a structural characterization of crosslinked alginate matrices, an accurate rheological investigation was carried out to highlight how the structural differences affected both flow properties and viscoelastic properties of each hydrogel. In detail, it was investigated how alginate concentration influence the properties of hydrogels produced with the same calcium amount (**Figure 6**). The first test performed was the flow sweep test, to investigate changes in the viscosity as consequence of increased shear rate. This evaluation allows to obtain information about hydrogels extrudability, that is a crucial aspect for hydrogels dispensing process. In fact, for practical applications, shear-thinning character of hydrogel ensures the matrix extrudability in a continuous flow, with high dispensing accuracy (**Lopez et al., 2021**). As shown in flow sweep graph, all batches showed a rapid decrease of viscosity when subjected to higher shear rate, typical of a shear-thinning behaviour. This remarkable characteristic is due to the alginate pre-crosslinking step that improve the polymer chain organization in comparison to the alginate raw material (**Falcone et al., 2022**). To obtain more detailed info about the flow properties of each produced hydrogel in function of alginate concentration, Power-Law model was used to perform a fitting study (**Adhikari et al., 2021**). This model is one of the most used approaches due to its ability to approximately describe the behaviour of a real non-Newtonian fluid using two parameters: the material consistency (c) and the flow behaviour index (p , $p < 1$ shear thinning behaviour, while $p > 1$ shear thickening behaviour) (**Table 2**). The regression coefficient higher than 0.90 confirm the applicability of this model to describe the properties of produced hydrogels showing that the increase in polymer concentration led to a relevant

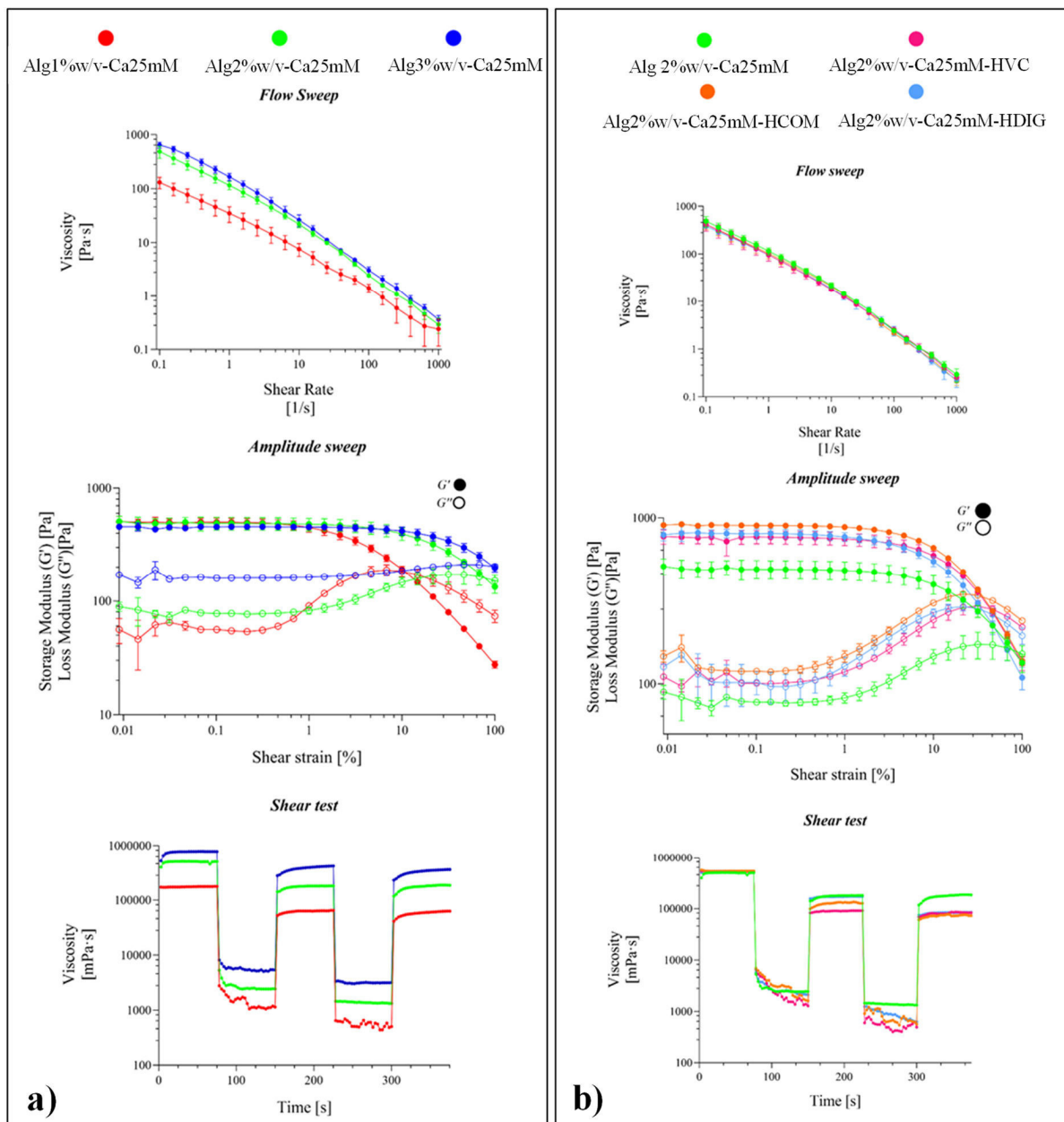


Figure 6: Rheological test on (a) HCa (25 mM) with different concentrations of alginate (1 %, 2 % and 3%) and (b) HCa 1% enriched with HSCOM, HSDIG, HSVC.

increase of hydrogel consistency (more than 2 times from 1% to 2% and 1.5 times to 3%) as well as in the flow index. The most interesting difference among samples is related to the evaluation of hydrogel viscoelastic behaviour carried out via amplitude sweep test (**Figure 6**). In fact, if all samples showed a gel-like character (storage modulus value > loss modulus

value) confirming the calcium-alginate network formation, moving from alginate concentration 1% w/v to 2% w/v, and 3% w/v, it is possible to observe an increase of limit of linear viscoelastic region (LVER) combined with a higher matrix homogeneity (underlined by a reduction of standard deviation in each point). Moreover, the use of lowest alginate concentration led to obtain an hydrogel with an excessively rigid matrix as confirmed by the loss factor value and the increase of loss modulus curve slope before crossover point that highlight the presence of microcrack in the polymeric structure. On the other hand, the increase of alginate concentration to the highest value (3% w/v) led to an excessive reduction of matrices consistency with a three time increase of loss factor, while hydrogel produced by 2% w/v alginate showed a good ratio matrix homogeneity and consistency, probably due to the more homogeneous egg-box structure formation as consequence of the better balance between alginate moles and calcium moles (**Fang et al., 2007**). Conversely, the hydrogels crosslinked with Fe^{3+} ions did not display a satisfactory structural organization under the same experimental conditions. The rheological data revealed unstable and poorly defined viscoelastic behavior, suggesting incomplete network formation and inhomogeneous crosslinking zones. These preliminary results indicate that the Fe-based systems require further optimization of both ionic strength and alginate concentration to achieve a stable and reproducible gel structure.

Finally, to obtain a complete overview of hydrogel rheological properties, the Time-dependent flow behavior was investigated to evaluate the thixotropic character, means the ability of hydrogels to return to its initial structure after the exposure to mechanical stress. The thixotropic feature is a quality control parameter, relevant in view of transforming an initially viscous hydrogel into a thin product, easy to spread at the site of administration with a well-organized structure influencing matrix erosion (**Gica et al., 2016**). As expected by the

previous evaluation of viscoelastic behaviour, all hydrogels showed a constant viscosity at lower shear rate indicating that the low stress are not sufficient to break the polymeric entanglement, with a direct correlation between alginate concentration and viscosity value. After that samples were subjected to higher shear rate (both 50 and 100 s⁻¹), hydrogels showed, in a short time, the ability to partial recover high viscosity value (up to 30%) confirming a good structural recovery due to the crosslinking process that improve the capacity to restore the polymeric network, in agreement with data reported about natural polymer based hydrogels (Chejara et al., 2013; Enoch et al., 2024).

Table 2. Summary of rheological results						
Samples	Rotational properties			Oscillatory properties		Transient properties
	Power-Law r^2 (1)	Concistency (Pa•s)	Flow index (1)	LVER (%)	Loss factor (1)	Thixotropic index (%)
HCa 1%	0.9392	32.81 ± 9.08	0.413 ±0.041	0.5	0.113 ±0.039	35.60
HCa 2%	0.9584	106.91 ± 19.45	0.363 ±0.038	1	0.176 ±0.004	36.97
HCa 3%	0.9796	150.29 ±25.72	0.393 ±0.044	3	0.374 ±0.006	39.90
HSDIG	0.9903	90.02 ±0.95	0.389 ±0.017	2	0.160 ±0.008	12.86
HSCOM	0.9547	96.72 ±19.37	0.380 ±0.038	2	0.162 ±0.009	15.39
HSVC	0.9341	91.46 ±20.78	0.356 ±0.017	2	0.160 ±0.020	16.88

The same methodological approach about hydrogel rheological characterization was applied to evaluate the effect of the addition of humic agent to the calcium-alginate matrix (**Figure 6b**). As it is possible to observe in the flow sweep graph, the incorporation of humic substance did not seem to lead variation in the hydrogel flow behaviour, with similar values for all samples independently from the substance added to the alginate matrix. The non-relevant effect was confirmed by the evaluation of Power-Law parameters, i.e., consistency and flow index, as well as the regression coefficient, which did not vary, although the introduction of a new component into the matrix could have altered the macroscopic flow properties (**Table 2**).

These results suggested the hypothesis that, at these specific concentrations, humic substances are physically incorporated in the polymeric entanglement. The hypothesis that was confirmed by the hydrogels viscoelastic evaluation. In fact, humic-loaded hydrogels showed higher LVER and a decrease in the loss factor in comparison to alginate, indicating a reinforcement effect of humic substance (**Le Goff et al., 2015**). On the other hand, the thixotropic character of calcium-alginate hydrogels decreases with the addition of humic substance showing a recover-ability of viscosity around 15% instead of 30%. The reason behind this reduction could be due to the physical obstruction of humic substance during the re-formation of calcium-alginate entanglement.

6.4.2.2 Characterization of Hydrogel before and after HS loading via MRI

Among the various techniques used to characterise H, Magnetic Resonance Imaging (MRI) was considered. While no significant visual differences were observed, the relaxation times and diffusion coefficients varied significantly depending on the formulation (**Supporting Figure S8**).

Figure S8a and Figure S8b display the transverse proton relaxation times (T2) and diffusivity data for the most representative samples in the study. The drastic decrease in T2 values confirms gelation for all the studied alginates compared to pure sodium alginate. When comparing calcium alginates with increasing alginate concentrations, it was observed, in line with the dehydration tests, that the 1% and 1.5% concentrations formed a more complex network (leading to slower water release through evaporation). This was also reflected in the diffusion coefficients, although at 1.5%, the values were too low, suggesting excessive water and nutrient retention. Hence, the 1% concentration was identified as the best compromise for the intended application.

Calcium alginates loaded with humic substances (HS) at 1% showed similar T2 values to the 1% control but significantly lower diffusivity values. This indicates that the humic

components interact with the water molecules, retaining them more effectively and limiting the amount of relatively free water. Importantly, these results demonstrate that the addition of HS does not substantially alter the hydrogel structure or its overall relaxation behavior, in agreement with rheological data. Therefore, humic substances can be incorporated without compromising the key structural and dynamic properties of the gels.

Overall MRI confirms that incorporating humic substances into both calcium and iron alginates does not fundamentally disrupt gelation or the structural framework. These findings support the conclusion that HS can be loaded into hydrogels while largely maintaining their native structural and dynamic properties.

6.4.3 Seed coating with alginates film

6.4.3.1 SEM analysis of seed coating experiment

The SEM micrographs reported in **Figure 7** show the surface morphology of *Lepidium sativum* seeds after immersion for 30 minutes in distilled water (CTRL) and in a 2% (w/v) calcium alginate hydrogel (HCa).

At both magnifications (100× and 500×), control seeds immersed in water (**Figure 7a**) displayed the typical reticulated epidermal pattern of the *L. sativum* seed coat, characterized by well-defined polygonal cells and evident intercellular ridges. The surface appeared clean and compact, without any noticeable external layer or structural modification due to immersion.

Conversely, the seeds treated with the HCa (**Figure 7b**) exhibited a clear modification of the surface morphology. The coating layer appeared as a continuous and adherent film covering the epidermal pattern, partially masking the underlying cellular contours. The surface texture

appeared smoother and more homogeneous compared to the control, indicating the successful adhesion of the hydrogel matrix to the seed surface.

Overall, SEM observations confirmed the effective formation and uniform adhesion of the alginate-based coating, demonstrating the success of the seed coating treatment and the ability of the hydrogel formulation to form a stable film on the seed surface.

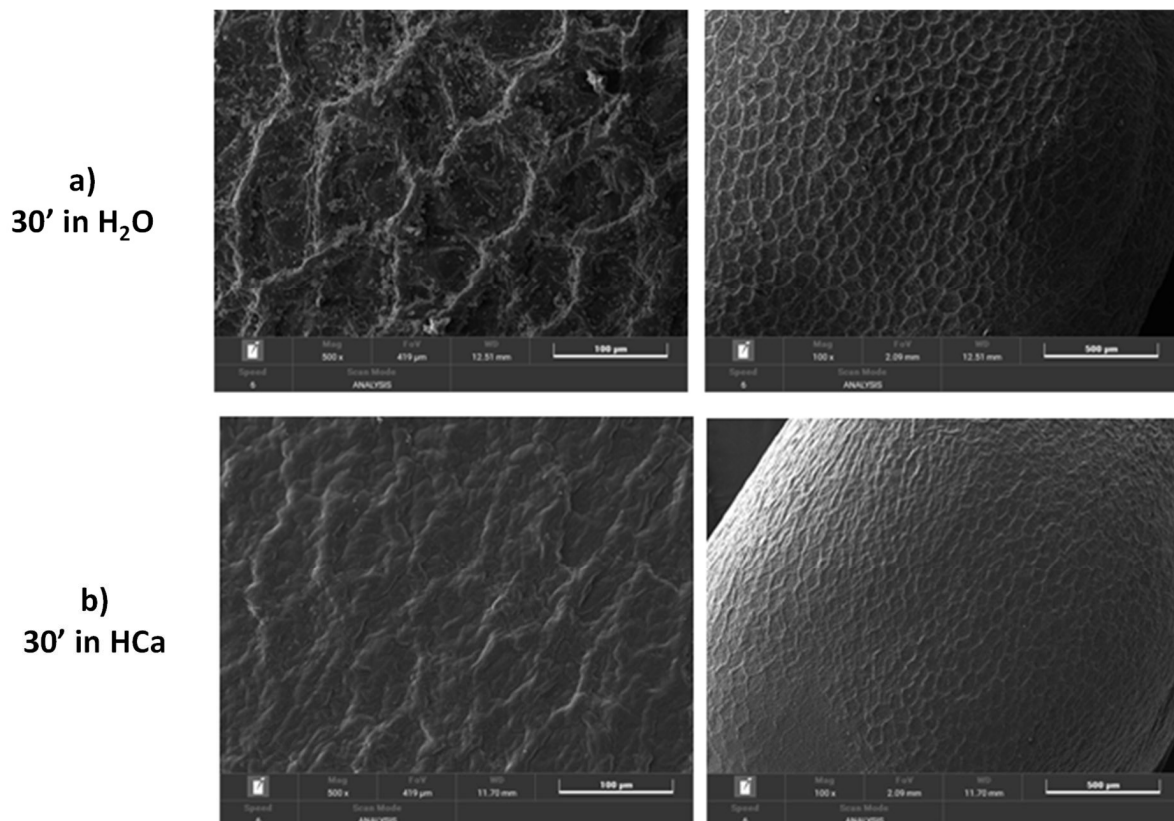


Figure 7. Scanning Electron Microscopy (SEM) micrographs of *Lepidium sativum* seeds after immersion for 30 minutes in distilled water (CTRL, a) and in a 2% HCa (w/v) calcium alginate hydrogel (HCa, b). Images were acquired at 100× and 500× magnifications. The untreated seed surface shows the characteristic reticulated epidermal pattern, while the HCa-treated seeds display a continuous and adherent hydrogel film covering the seed coat, confirming the successful formation of the coating layer.

6.4.3.2 Seed coating tests to assess their impact on germination

A *seed coating test* was conducted to assess the effects of 1% HCa (with and without humates) and 1.75% HFe on cress seed germination. Germination rates, root and shoot lengths, and total germination were measured using conventional indices.

No significant phytotoxicity was observed in any case, excluding potential phytotoxic effects from the alginate, extracts, or crosslinkers studied. In almost all cases, an average of 19 out of 20 seeds germinated within six days, with a significantly faster germination rate observed in all alginate-treated samples. **Table 3** indicates that, as compared to the control, calcium alginate alone resulted in slightly improved performance in terms of Relative Growth of Root (RGR), Relative growth of shoots (RGS), and Germination Index (GI). Notably, excepted HSCOM, in all cases the SG was 100%, The alginates with HSDIG and HSVC demonstrated beneficial effects on seed germination, In particular, HSDIG enhanced both root and shoot development, with the largest impact on roots. HCVC also provided a root increase, although with values lower than HSDIG. Conversely, HSCOM not only delayed the germination process but also negatively impacted shoot growth. The treatment with iron alginate (HFe) exhibited inhibitory effects on root development, likely due to excessive iron release from the coating, but a slight increase in the shoot. Future studies will adjust the iron concentration in the gel to mitigate this issue. Overall, these results highlight the potential of seed coating with alginate-based hydrogels to improve germination speed, promote early seedling growth, and modulate nutrient delivery, offering a promising strategy for enhancing crop establishment and uniformity in sustainable agriculture.

Table 3: Calculated indices for phytotoxicity tests on hydrogel-treated cress seeds					
	Germinated seeds in 6 days	SG %	RGR %	GI %	RGS %
CTRL	19 ± 0,00	100,00	100,00	100,00	100,00
ALG 1%	19 ± 1,41	100,00	108,23	108,23	102,65
ALG 2,5% Fe	19 ± 0,00	100,00	71,52	71,52	104,76
ALG 1% HSCOM	18 ± 1,41	94,74	101,27	95,94	75,13
ALG 1% HSVC	19 ± 0,71	100,00	108,23	108,23	100,00
ALG 1% HSDIG	19 ± 1,41	100,00	121,52	121,52	102,65

6.5 CONCLUSIONS

The systems investigated in this study represent innovative strategies with the potential to enhance soil fertility by acting as supplementary sources of water, calcium, iron, and humic substances. These strategies aim to improve soil health and resilience, while optimizing the use of valuable natural resources through a sustainable approach. While hydrogels may serve especially to increase the water holding content, the beads may serve to approach a quantitative release of nutrients and organic bioactive compounds. The proposed hydrogels and beads not only offer the capacity to retain and release water and nutrients, but also enable a gradual release of bioactives potentially useful for plants and soil microorganisms.

Moreover, SEM observations confirmed the successful formation and adhesion of the calcium alginate coating on seed surfaces, which, especially in case of alginates loaded with HSDIG, led to a significant and promising biostimulation on cress seeds, which can help to promote the germination in seeds also in challenging soil conditions.

Hydrogels crosslinked with Fe^{3+} ions did not display a satisfactory structural organization under the conditions tested, showing unstable rheological behavior and poorly defined network formation. However, the iron alginate beads exhibited a well-formed structure and stability, candidating the beads be more suitable for practical applications of iron-loaded systems and confirming it as a good solution for alkaline soils in which the pH makes not bioavailable this element. In conclusion, the use of alginate-based hydrogels and beads enriched with humic substances offers a promising pathway for soil fertility management. This approach supports eco-friendly and circular agriculture, as it leverages biodegradable materials and recycles organic waste, aligning well with global sustainability goals. With further development, these systems could provide effective tools for enhancing soil quality, promoting plant health, and ensuring more resilient and resource-efficient agricultural practices.

