

DOTTORATO DI RICERCA IN

Scienze Agrarie e Ambientali

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*Biotechnological procedures and "green extraction"
strategies for the sustainable conversion of by-products,
residues and wastes from agroforestry and agrifood sectors
into renewable resources to support innovative agriculture
and plant protection (Btech&green)*

Settore Scientifico Disciplinare

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Overview of the work

Climate change is one of the main systemic challenges of the 21st century, with profound and multidimensional implications for agricultural systems on a global scale. Rising average temperatures, increasing climate variability, and the intensification of extreme weather events are significantly altering the environmental conditions in which agricultural activities occur, affecting productivity, yield stability and the long-term sustainability of agri-food supply chains. Changes in rainfall and hydrological patterns, the increased frequency and intensity of droughts and heat waves, and greater seasonal unpredictability pose significant challenges for natural resource management and crop planning. At the same time, climate change is facilitating the spread of plant diseases and harmful organisms in areas previously unaffected, thereby amplifying the risks to food security and the resilience of rural communities. In this context, agriculture is both highly vulnerable to climate change and a strategic sector for implementing mitigation and adaptation measures. Its dual role requires a rethinking of traditional production models. Sustainable development is therefore central, aiming to balance ecosystem protection, economic efficiency, and social equity. Sustainable agriculture goes beyond reducing environmental impacts, seeking to strengthen the resilience of agri-food systems and ensure long-term resource availability and food security. Within this strategic framework, the circular economy emerges as a key paradigm for transforming agricultural and forestry systems. Moving beyond the linear model of resource extraction, use and disposal, the circular economy encourages the valorization of by-products and supply chain residues, promoting the closure of material and energy cycles. In particular, in the agricultural and forestry sector, the valorization of residual biomass represents a concrete opportunity to reduce the environmental impact of production processes and to create new value chains with a strong focus on innovation. Among the most interesting lignocellulosic biomasses, chestnut (*Castanea sativa* Mill.) residues are particularly notable due to their widespread distribution and their unique chemical properties. The chestnut tree is a species historically and culturally associated to the mountainous and hilly areas of the Tuscan Apennines, where it has played a central role in the rural economy and landscape management for centuries. Forest management practices, as well as wood and fruit processing activities, generate large quantities of residues, including wood, bark, husks and leaves, which represent a potentially valuable resource within the framework of the circular economy. From a chemical point of view, chestnut is particularly rich in phenolic compounds and polyphenols, including hydrolysable and condensed tannins, known for their antioxidant, antimicrobial and biologically active properties. Numerous studies have shown that extracts obtained from different chestnut matrices have high bioactive potential, making them suitable for a wide range of applications. In agriculture, these extracts can be used as biostimulants, natural defence agents or functional soil improvers, contributing to improved plant health, reduced use of synthetic chemical inputs and enhanced crop resilience to biotic and abiotic stresses.

The valorization of chestnut residues through sustainable extraction processes is fully in line with the principles of the circular economy and the bioeconomy, offering a concrete strategy for combining environmental protection, technological innovation and territorial development. In this sense, the study of the chemical and biological properties of chestnut residues and the potential applications of their extracts is an area of research of high scientific and practical relevance.

Another area for the exploitation of woody biomass, including chestnut residues, is represented by thermochemical processes, in particular pyrolysis. Various woody matrices from forestry and agro-industrial activities are used as feedstock in these processes, which aim to convert organic matter into products with higher added value. Pyrolysis yields three main fractions: biochar, synthesis gas and a condensable liquid phase, commonly known as wood vinegar. The latter is a by-product rich in organic compounds, including organic acids, phenols and other bioactive substances, whose composition depends strictly on the biomass of origin and the operating conditions of the process.

In agriculture, wood vinegar has attracted growing interest for its potential applications as a soil conditioner, biostimulant or crop defence support agent, in line with strategies to reduce chemical inputs and promote sustainable agricultural practices.

Therefore, the integration of climate change, sustainable development, circular economy and the valorization of chestnut residues offers a promising prospect for the transition to more resilient and sustainable agricultural systems, capable of responding to contemporary environmental challenges and generating economic and social value at local and global levels.

This PhD project focused on commercial wood vinegars derived from pyrolysis processes using chestnut as the starting biomass, as well as a laboratory-scale extract of chestnut wood chips. Wood chips are primarily used for energy production, as natural mulch in agriculture and gardening to control weeds and retain moisture, for compost preparation, as absorbent bedding in animal husbandry, and in sustainable construction. In this study, chestnut wood chips were supplied by Azienda Agricola Forestale Orlandini Antonio (Saturnana, Pistoia, Italy), which has been producing woodchips as mulch for nurseries for several years. The aim of the extraction procedure here developed was to obtain a tannin-rich product with high bioactive value, while preserving the spent woodchips for continued use as mulch.

The purpose of this thesis was to evaluate the biological value of these extracts, understand their potential, unveiling their effects at both microbial and plant levels. Wood vinegar served as a kind of positive control, providing a reference with well-characterized properties, although the distinction between biostimulants and corroborants is subtle, and deep scientific research is essential to confirm unequivocally its bioactivity. Assessing the potential and effective applications of these chestnut-derived products represent a valuable strategy for their direct use in marginal areas where this woody biomass is generated.

Critical insights on wood vinegar

Although there are virtually no previous studies on the application of woodchip extracts, the biological activity of its components is quite clear.

Wood vinegar, on the other hand, has been studied much more extensively, but all this work has produced a wide range of possible applications that need further clarification. Its chemical characterization depends on a wide variety of factors. Different temperatures, starting biomasses and timings can result in considerable variability in the final product. There are many areas of application, ranging from improving soil and plant health, through potential herbicidal activity, to use in plant defence management with control of both biotic and abiotic stress. To provide a thorough assessment of its potential applications, we conducted a systematic and exhaustive review of the literature. However, we took a critical approach, focusing on possible variables and the fact that it can have both species-specific and dose-dependent effects.

Starting from the bottom: an in vitro unveiling study

At the outset of our investigation, we designed a series of experiments aimed at elucidating the mechanisms of action of chestnut-derived products. To achieve a controlled evaluation, *in vitro* assays were conducted under aseptic conditions, allowing precise observation of interactions between the substances and both bacterial and plant systems. Chestnut wood vinegar by Biodea® (Arezzo, Italy) (WV) and chestnut woodchip extract (WCE) were systematically tested using a broad range of *in vitro* approaches, including antibacterial and anti-virulence assays against multiple microbial strains, as well as phytotoxicity analyses based on seed germination and early seedling development. Additionally, experiments with *in vitro* model plants were performed to examine morphological changes and the expression of selected genes involved in plant defense responses.

The combined use of these *in vitro* methodologies provided a rapid, reproducible, and cost-efficient framework for assessing the biological potential of the chestnut-derived products. Results indicated that WV exhibited pronounced dose-dependent effects, with significant antibacterial activity under conditions simulating plant infection, inhibitory effects at high concentrations, and growth-promoting activity at lower doses, highlighting its dual potential as a phytotoxic agent and plant growth enhancer. Conversely, WCE primarily functioned as a biostimulant, promoting plant growth and activating defense- and hormone-related pathways, while displaying only modest antimicrobial properties.

Chestnut extracts for soil health and plant response to abiotic stress

Following the initial *in vitro* evaluations, experiments were conducted under standard growth conditions to assess the effects of chestnut-derived products on soil properties and plant performance under abiotic stress, specifically drought and salinity. After characterizing the chemical composition of the products and analyzing their influence on soil, a plant-soil experiment was established using baby lettuce (*Lactuca sativa* var. baby) to investigate the impact of two different commercial chestnut wood vinegars (WV by Biodea® and NB by Nerabiochar® (Turin, Italy)), and WCE on plant growth and soil physicochemical parameters under stress conditions. Wood vinegar has drawn considerable attention as a potential agricultural input due to its reported ability to enhance crop yield, quality, and overall plant health. The results indicate that all products exhibit biostimulant activity, particularly under abiotic stress, without detrimental effects on soil quality. While no significant benefits were observed under optimal growth conditions, treated plants under drought stress achieved yields comparable to non-stressed controls. Under saline conditions, the most notable effects were observed at the soil level, including enhanced enzymatic activity, suggesting a positive modulation of soil functionality in response to the treatments.

Chestnut extracts and plant immunity

Building on the promising *in vitro* and plant-soil results, which highlighted the biostimulant and stress-mitigating potential of chestnut-derived WV, NB and WCE, we extended the investigation to field conditions. This study aimed to evaluate their effects on plant growth, physiology, and defense responses in tobacco (*Nicotiana tabacum*), used as a model species, and tomato (*Solanum lycopersicum*), an agronomically relevant crop. Plants were cultivated under both standard and salt stress conditions to assess treatment efficacy under abiotic stress. Physiological analyses, including biomass accumulation, chlorophyll content, and stomatal conductance, revealed dose-dependent responses. An accurate gene expression study indicated significant modulation of defense-related pathways, including activation of pathogenesis-related proteins. Overall, these findings confirm that wood vinegar and WCE can enhance plant defense mechanisms and support physiological performance under stress, reinforcing their potential as eco-friendly tools for sustainable crop management.

In summary, this work demonstrates that chestnut-derived wood vinegar and woodchip extracts possess significant biological potential, acting as biostimulants, growth enhancers, and modulators of plant defense. *In vitro*, soil, and field experiments demonstrated dose-dependent effects, improving plant physiological performances under abiotic stress without compromising soil health. WV exhibited two main features, that are antimicrobial and growth-promoting bioactivities, while WCE primarily stimulated plant growth and defense-related pathways. These findings highlight the value of integrating chestnut biomass valorization with sustainable agricultural practices. Overall, this study provides strong evidence that chestnut-derived products can serve as eco-friendly tools

for enhancing crop resilience, supporting circular economy principles, and fostering sustainable development in marginal and productive agricultural systems.

1. Introduction

Review

Is wood vinegar really all sunshine and roses?

Is wood vinegar really all sunshine and roses?

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Abstract

The increasing need for sustainable agricultural practices and the valorization of renewable resources has renewed interest in biomass-derived products. Among these, wood vinegar (WV), a liquid by-product of lignocellulosic biomass pyrolysis, has recently emerged as a promising bio-based input for agriculture. Although pyrolysis products such as biochar and syngas have been extensively investigated, WV has historically received comparatively less scientific attention, despite its long-standing use in traditional farming systems, particularly in Asia. WV is a chemically complex mixture, rich in organic acids and phenolic compounds, whose composition and bioactivity are strongly influenced by feedstock characteristics and pyrolysis conditions. In recent years, a rapidly expanding body of literature has highlighted the potential of WV as a plant growth promoter, soil amendment, and biotic and abiotic stress management tool. However, reported effects are often context-dependent and sometimes inconsistent, reflecting variability in WV composition, application rates, target organisms, and environmental conditions. In light of the sharp increase in scientific output and growing commercial interest, an updated and critical synthesis of WV bioactivity is needed. This review aims to provide a comprehensive and integrative assessment of the effects of wood vinegar on soil properties, plant performance, and stress mitigation, identifying key mechanisms, limitations, and knowledge gaps to support its rational and sustainable use in agriculture.

Keywords: wood vinegar; pyroligneous acid; sustainable agriculture; plant biomass pyrolysis

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1. Introduction

In response to growing global environmental and public health challenges, many countries are increasingly promoting the use of renewable natural resources. Among the most promising strategies is the valorization and the strategic management of plant biomass. It is estimated that approximately 200 billion tons of lignocellulosic biomass are generated annually worldwide from industrial, forestry, and agriculture activities, with forestry and agricultural residues representing a major fraction of this biomass in most agriculturally relevant countries [1].

Biomass can be converted into gaseous, liquid, and solid fuels through thermochemical processes such as carbonization, gasification, pyrolysis [2–4]. Among these, pyrolysis has attracted particular attention. During this thermochemical process, lignocellulosic macromolecules undergo thermal degradation, resulting in the formation of three main

products: pyrolysis gas, biochar, and wood vinegar (WV) [2]. Pyrolysis involves the industrial conversion of biomass at high temperatures (typically 400–500°C) under condition of limited oxygen availability [5]. While extensive research has been conducted on the properties and applications of pyrolysis gases and biochar [6–9], WV has received comparatively less attention and has only begun to be systematically investigated in recent years.

WV, also referred to as wood distillate, pyroligneous acid, or wood smoke is known by various names depending on the context and region [10]. In South Korea and Japan, it is commonly referred to as "Mokusaku-eki". WV is a dark-colored liquid composed primarily of water (80–90%) and contains more than 200 natural water-soluble compounds. The organic phase includes many chemical classes, such as organic acids, alcohols, aldehydes, ketones, ethers, esters, phenols, sugars, alkenes, furans, and other oxygenated compounds [2].

The chemical composition of the pyrolysis product is highly variable and depends largely on the nature of the raw material. During pyrolysis, three polymers of plant biomass - cellulose, hemicellulose, and lignin - are rapidly fragmented and depolymerized, generating molecules of varying size and chemical complexity [11]. Moreover, WV characteristics are influenced by several process parameters, including residence time, heating rate, temperature, particle size, and source of feedstock [12,13]. Acetic acid is the major constituent of WV, accounting for nearly 50% of its composition and conferring its characteristic low pH (<3) [14]. The strong aroma is primarily associated with ketones, esters and phenolic, furan, pyran, and nitrogen-containing derivatives, whereas its flavor is mainly related to phenol and alkyl phenol derivatives, as well as guaiacol, alkyl guaiacol, syringol, and alkyl syringol derivatives [13].

Despite the limited number of studies addressing WV application in agroecosystems, the product has long been regarded as a corroborant and a plant protection tool, particularly in Asia [15]. WV has been used in agriculture for centuries due to its wide range of beneficial properties. Several studies have demonstrated its ability to increase crop yields across multiple plant species, enhance seed germination, and promote plant growth. Its chemical composition confers antimicrobial activity, making WV effective against various fungal and bacterial pathogens and valuable in plant disease management. Moreover, an increasing body of evidence highlights its potential in deterring insect pests. Beyond biotic stress control, recent studies have also explored WV's role in mitigating abiotic stresses, including drought, salinity, and low-temperature conditions. WV further contributes to soil health by stimulating microbial activity and has been applied as an organic fertilizer, a pesticidal agent, and, more recently, as a natural herbicide.

The growing scientific and economic interest in WV is well documented by the bibliometric analysis conducted by Mhamdi (2022) [16], which examined publication trends from 2002 to 2022. Over this period, the number of publications increased markedly, from 2 in 1949 to 84 in 2025 (Figure 1). This geographical distribution of publications further highlights the uneven but rapidly expanding global interest in wood vinegar research. China clearly dominates scientific output, followed by several Asian countries, confirming the central role of Asia in both the historical development and current advancement of WV applications (Figure 2). This rising interest is mirrored by market data: in 2024, the global WV market was valued at USD 5.21 billion and is projected to grow at a compound annual growth rate (CAGR) of 5.30% between 2025 and 2033, reaching USD 8.32 billion by 2033. The Asia-Pacific region currently dominates the market, accounting for over 39.2%, driven by strong agricultural activity, abundant wood resources, and the widespread adoption of sustainable farming practices.

This review therefore aims to critically assess the current state of knowledge on WV bioactivity. Extensive work has already been devoted to WV production processes and

chemical characterisation [13,15,17], and further reviews on these aspects would be redundant. The most influential review on WV biological effects remains that of Grewal (2018) [14]. However, between 2019 and 2022 alone, 229 papers were published, representing nearly half of the total publications recorded between 2002 and 2022 [16]. This rapid expansion highlights the need for an updated and integrative overview to identify remaining knowledge gaps. Recent reviews published in 2024 have focused on plant disease management and termite control [18,19].