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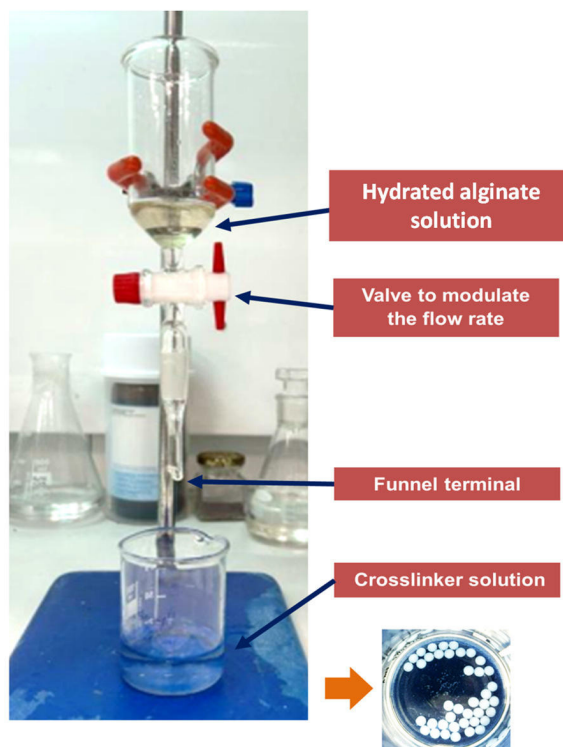
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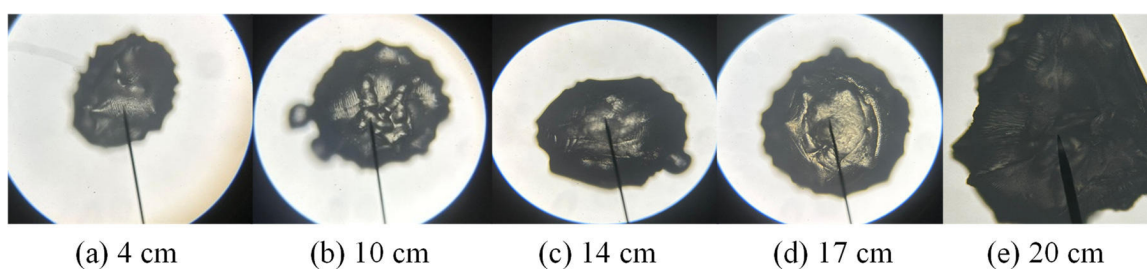
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Supporting Information

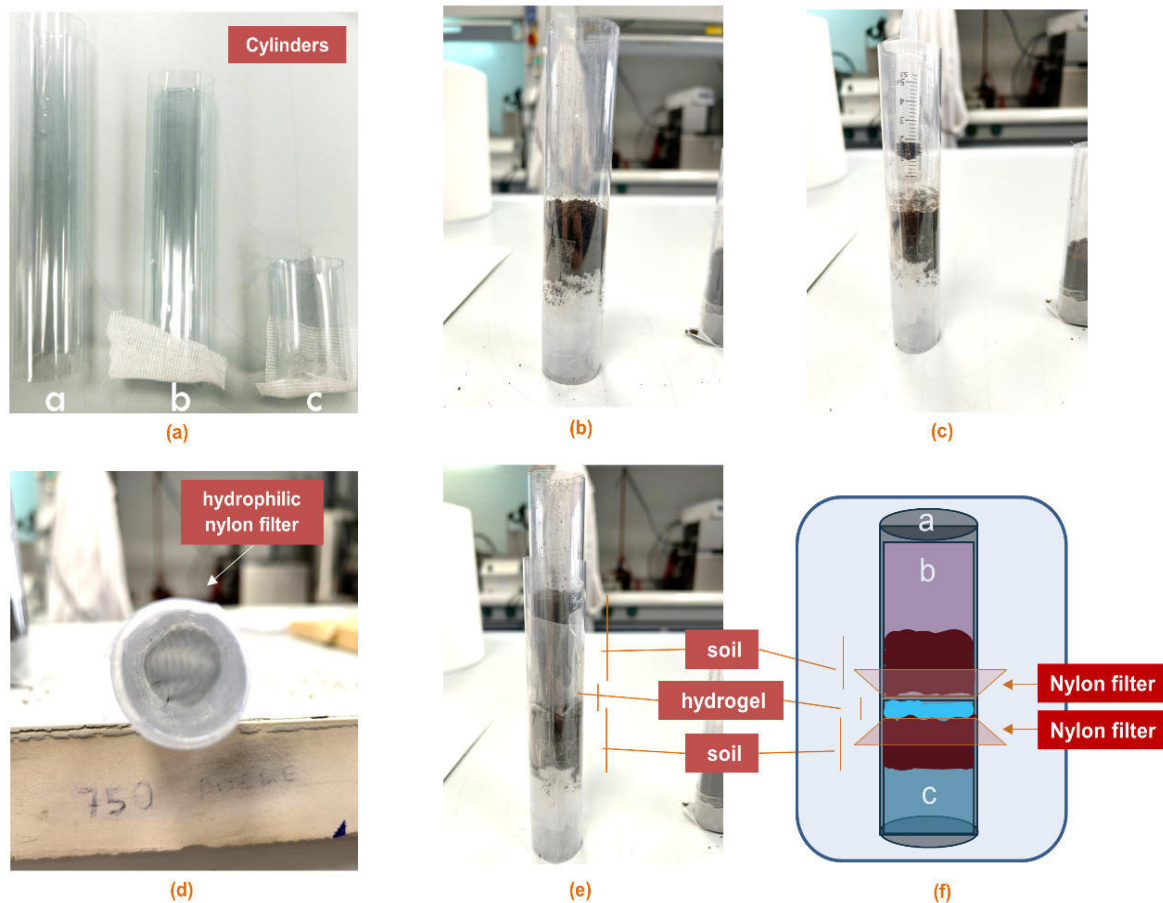
Supporting Figures



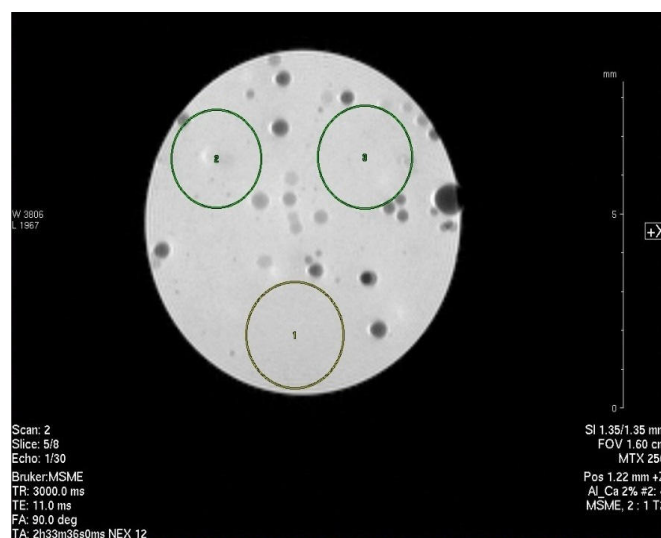
Supporting Figure S1: Glass funnel equipped with a stopcock in the end portion to modulate the drop flow rate during the beads formation.



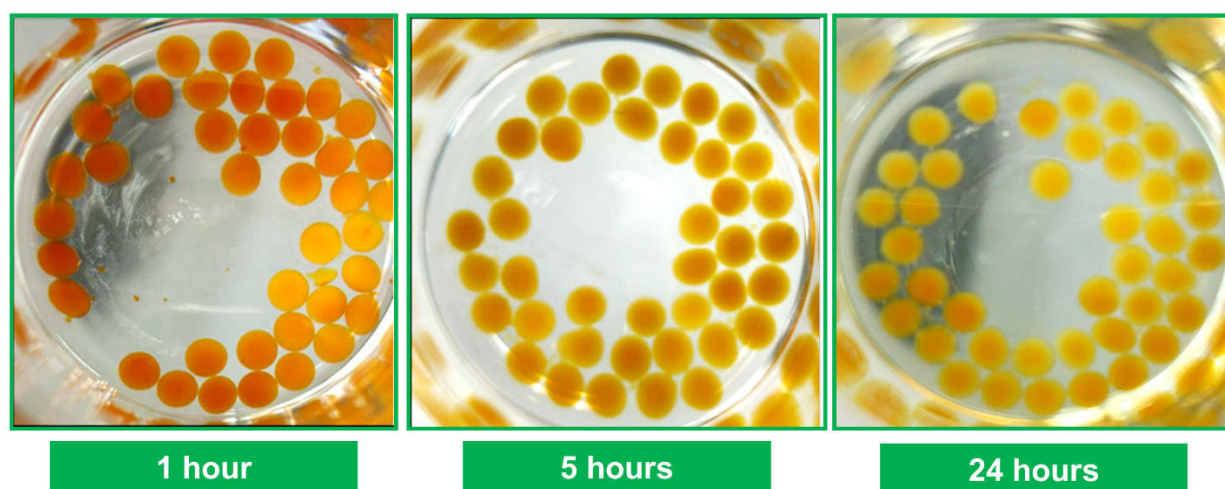
Supporting Figure S2: Morphologic evaluation of calcium beads produced by increasing the distance (4, 10, 14, 17 and 20 cm) between the end of the funnel and the surface of the crosslinker solution and observed by microscope (x4).



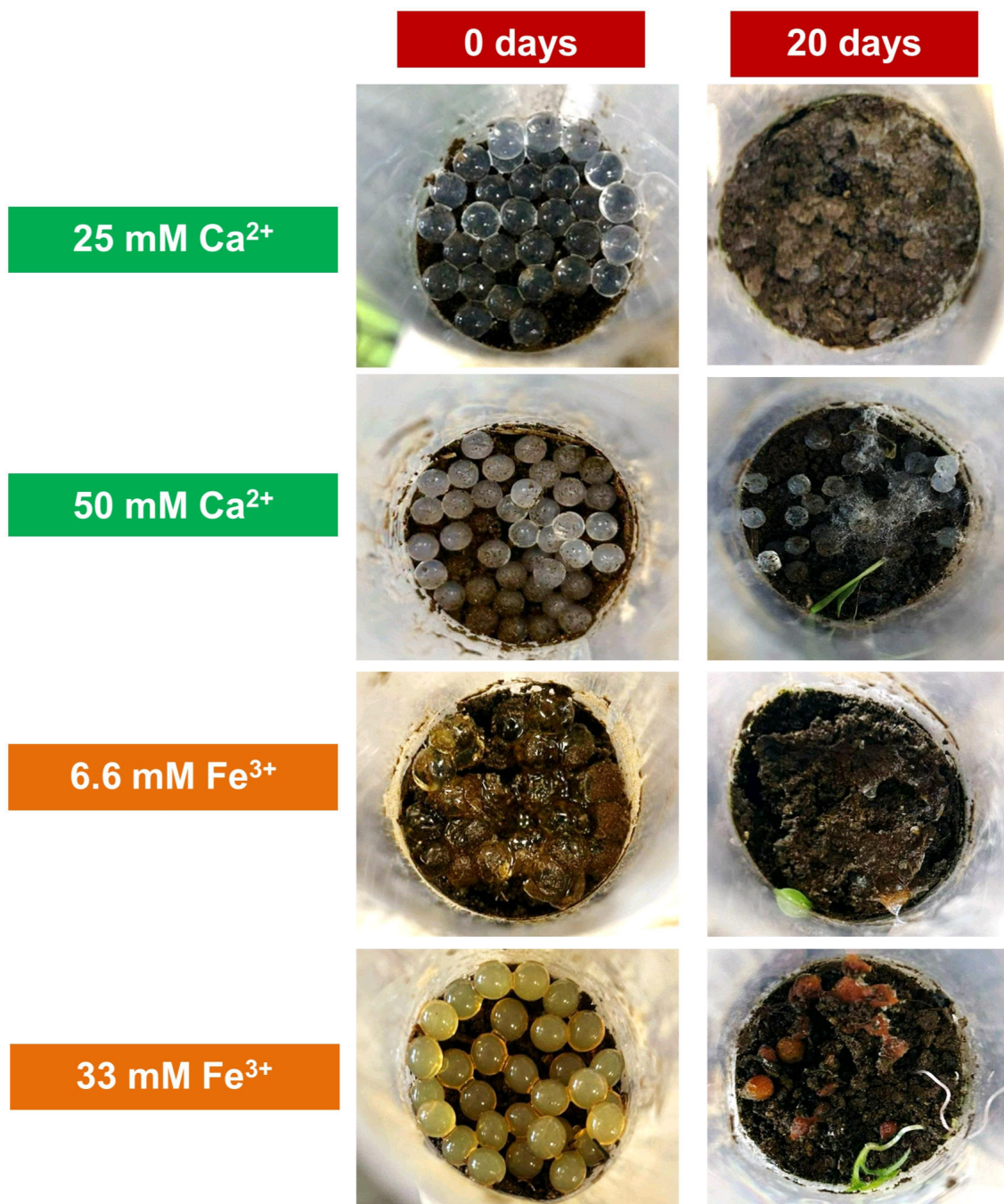
Supporting Figure S3: Artisanal system to confine hydrogels and beads between two layers of soil and, at the same time, permit a periodical and non-destructive visual monitoring of hydrogels. The system consisted of three cylinders, where one (a, the outer) had a diameter of 28.48 mm, while the other two (b and c, to be inserted into a) had a diameter of 26.16 mm. Two overlapped layers of a hydrophilic nylon filter (100 μm mesh) were placed on one of the margins of both cylinders b and c. The images show the progressive phases to set up the system (b-d) as well as a photo (d) and a scheme (e) of the assembled system.



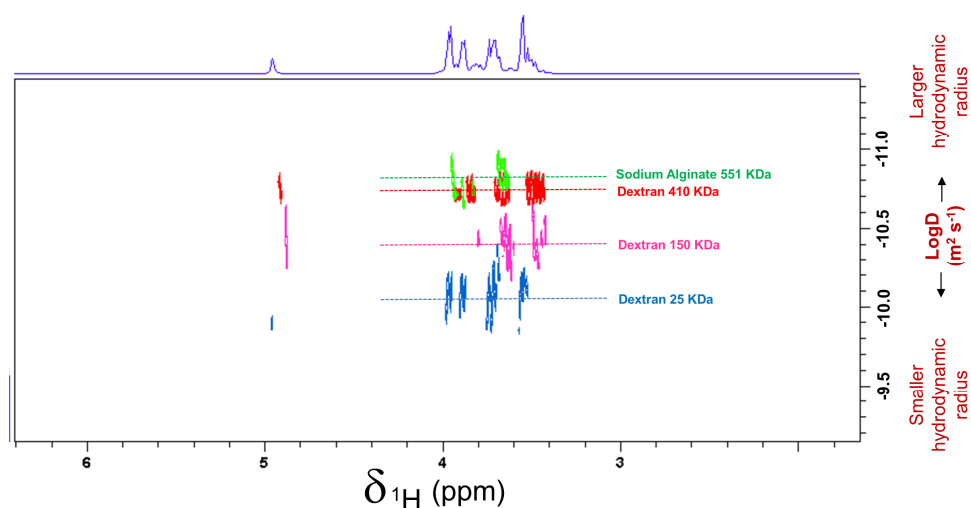
Supporting Figure S4: Representative axial slice of a HCa sample. The image shows a central slice depicting three ROIs (colored circular areas) used for calculations water spin-spin relaxatin times (T_2) and self-diffusion coefficient values. The small dark circles represent air microbubbles trapped within the H.



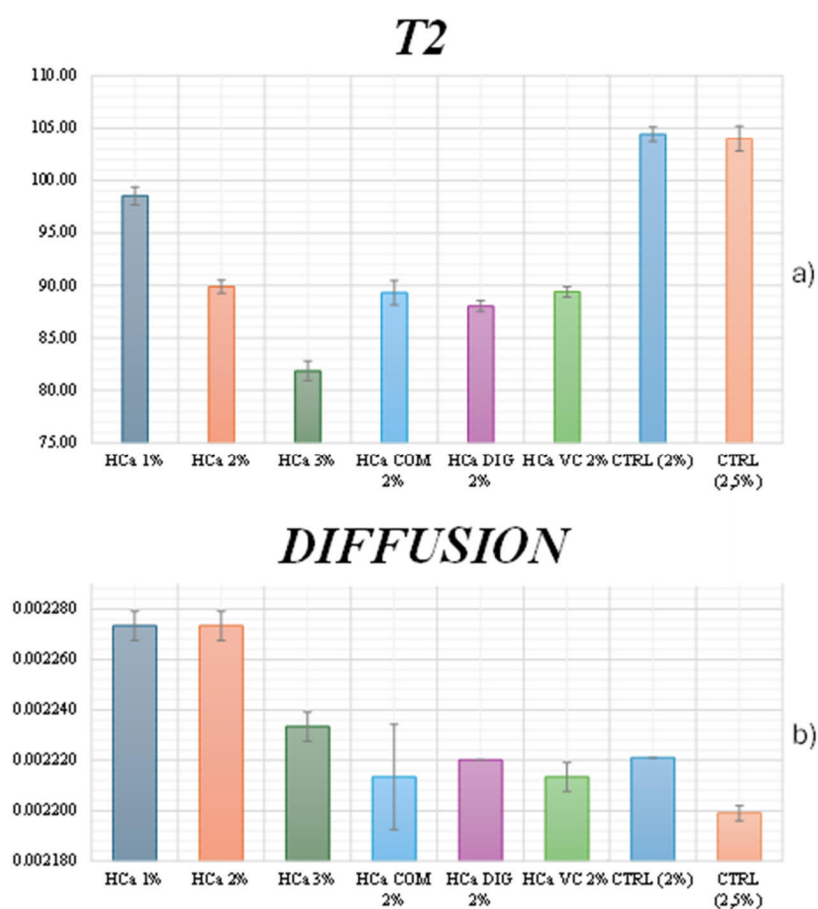
Supporting Figure S5: Progressive degradation of iron beads exposed to the action of citric acid for 1, 5 and 24 hours. The images highlight the gradual iron depletion moving from the surface of the beads towards the core, and revealed by a decrease in color intensity.



Supporting Figure S6: Calcium (25 and 50 mM) and iron (6.6 and 33 mM) alginate beads put in contact with a real soil in the cylinder system for 0 (left side) and 20 (right side) days from the beginning of the experiment.



Supporting Figure S7. Overlapping of ^1H DOSY 2D NMR spectra of standard dextrans (25, 150 and 410 KDa, blue, purple and red, respectively) and sodium alginate (green). The negative logarithm of the diffusivity coefficients is shown along the vertical axis. The figure depicts the spectral regions included between 1 and 5.2 ppm (on the right) and 6.2 and 7.35 ppm (on the left, x 32).



Supporting Figure S8: Values in transverse proton relaxation times (T_2 , a) and diffusion coefficients (b) calculated via MRI for all the most representative hydrogel samples in our study.

Supporting Tables

Table S1: Indices calculated after sixth day	
Seed germination (SG %)	$\frac{N.\text{seed germinated}}{N.\text{seed CTRL}}\%$
Germination index	$\frac{SG\%}{RGR\%}/100$
Relative growth of root (RGR %)	$\frac{\text{Mean root lenght samples}}{\text{Meand root lenght CTRL}}\%$
Relative growth of shoots (RGS %)	$\frac{\text{Mean shoot lenght samples}}{\text{Meand shoot lenght CTRL}}\%$
Germination rate coefficient (CVG)	$\frac{N.\text{seed germinated}}{\Sigma(n.\text{seed}*\text{day})}\%$

Table S2: Major Fourier transform infrared spectroscopy (FT-IR) absorption bands and assignments for of HSVC. HSDIG. HSCOM

Wave number (cm ⁻¹)	Assignment
3400–3300	O–H stretching, N–H stretching (minor), hydrogen-bonded OH
2935–2925, 2850	Alkyl C–H stretching
2925–2850	Methoxyl C–H stretching
1725–1710	C=O stretching of COOH
1640–1600	aromatic C=C skeletal vibrations
1512–1506	N–H deformation and C=N stretching
1420–1415	O–H deformation and C–O stretching of phenolic OH
1460–1450	C–H asymmetric bending of CH ₃ groups
1220	C–O stretching of aryl ethers and phenols
1184	C–O stretching of secondary alcohols and/or ethers
1130–1110	C–O stretching of polysaccharides and alcohols
960–900	OH bond in carboxyles

CONCLUSIONS AND FUTURE PERSPECTIVES

This work demonstrated that organic fertilizers derived from agro-industrial residues, including horticultural and livestock wastes, represent a viable and sustainable alternative to conventional mineral fertilizers, offering multiple benefits for soil fertility, plant growth, and environmental sustainability. The work highlights that proper valorization of such residues can contribute to circular bioeconomy strategies, reducing the environmental footprint of agricultural systems while maintaining or improving crop performance.

In line with these goals, the thesis pursued a progressive research pathway: from established organic amendments to innovative hydrothermal products and finally to advanced delivery systems. Each stage deepened the understanding of how the nature, structure, and formulation of organic matter influence plant metabolism and nutrient dynamics, ultimately showing how agro-industrial residues can be transformed into increasingly functional and high-value agricultural inputs.

The study shows that solid anaerobic digestate (AD) and its vermicomposted form (VC) possess distinct chemical and structural characteristics, which directly influence their effectiveness as fertilizers. AD rapidly enhances plant metabolism due to the high content in labile carbon, whereas VC, with its more recalcitrant and humified structure, not only results in very effective fertilizer but also provides long-term benefits to soil organic matter and nutrient cycling. Pot Greenhouse trials confirmed that both materials, when applied at the proper dose (ranging within 20 and 40%), can outperform mineral fertilizers in promoting growth and metabolic activity.

These findings reveal a fundamental concept that permeates the entire thesis: the agronomic performance of organic fertilizers depends not only on nutrient content, but on the *quality* of

their carbon pools and on their degree of biochemical stability. This insight establishes a foundational framework from which subsequent chapters logically emerge, guiding the exploration of materials with increasingly tunable and predictable properties.

Similarly, green composts from vegetable residues and its pelletized form demonstrated that the combination of labile and stable organic fractions improves nutrient availability and plant performance. Pelletization additionally offers practical advantages for storage, handling and field application, facilitating the adoption of sustainable amendments in horticultural practices.

Here, the thesis advances from characterizing biochemical functionality (as done with AD and VC) to addressing a second key dimension: *formulation and usability*. Fertilizers must be not only effective, but also manageable, transportable, and compatible with growers' needs. This chapter therefore bridges biological efficiency with technological feasibility, demonstrating how processing steps (such as pelletization) can influence both agronomic and logistic performance.

A key innovation of this work is the development of hydrochars (HCs), both solid and liquid fractions, as multifunctional bio-based fertilizers. This technique proves very promising, as it retains several advantages compared with other more established biomass valorization methods (compost, vermicompost, biochar, digestate), such as (i) it is rapid by requiring only few hours to convert the material in the final product (ii) it permits to work directly on fresh organic material (iii) the reaction occurs with water and does not require any organic and environmental pressant solvents (iv) the liquid hydrochar must be diluted at least 100 times with water, thus gaining a large amount of bioactive solution, per single reaction (v) HTC conditions sterilize the biomass (vi) the required energy is relatively lower than for those used

for biocharring (vii) high reproducibility of the reaction. Solid HCs provided a balanced source of nutrients while promoting root and shoot growth, whereas liquid hydrochar fractions contained soluble nutrients and bioactive molecules, including phytohormones, which enhanced germination, biomass accumulation, and nutrient use efficiency. These findings underline the dual role of hydrochars as nutrient providers and biostimulants, with mild hydrothermal carbonization conditions (180 °C) producing materials with the highest efficacy.

In this central phase of the thesis, the research performs a decisive conceptual leap: from biologically mediated amendments (AD, VC, compost) to thermochemically engineered products with controllable features. Hydrochars represent a new class of fertilizers where chemical structure, molecular composition, stability, and functional activity can be modulated through process parameters. This controllability introduces a level of precision and reproducibility not achievable with traditional organic fertilizers, positioning hydrochars as a potential “next-generation” technology for sustainable agriculture.

Finally, the integration of humic-rich amendments into alginate-based hydrogels and beads offers an additional level of innovation, enabling the controlled release of water, nutrients, and bioactive compounds, such as effective liquid hydrochar. Seed-coating and soil assays confirmed the ability of these systems to stimulate plant growth and improve nutrient availability, including iron mobilization in challenging soil conditions.

This last step is particularly significant because it reflects a shift from simply *producing* fertilizers to *engineering their delivery*. Encapsulation technologies allow organic amendments (whose dynamics are often difficult to predict) to behave in a more controlled and targeted manner. The thesis thus concludes by demonstrating that sustainable fertilizers

can be not only effective and circular, but also technologically sophisticated, opening pathways toward precision agriculture and resource-efficient soil management.

Overall, the results of this thesis support the strategic use of organic fertilizers from agro-industrial residues to enhance soil fertility, crop productivity, and resource efficiency. All experimental activities were carried out at the University of Salerno, Department of Pharmacy, within the AgrichemLab - Laboratory of Agricultural Chemistry, where all analytical and agronomic investigations were performed. By demonstrating the value and effectiveness of these materials compared to conventional mineral fertilizers, this work provides a framework for sustainable agricultural management and encourages the adoption of innovative, circular approaches that align with European Green Deal objectives and long-term environmental stewardship.

More broadly, the thesis illustrates that the biological, chemical, and technological transformation of agro-industrial residues can be seen as a continuum: from raw waste to biologically stabilized amendments, to thermochemically engineered fertilizers, and finally to smart delivery systems. This continuum not only addresses current soil fertility challenges, but also anticipates the needs of future agriculture: more efficient, more resilient, and more aligned with circular economy principles.

Future research perspectives may include testing the most promising hydrochars under open-field conditions to validate their efficacy in real agricultural systems, also by monitoring soil respiration and carbon dynamics over time. Additional studies could focus on the production of hydrochar-based or hydrochar/compost blended pellets to improve handling and application efficiency. The solid hydrochar obtained in this work could also be explored for environmental applications, such as the adsorption of heavy metals for wastewater purification, as previously

reported for similar materials. Further research should address the synthesis of hydrochars from alternative biomasses and the formulation of alginate-based carriers enriched with hydrochars, to evaluate their potential as combined nutrient and bioactive delivery systems. Moreover, the co-application of hydrochars with beneficial microorganisms may offer synergistic effects on soil health and plant growth, representing a promising direction toward integrated and sustainable soil management strategies.

Ultimately, this thesis demonstrates that the path toward truly sustainable agriculture does not lie in a single solution, but in re-imagining the entire life cycle of organic matter, transforming residues into resources, and resources into resilient systems capable of restoring the fertility of soils and the vitality of agroecosystems.

APPENDIX

ADDITIONAL RESEARCH PAPERS

Article

Short-Term Effects of Wood Biochar on Soil Fertility, Heterotrophic Respiration and Organic Matter Composition

Rossella Curcio ^{1,2} , Raffaele Bilotti ², Carmine Lia ³, Michele Compitiello ³, Silvana Cangemi ⁴, Mariavittoria Verrillo ^{4,5} , Riccardo Spaccini ^{4,5}  and Pierluigi Mazzei ^{2,*} 

¹ DAFE—Department of Agricultural, Forest, Food and Environmental Sciences, University of Basilicata, 10, Viale dell'Ateneo Lucano, 85100 Potenza, Italy; rossella.curcio@unibas.it

² Dipartimento di Farmacia (DIFARMA), Università degli Studi di Salerno, Via Giovanni Paolo II 132, 84084 Fisciano, Italy

³ Maniola Remote Sensing, s.r.l., 84015 Nocera, Italy

⁴ Dipartimento di Agraria, Università di Napoli Federico II, Piazza Carlo di Borbone 1, 80055 Portici, Italy; riccardo.spaccini@unina.it (R.S.)

⁵ CERMNU—Centro Interdipartimentale sulla Risonanza Magnetica Nucleare per l'Ambiente, l'Agro-Alimentare ed i Nuovi Materiali, Piazza Carlo di Borbone 1, 80055 Portici, Italy

* Correspondence: pmazzei@unisa.it; Tel.: +39-089-969175

Abstract: Biochar may represent a sustainable and eco-friendly strategy to recycle agro-forestry wastes, sequester carbon and improve soil health. With the aim of proving these benefits in a real scenario, we treated several soil parcels with 0 (CTRL), 1 (LOW) and 3 (HIGH) kg/m² of wood biochar, in open-field trials. The heterotrophic soil respiration (SR) was monitored continuously for two months via a Closed Dynamic Chamber (CDC) associated with an innovative pilot system, and the most important soil chemical parameters were measured 9 and 54 days after biochar application. Biochar induced an immediate dose-dependent increase in organic matter content and CEC (up to 41.6% and 36.8% more than CTRL, respectively), which tended to slightly and gradually decrease after 54 days. In all cases, biochar induced a more pronounced SR, although the most enhanced microbial response was detected for the LOW parcel (19.3% higher than CTRL). Fennels were grown in treated soils and only LOW microplots gave a significantly better response (weight and size). Finally, NMR, FT-IR and Pyr-GC/MS analyses of LOW SOM extracts revealed a relevant impact on the composition, which was accompanied by a higher content of carbohydrates, indole-based compounds and FAME species correlating with enhanced microbial activity. Our findings demonstrate that the proper biochar dose improves soil fertility by creating an environment favorable to plants and promoting microbial activity.

Keywords: soil sensors; biomass recycling; soil heterotrophic respiration; automated closed dynamic chamber; CPMAS NMR; thermochemiolysis



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1. Introduction

Serious and worrying environmental issues affect modern farming, including the intensive exploitation of soils resulting in a decline of their fertility and the widespread decrease in organic matter in agricultural soils [1], features associated with serious impacts on plant production, soil biodiversity and microbial quality. These dramatic conditions are worsened by the effects of climatic change and unsustainable soil management, which are detrimental for soil ecosystem services. Therefore, it is necessary to find useful, effective and innovative solutions to these problems, to make soil more resilient and to improve its quality and health. The recycling of waste biomass may produce new materials useful for soil and plants, and

represent an eco-friendly and sustainable strategy to manage forestry and agrofood wastes [2]. In fact, the proper recycling of biomass can produce new products for soil, capable of ameliorating its resilience and fertility, improving crop responses and serving as a slow releaser of important nutrients such as N, P and S [3]. Such a strategy would enhance the value of organic waste and, at the same time, decrease the demand for fertilizers and biostimulants, thus reducing costs in the management of agrosystems. Moreover, it would be perfectly in line with circular economy principles and sustainable development models [4]. Biochar has recently attracted a large amount of interest because it not only exhibits most of the above-mentioned properties, but also exhibits the potential to improve soil physical, chemical and microbiological quality [5]. Biochar results from the pyrolysis of organic material which consists of high thermochemical decomposition, carried out in the absence of oxygen and at temperatures ranging between 300 and 450 °C [6]. Solid biochar, which represents the solid phase resulting from this process, is a dark black solid fraction, rich in carbon and polyaromatic compounds [7]. Its composition can change drastically depending on factors such as the type of processed biomass, the pyrolysis temperature, the steepness of the heating gradient and the duration of pyrolysis [6]. During the biocharring process, the large amount of thermal energy supplied to the biomass cleaves chemical bonds in complex and polymeric organic molecules (i.e., cellulose, hemicellulose, lignin, tannins), altering their compositional characteristics and promoting their rearrangement into new bonds, especially via radical reactions [5]. One of the predominant syntheses that occurs during pyrolysis leads to the neoformation of complex molecules mostly characterized by their polyphenolic and hydrophobic nature [8]. Typically, the solid material resulting from this process exhibits up to 70% carbon content which, due to its recalcitrant nature, is only slowly degraded by soil microorganisms. For this reason, the biochar is considered an eco-friendly strategy to sequester carbon and limit the emissions of the greenhouse gas CO₂, with important environmental implications.

To date, an increasing body of research has proven biochar's effectiveness in increasing crop productivity and soil quality [9–13]. For example, Asai and coworkers [14] examined the effect of biochar, obtained from residues of teck wood (*Tectona grandis* L.) and rosewood, on both the physical properties of soil and the yields of mountain rice grains (*Oryza sativa* L.). They demonstrated an increase in soil productivity, although the effects were strongly dependent on the type of soil and biochar, as well as on the climatic conditions and the application rate [15]. Haefel and coworkers [16] tested the effects of rice biochar on both soil characteristics and rice crop growth, and reported an increase in total soil organic carbon and nitrogen and in available P and K, as well as demonstrating a soil type-dependent persistence in soil. It has been demonstrated that biochar also benefits tomatoes and lettuce by improving seed germination, root elongation, and biomass accumulation, and promotes enhanced soil fertility and microbial activity [17]. It has been also demonstrated that biochar can suppress phytopathogenic fungi, such as *Sclerotinia sclerotiorum*, by inhibiting hyphal growth [18]. Some studies have reported increased CO₂ emissions due to more pronounced microbial biomass and enzymatic activity elicited by biochar [19,20]. However, it has been proved that biochar's effects and the extent of its action are strictly dependent on factors such as feedstock type, pyrolysis conditions, and, ultimately, soil properties [21,22].

Another important aspect is represented by the impact which biochar may have on soil organic matter composition [23]. Due to the complex and heterogeneous nature of soil organic matter, such an investigation may be conducted via a preliminary extraction followed by the use of advanced and powerful analytical techniques, including Pyrolysis Gas Chromatography-Mass Spectrometry (Pyr-GC/MS) [24,25], ¹³C CPMAS NMR [26,27] and FT-IR spectroscopy [28,29]. However, a full understanding of biochar's properties and effects on soil, direct or indirect, is still lacking. In fact, studies concerning the effects

of biochar application on soil, with particular regard to open field trials and advanced evaluation of biochar's effects on soil organic matter composition, are still relatively limited and sometimes discordant.

Therefore, in this work we investigated the short-term effects of application of high-quality wood biochar on soil, supplied at different doses. The impact on soil quality and health was assessed by comparing time points before and 9 and 54 days after biochar administration, as well as by examining, in a continuous and innovative way, the soil's heterotrophic respiration through a pilot system based on an automated CDC (Closed Dynamic Chamber). Then, biochar's effects on crop productivity was estimated by assessing the relevant production parameters of fennels grown in treated soils. Finally, to delve into the impact of biochar on soil organic matter composition, the latter was extracted and evaluated in detail through a combination of advanced techniques, such as solid-state CPMAS-NMR, FTIR and Pyr-GC/MS.

2. Materials and Methods

2.1. Biochar

The biochar (Nera Biochar srl., Settimo Vittone, Italy) was obtained by the thermic treatment of PEFC (Program for Endorsement of Forest Certification schemes) certified wood. This biochar was produced in a modern production plant, capable of separating and removing most polycyclic aromatic hydrocarbons and guaranteeing a PHA content lower than 0.5 mg/kg. Briefly, it was produced by treating the wood at 650 °C, with a residence time of 1 h and a heating rate of 20 °C/min. According to the manufacturer's datasheet, the contents of heavy metals, such as Zn, Cu, Pb, Hg, Cd, Cr^{VI}, Ni, As, were below the required limits for agricultural applications. The main properties, including chemical and physical parameters as well as nutrient and heavy metals content, are reported in Supporting Figure S1 (information provided by the provider Nera Biochar).

2.2. Experimental Field and Microplot Preparation

The biochar-based open field experiments were carried out at the "Luigi Cerrato" farm, situated in Sarno (Salerno, Italy; latitude 14.6159011; longitude 40.7976669; altitude of 46 m a.s.l.). The farm's soil exhibited a sandy-loam texture, a depth ≥ 2 m and was slightly calcareous (≈ 60 g/kg of CaCO₃ equivalents). Nine squared soil microplots (1 m² each) were delimited within a total area of 20 m², according to a 3 $\times$ 3 randomized block design and considering a distance of 50 cm among each block.

The studied microplots were treated without (0 kg/m²; CTRL) and with biochar, at the doses of 1 (LOW) and 3 (HIGH, as recommended by the provider for sandy-loam textured soils) kg/m². The solid fragments of biochar were homogeneously distributed on the surface of treated soils and then physically incorporated within a depth of 20 cm (Supporting Figure S2a). In order to standardize the process, the soil particles were physically mixed, within 20 cm of depth, also in the CTRL microplots.

One soil respiration chamber (described in detail below) was installed in each CTRL, LOW and HIGH microplot (Supporting Figure S2b; three total respiration chambers). The chambers were considered operative only once (i) the system was physically stable and resistant to wind, (ii) temperature and humidity sensors provided stable responses and (iii) the data logger and photovoltaic panel responded to testing. The experiment with the respiration chambers lasted from October 2020 to December 2020. During this period, weeds were removed regularly (two times per week) from each microplot to minimize the autotrophic respiration. Grass removal was performed gently, to avoid compressing macropores and altering soil aeration. Throughout this period, the soil did not receive

irrigation water. Weather data concerning the whole experimental period (October 2020 and March 2021) are shown in Supporting Figure S3.

2.3. Soil Sampling and Characterization

A periodic sampling of soil was conducted for each of the nine microplots, at a depth in the range of 15–25 cm. For each microplot, four soil aliquots were collected and homogenized to take into account possible site-specific inhomogeneities.

Soil samples were collected immediately before biochar treatment (samples A, collected on 9 October 2020), and 9 days after (samples B, collected on 19 October 2020) and 54 days after (samples C, collected on 2 December 2020) biochar application. Samples were air-dried, at room temperature, sieved at 2 mm and stored until subsequent analysis.

2.4. Soil Chemical Analysis

Texture, total carbonates, salinity, pH, cation exchange capacity (CEC) and total organic matter (SOM) of the analyzed microplots were tested in triplicate, according to the official methods for soil analysis [30] and taking into account the variations proposed by the official revision of the Italian Soil Science Society. Briefly, the texture and the total carbonates were assessed by the Andreasen's pipette and Dietrich–Fruhling calcimeter methods, respectively. The soil pH and salinity tests were conducted by putting 4 g of soil in contact with 20 mL of either an aqueous solution of CaCl_2 [0.01 M] or ultra-pure water (dilution 1:5, *w:v*), respectively. The detection was conducted using a HANNA INSTRUMENT HI5221 detector (Levanchimica, Bari, Italy), equipped with probes suitable for measuring pH and electrical conductivity (in $\mu\text{S cm}^{-1}$). Total organic carbon (C_{org}) was measured by the Walkley–Black wet oxidation method. Briefly, 1 g of soil underwent oxidation by potassium dichromate (1 N) and concentrated H_2SO_4 (98%; Avantor, Milan, Italy) solutions. After 30 min, the reaction was stopped by adding 200 mL of cold water. The quantification of organic carbon was done by redox titration with a solution of ammonium iron (II) sulfate hexahydrate [0.5 M], by using ferroin as a redox indicator. The cation exchange capacity (CEC) was calculated by conducting the assay based on soil mono-saturation with a barium chloride solution at pH 8.2. Briefly, after mono-saturation of soil (2 g) with barium chloride (0.41 M; Avantor, Milan, Italy), a defined quantity of a magnesium sulphate solution was added to the Ba-saturated sample, thus leading to complete Ba/Mg exchange. The excess magnesium in solution was determined by complexometric titration by using Black Eriochrome T as a complexometric indicator (mixed with NaCl in the 3:1 W:W proportion), and ethylenediaminetetraacetic acid (2.5 cmol/L; Avantor, Milan, Italy) as titration solution. Soil analyses were conducted individually for each microplot (three replicates per microplot). The results shown are the average of the results achieved for each treatment (three measures per three field-replicates per thesis).

2.5. Carbon Mote System for Measuring Soil Respiration

The Carbon Mote device was manufactured by the company Maniola Smart Sensing s.r.l. (Nocera, Italy) and included a Closed Dynamic Chamber (CDC) intended to measure the carbon dioxide fluxes released by the soil. Briefly, the system was equipped with a self-powered system based on a photovoltaic panel to enable open-field evaluations for long periods in the absence of cabled energy sources (Supporting Figure S4a). In addition, it was coupled to sensors to continuously monitor parameters such as soil and air temperature, air humidity and atmospheric pressure.

In detail, the carbon mote device consisted of (i) a galvanized steel pole anchored to the ground and equipped with a solar panel (25 Wp, 12 V, 1.39 A), (ii) a device transmitting data to a central unit and allowing the control of peripheral devices, (iii) an external power supply, linked to solar panel/battery and controlling the node without power supply, (iv) a

module with an IRGA analyzer for CO₂ detection, equipped with an additional sampling pump, and (v) a dome-shaped chamber lid (Supporting Figure S4b; hole diameter of 28 cm, area of 615.7 cm²; chamber volume 5747 cm³). The latter was driven by a motor which, during the measurement phase, is lowered onto the hole-collar, thus sealing the accumulation chamber. The air accumulated in the chamber is then transferred to the IRGA analyzer, passing through a physical filter (to retain dust particles larger than 150 mm) and a chemical filter (based on soda lime and DRIERITE).

The CO₂ flux acquisition process consisted of 2 min-long accumulations/detections which were conducted daily (daytime, within the hours 13.00–15.00) for 54 days. The flux was calculated for each measure, according to the following Formula (1):

$$F_{CO_2} \text{ (ppm/s)} = \frac{(F_{end} - F_{beg.})}{t_{in}} \quad (1)$$

where t_{in} indicates the time interval (s) where the CO₂ development follows a linear evolution (generally lower than 90 s), while $F_{beg.}$ and F_{end} are the CO₂ contents revealed immediately after the beginning of the IR detection (10 s after lid closure) and at the end of the linear CO₂ evolution (indicating the CO₂ saturation of the chamber), respectively. The soil respiration values (SR) were calculated by applying the following Formula (2) [31].

$$SR \text{ (g CO}_2 \text{ m}^{-2} \text{ h}^{-1}) = \frac{V F_{CO_2}}{G S (T + 273.15)} \quad (2)$$

where V is the volume of the storage chamber (5747 cm³), F_{CO_2} is dCO_2/dt (expressed in ppm/h and obtained multiplying F_{CO_2} by 3600), G is the universal gas constant (8.31 J mol⁻¹ K⁻¹), S represents the area of the collar of the accumulation chamber (615.7 cm²), and T is the measured soil temperature (°C).

2.6. Effects of Biochar on Soil Fertility

On March 2021, fennels were transplanted into all of the studied microplots (three field replicates per treatment) to evaluate possible induced effects on soil fertility. We used the Preludio variety (F1 hybrids; Vivai le Georgiche, Brescia, Italy) because it is a high-quality spring-type fennel, characterized by good productive characteristics, a uniform size with a round section and a relatively small diameter of the taproot. It is an early cycle cultivar, the transplanting of which occurs between the end of March and the beginning of April, and which is usually harvested from May to June. Before transplantation, the soil was prepared in order to minimize compaction. After transplantation (which took place 5 months after the addition of biochar), constant and regular irrigation was applied through a drip system. The planting pattern consisted of single rows, with an average density of nine plants/m², with a simple pattern of 20 cm (intra-row) × 35 cm (inter-row). Fennels were harvested manually at the end of May 2021, before the beginning of sprout elongation. At harvest, the number of regularly developed fennels per parcel (27 per thesis) was evaluated, including their weight, axial diameter and length of the epigeal part. The results were expressed as averages and standard deviations per microplot and elaborated by ANOVA test. The differences in ANOVA results were considered significant at a p value of at least <0.05 for both Tukey and Benjamini–Hochberg tests (α confidence level of 0.05). All the statistical analyses were performed using XLSTAT Software (v. 2016, Addinsoft, Paris, France).

2.7. Extraction of Soil Organic Matter

The SOM was extracted from CTRL and LOW microplots 54 days following the application of biochar. For each thesis, the samples resulting from the three field replicates were merged and homogenized. The extraction was performed by placing 100 g of

2 mm sieved and dried soil in contact with 1 L of an aqueous solution containing NaOH (1 M; Avantor, Milan, Italy) and sodium pyrophosphate (0.1 M). The extraction process was carried out for 16 h on a rocking shaker, set at 150 rpm. Then, the supernatant was separated from the pellet through a 20 min centrifugation at 6000 rpm. The pellets underwent a second extraction which was performed for 2 h. The supernatant was again isolated by centrifugation and added to the first aliquot. The extract was filtered through a sterile gauze, to remove coarse and low-density organic material, then adjusted to pH 7, dialyzed against Milli-Q water (3500 kDa membranes; Levanchimica, Bari, Italy), frozen, and freeze-dried using an Edwards MODULYO 5289 freeze dryer.

2.8. FT-IR and CPMAS NMR Spectroscopies

The molecular composition of biochar and soil extracts was characterized in the solid state by Fourier Transform Infrared (FT-IR) and Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (CPMAS NMR) spectroscopies.

FTIR analysis was carried out via the ATR (Attenuated Total Reflection) technique on solid and dried samples, sieved at 0.5 mm. The analyses were conducted in duplicate on the PERKIN ELMER Frontier instrument, by setting 64 scans, a wavelength range between 4000 and 600 cm^{-1} , and a resolution of 4 cm^{-1} . For each acquisitions set, a background spectrum was examined. Spectra were converted in absorptive mode. A baseline correction was conducted for each spectrum in order to make them suitable for direct and reliable comparison. All spectra were processed using Spectragryph software (v. 1.2.16.1; F. Menges; Oberstdorf; Germany).