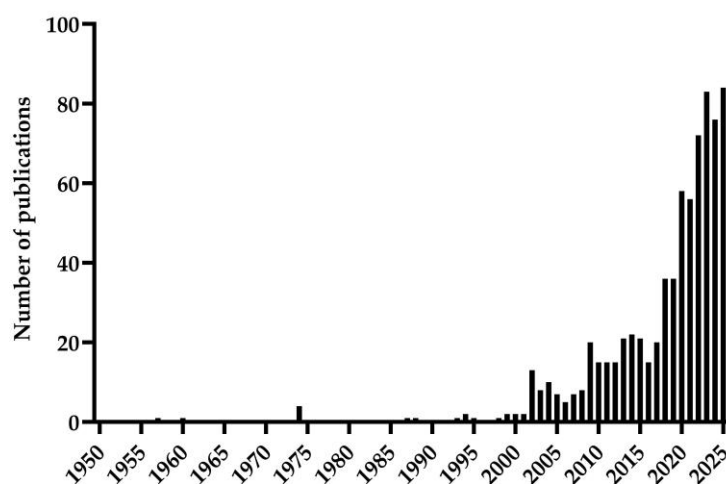


Figure 1. The number of publications per year reported in Scopus from 1949 to 2025, found by typing the keywords “Wood vinegar” in Title, Abstract or Keywords. It is interesting to note the progression from the two papers published in 1949—the first records on the topic indexed in Scopus—to 84 publications in 2025. (Data retrieved from Scopus in December 2025).

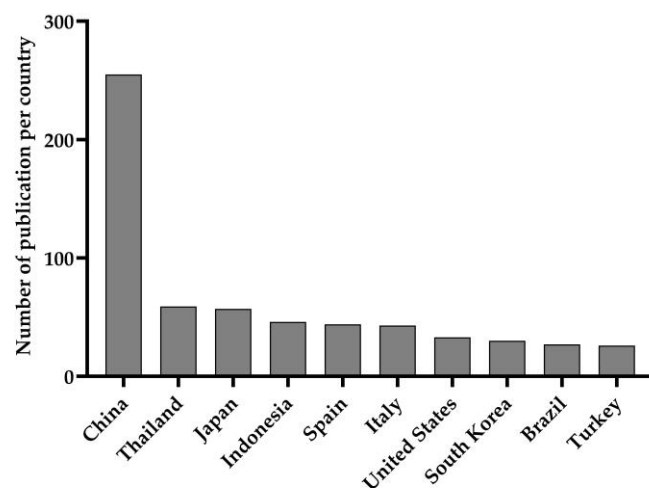


Figure 2. Number of publications on wood vinegar indexed in Scopus by country of publication. Data were retrieved by searching the keywords “Wood vinegar” in the title, abstract, or keywords fields. The figure shows the top 10 countries with the highest number of publications. China leads by a wide margin, followed by Thailand, Japan, and Indonesia. Data retrieved from Scopus in December 2025.

Building on these contributions, the present work provides a clear and concise synthesis with a specific focus on WV effects on soil, plant performance, and biotic and abiotic stress management. Particular emphasis is placed on a critical evaluation of the literature,

as numerous inconsistencies and context-dependent variables must be carefully considered despite the generally positive outcomes reported.

2. On soil health impact

For many years, scientific evidence regarding the effects of WV on soil health has been limited. Nevertheless, WV has long been hypothesised to improve soil physical and chemical properties, primarily due to its ability to stimulate soil microbial activity. Over the past five to ten years, interest in this topic has increased (Figure 3); however, the effects of WV on soil health remain difficult to generalise. This inconsistency is likely attributable to the wide variability in soil properties and to heterogeneous experimental conditions. WV is characterised by an acidic pH and a high content of organic compounds with potential bioactivity against microorganisms, as demonstrated by several studies [20]. By altering soil pH, this complex mixture may indirectly influence nutrient availability and microbial-mediated processes.

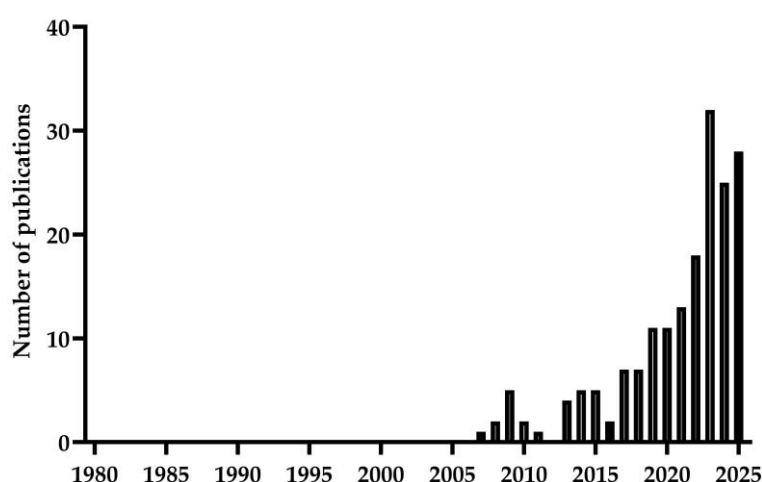


Figure 3. The number of publications per year reported in Scopus from 1949 to 2025, found by typing the keywords “Wood vinegar on soil” in Title, Abstract or Keywords. It is interesting to note that interest in the soil system essentially began around 2005. (Data retrieved from Scopus in December 2025).

Soil-applied WV has been shown to increase dissolved organic carbon (DOC) and microbial biomass, stimulate enzymatic activities, and enhance nitrate and available phosphorus concentrations in soil [21]. However, evidence regarding soil enzyme responses is contradictory. WV application significantly increased urease activity in bulk soil in some studies [22] whereas other authors reported a suppressive effect comparable to that of synthetic urease inhibitor [23]. The mechanisms underlying WV–enzyme interactions remain unclear; inhibitory effects on asparaginase [23] and stimulatory effects on β -glucosidase, invertase, and alkaline phosphatase have been reported [24].

WV addition has also been shown to modify soil bacterial community composition at the phylum, family, and genus levels. Several studies have documented increased growth [25] and metabolic activity of specific genera, such as *Pseudomonas* and *Nitrosomonas* [26]. According to Zhou *et al.* 2022, WV application increased the relative abundances of genera associated with plant growth promotion and metal mobilization, such as, *Bacillus*, *Gemmatimonas*, *Streptomyces*, *Sphingomonas*, and *Polycyclovorans* [27].

Also in microcosm, WV optimized the structure of the microbial community and enhanced system stability and resilience. Carbon and nitrogen metabolic processes were

significantly strengthened, with key genes and enzymes involved in nitrification and denitrification being highly enriched [28].

At lower concentration, WV improves soil physicochemical properties and supports the soil microbiome, particularly by enhancing populations of plant growth-promoting rhizobacteria (PGPR) [29]. Conversely, higher WV concentrations (5%) favour the proliferation of spore-forming bacterial genera such as *Bacillus*, which play a key role in plant protection against pests and diseases. These findings suggest that WV may enhance soil biological health by selectively promoting beneficial plant PGPB [29]. Moreover, WV application has been associated with increased densities of beneficial microorganisms such as actinomycetes and *Trichoderma*, which contribute to the suppression of damping-off diseases caused by *Rhizoctonia* and *Pythium* [30].

Finally, the combined effect of WV and biochar (BC) on soil health have been investigated. In saline-alkaline soils, the combined application of WV and BC improved soil physical and chemical properties [31], altered the composition of inorganic phosphorus-solubilising bacteria, and increased their diversity, particularly with more frequent WV application. Significant increases in available phosphorus and in alkaline, acid, and neutral phosphatase activities were also observed. In basil cultivation, WV and BC significantly increased organic carbon (OC) and microbial biomass carbon (MB-C), with BC preferentially retaining DOC species and WV-derived organic compounds in the BC-WV treatment. Their combined application notably stimulated phosphatase and urease activities, indicating that appropriate WV–BC combinations enhance soil microbial activity and overall soil biological quality [32]. Similar results were observed in cotton, where WV alone was more effective than BC alone, and the combined WV–BC treatment produced the strongest positive effects [33].

3. Direct effect on plant

WV is widely used in agriculture for its corroborating properties. It is the most studied aspect of WV in agriculture (Figure 4), however, its effects on plants are multifaceted and strongly influenced by several factors. Plant responses vary according to species, developmental stage, applied dose, application method, and cultivation strategy. WV effects have been evaluated at multiple levels, ranging from seed germination to plant growth and yield, and ultimately to the nutritional quality of harvested products.

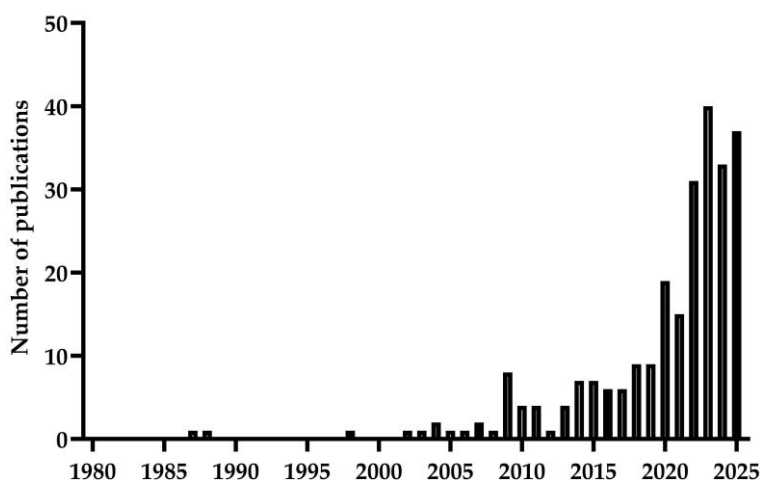


Figure 4. The number of publications per year reported in Scopus from 1949 to 2025, found by typing the keywords “Wood vinegar on plant” in Title, Abstract or Keywords. It is interesting to note that interest in plants emerged earlier than interest in soils. (Data retrieved from Scopus in December 2025).

Effects on germination. Germination assays are among the most commonly used tests to assess the hormonal and phytotoxic effects of WV. Numerous studies conducted on a wide range of plant species have consistently reported similar response patterns. Early germination tests performed in the early 2000s demonstrated that WV stimulates seed germination and radicle growth of watercress, chrysanthemum, lettuce and honewort [34]. More recently, different WV dilutions have been tested on several crops of agronomic interest, such as cucumber, rapeseed, wheat, basil, sage, parsley plants, white mustard, arugula, radish, dill, curly parsley, lettuce, seed peas, oats, pepper and tomato [35–39]. In these studies, the lowest concentrations consistently promoted both germination rate and early seedling growth.

Studies on threatened arable plant species have further highlighted that WV effects are dose-dependent and species-specific, producing divergent outcomes depending on the species, while still showing no negative effects at low concentrations [40]. Conversely, excessively high WV concentrations are clearly detrimental to plant performance. WV seed treatments have also been evaluated in combination with other products. When co-applied with biochar, WV individually stimulated pepper seedling growth, whereas the combined treatment more effectively enhanced tomato seedling growth [39]. In combination with eucalyptus extracts, WV enhanced the allelopathic effects of the latter due to their shared hormonal regulatory activity; nevertheless, growth-promoting effects were still observed at low concentrations [41]. WV has also been successfully employed in seed priming experiments, improving plant growth in rice, lettuce, basil and chickpea [36,38,42,43].

Effects on growth, yield, and nutritional quality. Given its long-standing use in agroecosystems, the impact of WV on plant development has been extensively investigated. However, comparison among studies remains challenging due to substantial variability in applied concentrations, application methods, and measured parameters. Experiments have been conducted under pot and field conditions, as well as in hydroponic, water-floating, and organoponic systems. Early studies identified esters such as methyl acetate and methyl formate as contributors to accelerated plant growth and development, and WV was shown to significantly enhance the productivity of rice [44,45], sorghum, sweet potatoes [46], rock melon, French marigold, zinnia, scarlet sage and tomato. Yield increases were also reported for soybean [47], while foliar WV applications improved growth and yield in lettuce, cucumber, and cabbage [48].

Subsequent research focused on fruit quality, revealing improvements in tomato and rock melon [49,50]. Nonetheless, appropriate dosage remains critical, as WV concentrations of 1–2% have been shown to negatively affect key agronomic parameters, even in perennial such as citrus [51]. Considerable attention has also been devoted to identifying optimal application methods. Comparative studies indicated that both foliar and soil-applied WV enhanced growth and yield in lettuce, basil, and pepper, while showing limited or no effects on tomato and carrot. Foliar application generally outperformed root application, although concentration-dependent and sometimes inconsistent responses were observed [50]. Conversely, soil irrigation with WV increased nitrate and available phosphorus levels, stimulated enzymatic activity, and enhanced microbial biomass and DOC by approximately 10% in beans, whereas foliar application exerted weaker soil-mediated effects [21].

In legumes, WV responses were species-specific. Significant increases in biomass, pod and seed weight, and total seed protein content were reported, particularly in lentil, along with increased concentrations of nutritionally valuable elements such as Fe, Ca, Mg, and K [51]. Positive effects were also observed in chickpea, where a 0.25% WV foliar spray increased seed diameter, weight, starch content, soluble proteins, total polyphenols, and antioxidant capacity [54]. Further studies on chickpea flour demonstrated that WV

treatment significantly increased polyphenol and protein content, including vicilin- and legumin-like proteins. Following simulated gastrointestinal digestion, a higher number of peptides were released from WV-treated flour-based cookies compared to controls [55].

Product quality improvements were also confirmed through NMR-based analyses in tomato and apple trees treated with WV. Tomatoes exhibited increased fresh weight, soluble sugars, antioxidant compounds, and lycopene content following 0.2% WV application, resulting in enhanced yield and nutritional quality [23]. Similarly, WV-treated apples showed significant increase in phenols, flavonoids, tannins, antioxidant capacity, sugars, pectin, free amino acids, and mineral content, particularly in the pulp [56]. Positive effects were also reported for tuber crops such as potato [57] were also reported for tuber crops such as potato [58].

WV is frequently evaluated in combination with other amendments, most notably biochar, another pyrolysis-derived product. Their co-application has resulted in improved performance in tomato, pepper, rice, basil, cucumber, strawberry, and blueberry [32,59–63]. In cucumber, the addition of palm vermicompost and seaweed extracts further enhanced yields compared to WV alone [64,65]. In rice, WV demonstrated significant improvements when applied in combination with various plant growth regulators (PGRs), particularly gibberellic acid and paclobutrazol [66].

Finally, increasing attention has been devoted to WV applications in hydroponic systems. Most studies have focused on lettuce grown on clay substrates, reporting significant improvements in yield and nutritional traits, with a clear dose-dependent response and negative effects above 0.2% WV concentration [67–69]. Onion cultivated in water-floating systems also benefited from WV, with optimal biological activity observed at concentrations of 250–500 $\mu\text{L/L}$, largely attributable to increased phenolic acids and flavonols [70]. Organoponic systems have likewise been tested in cucumber, where low WV concentrations applied both foliar and to the substrate resulted in growth stimulation [71].

4. Herbicide effect and weed control

In light of the dose-dependent responses observed in plant treatments, it is evident that WV effects shift from stimulatory to clearly phytotoxic once specific threshold concentrations are exceeded. This characteristic has been increasingly exploited in recent years, leading to a growing number of studies evaluating WV as a natural herbicide (Figure 5). WV therefore represents a potential alternative to synthetic chemical herbicides for weed control. Its herbicidal activity is mainly attributed to its content of phenols, organic acids, carbonyl compounds, and alcohols, which collectively contribute to tissue damage and growth inhibition.

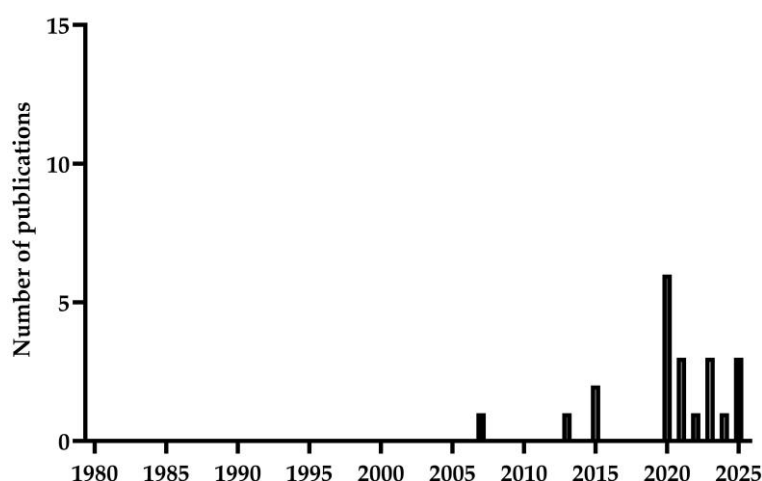


Figure 5. The number of publications per year reported in Scopus from 1980 to 2025, found by typing the keywords “Wood vinegar herbicide” in Title, Abstract or Keywords. A relatively modest number of studies emerged. (Data retrieved from Scopus in December 2025).