^{13}C CPMAS NMR spectroscopy was conducted on a 300 MHz Bruker Avance wide-bore magnet (Bruker Biospin, Rheinstetten, Germany), equipped with a CPMAS probe, at 25 °C and at the ^{13}C and ^1H frequencies of 75.47 and 300.13 MHz, respectively. Finely powdered solid samples (60–80 mg), sieved at 0.5 mm, were loaded into 4 mm zirconia rotors, sealed with Kelf caps, and spun at a rate of $10,000 \pm 1$ Hz. ^{13}C NMR spectra were obtained through the CPMAS experiment which included 1814 time domain points, a spectral width of 300 ppm (22,727.3 Hz), a recycle delay of 5 s, eight dummy scans, and 8000 scans. High-power proton decoupling was applied by the TPPM15 (time proportional phase modulation) decoupling sequence. Free induction decays (FIDs) were processed by Bruker Tospin (v 4.0.1; Bruker Biospin, Rheinstetten, Germany) software. Spectra underwent Fourier transformation by using a two-fold zero-filling and applying an exponential filter function of 400 Hz, before phase and baseline correction. ^{13}C CPMAS NMR spectra were separated into the following resonance intervals, the areas of which were then integrated and reported as absolute values per 100 mg of sample: alkyl-C (0–45 ppm); methoxyl-C and N-alkyl-C (45–60 ppm); O-alkyl-C (60–110 ppm); aryl-C (110–145 ppm); phenol-C (145–160 ppm); carboxyl-C (190–160 ppm); quinone-C and ketone-C (230–190 ppm). The hydrophobicity index (3), the lignin ratio (4) and the Alk/O-Alk ratio (5) were calculated by considering specific ^{13}C NMR intervals and according to the following equations:

$$\text{HB} = \frac{[(0-45) + (45-61) + (110-145) + (145-160)]}{[(61-90) + (97-110) + (160-190)]} \quad (3)$$

$$\text{LIGNIN RATIO} = \frac{(45-60)}{(145-160)} \quad (4)$$

$$\text{ALK/O-ALK RATIO} = \frac{(0-45)}{[(61-90) + (97-110)]} \quad (5)$$

2.9. Thermochemiolysis Followed by Gas Chromatography and Mass Spectrometry (Pyr-GC/MS)

Aiming to simplify the complex and heterogeneous nature of SOM and enable its analysis, the studied organic extracts underwent a preliminary thermochemiolysis inducing a depolymerization of large molecules and natural biopolymers, accompanied by a methylation of terminal hydroxyl and carboxyl groups. The procedure was applied according to the method of Verrillo et al. [32]. Briefly, the SOM extracts (500 mg) were placed into a quartz container and wetted with 1 mL of a tetramethylammonium hydroxide (TMAH) (25% in methanol). After two hours, the sample was introduced into a Pyrex tubular reactor (50 cm long $\times$ 3.5 cm i.d.), and heated at 400 °C in a furnace (Barnstead Thermolyne 21100; American Laboratory Trading; East Lime, CT, USA) for 30 min. During pyrolysis, the volatile components, including the ones deriving from depolymerization and derivatization, were transported by a helium flow (20 mL/min) and entrapped in two dichloromethane solutions, arranged in series and kept in an ice bath to maintain the temperature low, thus promoting compound solubilization. The dichloromethane solutions were combined and roto-evaporated to dryness. The residue was dissolved in 1 mL of dichloromethane and transferred to a glass vial prior to GC–MS analysis. The latter was performed using a Perkin Elmer Autosystem XL Gas Chromatograph, equipped with an RTX-5MS WCOT capillary column (Restek, 30 m $\times$ 0.25 mm; film thickness, 0.25 mm) and coupled, through a heated transfer line (250 °C), to a PE Turbomass-Gold quadrupole mass spectrometer. The chromatographic separation was achieved with the following temperature program: 60 °C (1 min isothermal), rate 7 °C min^{−1} to 320 °C (10 min isothermal). Helium was used as carrier gas at 1.90 mL min^{−1}, the injector temperature was 250 °C, and the split-injection mode had a 30 mL min^{−1} of split flow. Mass spectra were obtained in EI mode (70 eV) by scanning in the range 45–650 *m/z*, with a cycle time of 0.2 s. The identification of mass spectra of the eluted compounds was carried out by comparing the results with those of standard compounds, exploiting the mass spectra library NIST 05 (<https://www.nist.gov>; accessed on June 2024) with the support of the pertinent literature. The areas of signals detected in the pyrograms were integrated, grouped according to the specific compound class, and added up. The molecule categories dictating this grouping were the following: nitrogen-containing compounds, fatty acid methyl esters (FAME), branched FAME and hydroxyacids, unsaturated and branched FAME, aromatic compounds, N-containing aromatic compounds (including a subgroup referring to indole structures) and lignin moieties (including three subgroups discriminating P, G and S units). The respective cumulative integrations were reported both in absolute and in normalized (as % of the integration of the total chromatogram) forms.

3. Results and Discussion

3.1. Soil Chemical Analyses

Important parameters related to soil quality and fertility were evaluated for microplots treated without (CTRL) or with a high-quality wood biochar, supplied at relatively low (LOW, 1 kg/m²) and high (HIGH, 3 kg/m²; as recommended by the provider) doses. Evaluations were carried out before (A) and 9 (B) and 54 (C) days after biochar application. pH is a determinant factor for soil health and fertility, affecting both the solubility and bioavailability of several nutrients, and capable of influencing the activity and the survival of edaphic microorganisms and vegetable species. Due to its nature, biochar is generally characterized by a pH ranging from slightly alkaline to strongly alkaline, depending on the type of processed biomass and the conditions adopted in the production process [33,34]. Therefore, its administration in soil is expected to result in slight alkalization. The biochar used in this study had a pH of 8.4, while the soil exhibited a pH of 7.8, thus reflecting its appreciable limestone content ($\approx$ 60 g/kg CaCO₃; soil classified as slightly calcareous).

Biochar incorporation did not significantly change the soil pH, which invariably proved to be 7.8 ± 0.1 for each microplot, in both phases B and C (Figure 1a). In disagreement with previous conclusions [35,36], the investigated biochar, even when used at relatively high doses (3 kg/m^2), did not alter the soil pH, probably because of an efficient soil buffer effect. On the other hand, salinity also represents a problem in terms of soil quality, since it may have detrimental and harmful effects when it reaches excessive and unsustainable levels. In fact, high salinity impacts the bioavailability of water, limits the performance and survival of most plants and soil microorganisms, and may correlate with soil sodicity. Prior to treatment, the soil of all studied microplots was classified as non-saline, exhibiting an average electrical conductivity (EC) of $1688.3 \pm 37.9 \text{ } \mu\text{S/cm}$. However, the results observed in phase B (9 days after biochar application) revealed that biochar application was accompanied by the release of a significant amount of salts, which caused a general increase in EC (Figure 1b). In fact, the EC values for LOW and HIGH microplots were 1835 and $2187.5 \text{ } \mu\text{S/cm}$, respectively, while a value of $1650 \text{ } \mu\text{S/cm}$ was detected in the CTRL microplot. This means that, although the soil EC never exceeded the minimum salinity threshold ($4000 \text{ } \mu\text{S/cm}$), it increased by 11.2 and 32.6% more than that of the CTRL over the two subsequent time points, respectively. Such an outcome confirms previous works which state that the use of biochar can involve a salinization risk, especially when applied cyclically in clayey soils and in areas subject to low annual rainfall [33]. In phase C (54 days after treatment), a significant lowering of the electrical conductivity for both LOW and HIGH microplots was recorded, as a consequence of salt leaching promoted by rainfall, although the HIGH microplots still retained a higher content of salt than the other two microplot types.

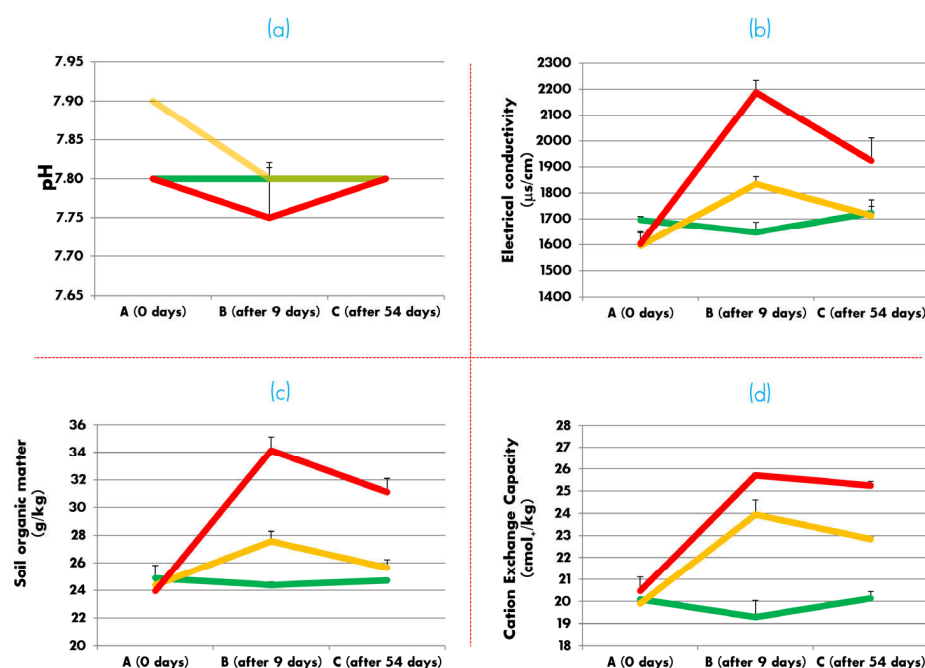


Figure 1. Soil pH (a), electrical conductivity (b) ($\mu\text{S cm}^{-1}$), organic matter (c) (g kg^{-1}) and cation exchangeable capacity (d) ($\text{cmol}_+ \text{kg}^{-1}$) of microplots CTRL (green), LOW (yellow) and HIGH (red), measured before (A), 9 (B) and 54 (C) days after treatment with biochar. The results refer to the mean values and include standard deviation bars.

The amount of soil organic matter is another important soil health indicator, as it can play an active role in chemical, biological and physical fertility. Figure 1c reports the SOM contents in the studied microplots, before and after biochar application. Before the treatment, the soil already exhibited a satisfactory organic carbon content corresponding to

24.65 g/kg. As expected [37,38], our results confirmed that the application of an exogenous organic product led to an increase in SOM, which occurred in direct correlation to the dose. This was recorded as 27.18 and 33.64 g/kg, respectively, for LOW and HIGH, which was respectively 10.27 and 36.4% higher than the CTRL microplots. After 54 days, a slow and gradual decrease in organic matter was observed. Given the soil's good hydraulic properties resulting from its loamy-sandy texture, this trend was mostly due to factors such as the leaching of soluble components, the physical translocation of the polymeric and low-soluble fractions, and microbial decomposition. Interestingly, microbial mineralization, although stimulated and enhanced by the biochar [39], involved mainly labile and semi-labile organic moieties already present in the studied soil, rather than the biochar itself. In fact, we expect that the biochar cannot serve as a direct source of labile carbon, due to its renowned recalcitrant nature [5]. Conversely, a discrete body of the literature agrees that many types of biochar are capable of favoring soil microorganisms by providing co-location of carbon and nutrients and providing a porous architecture which acts as an ideal micro-environment and physical protection for their growth [39]. Therefore, we must assume that the SOM fraction detected in LOW and HIGH microplots, and exceeding that of CTRL, was due to a more abundant microbial biomass elicited by the biochar.

The stabilized organic matter is rich in terminal functional groups capable of developing pH-dependent charges, such as carboxyls, phenolic hydroxyls and amines. Therefore, the incorporation of exogenous organic matter into the soil is expected to increase the net amount of electrical charges and, consequently, increase the soil cation exchangeable capacity (CEC). The latter is pivotal in determining the soil's ability to retain nutrients in their ionic form, and make them bioavailable for plants and microorganisms through reversible desorption processes. Before biochar treatment, the soil exhibited an acceptable CEC value, corresponding to 19.84 ± 0.48 cmol₊/kg. In line with previous results [40,41], biochar application led to an increase in CEC which was directly proportional to the dose. In fact, while the CEC value did not change for the CTRL microplots, it reached an average of 23.95 and 25.71 cmol₊/kg (20.35% and 25.53% more than the respective controls in phase A) for LOW and HIGH microplots, respectively (Figure 1d). These significant increases were directly ascribed to the phenolic and carboxylic terminal groups of biochar developing negative charges in neutral/alkaline conditions. After 54 days of experimentation, there was a progressive decrease in CEC for both LOW and HIGH. However, in all treated microplots, CEC remained significantly higher than the CTRL, thus guaranteeing a medium-term contribution ascribable to the recalcitrant organic moieties of biochar. It is important to emphasize that biochar may represent an especially advantageous solution for managing sandy soils, which cannot adequately retain either cationic nutrients or water, being typically characterized by fast organic mineralization and low content of charge-rich materials, such as clays and SOM.

3.2. Soil Heterotrophic Respiration

Carbon mote chambers were exploited to identify a possible impact exerted on soil heterotrophic respiration by the incorporation of the biochar under consideration. One respiration chamber was permanently installed per thesis (one representative microplot per treatment) to continuously monitor the carbon dioxide flux during the period October–December 2020. It is important to underline that, during this period, (i) spontaneous herbaceous species were periodically and gently removed from the microplot surfaces to minimize the contribution of autotrophic respiration; (ii) the measurements of installed and independent chambers were conducted almost simultaneously, to facilitate data standardization; (iii) only the measurements of CO₂ flows recorded from 13.00 to 15.00, during the afternoon, were considered. The latter choice was adopted to focus on CO₂ fluxes in the

interval exhibiting the most intense respiration rate, as a result of the combination of soil temperature, solar radiation and exposure [42,43].

The carbon flux data (F_{CO_2} , in ppm/s) acquired by the three respiration chambers in the period 30 October–2 December 2020 are shown in Figure 2. The fact that the minimum values of F_{CO_2} proved relatively low (around 0.43 ppm/s or 1.548 ppm/h) was expected, due to the average daily temperatures which ranged between 10 and 18 °C, in line with typical Italian temperatures recorded in late autumn. During the first set of measurements (Figure 2a), the F_{CO_2} values in CTRL ranged between 0.43 and 0.69 ppm/s, in LOW they were between 0.42 and 0.68 ppm/s, and in HIGH they were between 0.43 and 0.63 ppm/s. Interestingly, immediately after the biochar administration (30–31 November), we observed a significantly lower emission than the control (up to 31.1% less; Figure 2a). This behavior was presumably attributed to an initial biochar-induced perturbation in the soil's equilibrium, which implied an alteration in the soil's chemical properties and implicated the microbial component. However, from the 2nd of November onward, the LOW microplot exhibited a pronounced F_{CO_2} which, in most cases, was even higher than that of the CTRL microplot. The initial inhibition in HIGH lasted until the 4th of November, after which the F_{CO_2} values were almost constantly higher than CTRL, except for the 10th of November. During the second set of measurements, from the 12 November to the 2 December (Figure 2b), the F_{CO_2} values in CTRL ranged between 0.35 and 0.59 ppm/s, in LOW between 0.4 and 0.88 ppm/s, and in HIGH between 0.38 and 0.50 ppm/s. It is important to note that the flow tended to be higher in the case of the LOW microplot (up to 0.37 ppm/s more than CTRL), while in the case of HIGH, the values continued to be similar or slightly lower than CTRL. During this period, the LOW microplot not only exhibited the highest average carbon dioxide emission, but also the absolute highest values—in many cases, higher than 0.7 ppm/s, up to 0.88 ppm/s on the 20 November.

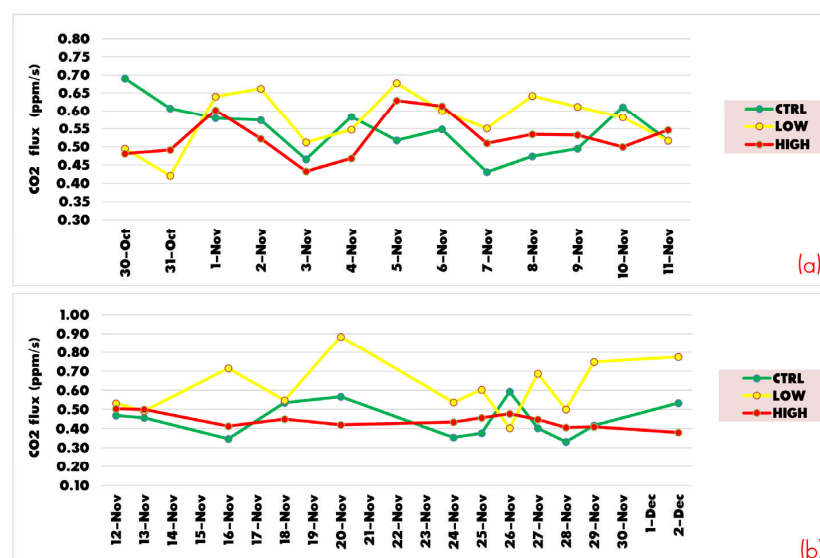


Figure 2. Carbon dioxide flux (ppm/s) released from soil and detected by the carbon mote system in the periods (a) 30 October–11 November and (b) 12 November–2 December from CTRL, LOW and HIGH microplots.

Supporting Table S1 shows the soil respiration values which were calculated accounting for F_{CO_2} (converted in ppm/h), the soil temperature and the physical characteristics of the respiration chamber. The examined microplots remained bare during the experimentation with the carbon mote systems, since we periodically removed each emerging herbaceous species. Therefore, we assumed that the carbon dioxide fluxes detected originated mostly from the soil's heterotrophic respiration and, thus, by the activity of edaphic microorganisms.

The measured respiration data ranged from 4.7 to 12.4 g CO₂ m⁻² h⁻¹. As can be seen, the values were proportional to the flux data shown in Figure 2 and, consequently, they confirmed the same trends already discussed above. However, the average respiration values for both measurements sets indicated that there were no significant differences between CTRL and HIGH (average values were 6.93 ± 0.13 gCO₂ m⁻² h⁻¹), while the LOW microplot showed a significantly higher value, corresponding to 8.4 gCO₂ m⁻² h⁻¹ (Supporting Table S1). Moreover, it is important to underline that, from 24 November to the end of the experimentation, the soil temperature reached values below 13 °C during the early afternoon, which was expected to slow down microbial activity. Interestingly, while the average SR decreased for CTRL and HIGH microplots, in the case of the LOW microplots it remained relatively higher, especially from 27 November onward.

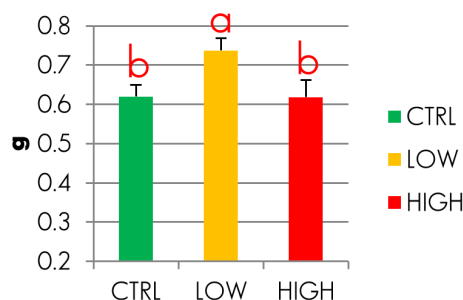
The data on soil heterotrophic respiration revealed that, at least in the short term, biochar-based treatment influenced the soil's respiration rate, with relevant and positive effects only in the case of the 1 kg/m² dose. Such a response was ascribed to greater and more efficient microbial activity and, therefore, to greater biological fertility of the soil, which is crucial for many fundamental processes, including the recycling of nutrients and plant production. From the literature, it seems that this improvement can be attributed to several reasons, including (i) the biochar-induced promotion of a favorable environment, making available a number of nutrients through desorption processes [44]; (ii) the microporous structure of biochar acting as a refuge for microorganisms and protecting them from predators (i.e., microarthropods) [45]; (iii) the fact that it promotes the bacterial rather than the fungal community [46]. Conversely, the dose of 3 kg/m² did not significantly affect the microbial activity, or even slightly inhibited it, as compared to CTRL. Such inhibitory action may be simply explained by the presence, in the biochar, of elements and/or compounds which, at relatively high concentrations, can affect the soil microbiota [47]. Another factor contributing to lower microbial activity in the HIGH microplot is the non-negligible saline content induced by the administration of 3 kg/m² of biochar (Figure 1). The fact that salinity can be increased with massive biochar applications has already been described in the literature [36,48].

3.3. Fennel Experimentation

The fennel variety Prelude F1 was grown in soils treated either without or with different doses of biochar, according to a 3 × 3 randomized block design. In order to assess possible positive effects induced on the fennel plants by the presence of biochar, we evaluated important crop productivity parameters, such as bulb weight, axial diameter and plant length (Figure 3). The fennels from LOW soils showed a significantly better response than for samples from both CTRL and HIGH soils, which, conversely, did not differ from each other (Figure 3). In fact, the fennels produced on LOW soils exhibited values of weight, diameter and maximum plant height of 0.74 g, 12.5 and 64.1 cm, respectively, which were significantly higher (ANOVA test, $p < 0.005$) than those of CTRL and HIGH. The fact that the best response was observed for fennels grown on LOW microplots suggests that the fennels benefited from more fertile soil conditions, which resulted in slightly higher productivity than the control, without resorting to the application of mineral fertilizers. This improvement indicated that the applied dose of biochar was very suitable and efficient for the soil under consideration. In fact, it may have favored fennel plants through a greater abundance of bioavailable nutrients and water, the potential release of compounds with biostimulant activity, and the promotion of beneficial microorganisms. Interestingly, the advantages observed in terms of fennel productivity were completely in line with the data on the soil's heterotrophic respiration, which exhibited a significantly higher trend for the LOW microplot during most of the experimental period (Figure 2). As SR is

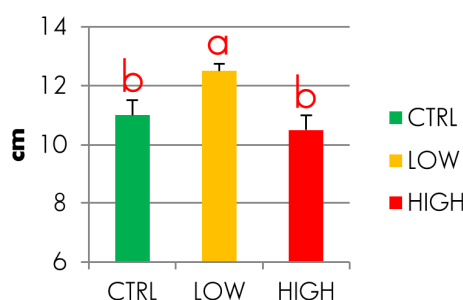
related to soil biological fertility, we can conclude that there is a strong correlation between crop productivity and microbial activity. Finally, our outcomes proved that, although soil chemical parameters such as CEC and soil organic matter content may serve as reliable markers of soil fertility, they are not sufficient to predict the productive response of a soil. In fact, despite the higher values of CEC and SOM detected in the HIGH microplots, the best results, in terms of fennel productivity and SR, were observed in LOW. This finding underlines the importance of considering a combination of analytical approaches to both objectively evaluate the real effects of exogenous organic matter on soil and develop reliable forecasting models.

Fennels bulb weight



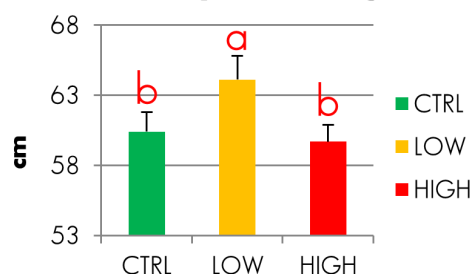
(a)

Bulb axial diameter



(b)

Fennels plant height



(c)

Figure 3. Bulb weight (a), diameter (b), maximal length of the epigeal portion (c) of fennel Prelude F1 grown on three microplots—CTRL (0 kg m²), LOW (1 kg m²) and HIGH (3 kg m²)—under the biochar-based treatment. The results are expressed as mean and standard deviation and the association of different letters on the histograms indicate that the differences are significant (Tukey and Benjamini–Hochberg test $p < 0.05$).