Experimental evidence indicates that WV efficacy varies among plant functional groups. WV generally exhibits stronger effects on broadleaf species than on narrowleaf species [72], and that dicotyledonous plants are more sensitive to acid-based herbicides than monocotyledonous plants [73]. In controlled experiments conducted on *Silibum marianum*, *Sinapis alba*, *Cardus tenuiflorus*, *Bromus madritensis*, *Bromus maximus* and *Hordeum murinum*, WV proved to be an effective an inhibitor of seed germination and seedling growth, particularly when applied through aerial contact. Sprayed WV concentrations of 50, 75 and 100 vol. % were effective against all plant species tested [74].

Field-scale studies further confirmed the herbicidal potential of WV. Application rates of 1,000, 2,000, and 4,000 L/ha reduced white clover shoot mass by 56%, 81%, and 98%, respectively, compared to untreated controls at 10 days after treatment [75]. WV has also demonstrated efficacy in controlling multiple broadleaf weed species under field conditions, with higher effectiveness observed at lower temperatures and under dark conditions [76]. However, WV efficacy is species-specific, as no inhibitory effects were observed in tests conducted on giant hogweed (*Heracleum mantegazzianum*) [77,78].

Beyond vascular plants, WV has also been evaluated for its effects on mosses and lichens. Short-term exposure to WV did not adversely affect non-target sensitive organisms; instead, WV treatments maintained lichen and moss vitality and resulted in minimal bioaccumulation of harmful compounds [79,80].

5. Abiotic stress tolerance and defence induction

Considering its hormonal regulatory potential, WV has been increasingly investigated as a stimulator and inducer of plant defence mechanisms. A key research focus is the evaluation of its role in mitigating abiotic stresses, an aspect of growing relevance under increasingly variable climatic conditions. Although studies addressing WV-mediated abiotic stress tolerance are still relatively limited, research activity has intensified in recent years (Figure 6). The main stress factors investigated to date include soil salinity, drought, low temperature, ozone exposure and heavy metal.

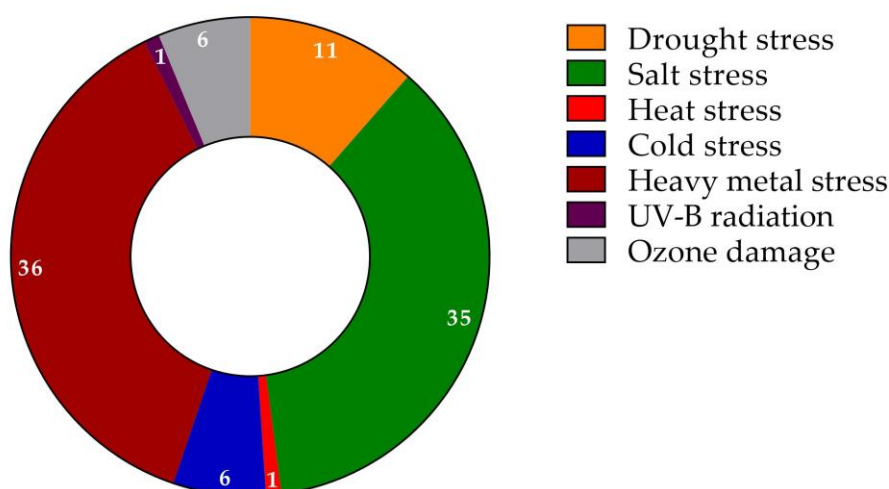


Figure 6. The number of different abiotic stress and WV effects reported in publications in Scopus from 1949 to 2025 in Title, Abstract or Keywords. (Data retrieved from Scopus in December 2025). Keywords used for each research: “wood vinegar drought”, “wood vinegar salt”, “wood vinegar heat stress”, “wood vinegar low-temperature AND cold stress”, “wood vinegar heavy metal”,

“wood vinegar UV-B radiation” and “wood vinegar ozone”. The results for some searches are redundant, but the keywords used allow users to view all papers of interest.

Salt stress. Soil salinization negatively affects seed germination and early seedling establishment, primarily due to the toxic effects of Na^+ and Cl^- ions, which impair water uptake [81]. Several studies have demonstrated the ability of WV to mitigate salt-induced damage. In rice, WV treatment increased K^+ accumulation, reduced Na^+ uptake in shoots, and alleviated the detrimental effects of salinity while promoting shoot growth [82,83]. WV application enhanced water uptake in seedlings, reduced osmotic stress, increased abscisic acid (ABA) levels and antioxidant activity in roots, and decreased Na^+ and Cl^- toxicity under saline conditions [84]. Consistently, treatment of *Vigna radiata* and rice seeds with 1:500 WV significantly improved germination rate, root and shoot length, seedling dry weight, vigor index, and yield under salinized conditions [81]. Foliar application of WV, alone or in combination with spermidine, also resulted in marked increases in yield, yield components, flag leaf development, and SPAD values in rice cultivars across all tested ratios [83,85]. In cucumber seedlings, use of WV (1500 ppm) via its nutrient and bioactive components is effective in improving the negative effects of the salinity (150 mM NaCl) [86]. In rapeseed, salinity significantly impaired growth, photosynthetic efficiency, and redox balance; however, pretreatment with WV at optimal concentrations alleviated salt-induced reductions in biomass, chlorophyll content, and gas exchange parameters. WV pretreatment also reduced oxidative stress and lipid peroxidation, enhanced antioxidant enzyme activities and proline accumulation, and was associated with reduced Na^+ uptake and enhancing expression of salt transporter genes [87]. In lettuce, WV significantly improved plant growth and survival under extreme salinity (>200 mM NaCl), despite the identification of a physiological tolerance threshold at 100 mM NaCl, beyond which oxidative stress markers increased and fresh biomass declined [88]. The combined application of WV and biochar (BC) further enhanced salt stress mitigation by promoting soluble salt leaching, improving soil structure and moisture content, and increasing maize growth in saline soils [89].

Drought stress. WV-mediated drought tolerance has been investigated in several plant species, including wheat, lettuce, and *Pandanus amaryllifolius* [36,90–92]. These studies consistently reported positive effects of WV on plant growth and stress tolerance under water-deficit conditions. Observed responses included increased leaf relative water content, root-to-shoot ratio, stem circumference, chlorophyll pigments, and antioxidant enzyme activities [90]. In lettuce, WV application resulted in a significant increase in total soluble protein content (14–28%) and enhanced antiradical activity, measured as DPPH scavenging capacity, reflecting improved reactive oxygen species (ROS) detoxification [91]. In wheat, WV treatment modulated ABA production, regulated stomatal closure to reduce water loss, and stimulated the expression of antioxidative genes in both roots and shoots, thereby enhancing drought tolerance. WV application prior to drought stress increased basal O_2 and H_2O_2 levels, whereas application after stress onset reduced ROS accumulation and malondialdehyde (MDA) content [90]. Seed pretreatment with WV (1:900) further strengthened root defence mechanisms and increased drought tolerance [36]. Similarly, WV seed priming in rice promoted seedling emergence and enhanced resistance to drought stress [90]. Although the precise mechanisms remain unclear, WV pretreatment appears to promote early defensive responses and root system development, thereby improving plant resilience to subsequent water deficits.

Ozone damage. Tropospheric ozone (O_3) represents a serious threat to vegetation, as it disrupts photosynthetic processes through excessive production of reactive oxygen species, accelerating senescence and reducing crop yields. WV has demonstrated protective effects against O_3 -induced oxidative stress in several plant species. In basil, WV treatment

reduced visible injury, preserved membrane integrity, and enhanced the accumulation of antioxidant compounds, including abscisic and salicylic acids, indicating an effective defence response. In lettuce, foliar WV applications preserved photosynthetic efficiency and antioxidant capacity under ecologically relevant O₃ concentration [93]. Further studies confirmed that WV-treated lettuce plants exhibited reduced damage to the photosynthetic apparatus, higher chlorophyll content, increased antioxidant power, elevated levels of caffeic acid and quercetin, and greater biomass accumulation[94]. Similar protective effects were observed in tobacco (BelW3), where WV application mitigated O₃-induced damage and maintained morphometric and photosynthetic parameters. Notably, WV-induced protection persisted for up to three weeks after treatment cessation, highlighting its short-term protective capacity against oxidative stress [95].

Cold stress. WV effects under low-temperature stress have been investigated in rapeseed. Foliar WV application improved plant performance, yield, and nutritional quality [96], and synergistic effects were also observed when WV was applied in combination with butyrolactone [97]. Also in alfalfa, WV enhanced cold tolerance [98].

Heavy metal stress. WV effectively mitigates heavy metal contamination in soils and plants, including Cd, Pb, Cr, Ni, Zn, Cu, and Al [99–104]. Its mechanisms include complexation, chelation, adsorption by organic compounds, and promotion of humification, which reduce metal bioavailability and mobility [99–101]. WV can facilitate phytoremediation by enhancing plant uptake in above-ground tissues while simultaneously stabilizing metals in soil or converting them into less toxic forms [100,104]. Additionally, it supports passivation and leaching processes, lowering ecological and human health risks [101–104]. By transforming heavy metals into more stable fractions and inhibiting their accumulation in edible parts, WV enables safer crop production on contaminated soils, offering a sustainable and eco-friendly approach to heavy metal management in agricultural systems.

6. Antimicrobial activity and disease control

The antimicrobial activity of WV represents one of the most extensively investigated aspects of this product and has attracted increasing attention in recent years. Several comprehensive reviews have already addressed this topic, in some cases with a specific focus on the application of WV in plant disease management [14,15,18,30,105]. The aim of this section is therefore not to replicate existing reviews, but rather to integrate previous findings and provide a clear and up-to-date overview of the current state of the art, with particular emphasis on recent experimental evidence. Antiviral activity is not discussed here, as the limited number of available studies has already been thoroughly summarised by Zhou *et al.* [18].

Antifungal activity. Fungal pathogens represent the most extensively studied targets of WV antimicrobial activity. The majority of investigations have been conducted under in vitro conditions, although studies involving whole plants and field experiments are increasingly reported. In many cases, multiple fungal pathogens have been tested against a single WV formulation, while other studies have compared the efficacy of WVs derived from different feedstocks or produced under varying pyrolysis conditions against the same pathogen. Given the large number of fungal strains evaluated and the heterogeneity of experimental approaches, the most relevant findings are summarised in Table 1.

Table 1. Pathogenic fungi studied with their corresponding findings.

Pathogen	Findings	Reference
<i>Alternaria panax</i>	WV at a concentration of 3.0% or more can inhibit mycelial growth of ginseng black spot fungus	[106]

	WV treatment significantly reduced grey mould in grapes by lowering hydrogen peroxide and MDA levels and enhancing antioxidant enzyme activity	[107]
<i>Botrytis cinerea</i>	At a concentration of 2.25% or more can inhibit mycelial growth and spore germination of ginseng gray mold	[106]
	<i>In vitro</i> assays showed that WV inhibited <i>B. cinerea</i> mycelial growth by approximately 0.70-fold. The 2% WV and 4% WV treatments, reduced average lesion length by 0.19-, 0.52-fold respectively, compared to the untreated plants	[108]
<i>Botryodiplodia theobromea</i>	Growth reduced by 50% (ED50) with 661.30 µ/mL	[109]
	Inhibitory effect by WV derived from different wood, especially bamboo and rubberwood	[110]
<i>Cochliobolus sativus</i>	100% inhibition with WV produced at high temperature (370-450°C)	[111]
<i>Colletotrichum coccodes</i>	Guava WV strongly inhibited pathogen mycelial growth, reaching 100% at 3% concentration, and foliar sprays at 2–3% greatly reduced potato black dot severity on stems, roots, and wilted plants.	[112]
<i>Colletotrichum orbiculare</i>	100% inhibition with WV produced at high temperature (370-450°C)	[111]
<i>Coriolus versicolor</i>	Growth reduced by 50% (ED50) with conc. 736.88 µg/mL	[109]
	Growth inhibition and wood weight loss was greatly reduced	[113]
	Inhibitory effect by WV derived from different wood, especially bamboo and rubberwood	[110]
<i>Fomitopsis palustris</i>	High inhibition at 1.5-2% WV concentration, but not complete	[114,115]
	Growth inhibition and wood weight loss was greatly reduced	[113]
<i>Fusarium oxysporum</i>	WV inhibited the <i>in vitro</i> growth of <i>Fusarium oxysporum</i> f. sp. <i>cubense</i> Tropical Race 4 (Foc TR4) at all tested concentrations. Lower WV concentrations (2% v/v) promoted banana plant growth, whereas higher concentrations caused severe phytotoxic effects, including leaf necrosis and chlorosis.	[116]
<i>Fusarium proliferatum</i>	MIC values of 3.5 µL/mL for mycelial growth and 1.0 µL/mL for conidial germination. Completely inhibited disease incidence at a concentration of 7.5 µL/g soil.	[117]
<i>Fusarium verticillioides</i>	WV had a high ability to inhibit the pathogen's growth at a concentration of 2% (78.32%)	[118]
<i>Gloeophyllum trabeum</i>	Growth was reduced by 50% (ED50) with conc. 640.77 µg/mL	[109]
	Inhibitory effect by WV derived from different wood, especially bamboo and rubberwood	[110]
	WV at 10% reduced pine weight loss by 77.7%, while 50% caused complete fungal mortality in 24 h.	[119]
	The toxic fungicide threshold was between 96 and 192 kg/m ³ of different WV	[120]
	The ethyl ether fraction (0.20 g/mL) totally inhibited the growth	[121]
<i>Helminthosporium sativum</i>	100% inhibition with WV produced at high temperature (370-450°C)	[111]
<i>Lentinus lepideus</i>	Growth inhibition at higher WV concentration and wood weight loss was greatly reduced	[113]
<i>Macrophomina phaseolina</i>	WV efficiency increased with concentration, with 2% achieving 100% suppression and reducing sunflower charcoal-rot by 61–67%.	[122]

<i>Magnaporthiopsis maydis</i>	WV efficiency increased with concentration, with 2% achieving 100% suppression and reducing maize late-wilt by 71%.	[122]
<i>Ophiostoma piliferum</i>	Growth inhibition at higher WV conc. and wood weight loss was greatly reduced	[113]
<i>Phytophthora capsici</i>	100% inhibition with WV produced at high temperature (370–450°C)	[111]
<i>Phytophthora infestans</i>	100% inhibition with WV produced at high temperature (370–450°C)	[111]
<i>Polyporus sanguineus</i>	81% inhibition at WV 0.25% and complete inhibition at 0.5%	[123]
<i>Pythium ultimum</i>	Tobacco WV showed growth inhibition against <i>P. ultimum</i> , and no against another tested pathogens	[124]
	Lignin and hemicellulose WV inhibited fungus growth	[125]
<i>Rhizoctonia solani</i>	WV (0.125–0.5%) significantly inhibited <i>R. solani</i> mycelial growth, reduced disease severity, and lowered pathogenicity by up to 87%.	[126]
<i>Rhizopus stolonifera</i>	100 mg/mL WV can inhibit mycelial growth	[18]
<i>Rigidoporus amylospora</i>	Inhibitory effect by WV derived from different wood, especially bamboo and rubberwood	[110]
<i>Trametes versicolor</i>	Complete inhibition at WV 1 and 2%	[114,115]
	90% inhibition at 0.25% WV conc and complete inhibition at 0.5%	[123]
	Yellow poplar WV produced by fast pyrolysis exhibited protective effect at concentration of 75%	[127]
	Growth inhibition and wood weight loss was greatly reduced	[113]
<i>Tyromyces palustris</i>	WV 10% reduced pine weight loss by 62.2%, while 50% caused complete fungal mortality in 24 h.	[119]
	The toxic fungicide threshold was between 96 and 192 kg/m ³ of different WV	[120]
	Yellow poplar WV produced by fast pyrolysis exhibited protective effect at concentration of 75%	[127]
	The ethyl ether fraction (0.20 g/mL) totally inhibited the growth	[121]
<i>Valsa mali</i>	100% inhibition with WV produced at high temperature (370–450°C)	[111]

Antibacterial activity. The antibacterial effect of WV has historically been investigated mainly against plant pathogenic bacteria rather than microorganisms relevant to human health. In recent years, interest in its environmental and agricultural applications has increased substantially, and a broad range of phytopathogenic bacteria has now been evaluated.