3.4. Characterization of Biochar and SOM Extracts

The ^{13}C CPMAS NMR spectrum (a) and the GC-MS pyrogram (b) of the biochar under consideration are shown in Supporting Figure S5. In line with the relevant literature, the carbon spectrum was dominated by a single, intense and broadened signal in the aromatic region (110–160 ppm), arising from the overlapping of aromatic and polyaromatic moieties composing the biochar. This evidence demonstrates the prevalently polyphenolic nature of this product, and explains most of its typical recalcitrant nature, making it a good strategy for carbon sequestration. Only minor and very weak components were appreciated at 20 ppm and in the range 50–70 ppm, and these were mostly attributed to alkyl and O-alkyl moieties (Supporting Figure S5a). The GC-MS pyrogram of biochar is shown in Supporting Figure S5b. Despite its indisputable power, the combination of thermochemiolysis with GC-MS fails in detecting low-volatile molecules and compounds which cannot be derivatized, such as polycondensed aromatic compounds. Therefore, and unlike what was revealed by the ^{13}C CPMAS NMR spectrum of raw biochar, the Pyr/GC-MS pyrogram only detected a few peaks, representing a small part of the biochar's components. However, a very intense peak was detected at 16.86 min, which was assigned to a lignan derivative, based on the literature [49] and on the prominent and diagnostic mass signal at 190 m/z (Supporting Figure S5c). The pyrogram also exhibited a few more peaks, including two peaks assigned to unsaturated compounds (1-Nonadecene and 1-Eicosene at 24.63 and 29.76 min, respectively) and two fatty acid methyl esters (tetradecanoic acid, 10,13-dimethyl-, methyl ester and heptadecanoic acid, 16-methyl, methyl ester at 28.052 and 33.17 min, respectively).

The compositional quality of organic matter in soil may provide a quantity of detailed information, useful for better understanding the mechanisms triggered in soil by the application of exogenous organic material, like wood biochar. Therefore, we compared the organic matter of LOW microplots with that of CTRL microplots (both isolated 54 days after the biochar application). Our choice was based on the fact that the dose of 1 kg/m² (LOW) resulted in the most effective treatment in enhancing SR and microbial activity. The ATR FT-IR (a) and ^{13}C CPMAS NMR (b) spectra of CTRL (green) and LOW (red) extracts are shown in Figure 4. The band at 3251 cm^{−1} was attributed to O–H stretching, N–H stretching, and hydrogen-bonded O–H, including hydration water. The adsorption bands at 1634 and 1594 cm^{−1} were assigned to aromatic C=C skeletal vibrations. The spectral region from 1220 to 1000 cm^{−1} referred mostly to C–O stretching, including the signals of carboxyls, where the sub-range 1130–1000 cm^{−1} was attributed to polysaccharides and alcohols. Finally, the region between 930 and 900 cm^{−1} was ascribed to O–H bending of carboxyls and C–H bending in alkenes [50,51]. By comparing the spectra, a single but relevant difference emerged in the spectral region 1200–1000 cm^{−1} which was significantly more intense for the LOW extract. This was ascribed to a more abundant carbohydrate content in LOW microplots, which not only agrees well with the SOM results (Figure 1c), but also suggests the presence of a larger microbial biomass as well as indicating greater soil heterotrophic respiration (Figure 2).

Figure 4b shows the ^{13}C CPMAS-NMR spectra of LOW and CTRL extracts along with a table with NMR integrations and the most relevant NMR-based indexes (Figure 4c). The carbon spectral region in the range 0–45 ppm is dominated by peaks of (CH₂)_n and CH₃ alkyl groups, mostly attributable to lipid compounds of plant origin, such as waxes and aliphatic polyesters. The region 45–60 ppm is characterized by CH₃O- groups, ascribable to aromatic methoxyls in syringyl and guaiacylic lignin units, as well as by C–N carbons in oligo- and poly-peptides. The region 60–110 ppm is assigned to the hydroxy-alkyl groups, prevalently represented by oligo- and poly-saccharides. Regarding this region, the most intense signal at around 70 ppm corresponds to the overlapping of C2, C3 and C5 carbons

in pyranosidic structures of cellulose and hemicellulose, and those at around 65 and 80 ppm are assigned to C6 and C4 carbons, respectively, while signals in the range 100–105 ppm correspond to anomeric carbons. The region 110–160 ppm is dominated by unsaturated alkyl groups and aromatic compounds (attributable largely to lignin moieties), whereas the sub-interval 145–160 ppm is generally assigned to quaternary aromatic carbons. In the region 160–190 ppm, carbonyls in carboxyl and ester groups resonate, mostly attributable to lignin biopolymers or lipids, whereas the region between 195 and 230 ppm is characterized by carbonyls in ketone groups (Figure 4b).

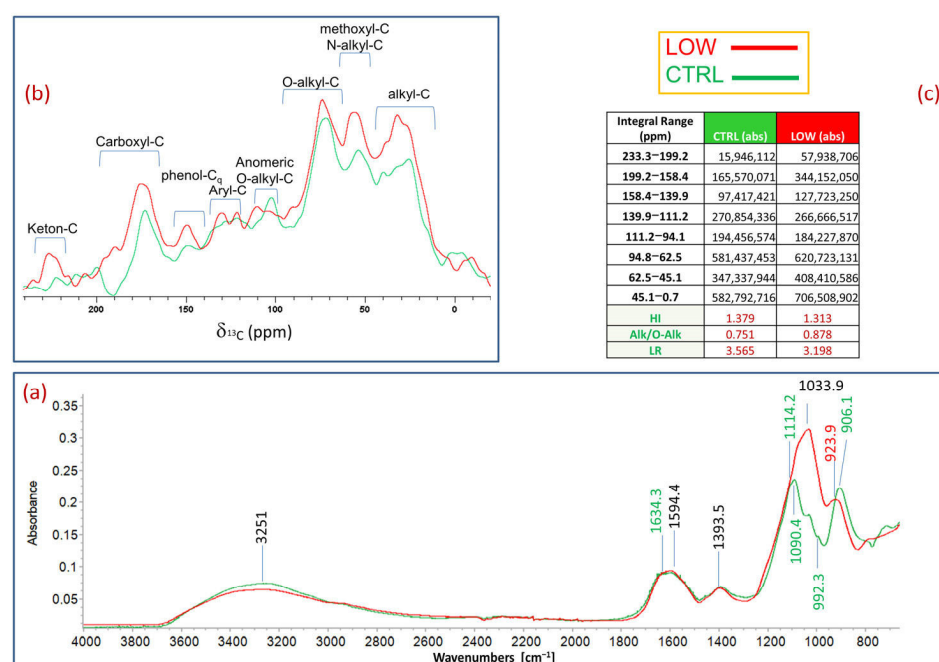


Figure 4. ATR FT-IR (a) and ^{13}C CPMAS NMR (b) spectra of CTRL (green) and LOW (red) extracts isolated from microplot soils 54 days after the beginning of the experiment; the box (c) reports the NMR integrations (absolute and normalized) and the most relevant NMR-based indexes.

The spectral comparison indicated a relatively larger abundance of carbohydrates in the LOW sample, thus confirming the results observed via FT-IR spectroscopy (Figure 4). Moreover, the relatively sharper signal in the peak at 80 ppm suggests a certain abundance of fast-moving compounds, such as mono- and oligo-saccharides, which represent most of the soil's labile carbon. The spectrum of the LOW extract also exhibits more intense peaks in both alkyl and carboxyl regions, attributable to lipids and to fatty acids, as well as a relevant ketone peak, partially arising from oxidized phenols and lignols. Although the LOW extract contained more carbohydrates and labile carbon than CTRL, the hydrophobic index (HI) was very similar to that of CTRL. This was due to the higher content of lipids in LOW which not only implied a higher Alk/O-Alk index as compared to CTRL, but also compensated for the carbohydrate abundance by conferring a certain hydrophobicity to LOW SOM. This response is partially explainable by the enhanced microbial biomass development in LOW (Figure 2). The lignin ratio (LR) index is indicative of the lignin content and reveals whether the peak resonating at 56 ppm is attributable to either G- and S-lignin units or to proteins and peptides. Typically, it is assumed that when the LR is higher than 2.5, the peptides are relatively abundant, thus providing a significant contribution to the intensity of the peak at 56 ppm, and vice versa. Therefore, although the content in proteins and peptides was estimated to be relatively high in both the extracts, the CTRL extract exhibited a slightly higher value.

The Pyr-GC/MS pyrograms of CTRL and LOW extracts are shown in Figure 5. Most of the signals were assigned on the basis of the NIST mass library, mass fragmentation pathways and comparison with standard compounds. The identification of signals and the most important information (compound class, compound name, molecular weight and dominant mass signals) are reported in Supporting Table S2. Unlike FT-IR and CPMAS NMR spectroscopies, which can provide a very powerful macroscopic vision of the main compound classes comprising the examined material, with the Pyr/GC-MS technique it is possible to go deeper into the molecular composition, identifying and quantifying single compounds.

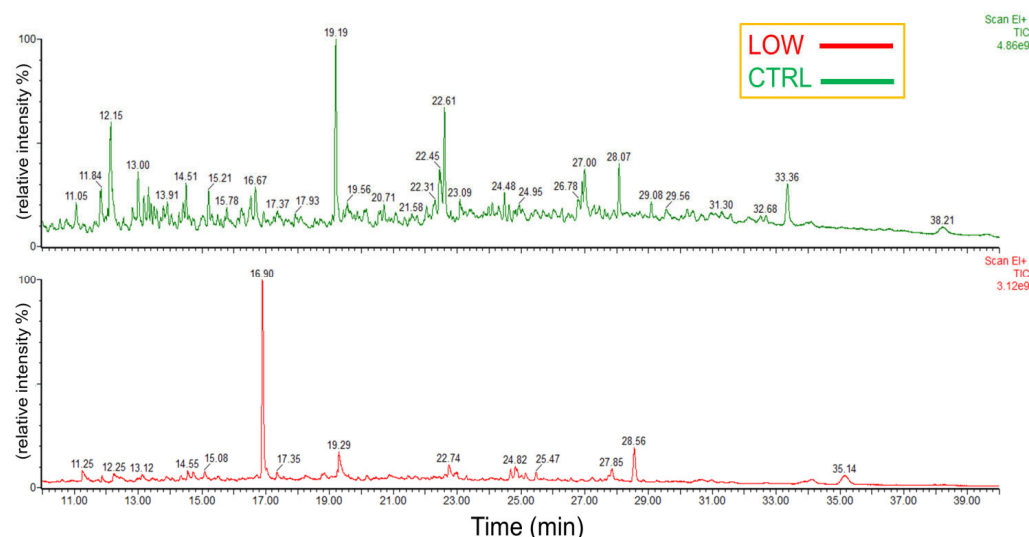


Figure 5. GC chromatogram of CTRL (green) and LOW (red) extracts isolated from microplot soils 54 days after the beginning of the biochar-based experimentation. Samples were pyrolyzed at 400 °C and derivatized via thermochemiolysis driven by tetramethylammonium hydroxide.

Except for a few compounds, which were detected in only one type of extract, most signals were identified in both CTRL and LOW extracts. However, the concentration of some compounds changed greatly from one extract to another (Figure 5). The peak at 16.9 min in the LOW extract corresponded to the lignan derivative already detected in the biochar, as confirmed by its mass spectrum [49] (Supporting Figure S5). Since this compound was totally absent in the CTRL sample, we concluded that it may serve as a reliable marker to trace the supply of this specific biochar in soil.

The most intense signals detected in both CTRL and LOW were assigned to benzoic acid, 3,4 dimethoxy-, methyl ester (Lignin G unit) at minute 19.29, to benzoic acid, 3,4,5 trimethoxy-, methyl ester (Lignin S unit) at minute 22.74, and to tetradecanoic acid, 10,13 dimethyl, methyl ester at minute 28.7. Other intense peaks detected in CTRL were the 2 propenoic acid, 2-(3,4 dimethoxyphenyl)-, methyl ester (Lignin G unit) at minute 27, and the 9-octadecenoic acid, methyl ester at minute 33.36. Conversely, a relevant and intense peak revealed in the LOW spectrum was that of heptadecanoic acid, 16 methyl, methyl ester, at minute 35.14.

All the signals detected in the pyrograms were integrated, grouped according to the specific compound class, and added up (Table 1). Interestingly, the CTRL extract contained a larger amount of lignin moieties than the LOW extract (26.31% vs. 16.47%), with the G units being the most abundant lignols in both cases. From a quantitative point of view, G units were followed by S units, while *p* units were relatively low in CTRL and almost negligible in LOW. Also, the N-compounds were slightly more abundant in CTRL than for LOW (3.62 vs. 1.64%). Such an outcome well agrees with the larger LR achieved by elaborating the NMR spectrum of CTRL (Figure 4b,c). In fact, part of the

N-compounds detected by Pyr GC/MS in CTRL are of peptidic nature and may have contributed to increasing the NMR signal at 56 ppm. The CTRL extract also exhibited a larger amount of aromatic compounds containing nitrogen (13.41% vs. 10.52%). However, it is important to underline that many of the N-aromatic molecules detected in LOW exhibited an indole-like structure (4.46 times more abundant than in CTRL), where part of them are expected to serve as biostimulants providing phytohormonal activity [52] (Table 1). This could explain part of the benefits observed for both heterotrophic respiration and fennel productivity under LOW treatment. On the other hand, the LOW microplots were characterized by a three-times higher content of aromatic moieties, presumably deriving from partial biochar depolymerization. Finally, all FAME types were more abundant in the LOW extract, as already observed in the NMR spectra. The fact that branched FAME, hydroxyacid FAME and unsaturated branched FAME were more abundant in the LOW extract is important, since most of these compounds have a microbial origin. This outcome indicates a more enhanced microbial activity in microplots treated with the lowest dose of biochar, which well agrees with the most pronounced heterotrophic respiration (Figure 2; Supporting Table S1). It also suggests that part of the higher SOM content in LOW compared to CTRL could, in part, be attributed to the microbial biomass.

Table 1. Integration (absolute and normalized % values) of GC-MS pyrograms of soil organic matter extracted from biochar-treated (LOW, 1 kg m²) and untreated soils. (N, FAME, G, P, and S labels correspond to Nitrogen-based, Fatty Acid Methyl Esters, *p*-hydroxyphenyl, guaiacyl and syringyl monomeric lignin units, respectively).

	C-CTRL	C-LOW	C-CTRL (%)	C-LOW (%)
N-compounds	94,314,231	13,212,714	3.62	1.64
Fame	10,168,751.3	18,439,959.5	0.39	2.28
Branched fame and Hydroxyacids	228,126,380	93,427,032.62	8.75	11.57
Unsaturated branched fame	0	47,709,987	0.00	5.91
Aromatic	233,967,191.2	212,946,751.5	8.98	26.37
N-Aromatic	349,283,102	84,987,775.75	13.41	10.52
Lignin-G	400,985,722	83,730,246.75	15.39	10.37
Lignin-P	28,572,688	0	1.10	0.00
Lignin-S	255,884,054	49,253,888.5	9.82	6.10
Total Lignin	685,442,464	132,984,135.3	26.31	16.47

Importantly, PyrGC-MS results also permitted us to conclude that the better response observed for fennels production (Figure 3) was not only due to higher CEC and SOM than CTRL, but also to both the optimal presence of indole-structured compounds, with potential phytohormonal action, and to microorganisms promoting plant growth, favoring SOM mineralization and the availability of nutrients. On the other hand, the fact that HIGH treatment, although it increased SOM and CEC to a level even higher than in LOW (Figure 1), did not produce a response different to CTRL in terms of SR and crop productivity, indicates that the dose of 1 kg m² (LOW) is the optimal one and the best compromise to increase chemical fertility and create the proper conditions to promote microbial activity, improve plant production and ameliorate soil health.

4. Conclusions

Our results permitted us to confirm, through open field trials, that wood biochar, when applied to soil at the proper dose (1 kg/m^2), serves as an efficient fertilizer to improve soil quality and health, in addition to performing efficient carbon sequestration. Our findings indicate that both tested biochar doses improved important chemical fertility parameters (SOM and CEC), the highest values appearing in the case of HIGH treatment. This notwithstanding, it was the LOW treatment which produced the best results in terms of soil respiration and fennel production. In particular, SR, which was monitored daily through a Closed Dynamic Chamber (CDC) associated with an innovative pilot system, revealed that the lower biochar dose elicited the most pronounced heterotrophic activity. We demonstrated, via NMR and Pyr/GC-MS techniques, that the lower dose correlated mostly with more enhanced microbial activity, since we detected a relevant presence of diagnostic FAME biomarkers in the SOM. Importantly, the proposed combination of advanced analytical techniques was useful for providing important information on SOM composition, including the facts that LOW SOM was characterized by (i) a greater abundance of SOM and labile carbon, (ii) a higher content of indole-structured compounds with potential phytohormonal activity, (iii) a more pronounced microbial biomass, presumably contributing to the promotion of soil biological fertility. Our findings demonstrated that the proper biochar dose improves soil fertility and creates an environment favorable to microbial activity, ultimately determining further benefits in plants. However, it is important to conduct more experiments in the future, to better optimize the application rate of the studied biochar, evaluate the biochar's effects in the medium and long term, and estimate the frequency with which the biochar application should be repeated. In conclusion, this study, albeit limited to a short experimental period, confirms that biochar, when administered in well-calibrated and not excessive doses, represents a sustainable and eco-friendly strategy for recycling forestry and lignin-rich wastes, promoting carbon sequestration, improving soil health and productivity and potentiating the ecosystem services offered by soil.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture15101091/s1>, Figure S1: Description of the main properties of the studied biochar, including chemical and physical parameters as well as nutrient and heavy metals contents (information provided by the provider Nera Biochar); Figure S2: (a) Soil microplots (1 m^2 each) selected for the evaluation of soil respiration, before the biochar-based treatment. (b) Soil microplots after the biochar treatment ($0, 1$ and 3 kg/m^2 , identified with the labels CTRL, LOW and HIGH, respectively) and equipped with the permanent installation of the Carbon mote system; Figure S3: Weather data concerning the whole experimental period (October 2020 and March 2021) and including the precipitations (mm/d), the minimum and maximum temperatures ($^{\circ}\text{C}$) and the solar radiation (kW/h); Figure S4: (a) Schematic representation of the complete Carbon Mote system; upper (b) and lateral (c) representation of the plate composed by a hole-collar, delimiting the examined soil area, and equipped with a module containing both the IRGA analyzer and the motor to control the chamber opening; Figure S5: (a) ^{13}C CPMAS NMR spectrum of raw and pulverized biochar, in the solid-state; (b) GC chromatogram of biochar (pyrolysed at 400°C and derivitized, via thermochemiolysis driven by tetramethylammonium hydroxide) and (c) the mass spectrum of the signal eluted at 16.86 min . Table S1: Soil respiration values (SR in $\text{gCO}_2 \text{ m}^{-2} \text{ h}^{-1}$) calculated accounting for soil temperature ($^{\circ}\text{C}$), the flow of carbon dioxide released in the unit of time (FCO_2 in ppm h^{-1}), the universal gas constant ($8.31 \text{ J mol}^{-1} \text{ K}^{-1}$), the chamber volume (5747 cm^3) and the soil area examined by the chamber (615.7 cm^2); Table S2: List of compounds identified in the GC-MS pyrograms of CTRL and LOW soil extracts, including compound class, compound name, molecular weight, the most intense and diagnostic signals and the retention time interval. The labels N, FAME, G, P, and S correspond to Nitrogen-based, Fatty Acids Methyl Esters, p-hydroxyphenyl, guaiacyl and syringyl monomeric lignin units, respectively, whereas the symbol >> indicates that the specific mass signal is predominant in the mass spectrum).

Author Contributions: Conceptualization, P.M.; Methodology, R.C. and R.B.; Validation, C.L. and M.C.; Formal analysis, R.C., R.B., S.C. and M.V.; Investigation, R.C. and P.M.; Resources, M.C., R.S. and P.M.; Writing—original draft, R.C. and R.B.; Writing—review & editing, P.M.; Supervision, C.L., M.C., M.V. and R.S.; Funding acquisition, P.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Authors Carmine Lia and Michele Compitiello were employed by the company Maniola Remote Sensing, s.r.l. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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An Integrated Multilevel Approach Unveils Complex Seed–Nanoparticle Interactions and Their Implications for Seed Priming

Elisa Cappetta,[■] Carmine Del Regno,[■] Marisa Conte, Christian Castro-Hinojosa, Susel Del Sol-Fernández, Chiara Vergata, Matteo Buti, Rossella Curcio, Anil Onder, Pierluigi Mazzei, Nicola Funicello, Salvatore De Pasquale, Mattia Terzaghi, Pasquale Del Gaudio, Antonietta Leone, Federico Martinelli, Maria Moros, and Alfredo Ambrosone*



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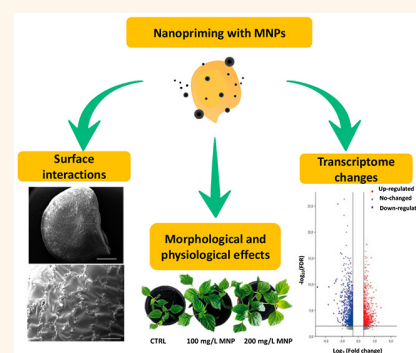
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Supporting Information

ABSTRACT: Nanotechnology has the potential to revolutionize agriculture with the introduction of engineered nanomaterials. However, their use is hindered by high cost, marginal knowledge of their interactions with plants, and unpredictable effects related to massive use in crop cultivation. Nanopriming is an innovative seed priming technology able to match economic, agronomic, and environmental needs in agriculture. The present study was focused on unveiling, by a multilevel integrated approach, undisclosed aspects of seed priming mediated by iron oxide magnetic nanoparticles in pepper seeds (*Capsicum annuum*), one of the most economically important crops worldwide. Inductively coupled plasma atomic emission mass spectrometry and scanning electron microscopy were used to quantify the MNP uptake and assess seed surface changes. Magnetic resonance imaging mapped the distribution of MNPs prevalently in the seed coat. The application of MNPs significantly enhanced the root and vegetative growth of pepper plants, whereas seed priming with equivalent Fe concentrations supplied as FeCl_3 did not yield these positive effects. Finally, global gene expression by RNA-sequencing identified more than 2,200 differentially expressed genes, most of them involved in plant developmental processes and defense mechanisms. Collectively, these data provide evidence on the link between structural seed changes and an extensive transcriptional reprogramming, which boosts the plant growth and primes the embryo to cope with environmental challenges that might occur during the subsequent developmental and growth stages.