WV has shown inhibitory activity against *Xanthomonas campestris* and *Erwinia carotovora*, two major pathogens of horticultural crops [128]. Phenols and guaiacols derived from Japanese cedar WV were effective against *Ralstonia solanacearum* [129]. Similarly, a 10% solution of pyroligneous acid also inhibited *Agrobacterium tumefaciens* and *X. campestris*, while WV obtained from pineapple biomass suppressed *Corynebacterium agropyri*, a bacterium associated with yellow gum disease [130].

The antibacterial potential of WV fractions derived from lignin, cellulose, and hemicellulose biomass was also evaluated against *X. campestris*, *Streptomyces scabies*, and *Clavibacter michiganensis* [125]. Among these, lignin-derived WV exhibited the strongest antibacterial activity, inhibiting all tested pathogens and additionally displaying antifungal and insecticidal properties. In contrast, cellulose-derived distillates showed limited antibacterial efficacy, inhibiting only *S. scabies* and moderately *X. campestris*.

In vitro antibacterial activity of three different PAs obtained from the pyrolysis of apricot kernels (AKPA), hazelnut shells (HSPA), and kermes oak (OPA) against bacterial plant pathogens species such as *Curtobacterium flaccumfacies* pv. *flaccumfaciens*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pseudomonas syringae* pv. *phaseolicola* and *Bacillus pumilus*. *C. flaccumfaciens* pv. *flaccumfaciens* was identified as the most susceptible species to the three WVs evaluated [131].

WV obtained from alternative feedstocks, including tobacco leaves and coffee grounds, and produced at different pyrolysis temperatures, has also been investigated [124,132]. Tobacco distillate produced at 450°C showed the best antibacterial effect, while coffee ground WV was most effective at 450–550°C for *S. scabiei* and 500–550 °C for *C. michiganensis*. These findings further confirm the critical role of pyrolysis temperature in determining WV chemical composition and bioactivity.

Feng *et al.* [133] reported that WV derived from *Eucommia ulmoides* inhibited several bacteria, including *Staphylococcus aureus*, *E. coli*, and *Bacillus subtilis*. Refined sorrel husk WV was particularly effective against soft rot pathogens in celery (*E. carotovora*) and fruit spot pathogens in melon (*Acidovorax avenae*), and also inhibited *Pseudomonas tolaasii*, the causal agent of bacterial brown spots.

A comprehensive study by Ahmadi *et al.* [134] investigated the efficacy of WV and wood tar, both produced from lignocellulosic biomass via pyrolysis, in controlling *Pseudomonas syringae*, the causal agent of bacterial canker in apricot orchards. Pyrolysis temperature was identified as a key factor influencing antimicrobial efficacy, with 350°C producing WV with optimal preservative and antibacterial properties. In contrast, higher temperatures (e.g., 400°C) promoted the formation of undesirable by-products that could compromise product safety and quality. Both laboratory and field trials confirmed a dose-dependent antibacterial response, with a 2% WV concentration providing the strongest inhibition. In orchard conditions, WV significantly reduced bacterial canker incidence, particularly when combined with physical barriers such as trunk bandaging. However, WV alone was insufficient to fully eradicate the pathogen, and bacterial regrowth was observed within three to six months, indicating the need for repeated applications or integrated disease management strategies. Beyond direct antibacterial effects, WV has also been shown to influence soil microbial communities. Application of diluted WV increased populations of beneficial soil bacteria, indirectly reducing disease incidence, such as tomato early blight (*Alternaria solani*) and improving melon resistance to soil-borne pathogens. Since soil bacteria represent 70–90% of total soil microorganisms, increasing their diversity can significantly contribute to plant health and pathogen suppression [18].

Mechanisms of action. At the mechanistic level, WV exerts antibacterial activity primarily by disrupting bacterial cell membranes, increasing membrane permeability, and inducing leakage of intracellular components, including proteins, sugars, and electrolytes. These alterations lead to metabolic dysfunction and ultimately bacterial cell death, with effects intensifying at higher WV concentrations [111]. Additional studies have shown that WV shortens bacterial growth phases, reduces intracellular protein content, and increases solution conductivity, further supporting its role as an effective natural antimicrobial agent [135]. WV mechanism of action against bacterial diseases involves slowing down the rate of bacterial division, destroying the bacterial cell membrane, which allows internal electrolytes to leak out, and inhibiting the synthesis of new proteins in bacteria. All these actions work together to prevent bacterial infection [111]. Excessive amounts of WV cause disease-causing bacteria to exude sugar, protein, and electrolytes, limit bacterial protein synthesis, disrupt cell metabolism, and ultimately result in cell death[18].

Against fungal pathogens, WV alters the morphological structure of spores, inhibits spore germination, and reduces fungal biomass accumulation[136]. WV may also disrupt fungal cell membrane permeability and inhibit cell division and protein synthesis, as

observed for *Fusarium graminearum*, leading to leakage of proteins, carbohydrates, Na⁺, and K⁺, and inducing metabolic disorders that suppress fungal growth [18].

In addition to its direct antimicrobial effects, WV enhances plant resistance through indirect mechanisms. WV application can activate plant antioxidant defence-related enzymes, reduce lipid peroxidation of cell membranes, lower hydrogen peroxide accumulation, and modulate soil microbial communities. At appropriate concentrations, WV improves nutrient availability and stimulates enzymes involved in nutrient cycling, thereby strengthening plant defence capacity [107].

WV-mediated disease resistance is closely linked to induced resistance (IR) pathways in plants, which include systemic acquired resistance (SAR) and induced systemic resistance (ISR) [137].

SAR is typically triggered by hypersensitive responses and is regulated by salicylic acid (SA), leading to the accumulation of pathogenesis-related proteins [138,139]. In contrast, ISR is induced by beneficial root-associated microorganisms and relies on ethylene (ET) and jasmonic acid (JA) signalling pathways [140,141].

WV seed priming and application have been shown to enhance systemic resistance by stimulating the activity of key defence-related enzymes, including polyphenol oxidase (PPO), peroxidase (PO), phenylalanine ammonia-lyase (PAL), chitinase, superoxide dismutase (SOD), and β -1,3-glucanase [110,132]. PO and PPO contribute to reactive oxygen species detoxification and cell wall strengthening, while PAL plays a central role in phenolic compound biosynthesis, reinforcing plant resistance [142]. For instance, the combined application of WV and peach gum in blueberry significantly enhanced PO, PPO, and PAL activities, reduced oxidative stress, and effectively suppressed *Botrytis cinerea* infection [122].

7. Insecticidal properties

The most widely adopted strategy for managing plant pests relies on synthetic chemical pesticides. While their extensive use has significantly increased agricultural productivity, it has also caused adverse environmental and ecological effects. As a result, sustainable agricultural practices aimed at reducing dependence on chemical pesticides are gaining increasing attention. In this context, the insecticidal potential of WV has attracted interest, largely attributed to its phenolic constituents. WV has demonstrated broad-spectrum insecticidal activity across various studies (Figure 7).

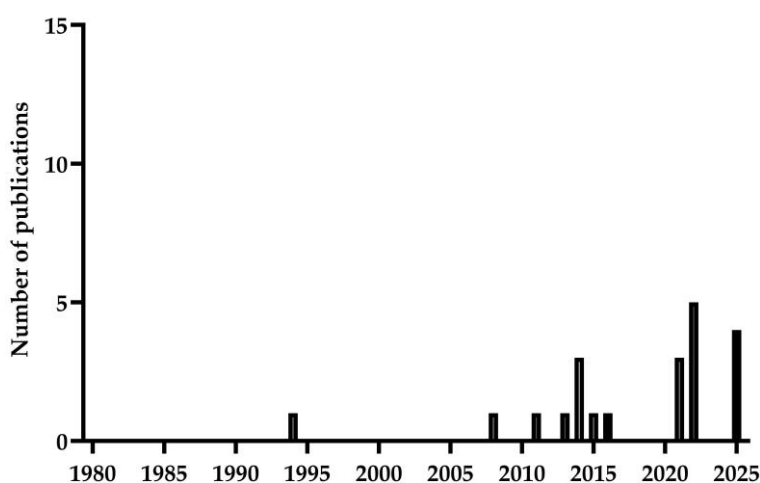


Figure 7. The number of publications per year reported in Scopus from 1949 to 2025, found by typing the keywords “Wood vinegar insect” in Title, Abstract or Keywords. A relatively modest number of studies emerged. (Data retrieved from Scopus in December 2025).

High efficacy levels, often exceeding 90% mortality, have been reported against pests such as the green peach aphid (*Myzus persicae*), the red spider mite (*Tetranychus urticae*), and aphids infesting *Solanum melongena* [143,144]. In addition to direct toxicity, WV has exhibited repellent or lethal effects against a wide range of invertebrate pests, including slugs (*Arion lusitanicus*), snails (*Arianta arbustorum*), houseflies (*Musca domestica*), striped mealybugs (*Ferrisia virgata*), the Colorado potato beetle (*Leptinotarsa decemlineata*), and the tobacco beetle (*Lasioderma serricornis*) [15,145–147].

WV produced at pyrolysis temperatures of 450–550°C showed marked toxicity against *L. decemlineata*, *Trichoplusia* and *Acyrtosiphon pisum* [125]. Moreover, WV may act synergistically when combined with other control agents, such as carbosulfan or neem oil, further enhancing its insecticidal efficacy and suggesting its potential role within integrated pest management strategies [148,149].

Several studies have also highlighted the larvicidal activity of WV. In the case of the fall armyworm (*Spodoptera frugiperda*), a 10% WV concentration achieved approximately 80% larval mortality [150]. Conversely, WV treatments ranging from 1.5% to 3.0% exhibited only limited larvicidal effects against *Spodoptera litura* [151]. WV has also shown insecticidal activity against almond leaf bees, with the greatest reduction in larval abundance observed at concentrations between 10% and 25% [152]. At lower concentrations, WV demonstrated strong efficacy against mosquito larvae; a 2% solution applied via the dripping method eliminated 73.75%, 82.50%, and 96.25% of mosquito larvae after 24, 48, and 72 hours, respectively [153].

WV and its nanoemulsion formulations exhibited strong larvicidal activity against *Culex pipiens*. Nanoemulsification significantly enhanced toxicity compared with bulk formulations. Among the identified components, 2-methoxy-*p*-cresol showed the highest potency, with an LC₅₀ of 0.09 mg/L after 48 h of exposure. Treated larvae displayed clear biochemical disruptions, including elevated oxidative stress markers and inhibition of key neuroenzymes, indicating combined oxidative and neurotoxic modes of action [154].

Beyond lethality, WV has been shown to influence insect behaviour. Against *Ceratitis capitata*, WV significantly reduced visitation and oviposition rates on treated orange fruits by 53% and 62%, respectively [155]. Similarly, WV exhibited strong repellent activity against rice weevils, achieving 98.3% repellence at a concentration of 200 ppm, with a lethal concentration (LC) of 77.62 ppm [156]. Mosquito repellence varied depending on both WV concentration and mosquito species; WV was highly effective against *Culex pipiens pallens* even at low concentrations, whereas higher doses were required to repel *Aedes togoi* [157].

With regard to termiticidal activity, an extensive and comprehensive review by Trabelsi and Mhamdi (2024) [19] provides an in-depth evaluation of the available evidence. In summary, WV can exert both repellent and toxic effects against termites, with increased concentrations generally leading to stronger termiticidal responses. A concentration of approximately 10% is commonly regarded as the threshold for effective termite control, although optimal concentrations vary depending on the WV type and termite species. Nevertheless, the overall efficacy of WV as a termiticide remains difficult to generalize due to heterogeneous experimental approaches and inconsistent results. Moreover, despite extensive characterization of WV chemical composition, limited information is available regarding the specific compounds responsible for its repellent and antifeedant effects, as few studies have focused on isolating and identifying the active ingredients underlying its termiticidal properties.

8. Is WV really all sunshine and roses?

Before drawing definitive conclusions, it is essential to address several critical issues that have emerged from literature. First, the wide range of terms used as synonyms for

WV represents a significant limitation for its functional assessment. The use of different keywords can hinder comprehensive literature retrieval, increasing the risk of overlooking previously published studies. In this review, attention has not been devoted to production-related aspects, given the extensive body of work already available; nevertheless, several key considerations must still be highlighted.

It is evident that numerous variables influence the final effects resulting from WV application. Across the analyzed studies, neither a clearly defined optimal dosage nor standardized application methods have been consistently identified. In addition, the production process itself plays a pivotal role, particularly with respect to both the nature of the starting plant matrix and the pyrolysis temperature. The biological effects of WV are intrinsically linked to its chemical composition, which in turn depends on the biochemical characteristics of the feedstock and the conditions applied during production. Despite efforts to conduct detailed comparisons and cross-analyses among available studies, the heterogeneity of experimental variables makes it extremely challenging to assign a specific weight to each factor and to clearly determine its relative contribution. To address these issues, several studies have investigated compositional changes as a function of both biomass type and pyrolysis temperature. To better illustrate these concepts, some representative studies are discussed. One study examined WV obtained from hardwood, softwood, and bamboo waste subjected to pyrolysis at different temperatures (200, 300, 400, and 500°C) [158]. The lowest concentrations of acids and phenols were observed at 200°C, whereas the highest levels were detected at 400–500°C. Hardwood-derived WV was dominated by lignin-derived phenols, while softwood WV primarily contained guaiacyl lignin derivatives. In contrast, bamboo WV exhibited phenolic compounds derived from guaiacyl, syringyl, and p-hydroxyphenyl units. These results clearly demonstrate that pyrolysis temperature stratification leads to marked differences in WV chemical composition.

Similarly, a study based on pine biomass evaluated the effects of pyrolysis temperature across four ranges between 300 and 700°C [159]. Higher temperature resulted in increased production of water-dissolved organic matter (WOM), characterized by lower molecular weight and a higher degree of oxidation. Moreover, WOM derived from lower temperatures (< 400°C) promoted wheat seed growth, whereas WOM obtained at higher temperatures (> 400°C) exerted inhibitory effects. Conversely, investigations involving multiple biomass sources (poplar, pine, pruning litter, and forest waste) reported no significant differences in WV composition [160]. Taken together, these findings—along with the evidence discussed throughout this review—suggest that pyrolysis temperature may represent the most influential factor determining WV properties, while the source biomass, although still relevant, may play a secondary role.

Another important aspect deserving consideration is the discrepancy between research activity and market availability. As noted in the introductory section, WV has been used for years and is increasingly commercialized, with a projected exponential growth in its diffusion. However, identifying major industrial producers remains challenging. Most WV products investigated in the literature are generated on a small scale within academic settings or through experimental protocols developed in collaboration with private companies. This situation results in a paradox in which many commercial formulations reach the market without undergoing rigorous experimental validation, while scientific studies often focus on products whose long-term commercial availability is uncertain. It is important to highlight the major gaps that remain and to outline future research directions. Continued investigation across the various tested applications is essential to clarify optimal dosages and administration strategies, particularly given the increasingly evident species-specific and dose-dependent responses. Future efforts should progressively shift the focus from describing observed effects to elucidating their underlying

mechanisms. Although WV is an extremely complex mixture, isolating individual compounds will be crucial to identify those primarily responsible for biological activity. In parallel, greater attention should be devoted to metabolic and molecular processes, elucidating how WV components interact with plant and microbial cells. While some progress has already been made, the adoption of omics-based approaches remains highly promising. Metabolomic analyses can reveal the diversity of compounds present in living-cell extracts [161], while RNA-Seq techniques—widely used to characterize transcriptomic profiles—should be more frequently integrated into experimental designs. Such approaches would enable a deeper understanding of plant responses, including hormonal regulation, activation of defense mechanisms against pathogens, and gene expression pathways associated with abiotic stress responses.

9. Conclusion and perspectives

WV is emerging as a promising and sustainable by-product of lignocellulosic biomass pyrolysis, with increasingly relevant applications in modern agriculture. Rich in bioactive compounds such as phenols and organic acids, WV exhibits antimicrobial, pesticidal, antioxidant, herbicidal. It appears as a versatile tool for plant protection, growth enhancement, and the reduction of synthetic agrochemical use. Moreover, WV offers a valuable pathway toward more sustainable agricultural systems by improving soil health and resilience to environmental stress.

The chemical composition and biological efficacy of WV are strongly influenced by both the feedstock and the pyrolysis conditions, which ultimately determine its functional outcomes. Despite the growing body of evidence supporting its potential, the responsible and science-based application of WV requires a more comprehensive understanding of its mechanisms of action. In this context, molecular biology approaches will play a pivotal role in elucidating the interactions between WV components and plant or soil systems.

Advancing research in this direction will be essential to establish WV as a reliable natural corroborant and to support its integration into eco-friendly and resilient farming practices.

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2. Methodologies and Results

2.1. Scientifically validated *in vitro* bioactivity assessment

Article

“How to” but *in vitro*: integrated and advanced biotechnological assays to predict *in vivo* potential of wood-derived extracts

“How to” but *in Vitro*: Integrated and Advanced Biotechnological Assays to Predict *in Vivo* Potential of Wood-Derived Extracts

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Abstract

In the context of climate change and the increasing need to reduce agrochemical inputs, this study explores sustainable alternatives for the control of plant pathogenic bacteria using *in vitro* experimental systems. The progressive restriction of copper-based compounds and the widespread reliance on chemical pesticides emphasize the urgency of identifying eco-friendly plant protection strategies. Chestnut-derived wood vinegar (WV) and chestnut woodchip extract (WCE) were evaluated through a comprehensive set of *in vitro* assays, including antibacterial and anti-virulence tests against different microorganisms, phytotoxicity assessments based on seed germination and early growth, as well as experiments on *in vitro* model plants to analyze morphological responses and the expression of selected defense-related genes. The integration of multiple *in vitro* approaches enabled a rapid, controlled, and cost-effective evaluation of the biological potential of chestnut-based products. Overall, WV exhibited a strong dose-dependent biological activity, showing notable antibacterial efficacy, particularly under conditions simulating plant infection. At high concentrations, WV displayed phytotoxic effects, whereas at low concentrations it promoted plant growth, supporting its potential use as both an herbicide and a plant corroborant. In contrast, WCE mainly showed biostimulant properties, enhancing plant growth and development through the activation of hormonal and defense-related pathways, while exhibiting limited antimicrobial activity.

Keywords: wood vinegar, woodchip extract, chestnut biomass, *in vitro* assay, biostimulant, antimicrobial, phytotoxicity, *in vitro* plant, gene expression.

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1. Introduction

Human-driven climate change, driven by rising greenhouse gas emissions, threatens global agriculture and food security by harming plant growth. At the same time, agriculture contributes to these emissions through heavy agrochemical use, unsustainable livestock practices, resource overexploitation, and deforestation [1,2].

Plant pathogenic bacteria cause significant damage and economic losses in agriculture and remain difficult to control, despite being less prevalent than fungal or viral pathogens. Current management strategies rely heavily on prevention measures such as quarantine and eradication, which can be costly, as well as on chemical treatments, mainly copper-based compounds and antibiotics [3]. However, antibiotics are banned for plant

protection in Europe, and the use of copper has been progressively restricted due to environmental concerns and its role in promoting antibiotic resistance. While sustainable alternatives to copper are available for fungal diseases, comparable solutions for bacterial pathogens are still limited.

To address these challenges, many countries are promoting green and circular economy strategies, including the efficient use of plant biomass [4]. Certain plant materials are particularly rich in polyphenols, such as tannins, which are well known for their antimicrobial and insect-repellent properties [5]. Identifying extraction methods capable of preserving and enhancing these beneficial traits is therefore essential.

Wood vinegar, a by-product of biomass pyrolysis, represents a sustainable approach for valorizing lignocellulosic residues. This liquid—composed mainly of water and hundreds of natural organic compounds—has long been used in agriculture to promote plant growth, protect against pests and diseases, and improve soil health [6–8]. Its biological activity is strongly dose-dependent, and at high concentrations it can also exert herbicidal effects [9–11].

Chestnut biomass has been widely studied due to its high tannin content. Chestnut tissues such as leaves, galls, bark, and wood are rich in hydrolysable tannins (HTs) and condensable tannins (CTs), polyphenolic metabolites that play key roles in plant defense through antimicrobial, insecticidal, and anti-herbivory activities. In addition, these compounds are increasingly investigated for their beneficial effects on plant development, soil quality, irrigation water, pest resistance, and tolerance to abiotic stress [12–14].

In vitro approaches, widely used in biomedical research, offer several key advantages, including highly controlled and reproducible conditions, high-throughput screening capabilities, reduced costs, and shorter experimental times compared to *in vivo* studies. Moreover, these methods provide valuable mechanistic insights into cellular and molecular processes [15].

In vitro antimicrobial assays are essential tools for the rapid screening and evaluation of drugs or natural compounds, offering benefits like cost-effectiveness, speed, and versatility [16,17]. In this context, increasing attention has been paid to inhibitors targeting bacterial virulence and pathogenicity, particularly those interfering with quorum sensing (QS) and the type three secretion system (T3SS) [18–20]. Similarly, *in vitro* plant assays allow precise investigation of plant responses under controlled conditions, facilitating the analysis of growth parameters and chemical effects and supporting compound screening and physiological studies [21,22]. Germination-based phytotoxicity assays are especially valuable due to their simplicity, low cost, and rapid output, providing early insights into the harmful effects of substances on plant life by assessing germination rates and root/shoot growth inhibition - parameters of key relevance for agriculture and environmental monitoring [23,24].

In this study, chestnut-derived wood vinegar and laboratory-prepared chestnut extracts were investigated using a range of *in vitro* tests. Antibacterial and anti-virulence activity were evaluated against several microorganisms, while phytotoxicity tests were performed on different plant species of agronomic interest. Finally, *in vitro* plant model systems were employed to assess the effects of these products at both the morphological level and on the expression of selected plant defense-related genes.

2. Materials and Methods

2.1. Tested Products

In this study, a commercial wood vinegar and a chestnut woodchip extract were tested. The selected commercial product was Biodea® (Arezzo, Italy) wood vinegar (WV), produced using chestnut wood as the starting material. The chestnut woodchip extract (WCE) was prepared by infusing 2 L of chestnut woodchips (approximately 1.2–1.3 kg) in 4 L of water. The process lasted 3 hours at 80–85°C [25]. After extraction, the liquid was sequentially filtered and centrifuged to separate macro residues and contaminants.

Both products were characterized by acidic pH and a high phenol content. Specifically, WV and WCE exhibited pH values of 2.29 and 3.31, respectively, and total phenolic contents of 8.31 and 7.06 mg GAE/mL, respectively.

2.2. In vitro workflow

For a complete and exhaustive analysis of the products of interest, a series of *in vitro* tests was set up, starting from the effect on bacteria and ending with the evaluation of several hormonal effects on *in vitro* plant systems (Figure 1).

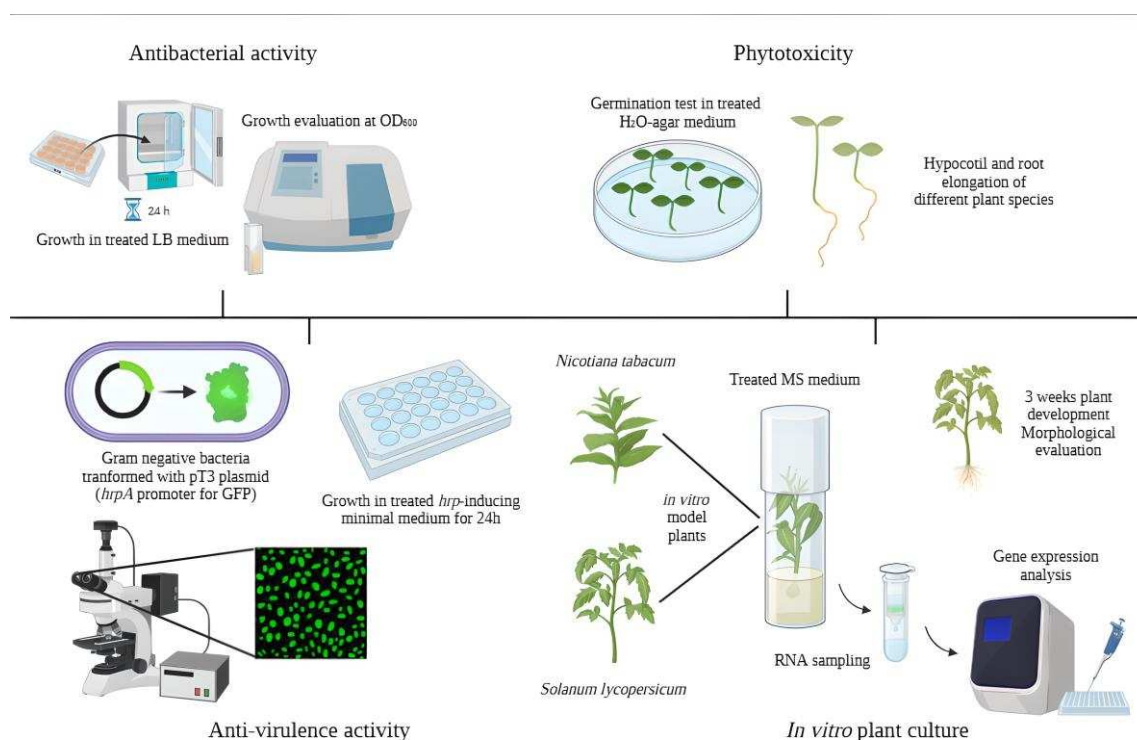


Figure 1. *In vitro* assay workflow.

2.3. Antibacterial Activity Assay

Antibacterial activity was evaluated against a broad panel of bacterial strains, including pathogenic and non-pathogenic species as well as Gram-negative and Gram-positive bacteria. All tested strains are listed in Table 1 and belong to the collection of the Laboratorio di Patologia Vegetale Molecolare (DAGRI, University of Florence, Italy).

Bacterial cultures were grown overnight in Luria-Bertani (LB) medium [26] at 26°C, 180 rpm, overnight, with the exception of *Sinorhizobium meliloti*, which was cultured at 30°C. Cells were harvested by centrifugation (8,000 $\times$ g, 5 min), washed twice with sterile physiological solution (SPS, 0.85% NaCl in distilled water) and resuspended in LB medium to a final optical density of OD₆₀₀ = 0.1, as determined using the spectrophotometer Infinite1 M200PRO Quad4MonochromatorsTM-based (TECAN, Switzerland).

The samples were supplemented or not (control) with WV and WCE at different concentrations (1:100, 1:500 and 1:1,000). The antibacterial activity of WV and WCE was evaluated *in vitro* by monitoring the bacterial growth as optical density at 600 nm (OD₆₀₀) using 24-multiwell plates (BIOFIL1, Guangzhou, China). Measurements were taken at different time-points during a 24 h incubation period in the presence of the tested extracts.

Table 1. Bacterial species tested for antibacterial activity

Species	Strain	Abbreviation	References
<i>Pseudomonas amygdali</i> pv. <i>tabaci</i>	ATCC 11528	<i>Pst</i>	[27]
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	DC3000	<i>Pto</i>	[28]
<i>Pseudomonas savastanoi</i> pv. <i>savastanoi</i>	Psv5	<i>Psv</i>	[29]
<i>Pseudomonas savastanoi</i> pv. <i>phaseolicola</i>	IPV-BO 2325	<i>Psp</i>	[30]
<i>Pseudomonas savastanoi</i> pv. <i>nerii</i>	Psn23	<i>Psn</i>	[29]
<i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i>	Type Strain	<i>Cff</i>	[31]
<i>Sinorhizobium meliloti</i>	RM1021	<i>Sm</i>	[32]
<i>Bacillus amyloliquefaciens</i>	MBI600	<i>Ba</i>	BASF

2.4. Anti-Virulence Activity Assay

2.4.1. GFP-Transformed Bacteria

To evaluate the effect of the tested bioproducts on bacterial virulence, a biotechnological artefact was used, focusing on GFP-based transcriptional screening. Two transformed strains of *Psn* were used: one carrying the plasmid pLPVM_T3A and the other carrying pLPVM_QS [20]. Both plasmids contain the *gfp* reporter gene; in pLPVM_T3A, *gfp* is under the control of the *hrpA* promoter, while in pLPVM_QS it is driven by the *psnI* promoter.

Plasmid pLPVM_T3A was extracted from *Psn* using NucleoSpin® Plasmid/plasmid (NoLid) kit (Macherey-Nagel, Düren, Deutschland), according to the manufacturer's instructions for low copy plasmids. Subsequently, the plasmid was inserted into *Pst* and *Sm* cells by electroporation. For *Pst*, 40 µL of electrocompetent cells were mixed with 1 µL of plasmid at a concentration of 100 ng/µL, and were subsequently electroporated at 25 µF, 200–500 Ω, 2,500 V. To transform the *Sm*, 50 µL of electrocompetent cells were mixed with 50 ng of plasmid and electroporated at 3,000 V with a time constant of 5 ms. Both transformations were performed with Gene PulserXCell™ (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.4.2. Experimental Setup

Bacterial cells were cultured overnight in LB medium, harvested by centrifugation, and washed twice with SPS. Cells were then inoculated into *hrp*-inducing minimal medium (MM) [33] to a final OD₆₀₀ of 0.2 and supplemented with WV or WCE at dilutions of 1:100, 1:500, and 1:1,000. *Psn*, *Pst*, and *Sm* strains carrying the empty vector were used as controls.

Experiments were carried out into 24-multiwell plates (BIOFIL®, Guangzhou, China) over a 24h incubation period. Promoter activity of *hrpA* and *psnI* was quantitatively assessed using an Infinite® M200PRO Quad4Monochromators TM-based (TECAN), by simultaneously measuring the GFP fluorescence (Abs Ex.485nm; Em.535nm) and the bacterial growth as OD₆₀₀.

2.5. Phytotoxicity Assay (Germination Test)

Germination tests were performed on plant species commonly used as model systems or for their agronomic relevance: *Nicotiana tabacum* (tobacco), *Solanum lycopersicum*

(tomato), *Cucumis sativus* (cucumber), as well as meadow species *Lepidium sativum* (cress) and *Medicago sativa* (alfalfa). Seeds were surface-sterilized with 1% NaClO, rinsed several times in sterile distilled water, and placed in Petri dishes containing H₂O-agar medium (18 g/L).

WV and WCE were tested at application rates equivalent to 4.5 L/ha, 45 L/ha and 450 L/ha, corresponding to dilutions of 1:10,000, 1:1,000 and 1:100, respectively, relative to the applied volume. Treatments were evenly distributed on the agar surface. After incubation for two nights at 26 ± 2 °C, germination rates were recorded.

For root and shoot elongation assays (except for tobacco), five germinated seeds per plate were aligned in the center of fresh H₂O-agar media and treated as described above. Plates were positioned vertically in a controlled growth chamber. After one week, root and shoot length were measured for each treatment.

2.6. In Vitro Plants Assay

2.6.1. Experimental Design

To further investigate the biological activity of the products, an *in vitro* plant assay was conducted using *N. tabacum* and *S. lycopersicum*. Plants were grown in Murashige & Skoog medium (MS) [34], supplemented with 18 g/L sucrose and 4 g/L Phyto agar, adjusted to pH 5.8. All plant material was obtained through callus induction and subsequent dedifferentiation using phytohormones, as previously described [35,36].

Plants were cultivated in LA-Round containers (Lab Associates, Oudenbosch, Netherlands) under controlled growth chamber conditions at 24 ± 2 °C with 16 h light/8 h dark photoperiod. WV and WCE were added to the MS medium at dilutions of 1:200 and 1:500. Apical explants (1 cm for tobacco and 2 cm for tomato) were excised and transferred to treated media. Nine plants were used for each treatment.

Plants were maintained in the growth chamber for 3 weeks. Every 7 days, three plants per treatment were sampled for morphological evaluation. Leaves were immediately collected, frozen in liquid nitrogen, and stored for subsequent gene expression analysis.