KEYWORDS: iron oxide magnetic nanoparticles, nanopriming, seed–nanoparticle interactions, RNA-seq, *Capsicum annuum*



INTRODUCTION

Climate change, population growth, and the reduction of arable land pose significant threats to agriculture and undermine food security worldwide. Innovative actions are needed to overcome these constraints and ensure the sustainability and protection of major crops in the near future.¹ Nanotechnology, if properly employed, has the potential to revolutionize the agricultural sector.

In the past decade, thanks to the small size and tailorable surface properties, engineered nanomaterials (NMs) have been used to overcome biological barriers in plants and to efficiently deliver micronutrients, antioxidants, and genetic materials, with the aim of improving plant productivity, resilience, and other agronomic traits.^{2–8} Furthermore, nanoenabled products can be used as components of smart sensors to advance the agronomic management of high-value crops.^{7,9}

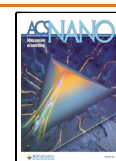
Irrespective of their different composition, the potential benefits of NM application in plant science and agriculture have not yet been fully exploited due to a limited understanding of biological interactions of nanoparticles with plants, including uptake, translocation, transformation, and bioavailability.¹⁰ High production costs and concerns about potential unpredictable environmental effects further restrict nanoenabled extensive farming.

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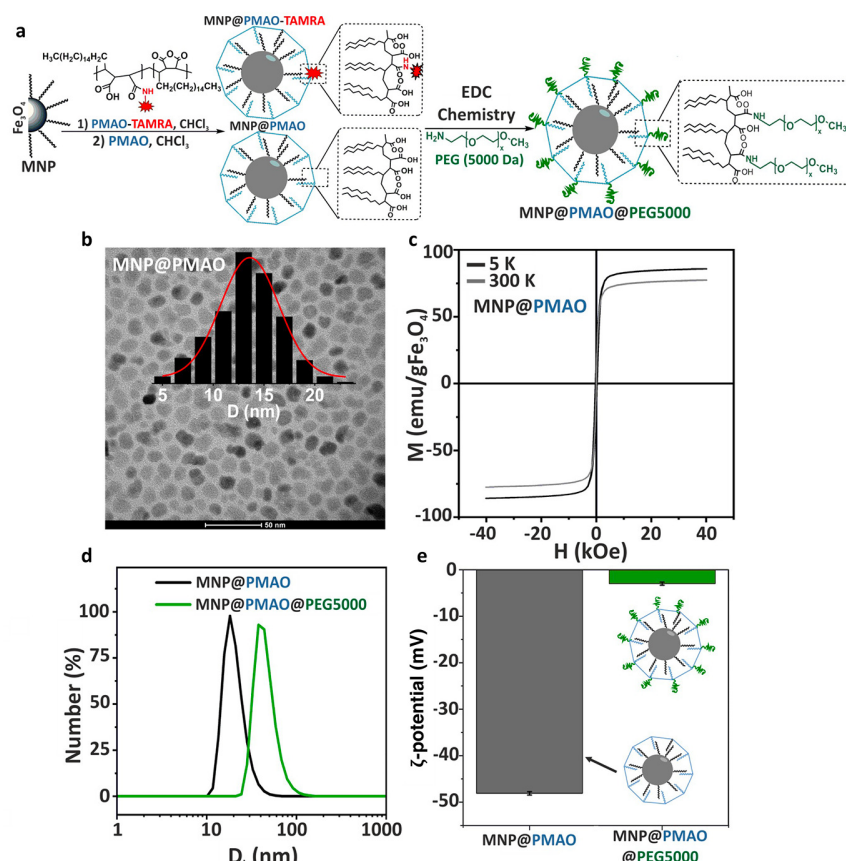


Figure 1. MNP synthesis and characterization. (a) Scheme of MNP transfer into water functionalization with PMAO or modified with TAMRA and functionalized with PEG (5000 Da) molecules by carbodiimide (EDC) chemistry. (b) TEM image of MNPs transferred to water and their size distribution. (c) Hysteresis loops recorded at 5 and 300 K. Inset: low-field hysteresis loops. (d) Hydrodynamic diameters measured by DLS and (e) ζ -potential measurements of MNPs@PMAO and after functionalization with PEG, MNPs@PMAO@PEG5000.

In this scenario, seed nanopriming may be an opportunity to meet plant nanotechnology advances with economic and environmental needs. Basically, seed priming is a complex adaptive mechanism achieved through nonlethal exposure of seeds to biotic or abiotic stresses in a presowing stage. This process confers multiple advantages to the primed plants, including higher germination rates, vigorous development, enhanced nutrient utilization, and improved ability to respond to environmental constraints more quickly and robustly.^{11,12} Numerous successful strategies for seed priming have been described, including seed treatment with salt solutions (halo-priming), water (hydro-conditioning), osmotic agents (osmo-priming), plant hormone solutions (hormonal priming), exposure to high temperatures (heat priming), magnetic fields (magneto-priming), and, more recently, nanoparticles, known as seed nanopriming.^{13,14} Seed nanopriming technology is expected to minimize the input of NMs in agriculture, as low amounts of nanoparticles (NPs) can be used to pretreat thousands of small seeds, which are then washed to eliminate the excess of NPs that could be released into the environment. Seed nanopriming has been associated with enhanced germination rates, improved seedling growth, increased stress tolerance (such as drought or salinity), and better nutrient uptake in many crops.^{13,15} Nanopriming can also contribute to increase crop yields and overall plant health. Nonetheless, a substantial gap exists in our understanding of the physical interactions between seeds and NPs. Moreover, unraveling the regulatory networks

responsive to nanoparticle stimuli holds the potential to elucidate their effects, mitigate undesired outcomes, and optimize the application of nanomaterials in agriculture. In this context, the use of magnetic iron oxide nanoparticles (MNPs) to prime plants has shown promise in boosting both growth and defense mechanisms in various plant species.^{16–18} However, the underlying mechanisms of these effects are still not fully understood.

In this study, we provide a comprehensive and multi-disciplinary evaluation of MNP-mediated seed priming. Our goal was to gain a deeper understanding of how and to what extent MNPs influence seed germination and plant development using a range of analytical approaches including structural, physiological, phenotypic, and transcriptomic analyses.

The study was conducted in pepper (*Capsicum annuum*), one of the world's most economically and nutritionally important horticultural crops. We synthesized MNPs conjugated with a fluorophore (TAMRA) and tracked their internalization in the seeds using fluorescence microscopy. Analytic and imaging techniques, including Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) and scanning electron microscopy (SEM), were used to quantify MNP internalization and to analyze surface structural modifications of the seeds. Moreover, magnetic resonance imaging (MRI) was employed to create a map of MNP spatial distribution. We subsequently evaluated the impact of MNPs on embryo morphology and plant development, demonstrating their safety profile and positive

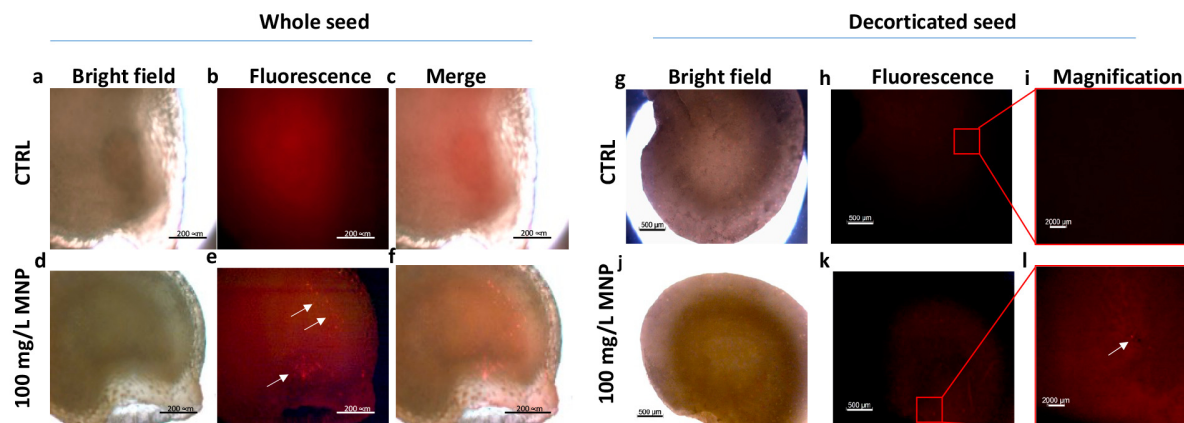


Figure 2. Fluorescence microscopy of *C. annuum* seeds treated with TAMRA-conjugated MNPs. (a) Bright field image of untreated seed. (b) Fluorescence imaging of a. (c) Overlap of (a) and (b). (d) Bright field image of a seed treated with 100 mg/L of NPs for 24 h. (e) Fluorescence imaging of (d). (f) Overlap of (d) and (e). (g) Decorticated control seed. (h) and (i) Fluorescence imaging of g at increasing magnification. (j) Seed treated with 100 mg/L of NPs for 24 h imaged after coat removal. (k) and (l) Fluorescence imaging of j at increasing magnification showing the presence of MNPs@PMAO@TAMRA@PEG in the endosperm. White arrows indicate MNP in the seed coat. Scale bars: 200 μm (left panel, a–f), 500 μm (g, h, j, k), and 2000 μm (i, l).

influence on vegetative growth, not observed in plants primed with FeCl_3 , used as control to test the effects of the iron ionic counterpart. RNA sequencing revealed global gene expression changes induced by MNPs in pepper seeds. Considering the limited understanding of molecular impacts resulting from nanomaterial exposure in seeds, our approach contributes to gaining insights into the genetic aspects of plant nanobiotechnology. As a final remark, our data prove that MNP exposure modulates the expression of many biotic and abiotic stress-responsive genes, disclosing important implications for seed nanoprimering.

RESULTS AND DISCUSSION

MNP Synthesis and Characterization. Iron oxide MNPs are widely employed for various applications, owing to their exceptional properties. The established use of MNPs in the biomedical field as contrast agents for magnetic resonance imaging (MRI), drug delivery systems, and hyperthermia treatments for cancer has not revealed substantial hazards to human health. As a result, MNPs are now considered relatively safe.^{19–21} Moreover, the cost-effective synthesis of MNPs enables their application in agriculture, where they have been shown to have promising effects in augmenting crop production, enabling targeted delivery of agrochemicals and nutrients, enhancing plant development and resilience to different abiotic and biotic stress conditions, and ameliorating the decomposition of organic matter and the activities of microbial enzymes during the composting of agricultural waste.^{22–24}

In this work, MNPs were synthesized by a one-step thermal decomposition method, obtaining MNPs coated with oleic acid soluble in organic solvents. These MNPs were transferred from hexane to water by coating them with an amphiphilic polymer poly(maleic anhydride-*alt*-1-octadecene) (PMAO) modified or not with tetramethylrhodamine-cadaverine (TAMRA), a fluorescent dye that facilitates MNP tracking (Figure 1a). MNPs@PMAO showed a mean diameter of 13.6 ± 2.9 nm, as determined from transmission electron microscopy (TEM) images (Figure 1b). The magnetization variation curve as a function of the applied magnetic field M (H) recorded at 300 and 5 K is shown in Figure 1c. At 300 K, the sample shows

typical superparamagnetic behavior with negligible coercivity and remanence (see Table S1 in the Supporting Information). In contrast, the MNPs display a clear ferrimagnetic behavior at 5 K. Hydrodynamic diameter (D_h) and ζ -potential were determined with Malvern Zetasizer equipment, obtaining a size of 22.7 ± 0.9 nm and a surface charge of -48.0 ± 0.4 mV.

Although these MNPs are stable in aqueous media, they aggregate in more complex media due to the large number of carboxylic groups present on the PMAO surface. Thus, the MNP surface was passivated by functionalizing the polymer with α -methoxy- ω -amino polyethylene glycol (PEG, 5000 Da), increasing in this way their stability in more complex media.²⁵ This step was performed by activating the carboxylic groups of the MNPs@PMAO with 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDC) to bioconjugate the aminated PEG. The functionalization was evaluated by gel electrophoresis (agarose 1% w/v), evidencing a variation on the electrophoretic mobility promoted by the presence of PEG molecules (Figure S1). Furthermore, an increment in the D_h to 45.9 ± 2.3 nm accompanied by a neutralization of the negative surface charge to -3 ± 0.4 mV was observed (Figure 1d,e), corroborating the functionalization of the MNPs@PMAO with PEG, named MNPs@PMAO@PEG5000. In addition, the successful PMAO coating and PEG coordination were confirmed by Fourier transform infrared (FT-IR) spectroscopy in MNP@PMAO and MNP@PMAO@PEG5000 samples (Figure S2).

Surface Interactions and MNP Uptake in Pepper Seeds. While tangible progress has been made in understanding plant–NP interactions over the past decade, numerous challenges still need to be addressed before nanoenabled products can become widespread tools for studying and modulating plant functions. For instance, there is still a lack of understanding regarding the influence of NPs on seed structures and the mechanisms by which they enter the seeds, as well as the subsequent triggered biological effects.

In this study, we have utilized seeds of pepper, considering its significance as one of the most important horticultural crops. We initially used 100 mg/L of MNPs@PMAO@TAMRA@PEG to track the internalization of the MNP at the seed surface. After a 24 h incubation period, the seeds underwent thorough washing with distilled water to remove any MNPs that were not

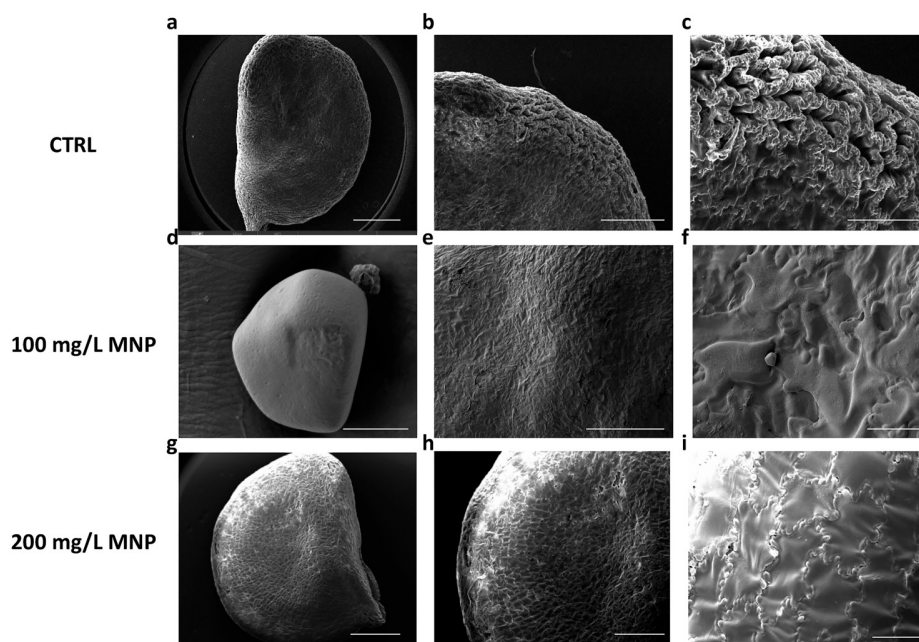


Figure 3. Scanning electron micrographs of untreated seeds (a–c) and seeds treated for 24 h with 100 mg/L of MNPs and (d–f) 200 mg/L of MNPs 200 mg/L (g, h, i). Scale bars: 1 mm in a, 500 μ m in b, 200 μ m in c, 2 mm in d, 500 μ m in e, 50 μ m in f, 1 mm in g, 500 μ m in h, 100 μ m in i.

internalized. Subsequently, the seeds were dissected in order to separately image the tegument (Figure 2a–f) and the endosperm (Figure 2g–i). This examination revealed the presence of a diffuse staining pattern and numerous fluorescent spots on the outer layer of the seed coat (Figure 2e), which were not observed in the control seeds under the same microscope settings (Figure 2b). Additionally, a few fluorescent spots were detected within the endosperm of the MNP-treated seeds (Figure 2k and l and Figure S3). These spots may indicate accumulations of MNPs, as generally observed in animal cells or tissues exposed to fluorescent NPs. These findings suggest that, following a 24-h exposure, the MNPs primarily localize in the seed coat of pepper seeds, with partial penetration observed in the endosperm. These results underline the potential implications for utilizing MNPs in the delivery of biomolecules into the seeds.

To quantitatively estimate the MNP internalization in the seeds, we determined the Fe content by ICP-AES analysis. To this aim, seeds treated with 100 and 200 mg/L of MNP@PMAO@PEG for 24 h and untreated seeds were washed thoroughly, macerated in liquid nitrogen, and analyzed by ICP-AES for the iron content determination which was 1.3 ± 0.6 μ g/seed in the control and 2.9 ± 0.5 μ g/seed and 3.5 ± 0.8 μ g/seed, respectively, upon treatments with 100 and 200 mg/L of MNPs.

To investigate further the structural effects of MNPs on seed coat, we performed a scanning electron microscopy (SEM) analysis after treating the seeds for 24 h with MNPs@PMAO@PEG at two different concentrations. These observations showed that the tegument structure was drastically altered by the MNP exposure (Figure 3). In more detail, SEM micrographs revealed that the typical mesh-like structure of the control seed coat (Figure 3a–c) was modified upon 24 h exposure to 100 mg/L of MNP (Figure 3d–f) and 200 mg/L (Figure 3g–i), converting the tegument of MNP-treated seeds to a smooth and uniform surface rather than the typical wrinkled shape of control seeds kept for 24 h in MilliQ water. Previous studies have described a similar coat modification in *Salvia lerifolia* upon

hydropriming.²⁶ Although the biological significance of these structural changes of the seed coat is not fully understood, it is possible to hypothesize that the physical interactions between seeds and nanoparticles and consequent structural modifications might modify the coat permeability and likely the seed imbibition kinetics at an early stage of germination. Moreover, the structural modification might also contribute to the MNP internalization in the endosperm.

NMR Imaging of Nanoprimered Seeds. To integrate the uptake study and profile the spatial distribution of MNPs in pepper seeds, nuclear magnetic resonance imaging (MRI) analysis was performed. ¹H MRI represents a noninvasive NMR spectroscopic technique capable of reconstructing images and revealing inner morphological characteristics of intact samples at a micrometer level. Proton MRI provides structural and conformational information based on amount, location, and mobility of water molecules.^{27,28}

In this work, the MRI technique was used to prove and localize the presence of MNPs on seeds treated with 100 mg/L of MNPs. To this aim, pepper seeds were treated with MNPs@PMAO@PEG, extensively washed, and dissected in order to analyze independently whole seeds, isolated teguments, and decorticated seeds. The disposition of whole seeds in the NMR tube is visible through a 3D reconstruction (Supporting Information, clip 1) and representative 2D axial slices (Figure S4). Representative spin density images of all samples are displayed in Figure 4 (upper panel, a–f).

Interestingly, although no significant difference in morphology emerged by a visual comparison between control and MNP-treated whole seeds, the spin density integrations revealed two types of profiles (Figure 4g and j), with a much lower response in the case of MNP-treated seeds and for the intensity histograms ranging within 115–135 (Figure 4j). The lower spin density integration values observed in the MNP-treated seeds may be likely attributed either to a lower content of water in examined seeds or to the presence of iron MNP, responsible for partially broadening and quenching proton signals.

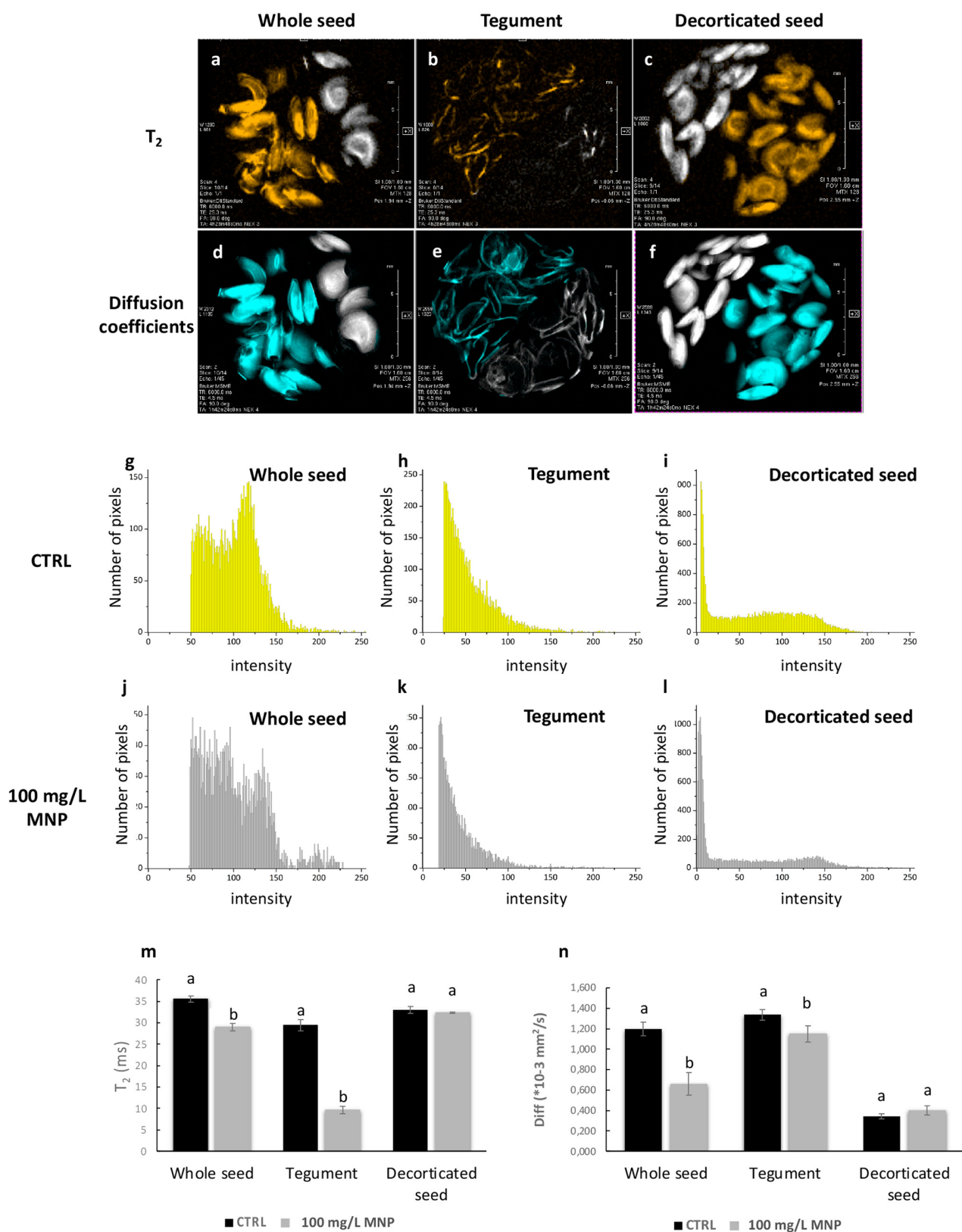


Figure 4. Upper Panel. Central axial slices for whole seed (a,d), tegument (b,e), and decorticated seed (c,f). The 3 images on the top are T_2 experiments, while those on the bottom are diffusion experiments (yellow and cyan colors identify the control samples in T_2 and Diff experiments, respectively). Central Panel. Spin density integrations (number of pixels per intensity) of axial slices of whole seed (g,j), tegument (h,k), and decorticated seed (i,l). The histograms on the top and bottom refer to control and MNP-treated samples, respectively. Bottom Panel. ^1H NMR Spin–spin relaxation times (m) and self-diffusion coefficients (n) of whole seed, tegument, and decorticated seeds. Figures

Figure 4. continued

include, for each measure, the standard deviation, and the results of ANOVA Tukey's test (0.05 confidence level and 95% of significance; letters a-b) on the histograms refer to the comparison between control and treated samples.