2.6.2. Analysis of Plant Physiological Parameters

Before leaf sampling for gene expression analysis, chlorophyll (chl) *a* and *b* contents were determined. Specifically, the fresh tissue from the third fully expanded leaf of each plant was collected and processed according to the method described by Wang *et al.* [37]. Briefly, 0.05 g of leaves (fresh weight, FW) were incubated in 2.5 mL (V) of 80% (v/v) acetone for 24 h in the dark at room temperature. The chl content was determined by measuring the optical density at 663 nm (OD₆₆₃) and 645 nm (OD₆₄₅) for chl *a* and *b*, respectively, with the Infinite1 M200PRO Quad4MonochromatorsTM-based (TECAN). Afterward, the chl concentration was calculated using the following formulas:

$$\text{chl } a \text{ (mg/g)} = \frac{(12.72 \times \text{OD}_{663} - 2.59 \times \text{OD}_{645}) \times V}{1,000 \times \text{FW}}$$

$$\text{chl } b \text{ (mg/g)} = \frac{(22.88 \times \text{OD}_{645} - 4.67 \times \text{OD}_{663}) \times V}{1,000 \times \text{FW}}$$

After chl determination, plants were removed from the culture containers, and morphological parameters were recorded. Plant height, primary root length, and the number of roots were measured for each individual plant.

4.5.3. RNA Extraction and Plant Gene Expression Analysis

Leaf samples were ground in liquid nitrogen, and RNA extraction was performed using the NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Deutschland) according to the manufacturer's instructions. The total RNA was eluted in 60 µL of nuclease-free water.

RNA concentration and quality were assessed using the NanoDrop Spectrophotometer ND-100 (Nanodrop Technologies Inc., Wilmington, DE, USA), and RNA integrity was verified by agarose gel electrophoresis (1% agarose in 1x TAE buffer).

To remove residual genomic DNA (gDNA), RNA samples were further purified using the RapidOut DNA Removal Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Then, cDNA was synthesized using the RevertAid RT Kit (ThermoFisher Scientific) following the manufacturer's protocol.

All primers used in this study were selected from the literature and are listed in the Supplementary Materials (Table S1). Expression profile of the following genes was analyzed in both tested plants: auxin-responsive gene 1 (*ARF1*), transport inhibitor response 1 (*TIR1*), phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*) and a pathogenesis-related proteins (*PR1*).

Real-time quantitative PCR (RT-qPCR) was performed with the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific), according to manufacturer's protocol. Gene expression levels were normalized using *Act* and *Ubi3* as housekeeping genes for tomato [38], and *L25* for tobacco [39]. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [40]. Data from the treated plants were compared with those from control. For each sample, three technical replicates were processed in each of the three independent RT-qPCR runs carried out.

4.7. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 8 (GraphPad Software, Boston, MA, USA). After verifying the normal distribution of the data, either one-way ANOVA or the Kruskal–Wallis test was applied, with significance set at $p < 0.05$. *Post hoc* comparisons were then performed using Tukey's or Dunn's test, evaluating differences between all treatment pairs for each plant species.

3. Results

3.1. Antibacterial Activity

The antibacterial activity assay showed species-specific responses to the tested bioproducts (Table S3). Overall, WV exhibited a markedly stronger inhibitory effect than WCE. In particular, WV at a dilution of 1:100 significantly inhibited the growth of *Psp*, *Psn* and *Sm* compared to the control. A significant reduction in growth was also observed for *Cff* and *Ba* with WV 1:100. WCE, on the other hand, never exhibited a significant inhibitory effect, inducing only a slight decrease in the growth of *Psp* and *Psn*.

3.2. Anti-Virulence Activity

Fluorescence analysis of transformed bacterial strains revealed pronounced biological activity and species-specificity of WV and WCE. Specifically, WV strongly affected the induction of the *hrpA* promoter in a dose-dependent manner across the tested species. In *Sm*, all WV dilutions resulted in a marked reduction in emitted fluorescence, with the strongest effect observed at 1:100 (Figure 2C). In *Pst*, a significant reduction was detected only at the 1:100 dilution, whereas in *Psn* a slight decrease in fluorescence was also observed at 1:500 and 1:1,000 compared to the control (Figures 2A–B). Notably, *Psn* carrying the pQS plasmid exhibited a clear reduction in fluorescence at all WV dilutions tested (Figure 2D).

WCE also showed a significant reduction in fluorescence in all tested species, although its overall effect was weaker than that of WV. The most pronounced reduction was observed at the 1:100 dilution in *Pst* and *Sm* (Figures 2F–G). In contrast, *Psn* with pQS did not show any change in fluorescence in response to WCE treatments (Figure 2H).

In addition to fluorescence, bacterial growth was influenced by the treatments. Exposure to WV 1:100 maintained all bacteria populations at their initial levels, whereas bacteria treated with lower WV concentrations grew similarly to the control (Figures S1A-D). A comparable trend was observed for WCE, except for *Pst*, which showed reduced growth in the presence of WCE 1:100 compared to the control (Figures S1E-H).

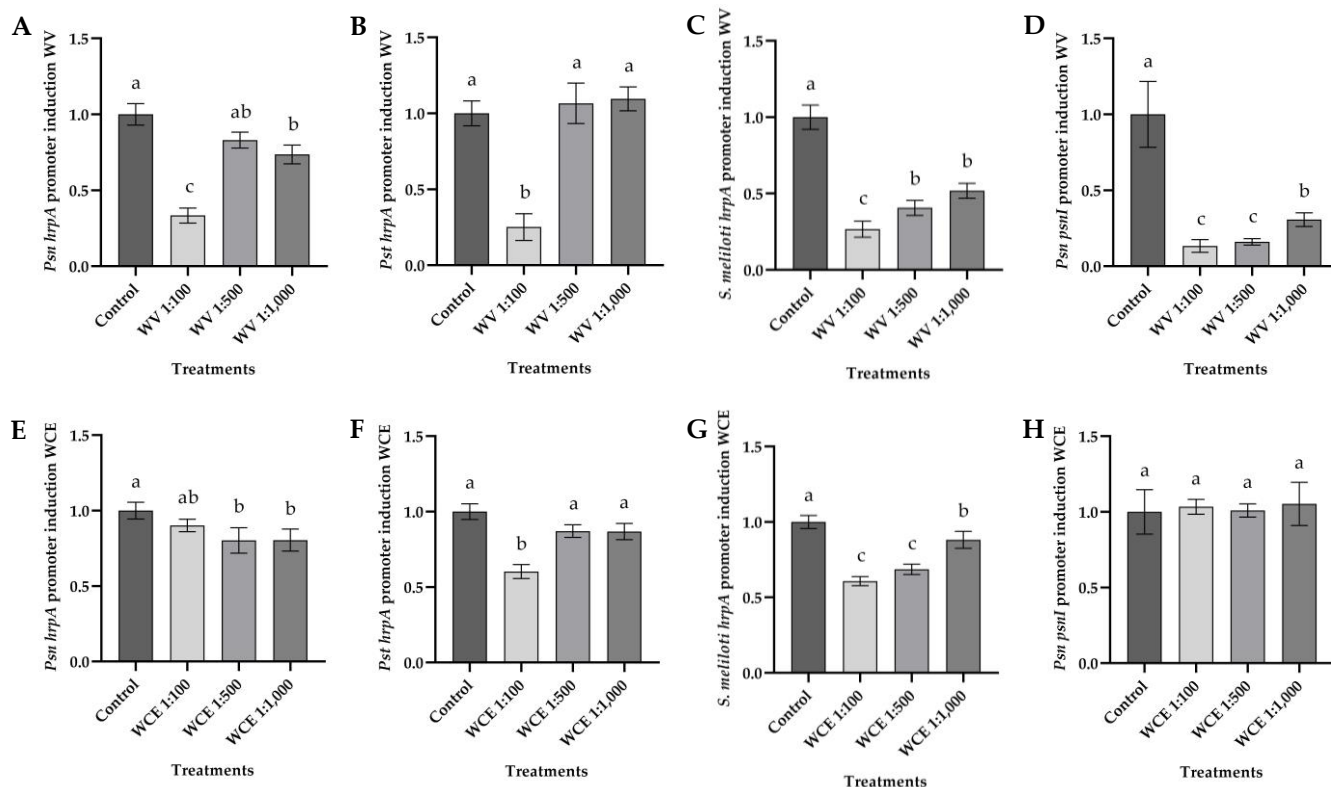


Figure 2. Effects of WV and WCE on the trans-activation of *hrpA* and *psnI* promoters. OD₆₀₀ was recorded after 24 h growth and data are calculated as GFP Abs (Ex.485nm; Em.535nm) / Abs (600nm) ± SD, and as normalized fold versus untreated bacterial cultures. A-D: *Psn*, *Pst* and *Sm* with *hrpA* promoter, *Psn* with *psnI* promoter treated with WV; E-H: *Psn*, *Pst* and *Sm* with *hrpA* promoter, *Psn* with *psnI* promoter treated with WCE. Error bars represent the standard deviation (SD). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (*p*-value < 0.05).

3.3. Phytotoxicity assay

The phytotoxicity assay revealed distinct effects between WV and WCE, highlighting both dose-dependent and species-specific responses. Concerning germination, WCE generally maintained rates comparable to the control across all tested species (Figure 3). A slight reduction in germination was observed only in tobacco and cress at high concentrations. WV, on the other hand, tended to inhibit germination when applied at high doses (450 L/ha) in tobacco, cress and alfalfa (Figures 3C-E). Tomato also showed a sharp decline in germination, whereas cucumber germination was not significantly affected by WV treatments (Figures 3A-B).

Root and shoot elongation responses varied among species and treatments. WCE had minimal effects on root elongation regardless of concentration, with the exception of cucumber, which showed a significant increase at the highest dose, and cress, which exhibited reduced root growth at 450 L/ha (Figure 4). A similar trend was observed for shoot elongation, with enhanced growth in cucumber and reduced elongation only in alfalfa (Figure 5).

Conversely, WV at 450 L/ha inhibited root development in all tested species. WV at 45 L/ha also significantly reduced root elongation in tobacco and cress (Figure 4). At the apical level, the inhibitory effect of WV was less pronounced; hypocotyl was still able to develop but remained significantly shorter than those of the control. WV at 4.5 L/ha did not negatively affect growth compared to control, except for a slight increase in cucumber (Figure 5).

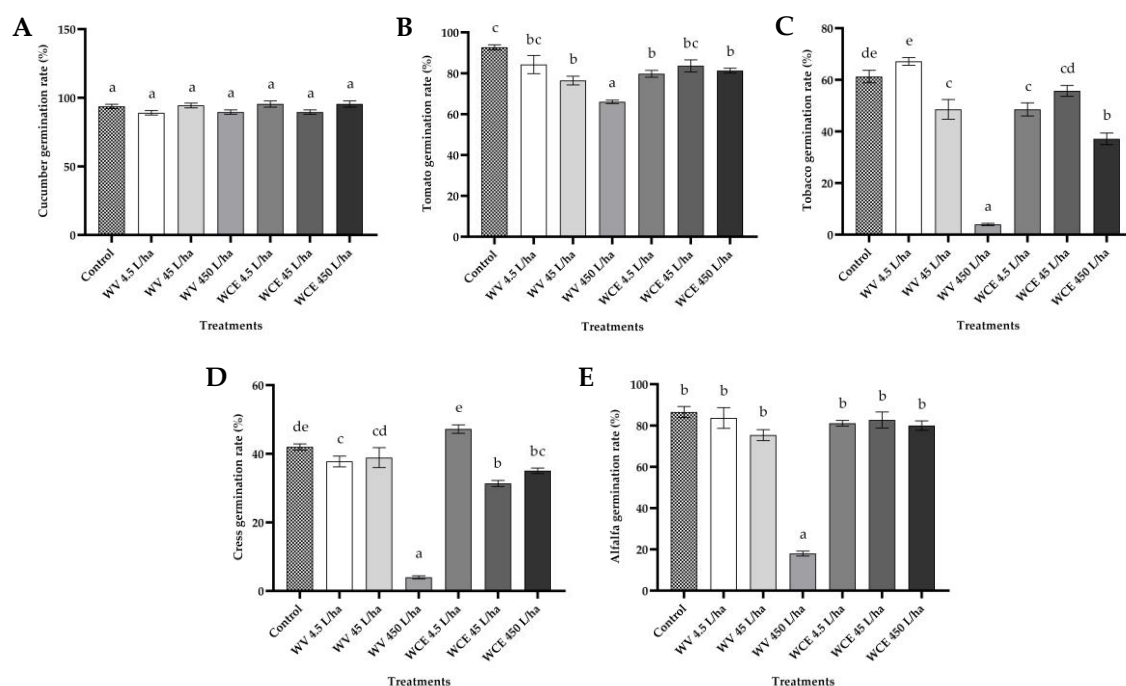


Figure 3. Germination rate after treatment with WV and WCE of cucumber (A), tomato (B), tobacco (C), cress (D) and alfalfa (E). Error bars represent the standard error (SEM). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's post hoc tests (p-value < 0.05).

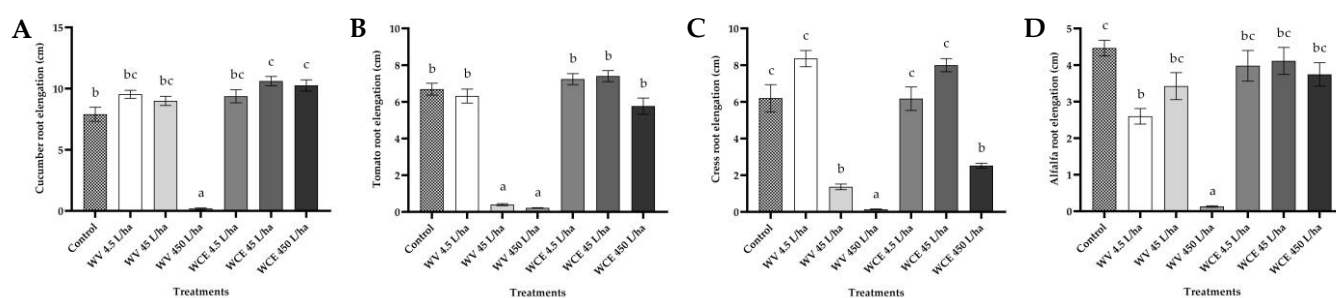


Figure 4. Root elongation after 7 days treatment with WV and WCE of cucumber (A), tomato (B), cress (C) and alfalfa (D). Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's post hoc tests (p-value < 0.05).

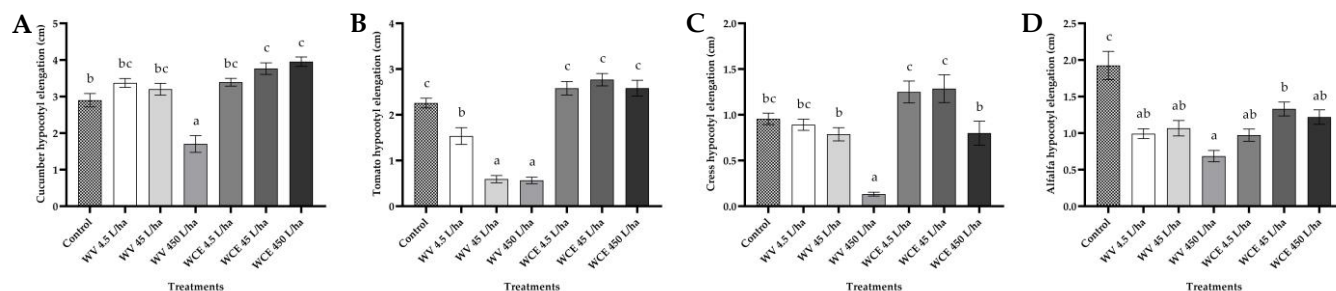


Figure 5. Root elongation after 7 days treatment with WV and WCE of cucumber (A), tomato (B), cress (C) and alfalfa (D). Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

3.4. WV and WCE Effect on In Vitro Plants

3.4.1. Plant Development and Physiology

In vitro-grown tobacco and tomato plants were sampled every 7 days; however, the results discussed here refer to the final sampling time (Figure S2). In tobacco, stem and root growth exhibited similar trends across treatments. WCE, regardless of concentration, maintained growth comparable to the control and significantly enhanced development at the 1:1,200 dilution. WV at 1:500 negatively affected tobacco growth, while WV 1:1,200 did not significantly alter development compared to the control (Figures 6A–C). Chlorophyll content in tobacco leaves was significantly higher in plants treated with WV 1:500 and with both WCE concentrations compared to both the control and WV 1:1,200 (Figure 6D).

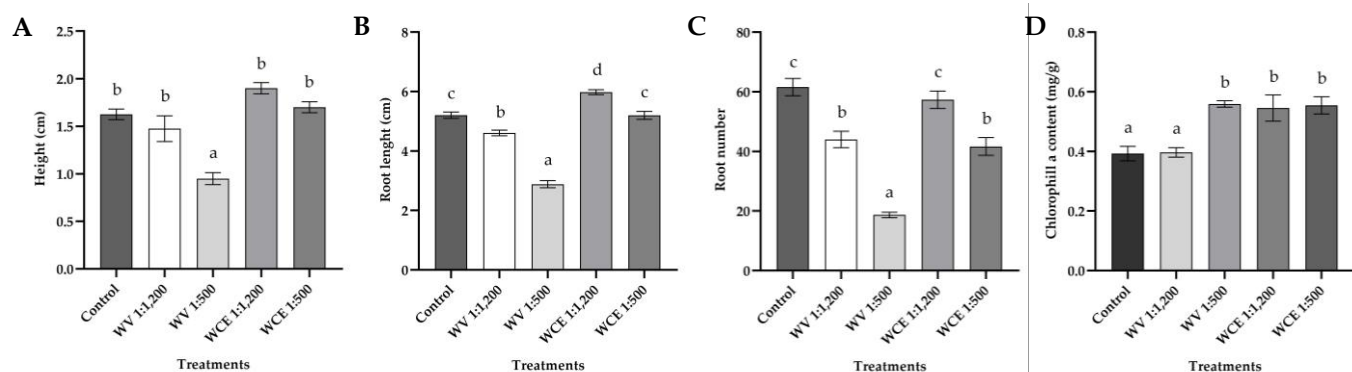


Figure 6. Morphological parameters of treated *in vitro* tobacco plants after 21 days: stem height (A), root length (B), root number (C) and chlorophyll *a* content (D). Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

In tomato, WCE treatments did not negatively affect any of the evaluated parameters compared to the control (Figure 7). Both tested concentrations promoted enhanced root development, particularly in terms of secondary roots. In contrast, WV at 1:500 had a strong phytotoxic effect, severely impairing root growth and secondary root formation. WV at 1:1,200 caused a slight reduction in performance compared to the control without compromising plant health.

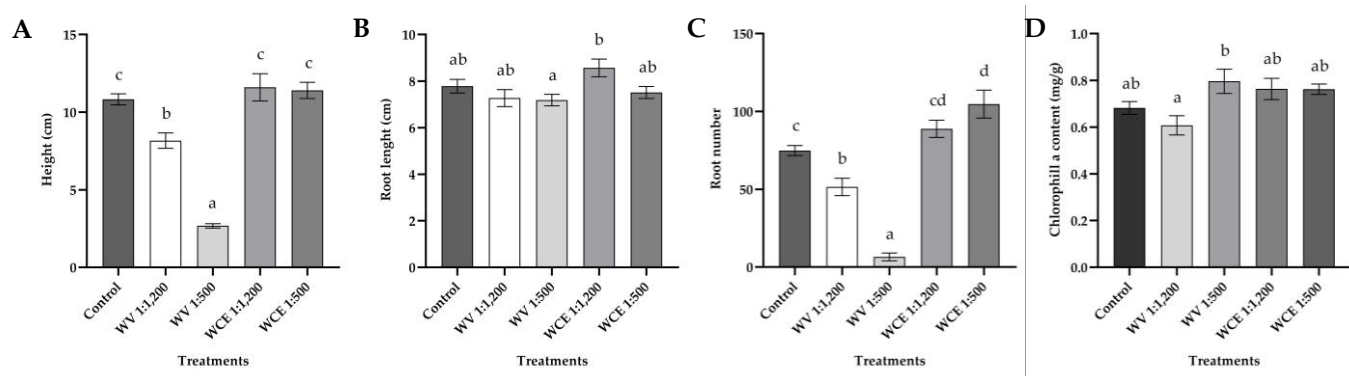


Figure 7. Morphological parameters of treated *in vitro* tomato plants after 21 days: stem height (A), root length (B), root number (C) and chlorophyll *a* content (D). Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

3.4.2. Gene Expression of Defense Related Genes

Bioproduct treatments modulated the expression of defense-related genes in a time-, dose-, and species-dependent manner. In tobacco, *ARF1* expression remained comparable to the control even after treatments lasting up to 14 days. However, after 21 days a clear upregulation was observed in plants treated with WV 1:500 and WCE 1:500 (Figures 8A-C). In tomato, *ARF1* responded more rapidly: it was significantly overexpressed after 7 days in response to 1:500, while at 14 days an upregulation was detected for all treatments. After 21 days, *ARF1* expression was downregulated in plants treated with WV 1:500 (Figures 8D-F).

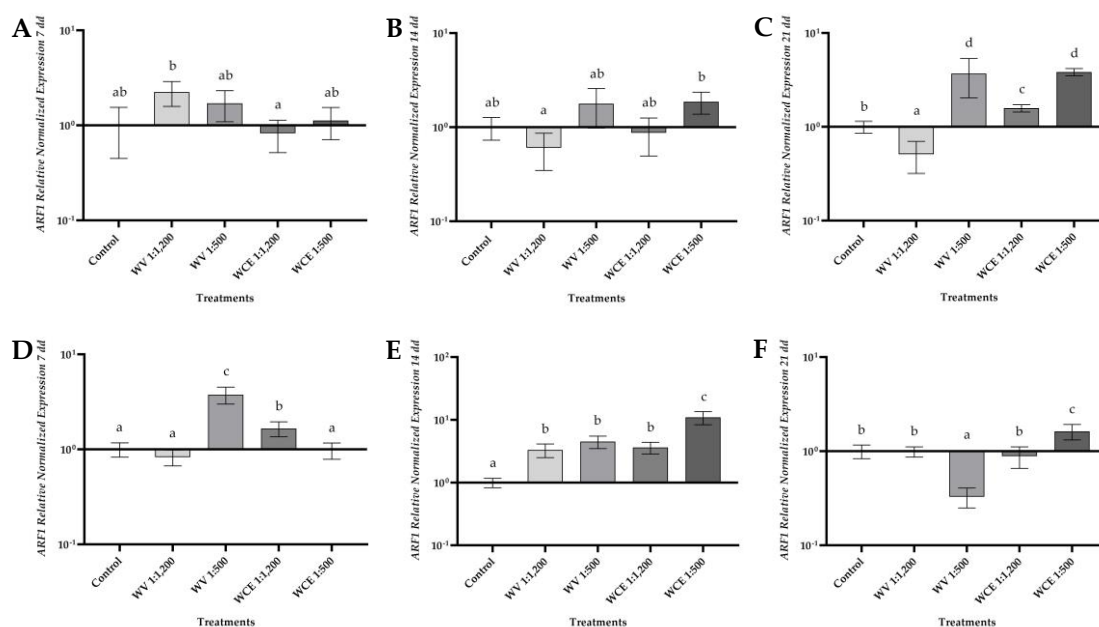


Figure 8. *ARF1* gene expression analysis at 7, 14 and 21 days after treatment with WV and WCE in *N. tabacum* (A-C) and *S. lycopersicum* (D-F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

In tobacco, *TIR1* was downregulated after 7 days of treatment with WCE 1:500, and this trend persisted at 14 days, whereas WV 1:1,200 induced overexpression at this time point. By the end of the experiment, *TIR1* was upregulated in response to all treatments

compared to the control (Figures 9A-C). In tomato, *TIR1* was overexpressed after 7 days in response to WV 1:500 and downregulated following WCE 1:500 treatment. At both 14 and 21 days, *TIR1* expression was consistently upregulated by WV 1:500 and by both WCE concentrations (Figures 9D-F).

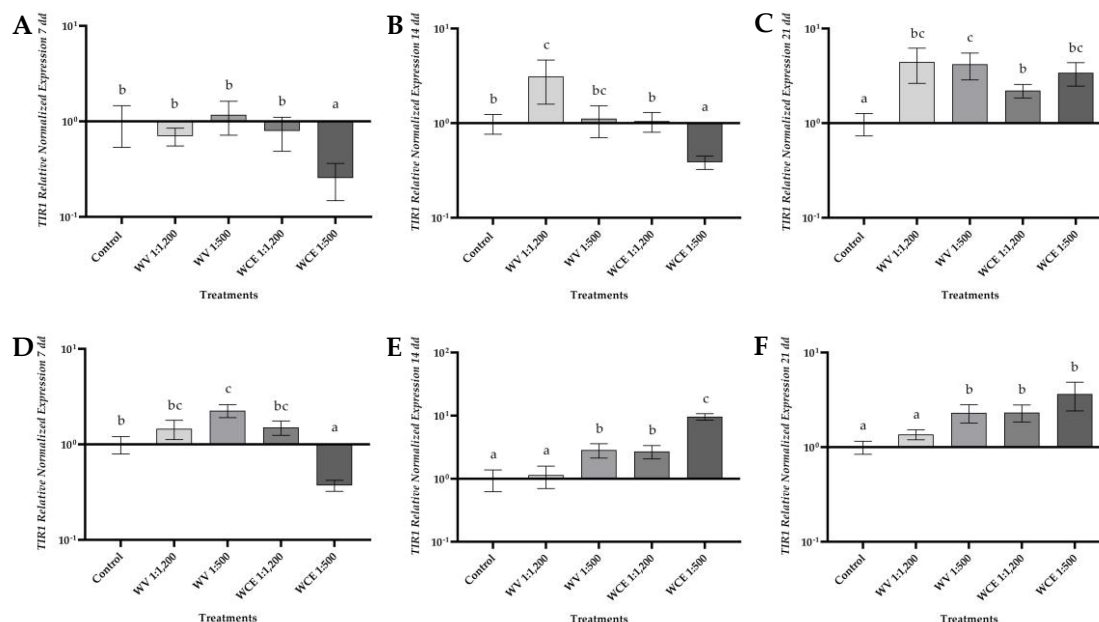


Figure 9. *TIR1* gene expression analysis at 7, 14 and 21 days after treatment with WV and WCE in *N. tabacum* (A-C) and *S. lycopersicum* (D-F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

In tobacco, *PAL* expression was induced after 7 days in response to WCE 1:1,200. At both 7 and 14 days, *PAL* was also overexpressed following WV 1:500 treatment and downregulated by WCE 1:500. By the end of the experiment, it was downregulated under all treatments (Figures 10A-C). In contrast, in tomato no differences were observed after 7 days compared to control, while a general upregulation was observed at both 14 and 21 days (Figures 10D-F).

Expression of *CHS* in tobacco showed marked temporal fluctuations. After 7 days, *CHS* was overexpressed in all treatments except WCE 1:500, which caused downregulation. After 14 days, a general overexpression was observed, particularly with WV 1:500 and WCE 1:500. At the final sampling time, *CHS* was overexpressed in plants treated with WV 1:1,200 and WCE 1:500, while it was downregulated under the other treatments (Figures 11A-C). In tomato, a general overexpression was observed throughout the experiment, with the highest levels detected after 21 days (Figures 11D-F).

In tobacco, *PR1* expression was downregulated after 7 days in response to WCE 1:500, whereas at 14 days it was overexpressed following treatment with WV and WCE 1:500. At the end of the experiment, *PR1* was upregulated in plants treated with WV 1:1,200 and WCE 1:500 (Figures 12A-C). In tomato, *PR1* was upregulated after 7 days in response WV and downregulated with WCE. After 14 days, downregulation was observed with WV and WCE 1:500, while no significant differences compared to the Control were detected at the final time point (Figures 12D-F).

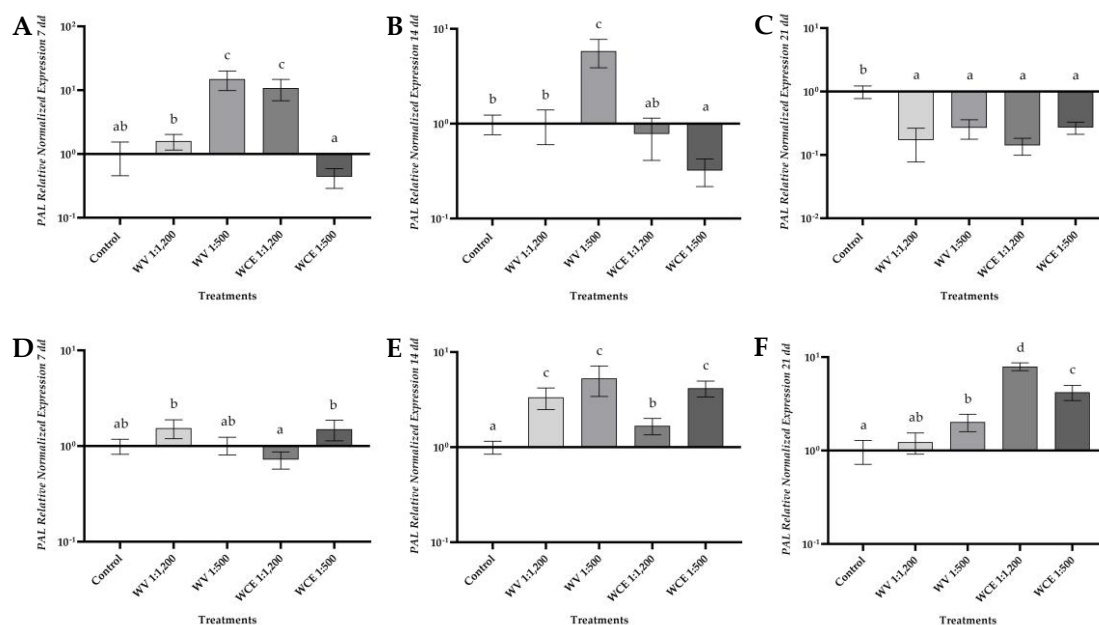


Figure 10. PAL gene expression analysis at 7, 14 and 21 days after treatment with WV and WCE in *N. tabacum* (A-C) and *S. lycopersicum* (D-F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05)

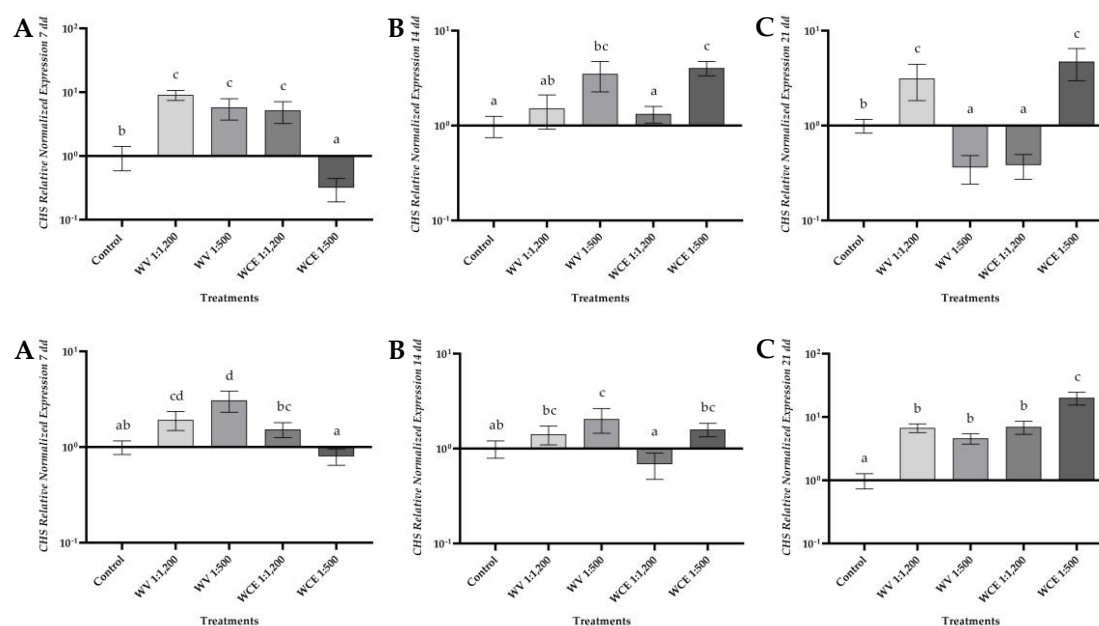


Figure 11. CHS gene expression analysis at 7, 14 and 21 days after treatment with WV and WCE in *N. tabacum* (A-C) and *S. lycopersicum* (D-F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05)