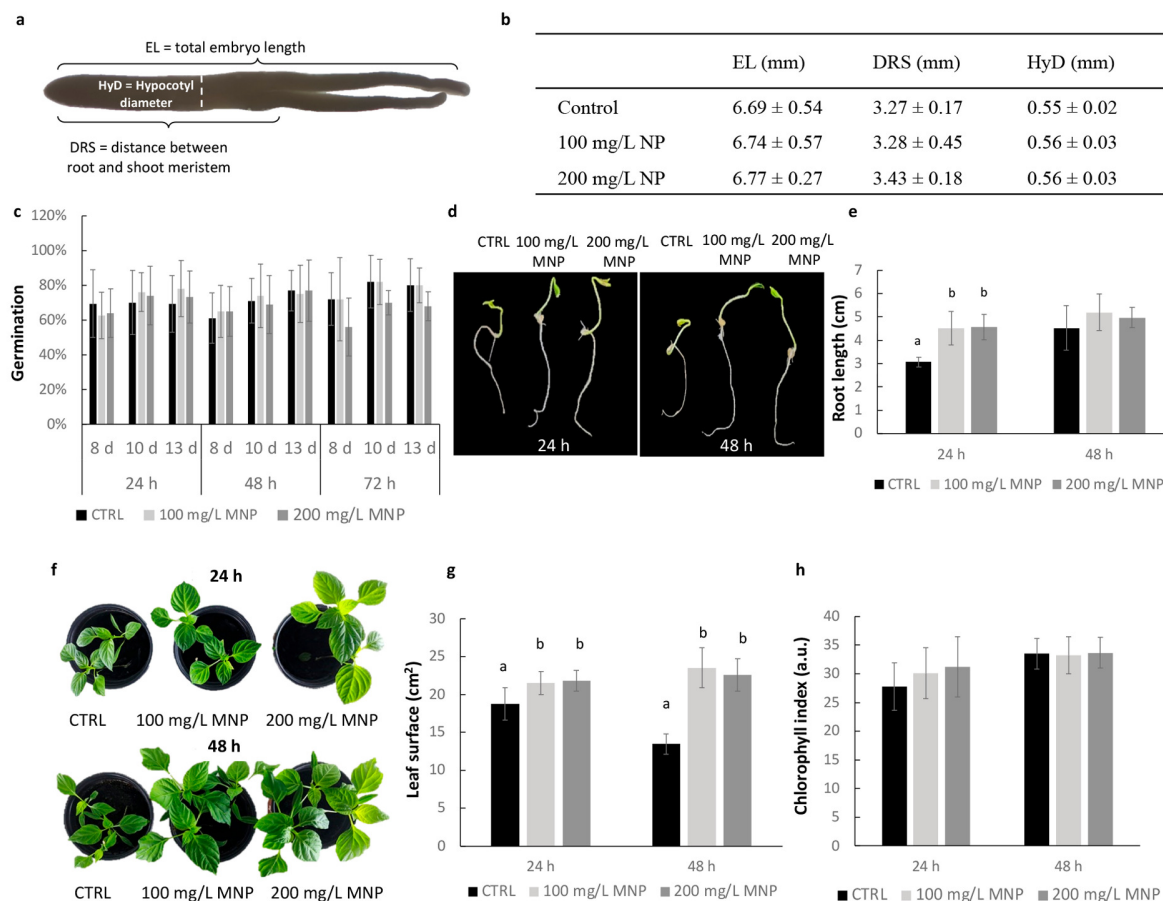


Figure 5. Physiological and morphological analyses after seed priming with MNPs. (a) Representative image of pepper embryos. (b) Morphometric analysis of pepper embryos after 24 h MNP exposure. EL, Total embryo length; DRS, Distance between root and shoot meristems; HyD, diameter of the hypocotyl. Values are means $\pm$ SD ($n = 5$). (c) Germination analysis of control and nanoprimered seeds. Seeds were treated with 100 and 200 mg/L of MNP for 24, 48, and 72 h, and germination data were collected after 8, 10, and 13 days. (d) Representative images of seedlings subjected to seed nanoprimering with 100 mg/L and 200 mg/L of NP for 24 and 48 h. (e) Primary root length in control and nanoprimered seedlings. (f) Representative plants employed to determine leaf surface area. (g) Leaf surface area in control and MNP-treated plants. (h) SPAD measurements in control and MNP-treated plants to determine the chlorophyll index expressed as arbitrary units. The images and graphs shown are representative of at least three independent biological experiments. Statistical comparisons were performed using one-way ANOVA. The letters denote statistical significance with respect to untreated seeds ($P \leq 0.05$).

Afterward, an evaluation of the proton spin–spin relaxation time (T_2) of the seeds was also conducted. Overall, a shortening of water proton T_2 relaxation times is indicative of a change in the sample structure, at a nanoscopic level.²⁹ Importantly, it is expected that MNP presence in seeds may strongly influence the nuclear chemical environment, by promoting and accelerating a faster T_2 relaxation, due to the ferromagnetic nature of iron.³⁰

Data reported in Figure 4m show that MNP-treated whole seeds exhibited a slightly lower T_2 value with spin–spin relaxation times of 29 ms compared to 36 ms of control seeds. The same evaluation was conducted on both the isolated teguments and the seeds deprived of tegument (decorticated seeds). While the spin–spin relaxation times in decorticated seeds resulted in constant and unaltered by MNP treatment, teguments exhibited a significant decrease in T_2 values. In fact,

the T_2 relaxation was dramatically hastened in the isolated tegument of MNP-treated seeds, as compared to the isolated teguments of control seeds, since the values decreased from 29.5 to 9.7 ms (a 67% decrement). Accordingly, the T_2 image of isolated teguments shown in Figure 4b revealed an almost total quenching of tegument signals in presence of MNPs. Such a variation is unequivocally due to the ferromagnetic action exerted by the iron contained in MNP. Altogether, these data provide clear evidence that MNPs were prevalently retained by the seed. Moreover, the low decrease in the T_2 relaxation time of MNP-treated decorticated seeds compared to control untreated seeds confirmed that MNPs were mostly localized on the tegument.

The MNP-induced structural changes may affect the water dynamics in the seeds, and this possibility was evaluated by

diffusion coefficients (DCs), which allow to estimate the apparent translational self-diffusion of water molecules within a plant tissue, thus indirectly estimating the extent of free diffusion within the studied matrix.³¹ Consequently, DCs are expected to decrease when water molecules experience barriers to diffusion, interact strictly with the matrix, or are forced to diffuse within relatively small-sized pores. Conversely, DC values increase if water molecules can move more freely into matrixes, characterized by longer free diffusion distance.^{28,32}

DC values also proved to be very similar between control and MNP-treated decorticated seeds (Figure 4p). Conversely, a significant decrease of DC values was detected in the whole seeds and in the isolated teguments, indicating a slower mobility and a lower content of water at seeds' surface level. This might be the consequence of the adsorption of MNPs on the seed tegument surface, which restricts the retention of water on it and limits the penetration of water at the tegument–seed interface level, as shown in Figure 4n. Moreover, the decrease of tegument roughness revealed by SEM images in MNP-treated seeds suggests a decrease in cavities capable to retain water on the tegument surface, further contributing to lower diffusion coefficients at the surface level.

Interestingly, the latter aspect suggests that the difference was much more enhanced in the case of the whole seeds, as MNP-treated seeds received a lower penetration of water. In fact, in the case of control whole seeds, a larger amount of free-like water penetrated at the tegument–seed interface, thus implying a faster average diffusivity.

In summary, MRI experiments allowed us to prove the presence of MNPs on treated seeds and obtain indirect information on the tegument structural changes. In particular, T_2 relaxation times suggested that most of the MNPs were concentrated on the tegument. However, the internalization of a few MNPs in seed endosperm cannot be completely excluded, as also shown by fluorescence microscopy. On the other hand, diffusion experiments indicated that the water behavior and its penetration extent into the seed changed drastically, as a function of the treatment, especially at both the tegument and tegument–seed interface level.

MNP Effects on Embryo and Plant Development. We next investigated whether treatment with MNPs on pepper seeds might influence embryo and subsequent plant development. Quantitative measurements were conducted on the embryos by evaluating the diameter of the hypocotyl (HyD), the distance between root and shoot meristems (DRS), and the total embryo length (EL). As reported in Figure 5b, EL, DRS, and HyD did not show any significant changes in treated seeds compared to the control, indicating that the MNPs did not compromise the integrity of the pepper embryos.

To obtain an in-depth evaluation of nanoprimer effects on pepper plant development, important physiological and morphological parameters, including germination, root length, leaf surface area, and chlorophyll index, were monitored in young plantlets from MNP-treated or control seeds.

Data reported in Figure 5c show that pepper seed germinability was unaffected by nanoparticle exposure, although a slight decrease in germination rate was observed in seeds after 72 h of priming with 200 mg/L of MNPs. Therefore, the analysis of other morpho-physiological parameters was conducted 24 and 48 h after nanoprimer.

To evaluate the impact of MNPs on the seedling growth of *C. annuum*, we measured the root length of 14-day-old seedlings that were subjected to seed nanoprimer at concentrations of

100 and 200 mg/L of MNP for 24 and 48 h. The results indicated a significant enhancement in relative root length after both 24 h of nanoprimer, as depicted in Figure 5d–e. Notably, a 24 h nanoprimer treatment led to an increase in root length of over 50% compared to the control seeds, as shown in Figure 5e.

Afterward, the effects of seed nanoprimer on vegetative growth were monitored by measuring leaf surface area, a key parameter directly linked to the quantity of light intercepted for photosynthesis, impacting plant growth and yield.³³ Leaf areas of plantlets from control or nanoprimered seeds were measured 20 days after seedling transplanting. As reported in Figure 5g,h, seed nanoprimer promotes a significant increase in leaf expansion in all treatments. Finally, we estimated the chlorophyll index of leaves which is commonly influenced by a variety of factors including nutrient availability and environmental stresses.³⁴ Measurements by Soil Plant Analysis Development (SPAD) showed chlorophyll content values comparable to those of all treatment groups, indicating that MNP treatments did not significantly affect the chlorophyll levels in the leaves. Moreover, as SPAD values are commonly used as an indicator of leaf nitrogen status,³⁵ our findings further support that MNPs did not have an impact on nitrogen content in pepper plants. Finally, we compared the effects of MNPs to those of their iron ionic counterpart by priming the seeds with FeCl_3 . As reported in Figure S5, ionic iron did not produce significant effects on seed germination, root elongation, or leaf expansion after 48 h of exposure at both 100 and 200 mg/L Fe concentrations. Conversely, leaf expansion was negatively affected in plants primed for 24 h with 200 mg/L of Fe. These findings demonstrated that the influence of MNPs on plants diverges from that of their ionic counterpart, suggesting that growth-promoting effects cannot be solely attributed to the direct delivery of Fe ions by MNPs.

It is worth noting that the impact of seed nanoprimer on physiology and development of pepper plants has been poorly investigated so far. However, a recent work reported that ZnO NPs applied to seeds inhibited seedling radical growth and promoted accumulation of phenolic compounds showing phytotoxic effects.³⁶

Altogether, our data indicate that seed priming with iron oxide nanoparticles is able to stimulate root growth as well as to increase the plant vegetative growth in *Capsicum annuum*, confirming the stimulatory effects of nanoparticle-mediated seed priming already reported in different plant species.^{14,37,38} Therefore, as seed priming is a pivotal phase in seed preparation, focused on achieving optimal germination and early seedling establishment, the adoption of MNPs in this step may offer substantial benefits to seed companies and nurseries.

To better understand the underlying mechanisms of such a growth stimulation on plant growth of MNPs and to uncover their unexplored molecular effects, we also conducted a transcriptome analysis on pepper seeds exposed to MNPs.

Transcriptome Analysis in Nanoprimered Seeds. Overall, global gene expression analysis might reveal cellular responses to specific environmental challenges. There is a lack of knowledge regarding how nanoparticles affect the plant transcriptome. Previous works have mostly concentrated on studying the effects of diverse nanoparticles, such as Ag NPs and Zn NPs, on adult plants in the model species *Arabidopsis thaliana*³⁹ and tomato.⁴⁰ In contrast, how priming with nanomaterials influences seed transcriptome has not yet been thoroughly investigated.

In this work, RNA sequencing (RNA-seq) was carried out to profile the transcriptomic changes associated with nanoparticle

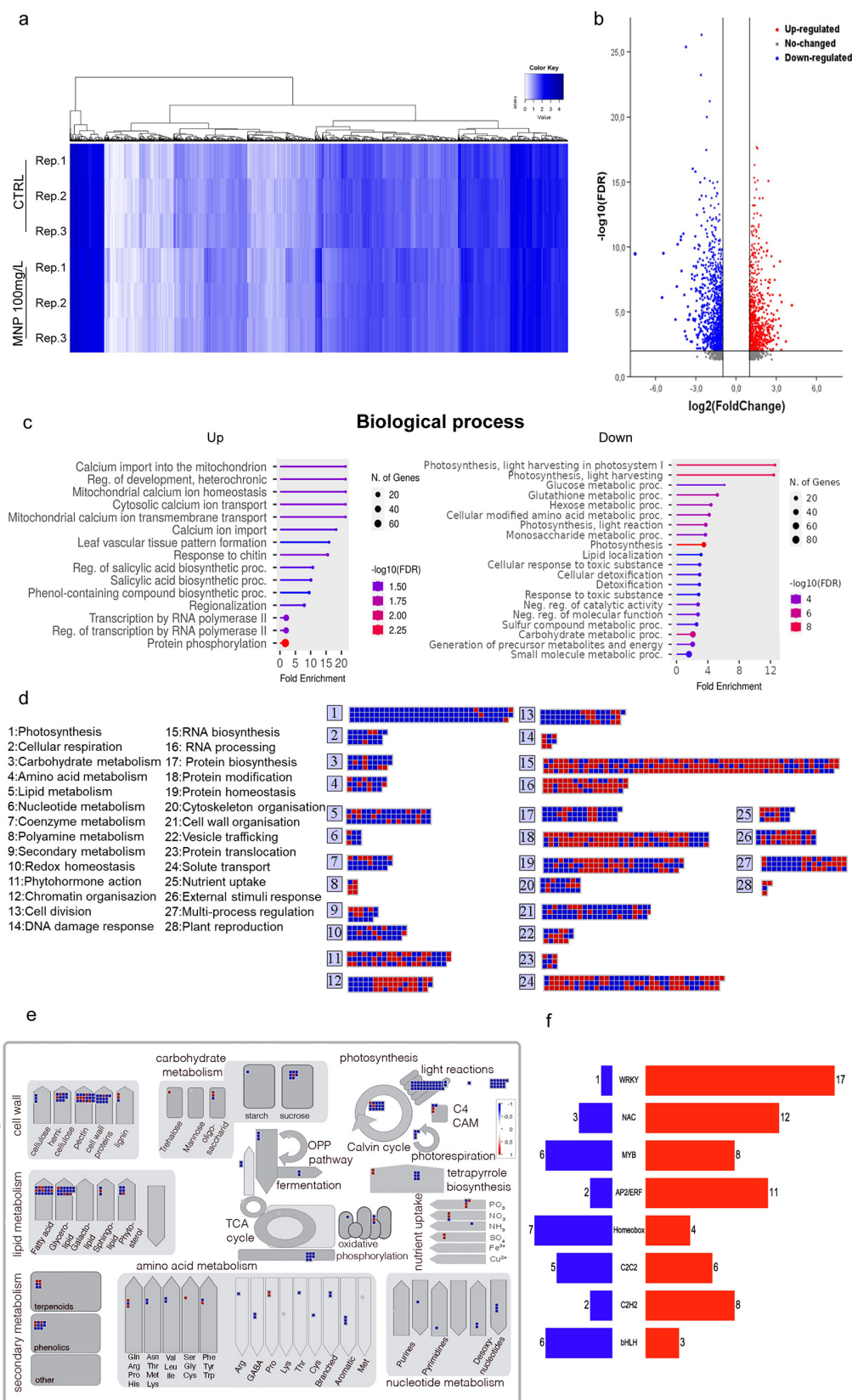


Figure 6. Transcriptome analysis of MNP-nanoprimered seeds. (a) Heatmap showing the expression levels in $\log_{10}(\text{FKPM} + 1)$ of differentially expressed genes ($\text{FDR} < 0.05$, $|\log \text{FC}| > 1$) upon MNP priming. (b) Volcano plot showing the differential gene expression in nanoprimered (100 mg/L) vs control (not treated) seeds. The data points above the significance threshold ($\text{FDR} < 0.05$, $|\log \text{FC}| > 1$) are marked in blue (downregulated in 100 mg/L) and red (upregulated in 100 mg/L), and others are marked in gray (not significant). (c) GO biological process enrichment analysis ($\text{FDR} < 0.05$). (d) MapMan pathway (bin) representation comparing up- (red) and down- (blue) regulated genes. (e) The

Figure 6. continued

“Metabolism overview” MapMan pathway employed to display expression changes of genes involved in primary and secondary metabolism upon exposure to 100 mg/L of MNP. (f) Identification and classification of the most abundant transcription factor families among the DEGs (red: upregulated; blue: downregulated).

exposure in pepper seeds. Briefly, total RNA was extracted from seeds primed with 100 mg/L of MNP for 24 h and control seeds (hydroprimed). RNA sequencing generated 12–16 million raw read pairs per library, which were deposited in the EMBL-EBI ArrayExpress database under accession number E-MTAB-13087. After trimming and filtering, 92–93% of read pairs remained, and 85–95% aligned to the ASM51225v2 reference genome (Table S2). Out of 35,845 predicted pepper transcripts, 18,395 were found active and used for subsequent analyses. RNA library counts were normalized by their size, and multidimensional scaling (MDS) revealed clear separation between control and treated conditions (Figure S6). A differential expression analysis was carried out comparing the MNP-primed seeds and the control (hydroprimed) seeds (Figure 6a). We identified 2,204 differentially expressed genes (DEGs) in MNP-treated seeds ($\text{FDR} \leq 0.05$), with 977 upregulated and 1,227 downregulated transcripts (Figure 6b and Supporting Information data set 1). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) confirmed the expression patterns of 7 genes (4 upregulated and 3 downregulated). High consistency between the RNA-seq and RT-qPCR results was found using linear regression analysis of the fold change of gene abundance in treated versus control samples ($R = 0.92$, Figure S7), indicating that the data produced through RNA-seq are reliable. Conversely, the expression changes of selected genes observed via qRT-PCR in seeds exposed to FeCl_3 were unrelated to those we observed by the RNA-seq analysis in MNP primed seeds (Figure S8), further proving that MNPs evoke a distinct biological response compared to the iron ionic counterpart.

Overall, we found that the molecular response of pepper seeds to MNPs involves an impressive higher number of genes with respect to those previously observed in adult plant tissues treated with different metallic and nonmetallic nanomaterials.^{39–42} These data suggest that, compared to treatments applied to adult plants, MNP-seed priming prompts a more extensive transcriptome reprogramming, which may prepare the embryo and the young seedling/plantlet to face unpredictable environmental conditions that might occur during the growth stages, optimizing the use of external resources and tuning their growth and development, accordingly.

In our study, the most upregulated genes in pepper seeds exposed to MNPs were *C2H2-type domain-containing protein*, *ribosomal silencing factor RsfS*, *MLO-like protein*, *Pistil-specific extensin-like protein*, *interferon-related developmental regulator N-terminal domain-containing protein*, *WRKY domain-containing protein*, and *Brr2 N-terminal helicase PWI domain-containing protein*. Conversely, among the most repressed transcripts, we found many genes involved in the photosynthesis such as *Chlorophyll a-b binding protein*, *photosystem I reaction center subunit N*, and *photosystem II reaction center X protein* (Supporting Information data set 1).

To understand the potential biological significance of the extensive set of DEGs identified, we conducted a gene ontology (GO) enrichment analysis, focusing on the “biological process” (GO-BP) categories. Results displayed in Figure 6c show that the categories relative to calcium import, transport, and

homeostasis were significantly enriched ($\text{FDR} \leq 0.05$). Besides maintaining the intracellular homeostasis, Ca^{2+} acts as a key messenger in nutrient and stress signaling, as well as in plant development and immunity.^{43,44} This supports the notion that the activation of genes involved in calcium homeostasis and transport may be interconnected with the subsequent induction of gene sets involved in plant development and stress responses (e.g., response to chitin, response to salicylic acid) observed in nanoprimed seeds. A significant enrichment was found also for the GO-BP terms related to leaf vascular tissue formation, suggesting that MNP exposure might stimulate plant differentiation processes. Not least, the significant enrichment of GO terms related to the regulation of salicylic acid biosynthetic processes and phenol-containing compound biosynthetic processes points out a potential role of MNP priming in improving plants’ defense mechanisms. We also observed that in the subset of upregulated genes “Transcription of RNA polymerase II”, “Regulation of transcription by RNA polymerase II”, and “Protein phosphorylation” were the most represented categories, which are likely responsible for the extensive remodeling of transcription and signaling processes required to cope with MNP exposure.

By contrast, among the downregulated genes, we found a very strong enrichment of GO terms related to photosynthesis and other metabolic processes such as sugar and lipid metabolism (Figure 6c). Interestingly, a significant enrichment in downregulated genes was observed for categories related to cell detoxification.

Consistent with the GO analysis, an analysis by MapMan software confirmed that downregulated genes were predominantly associated with pathways (bins) related to photosynthesis (PS I, PS II, Calvin cycle) and metabolic processes such as lipids (e.g., glycerolipids, fatty acids, and phytosterols), amino acids, sucrose metabolism, and tricarboxylic acid (TCA) cycle, as also schematized in the metabolism overview (Figure 6e). The shutdown of this class of genes in a developmental embryo phase, depending exclusively on the seed storage substances, indicates that the cell energy may converge preferentially toward the transcriptional regulation of a network of genes related to plant defense. Interestingly, MapMan analysis also revealed a repression of many genes (e.g., pectinesterases, Alpha- and Beta-galactosidases, GDSL esterases/lipases, and endoglucanases) known to be involved in cell wall organization. This is an important aspect as the cell wall is a frontline between the plant cell and the environment, and its structure and composition rapidly change when plants are subjected to biotic or abiotic stress.⁴⁵

MapMan results also showed that several DEGs mapped in the Redox homeostasis pathway. In more detail, our analysis specifically identified 19 DE glutathione S-transferase (GST) genes, with 14 being downregulated and 5 upregulated. Concomitantly, MNPs are likely to downregulate expression of other genes known to be involved in the detoxification mechanisms such as the *putative L-ascorbate peroxidase 3*, *peroxiredoxins*, and *thioredoxins*.

Based on these data, it is reasonable to hypothesize that MNP treatment induces a mild not toxic accumulation of ROS in

seeds, as generally occurs in response to various factors, including water uptake during seed imbibition, seed priming, or exposure to different environmental stimuli. In this context, ROS are intended to act as signaling molecules that regulate seed germination, dormancy, and the growth of seedlings.^{46,47}

It is important to note that the precise mechanisms through which MNPs induce ROS and the subsequent downstream effects require further investigation and elucidation.

Also, we found that the category related to solute transport exhibited a significant number of DEGs in treated seeds. In particular, calcium-permeable channel (OSCA) members serve as osmosensors in plants, enabling them to detect and react to both external and internal osmotic fluctuations. They play a crucial role in regulating plant growth and enhancing adaptability to environmental stressors.^{48,49} Not least, BIG and PIN, two key auxin transporters, were found to be upregulated in MNP-treated seeds. A previous study suggested that BIG may act synergistically with PIN1 to affect leaf growth.⁵⁰ These data, together with the overexpression of genes involved in leaf vascular formation, may in part explain the positive influence of MNPs on the vegetative growth observed in young pepper seedlings and plantlets from primed seeds.

Finally, MapMan analysis confirmed that upregulated genes were mostly involved in RNA biosynthesis and processing (including an impressive number of transcription factors), protein modification (e.g., phosphorylation), and chromatin organization processes (Figure 6d).

A total of 152 transcription factors were differentially expressed in response to MNPs. Among them, 102 were found to be upregulated and 50 downregulated. Most of the TFs belong to the bHLH, C2H2, C2C2, WRKY, MYB, Homeobox, NAC, and ERF families (Figure 6f and Supporting Information Table S3). In particular, WRKY, ERF, and NAC TFs, mostly involved in plant development and abiotic stress responses,^{51,52} were prominently induced under MNP treatment, highlighting their potential pivotal role in managing the plant response to fluctuating external conditions in advanced growth stages.

To further understand the regulatory processes governed by the identified TFs in MNP-primed seeds, we constructed a protein–protein interaction (PPI) network using the STRING database (<https://string-db.org/>), based on the integration of various approaches, including experimental validation, text mining, databases, coexpression, and neighborhood interactions.

Analyzing the downregulated TFs in the network, no significant connections or clusters were observed (Figure S9a). However, within the upregulated TFs, we identified a large cluster comprising 39 genes, including 15 WRKY, 6 AP2/ERF, and 5 NAC TFs, among others (Figure S9b and Supporting Information Table S4). This cluster exhibited strong relationships and enriched functional categories associated with the response to chitin, various stimuli, abiotic stress, as well as defense response and hormone-mediated signaling pathways such as salicylic acid (SA), jasmonic acid (JA), and ethylene (Figure S9c). These findings suggest that the use of nanoparticles could potentially induce a surge of hormone-mediated defensive responses. Accordingly, previous works have provided similar evidence, reporting that the application of NPs in plants can trigger defensive responses driven by increased levels of phytohormones (SA, JA, and abscisic acid) and the upregulation of genes involved in hormone-mediated defense signaling pathways.^{24,53–56}

CONCLUSIONS

Herein, we showed that seed priming with MNPs is a safe technology and stimulates vegetative growth, an effect not observed in pepper seeds primed with FeCl₃. This underlines the significant impact of MNP physicochemical properties on exerting distinct biological effects with respect to the ionic counterpart during seed priming, a trend often described across various fields of application. Then, we also investigated at the molecular level how seeds respond to MNP treatment, reporting an extensive gene expression change. Nanopriming led to the suppression of photosynthesis-related genes and many energy pathways associated with ATP production. Moreover, MNP exposure downregulated genes encoding enzymes involved in the detoxification of ROS, suggesting that the maintenance of a sublethal level of ROS in germinating seeds may act as plant signaling molecules that participate in various plant growth and developmental programmes. On the other hand, seed nanopriming enhances the expression of several genes encoding TFs known to regulate different defense pathways, indicating that the appropriate length and time of MNP exposure is sensed by plant cells as a sublethal stress-inducing event and triggers transcriptional changes that prime the embryo to better respond to external clues. Based on these results, we hypothesize that MNP-mediated priming may likely establish a somatic memory in primed plants that confers a greater ability to rapidly and effectively face unpredictable environmental changes that might occur during the following developmental stages. Many questions remain unanswered, including the duration of the priming effects which may vary based on plant genotypes, the potential involvement of epigenetic modifications, and the connection between MNP-induced transcriptional reprogramming and variations in seed hormonal homeostasis or ROS levels. The biotransformation of MNPs throughout various stages of plant development and their long-term effects on plant productivity are crucial aspects that demand comprehensive investigation, as well. These considerations are essential for gaining insights into the safety and sustainable utilization of MNPs in agriculture.

Not least, from a technological point of view, the results of this work have expanded the basic knowledge of the physical interaction between seeds and nanomaterials, providing valuable information for the MNP-mediated delivery of biomolecules (e.g., nucleic acids) into the seeds.

METHODS

Synthesis and Functionalization of Iron Oxide Magnetic Nanoparticles. Magnetic nanoparticles (MNPs) were synthesized following a thermal decomposition method.⁵⁷ Fe(III) acetylacetonate (15 mmol) was dissolved in dibenzyl ether (150 mL) in the presence of oleic acid (45 mmol) and 1,2-hexadecanediol (30 mmol) under a nitrogen flow and mechanic stirring. The mixture was heated to 200 °C and kept for 2 h. Then, the temperature was increased to reflux and kept for another 2 h. The mixture was cooled to room temperature under inert atmosphere, and the MNPs were washed with a mixture of hexane/ethanol four times, by magnetic separation.

To transfer the MNPs into water, 240 mg of poly(maleic anhydride-alt-1-octadecene) (PMAO) (MW: 30–50 kDa) was dissolved in a round-bottomed flask with 100 mL of CHCl₃ and MNP (10 mg of Fe) added dropwise.²⁵ When fluorescent MNPs were needed, the polymer was previously modified with TAMRA as previously reported.⁵⁸ The final mixture was sonicated for 15 min at room temperature, and thereafter CHCl₃ was evaporated using a rotary evaporator at 40 °C. In order to hydrolyze the anhydride groups present in the polymer and to confer stability in water to the MNPs, 30 mL of NaOH 0.05 N was

added to the mixture, heated at 70 °C, and evaporated until reaching a final volume of 20 mL. The suspension was filtered using a Millipore filter (0.22 μ m) to remove MNP aggregates, and the excess of polymer was eliminated by four consecutive ultracentrifugations at 70000g per 2 h each. The resultant nanoparticle suspension MNP@PMAO was stored at 4 °C.

To functionalize the MNPs with PEG, MNPs (0.5 mg of Fe) previously coated with PMAO were mixed with 18.5 μ mol of α -hydroxy- ω -amino PEG (MW: 5000 Da). Functionalization was carried out using 40 μ mol of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in a borate-buffered saline (SSB) solution (50 mM, pH 9).²⁵ The mixture was kept at 37 °C for 3 h. At that point, the MNPs were washed from the excess of reagents using ultracentrifugal filters (Amicon, Millipore, 100 kDa cutoff) and stored at 4 °C.

Seed Nanoparticle Treatment. Pepper seeds (*Capsicum annuum*, cv Friariello, TopSeed) were treated with 100 and 200 mg/L of MNPs. Typically, 25 seeds were placed into a tube containing 1 mL of a given MNP suspension (Figure S10) and incubated on a rotator at room temperature in the dark for 24, 48, or 72 h. Control seeds were kept in Milli-Q water and maintained as treated seeds (Figure S10). After the treatment, the seeds were surface sterilized using a 1% sodium hypochlorite solution for 2 min to prevent microbial contamination and then extensively rinsed in distilled water.

Fe Quantification by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). To estimate the extent of MNP uptake, five seeds were incubated for 24 h with 100 mg/L or 200 mg/L of Fe supplied as MNPs, then washed, ground in liquid nitrogen, and digested with 250 μ L of HNO₃ (65% purity) and incubated during 2 h at 95 °C. After this time, 150 μ L of HNO₃ and 100 μ L of H₂O₂ (33% w/v) were added to the samples and digested overnight at 95 °C. Once digested, the samples were diluted up to a volume of 15 mL and were quantified by ICP-AES (Horiba Jobin Yvon) in duplicate and analyzed with the software Activa Analyst 5.4.

Fluorescence and Scanning Electron Microscopy. The internalization of MNPs@PMAO@TAMRA@PEG in pepper seeds was investigated by fluorescence microscopy (Axioplan 2, Zeiss). Images were acquired with an Axiocam (Zeiss) camera and analyzed by Axiovision software (rel. 4.8, Zeiss). For scanning electron microscopy, three seeds for each treatment were extensively washed, fixed with 1 mL of 4% paraformaldehyde, and kept at 4 °C for 24 h. Seeds were spotted on carbon-coated electron microscopy grids. Samples were dried with nitrogen flux under a chemical fume hood. SEM images of the samples were then acquired using the Tescan S8000 microscope equipped with secondary electron and backscattered electron detectors (TESCAN s.r.o., Brno, CZ). Analyses were conducted at 1.5 and 5 keV without any coating of the particles, respectively. SEM images were taken from three independent experiments.

Magnetic Resonance Imaging (MRI) of Pepper Seeds. All ¹H MRI experiments were performed at 298 $\pm$ 1 K on a 300 MHz Bruker Avance wide-bore magnet (BrukerBiospin, Rheinstetten, Germany), equipped with a 1.5 mm μ -imaging MICRO 5 probe working at a ¹H frequency of 300.13 MHz. Seed samples were loaded into 15 mm sterilized NMR tubes (Norell – borosilicate). Treated and control seeds were loaded in the same NMR tube (at least 20 seeds per treatment) and examined concomitantly, to minimize any instrumental influence and emphasize possible evidence ascribable to the NP presence. In all cases, a rigid and transparent polypropylene layer (126 μ m thick) was inserted into the tube to separate control from treated seeds, and a blue spongy and inert material with a semicircular shape was put to the tube bottom to easily recognize the tube side containing the control seeds (Figure S11). NMR tubes were tightly closed with a cap during the experimental acquisitions to limit the extent of water evaporation.

Evaluation of Embryo Morphology. Under a stereomicroscope, the coat of treated and control seeds (five for each treatment) was removed by making a longitudinally shallow cut all around the seed with a razor blade. Secondary cuts were performed to free the embryo from the endosperm. Images of embryos were obtained under an optical microscope, and morphological traits (diameter of the hypocotyl, HyP;

total length of the embryo, EL; distance between root and shoot apical meristems, DRS) were measured with ImageJ software.

Germination Analysis and Plant Growth Measurements. Seeds were treated with 100 and 200 mg/L of MNPs and equivalent Fe concentrations supplied as salt (FeCl₃) for 24, 48, and 72 h. After the treatments, the seeds were mildly surface-sterilized using a solution of sodium hypochlorite (0.45% NaOCl) and then placed in 10 cm Petri dishes on a filter paper (Whatman International Ltd., Maidstone, UK) and soaked in 2 mL of distilled water renewed every other day. The seeds were kept in the dark for 13 days at 23 °C in a growth chamber. Fifty seeds (10 seeds per plate) were sown for each treatment, and the percentage of germination was calculated. The data were collected over a period of 13 days post-treatment. Germination was evaluated as protrusion of the primary root. Germination tests were conducted in 5 independent biological experiments, each comprising 5 technical replicates.

To evaluate the root elongation after NP treatment, photographs of 13-day-old pepper seedlings grown on filter paper were taken and analyzed by the ImageJ software. For each treatment, the root length of ten seedlings was measured. Five independent experiments in technical triplicate were carried out. Leaf area was measured on plants 40 days after transplanting, using the ImageJ software to analyze the photographs of the displaced leaves on a white background.

Chlorophyll Index. Four-week-old pepper plants were grown in pots containing universal soil (Triplo Natural Bio, TerComposti, Italy) in a growth chamber at 23 °C under a 16 h light/8 h dark photoperiod. Pepper plants of the same developmental stage were maintained in the condition of a regular water regime. Plants of the same developmental stage were maintained in condition of regular water regime. Soil Plant Analysis Development chlorophyll meter (SPAD, Minolta Camera Co., Osaka, Japan) was employed to determine the chlorophyll index. SPAD readings were taken at least in technical triplicate in five fully expanded upper leaves for each plant. At least three plants from each experimental condition were used in three independent experiments.

RNA Extraction, Library Preparation, and Sequencing. Pepper seeds were incubated with 100 mg/L of MNPs and placed on a rotator at room temperature in the dark for 24 h. Control seeds were kept in Milli-Q water and maintained as treated seeds. After the treatment, seeds were rinsed in distilled water and placed in 10 cm Petri dishes on filter paper (Whatman International Ltd., Maidstone, UK) soaked in 2 mL of distilled water. After three days, total RNA was extracted from 10 seeds using 1 mL of TRIzol extraction buffer (Thermo Fisher Scientific, Wilmington, DE, United States) according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States). The RNA extracted from each sample was treated with DNaseI (NEB) and purified using a specific silica-based method following the manufacturer's instructions (Monarch RNA Cleanup kit – NEB). The quality of the processed extracts was assessed using a Bioanalyzer 2100 system (Kit RNA 6000 Nano – Agilent Technologies). A total of six extracts (three biological replicates for each treatment) were used for library preparation. Libraries were processed according to Illumina mRNA Library Prep kit instructions, and a dual index combination was used for each sample for barcoding. The concentration and quality of the libraries were evaluated using a Qubit 4 Fluorometer (dsDNA High Sensitivity Kit, Invitrogen) and Bioanalyzer 2100 system (HS DNA kit – Agilent Technologies). A sequencing was then run on the Illumina Novaseq6000 platform using a Novaseq 6000 S1 Reagent Kit (2 $\times$ 100 + 10 + 10 bp parameters).

RNA-Seq Data Elaboration and Differential Expression analysis. The raw reads quality of the six RNA-Seq libraries was assessed using FastQC v0.11.9,⁵⁹ while adaptor sequences and low quality bases were removed using Trimmomatic v0.39⁶⁰ with these parameters: HEADCROP:1 LEADING:3 TRAILING:3 SLIDING-WINDOW:4:18 MINLEN:40. The filtered RNA reads of the six libraries were mapped to *Capsicum annuum* CM334 reference genome assembly ASM51225v2⁶¹ downloaded from the Ensembl Plants Web site (<https://plants.ensembl.org/>, accessed on 08 March 2023). Reads counts were generated from the alignment files using featureCounts v2.0.3⁶² with default parameters based on the “exon” feature and

“transcript_id” meta-feature of Pepper Genome Annotation (PGA) gene predictions retrieved from the EnsemblPlants Web site (<https://plants.ensembl.org/>, accessed on 08 March 2023).

Differential expression analyses were carried out using Bioconductor EdgeR v3.38.1⁶³ that was used to filter out the not expressed or poorly expressed transcripts (a transcript was considered “active” if reads per million mapping to that transcript was >1 in at least two RNA libraries), normalize the RNA libraries, and do the differential expression analysis between treated and control samples. The transcripts with resulting false discovery rate (FDR) lower than 0.05 and log₂(Fold Change) (LFC) lower than −1 or higher than 1 were considered as differentially expressed.

Functional Annotation and Enrichment Pathway Analyses of DEGs. Gene ontology analysis was performed using ShinyGO V0.77 (Ge, S.X.; Jung, D.; Yao, R. *Bioinformatics* **2020**, *36*, 2628–2629) with a FDR cutoff 0.05. The MapMan software (version 3.5.1 R2)⁶⁴ was used to annotate functional MapMan bin codes (BINs) of DEGs. The protein–protein interaction (PPI) profile of the 102 upregulated and 50 downregulated TFs was obtained from the STRING webserver (<https://string-db.org/>). To cluster the most similar nodes of the network into an easily distinguishable function-based classification, we employed the Markov Cluster Algorithm (MCL) for graphs. This is based on simulation of stochastic flow in the obtained graph. For achieving a balance between sensitivity and selectivity, we set the inflation factor at 1.1.

Validation of Differentially Expressed Genes by Quantitative Polymerase Chain Reaction. Reverse transcribed DNase-treated total RNA (1 μg) was obtained using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, United States). qRT-PCR was performed in a QuantStudio 5 Real-Time PCR Instrument. Each PCR reaction consisted of 1 μL of 1:20 diluted cDNA, 5 μL of 2X PowerUp SYBR Green Master Mix (Applied Biosystems, CA, United States), and 0.4 μM of each gene-specific primer in a total volume of 10 μL. All specific primer pairs were listed in Table S5. The thermal cycling conditions were 50 °C for 2 min (one step), one cycle at 95 °C for 2 min, followed by 40 cycles of two steps at 95 °C for 15 s, and 56 °C for 15 s. Three technical repetitions were performed for each sample. To normalize gene expression values, *actin* (AY572427) and glyceraldehyde-3-phosphate dehydrogenase (AJ246013) genes were used as internal control, whose expression upon MNP treatment was confirmed to be stable by RNA-seq. Expression was calculated by the 2^{−ΔΔCT} method.⁶⁵ Expression levels of selected transcripts were measured in seeds primed with 100 mg/L of MNPs or with an equivalent Fe concentration supplied as FeCl₃. Control seeds were used as calibrator samples.

Statistics and Reproducibility. Sample size, number of replicates, and statistics tests for each experiment were described in the figure legends, where possible. Otherwise, these data were properly reported throughout the Methods section. Error bars in the graphical data represent standard deviations calculated on the mean of at least three independent experiments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.3c06172>.

Additional data (images and tables) about MNP characterization, MNP internalization in seeds, MRI setup, physiological and morphological effects of seed priming with FeCl₃, RNA-seq analyses, qRT-PCR analyses, and the list of primers (PDF)

Movie of the disposition of all whole seeds in the NMR tube, appreciable through a 3D reconstruction (MOV)

List of 2,204 differentially expressed genes (DEGs) in MNP-treated seeds (FDR ≤ 0.05), with 977 upregulated and 1,227 downregulated transcripts (XLSX)

AUTHOR INFORMATION

Corresponding Author

Alfredo Ambrosone — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy; orcid.org/0000-0002-1897-4028; Email: aambrosone@unisa.it

Authors

Elisa Cappelletta — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy

Carmine Del Regno — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy

Marisa Conte — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy

Christian Castro-Hinojosa — Instituto de Nanociencia y Materiales de Aragón, INMA (CSIC-Universidad de Zaragoza), Zaragoza 50009, Spain

Susel Del Sol-Fernández — Instituto de Nanociencia y Materiales de Aragón, INMA (CSIC-Universidad de Zaragoza), Zaragoza 50009, Spain

Chiara Vergata — Department of Biology, University of Florence, Sesto Fiorentino 50019, Italy

Matteo Buti — Department of Agriculture, Food, Environmental and Forestry Sciences (DAGRI), University of Florence, Firenze 50144, Italy

Rossella Curcio — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy

Anil Onder — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy; orcid.org/0000-0002-7407-6409

Pierluigi Mazzei — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy; orcid.org/0000-0002-5312-4969

Nicola Funicello — Department of Physics ‘E.R. Caianiello’, University of Salerno, Fisciano 84084, Italy

Salvatore De Pasquale — Department of Physics ‘E.R. Caianiello’, University of Salerno, Fisciano 84084, Italy

Mattia Terzaghi — Department of Biosciences, Biotechnologies and Environment, University of Bari Aldo Moro, Bari 70121, Italy

Pasquale Del Gaudio — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy

Antonietta Leone — Department of Pharmacy, University of Salerno, Fisciano 84084, Italy

Federico Martinelli — Department of Biology, University of Florence, Sesto Fiorentino 50019, Italy

Maria Moros — Instituto de Nanociencia y Materiales de Aragón, INMA (CSIC-Universidad de Zaragoza), Zaragoza 50009, Spain; Centro de Investigación Biomédica en Red de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Madrid 28029, Spain; orcid.org/0000-0002-2861-2469

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acsnano.3c06172>

Author Contributions

■ These authors contributed equally.

Notes

The authors declare no competing financial interest.

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