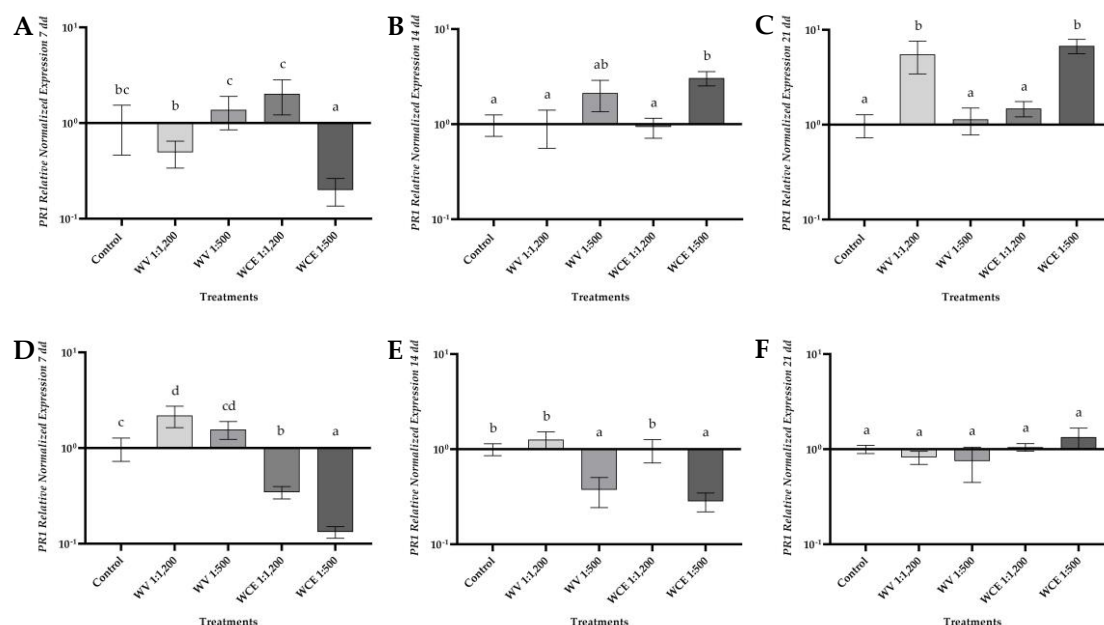


Figure 12. PR1 gene expression analysis at 7, 14 and 21 days after treatment with WV and WCE in *N. tabacum* (A–C) and *S. lycopersicum* (D–F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey’s or Dunn’s *post hoc* tests (*p*-value < 0.05)

4. Discussion

In the context of climate change and the urgent need to reduce agrochemical inputs, this study investigates sustainable strategies for the control of plant pathogenic bacteria using *in vitro* experimental approaches. The increasing restrictions on copper-based compounds and the extended use of chemical pesticides for plant protection highlight the necessity of identifying alternative solutions. In this framework, chestnut-derived wood vinegar and woodchip chestnut extracts were evaluated through a comprehensive set of *in vitro* assays. These included antibacterial and anti-virulence tests on different microorganisms, phytotoxicity assays based on seed germination and early plant growth, and experiments on *in vitro* model plants to assess both morphological responses and the expression of selected defense-related genes. By integrating multiple *in vitro* systems, this study provides a rapid, controlled, and cost-effective evaluation of the potential of chestnut-based products as eco-friendly tools for plant disease management.

Overall, the tested bioproducts exhibited distinct and context-dependent biological activities across all experimental systems. Antimicrobial activity was high, particularly for WV. Although the antimicrobial potential of WV has been investigated for several years, most studies have focused primarily on fungal pathogens, with comparatively less attention given to the antibacterial activity [8]. In this study, special emphasis was placed on bacterial species belonging to the *Pseudomonas* genus. Inhibitory effects against members of this genus have been previously reported [41], and our results further confirmed a pronounced species-specific response dependent on WV concentration (Table S3). Among the tested dilutions, only WV 1:100 showed clear biological activity, causing complete growth inhibition in *Psp* and *Psn*. Growth inhibition was also observed in *Sm*, a Gram-negative, nitrogen-fixing bacterium [42]. A similar trend was observed in the Gram-positive bacteria tested, with a significant reduction in growth with WV 1:100 for both *Cff* and *Ba*. *Cff* is a pathogen of legumes [43,44], phylogenetically close to *Clavibacter michiganensis* (*Cm*) [45], a species for which WV inhibitory activity has been consistently reported [46–48].

To investigate effects on bacterial virulence, a biotechnological reporter system originally developed by Biancalani *et al.*, 2016 [20] was employed. In bacteria belonging to the *P. syringae* group, the *hrpA* gene encodes the main structural protein of the T3SS pilus, while *psnI* encodes the *luxI*-homolog lactone synthase involved in canonical QS. The presence of T3SS in symbiotic bacteria revealed that its function is not limited to virulence. While most T3SS-containing pathogens require this system to cause disease, in rhizobia T3SS is not essential for nodulation and may exert either beneficial or detrimental effects on their symbiosis with host plants. Although the T3SS in rhizobia is not based on *hrp* genes but rather on *rhc* (Rhizobium conserved) genes [49], the high homology between the two systems has been confirmed by the recognition and activation of the *hrpA* promoter. In this study, the plasmid containing the *hrpA* promoter was also inserted into *Pst* and *Sm*. This methodology had already proved interesting and useful for the study of certain plant extracts [20], which had been found to be effective in compromising the infectious and communicative abilities of *Psn*. Our results demonstrate that WV at 1:100 was highly effective across all tested species, strongly reducing GFP expression driven by the *hrpA* promoter (Figures 2A–C). Notably, QS-regulated activity in *Psn* was compromised at all WV concentrations tested (Figure 2D). In contrast, WCE induced only a moderate reduction in reporter expression, consistently lower than that observed for WV (Figures 2E–H).

In addition to virulence-related traits, significant effects on bacterial growth were observed. The MM is not specific for growth but mimics plant conditions to stimulate bacterial interaction and signaling [33]. Under these conditions, control cultures approximately doubled their OD₆₀₀ values. In contrast, all bacteria treated with WV 1:100 and WCE 1:100—except for *Pst* in the case of WCE—maintained OD₆₀₀ values close to their initial levels, indicating a strong limitation of the growth (Figure S1). These findings suggest that the tested bioproducts may exert a dual effect in planta, simultaneously impairing bacterial growth and interfering with virulence and communication pathways, thereby limiting pathogen spread within plant tissues. The effectiveness observed under plant-mimicking *in vitro* conditions indicates that these mechanisms may also be relevant in field applications, potentially supporting the use of chestnut-derived products in plant protection strategies such as endotherapy [50].

Germination assays are widely employed to evaluate the hormonal and phytotoxic effects of WV. Previous studies across a broad range of plant species have consistently shown that low concentrations of WV stimulate seed germination and early seedling growth, while higher concentrations exert phytotoxic effects [51–56]. The results of the present study fully confirm this dose-dependent trend. At higher application rates (45 and 450 L/ha), seeds exhibited clear phytotoxic responses, affecting both germination and subsequent shoot and root elongation (Figures 3–5). The lowest concentration was always comparable to the control.

Among the tested species, cucumber displayed a reduced sensitivity to WV-induced phytotoxicity and even showed growth enhancement at low concentrations (Figures 4A, 5A). This response may be related to seed size, as cucumber seeds were substantially larger than those of the other species examined. These results confirm the potential application of WV as an herbicide when applied at high concentrations [9]. This may be relevant for grassland plants such as cress and alfalfa, as herbicidal effects had already been observed on white clover [10].

WCE, on the other hand, showed no inhibitory effects on the species tested, even at high concentrations (Figures 3–5). At low doses WCE consistently promoted both shoot and root development, confirming its strong biostimulant potential and indicating a low risk of phytotoxicity. Considering the different biological activity of the two products, it

can be assumed that the effect obtained was not directly associated with pH, as both substances are extremely acidic, but rather with their composition.

These trends were also observed in treated *in vitro* plants. Although *in vitro* culture systems are traditionally used for the conservation, propagation and regeneration of plant species [57], they are less frequently applied to assess the biological activity of compounds. While such systems do not fully reproduce whole-plant physiology, they provide a sterile and highly controlled environment that minimizes experimental variability, thereby allowing a clearer evaluation of treatment effects. In this context, WCE stimulated the development of both tobacco and tomato across all evaluated parameters and at both tested concentrations, further corroborating its biostimulant potential (Figures 6-7). Conversely, WV exhibited a pronounced dose-dependent response: WV 1:500 proved to be unsuitable for *in vitro* cultivation, severely impairing both shoot and root development, whereas WV 1:1,200 slightly reduced shoot elongation without compromising overall plant health in either tobacco or tomato (Figures 6-7).

Once absorbed by plant tissues, WV is known to trigger defense-related signaling processes, including the production of reactive oxygen species. Previous molecular studies have mainly focused on the activity of antioxidant enzymes [37,58,59]. Tannins are also known to contribute to plant defense through antimicrobial activity and the mitigation of oxidative stress [60,61]. Here, the expression of key genes involved in hormone signaling, secondary metabolism and salicylic acid-dependent pathways was examined to clarify the molecular effects of WV and WCE. For the selected genes, different trends over time were observed between tobacco and tomato.

Auxin signaling was investigated by analyzing the expression of *ARF1* and *TIR1*. ARF proteins regulate auxin-responsive gene expression through interactions with Aux/IAA proteins, with *ARF1* acting as a transcriptional repressor [62,63]. In tobacco, the highest peaks of *ARF1* overexpression were observed at the end of the trial, while in tomatoes they were observed after 14 days of treatment (Figure 8). *TIR1*, on the other hand, reached overexpression in both plant species at 21 days with both products tested (Figure 9).

PAL is a key enzyme in the phenylpropanoid biosynthesis pathway [64], and it is also involved in salicylic acid (SA) biosynthesis. In tobacco, *PAL* expression was downregulated at the end of the experiment across all treatments, whereas in tomato it was consistently upregulated at both 14 and 21 days, with a stronger response to WV and then with WCE (Figure 10).

CHS is a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway. Besides being part of the plant developmental program, the *CHS* gene expression is induced in plants under stress conditions; it is also involved in SA defense pathway [65]. In both tobacco and tomato, *CHS* showed a general trend of increasing overexpression over time. At 21 days, the strongest upregulation was observed with WV 1:1,200 and WCE 1:500 in both species (Figure 11).

Finally, expression of the pathogenesis-related gene *PR1* exhibited species-specific patterns (Figure 12). In tobacco, a significant modulation was observed mainly in response to WCE 1:500, with downregulation after 7 days followed by upregulation at 14 and 21 days. At the final time point, WV 1:1,200 also induced significant changes in *PR1* expression. In tomato, *PR1* expression tended to be downregulated by WCE relative to the Control but returned to basal levels by the end of the experiment. Overall, tomato appeared to respond more strongly through flavonoid-related pathways, whereas the combined overexpression of *CHS* and *PR1* in tobacco suggests that WV 1:1,200 and WCE 1:500 are the most effective treatments in stimulating the plant defense responses after prolonged exposure.

5. Conclusions

In conclusion, this extensive series of *in vitro* experiments allowed the distinct biological activities of WV and WCE to be elucidated. WV consistently exhibited a clear dose-dependent effect across all assays carried out. It demonstrated strong antibacterial activity, particularly under conditions simulating plant infection, suggesting potential applications in plant protection strategies such as endotherapy. In plant-based assays, WV confirmed its phytotoxic effect at high concentrations and its hormone-like stimulatory effects at low concentrations, supporting its dual potential as both an herbicide and a plant corroborant.

In contrast, WCE displayed predominantly biostimulant properties, enhancing plant development and growth through the activation of multiple hormonal and defense-related pathways. Its antimicrobial activity appeared limited under the conditions tested.

Overall, these findings highlight the distinct and complementary biological profiles of WV and WCE. Further studies will be required to validate and expand upon these observations and to better define their practical applications in agricultural systems.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/doi/s1>, Table S1. Primer information for *Nicotiana tabacum*; Table S2. Primer information for *Solanum lycopersicum*; Table S3. Bacterial growth in LB after 24h incubation. The OD₆₀₀ values were all normalized using the respective control. Error values represent the standard deviation (SD). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (*p*-value < 0.05); Figure S1. Bacterial growth in MM after 24h incubation. A-D: *Psn*, *Pst* and *Sm* with *hrpA* promoter, *Psn* with *psnI* promoter treated with WV; E-H: *Psn*, *Pst* and *Sm* with *hrpA* promoter, *Psn* with *psnI* promoter treated with WCE. Error bars represent the standard deviation (SD). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (*p*-value < 0.05); Figure S2. Most representative plant of each treatment of tobacco (A) and tomato (B).

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“How to” but *In Vitro*: Integrated and Advanced Biotechnological Assays to Predict *In Vivo* Potential of Wood-Derived Extracts

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Table S1. Primer information for *Nicotiana tabacum*.

Gene		Primer sequence 5′ – 3′	Tm (°C)	Designed by
<i>L25</i>	Forward	TATCCAAATGGCTCCAGC	59	[1]
	Reverse	TCTTCAAAGTCTTAGGTCCG		[2]
<i>ARF1</i>	Forward	CGGCAAATCACTTCGGTGG	60	[1]
	Reverse	AATCCCTGATGTGTCGATGC		
<i>TIR1</i>	Forward	GCTCCAGACTCACCTCTT	57	[1]
	Reverse	GGCAGCAAGTTCCTCAAG		
<i>PAL</i>	Forward	TAGACAAACCAACCCCTTTACACTT	60	[1]
	Reverse	GCCACCATTTTCTTCACTTCATC		
<i>CHS</i>	Forward	ATGTGGATAGTATGGTTGGAC	57	[1]
	Reverse	CAAGTGAAATGTTAGCCCGA		
<i>PR1</i>	Forward	CCTCGTACATTCTCATGGTCAAT	57	[1]
	Reverse	CCATTGTTACTGAACCCTAGC		

Table S2. Primer information for *Solanum lycopersicum*.

Gene		Primer sequence 5′ – 3′	Tm (°C)	Designed by
<i>Ubi3</i>	Forward	TCGTAAGGAGTGCCCTAATGCTGA	60	[3]
	Reverse	CAATCGCCTCCAGCCTTGTGTAA		
<i>Act</i>	Forward	AGGCAGGATTTGCTGGTGATGATGCT	60	[3]
	Reverse	ATACGCATCCTTCTGTCCCATTCCGA		
<i>ARF1</i>	Forward	CGGCAAATCACTTCGGTGG	54	[1]
	Reverse	AATCCCTGATGTGTCGATGC		
<i>TIR1</i>	Forward	AGGGGTCCTCCAGATACAAG	60	[4]
	Reverse	CGCTAATACCTGCCCATCTTT		
<i>PAL</i>	Forward	ATGGAACACATTTTGGATGG	58	[1]
	Reverse	TCTCGATCATCTTAGTTGC		
<i>CHS</i>	Forward	ATGTGGATAGTATGGTTGGAC	58	[1]
	Reverse	CAAGTGAAATGTTAGCCCGA		
<i>PR1</i>	Forward	GGCATCCCAGACAAAA	60	[5]
	Reverse	CCCAGCACCAGAATGAATCA		

Table S3. Bacterial growth in LB after 24h incubation. The OD₆₀₀ values were all normalized using the respective Control. Error values represent the standard deviation (SD). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (*p*-value < 0.05).

	WV Bacterial Growth	WCE Bacterial Growth
<i>Pseudomonas amygdali</i> pv. <i>tabaci</i>		
Control	1 + 0.11 a	1 + 0.12 a
1:100	0.96 + 0.12 a	1.00 + 0.12 a
1:500	1.01 + 0.12 a	0.99 + 0.11 a
1:1000	1.01 + 0.12 a	1.01 + 0.13 a
<i>Pseudomonas syringae</i> pv. <i>tomato</i>		
Control	1 + 0.13 a	1 + 0.11 a
1:100	0.856867 + 0.12 a	0.94 + 0.12 a
1:500	0.93 + 0.12 a	0.92 + 0.11 a
1:1000	0.94 + 0.13 a	0.92 + 0.13 a
<i>Pseudomonas savastanoi</i> pv. <i>savastanoi</i>		
Control	1 + 0.24 a	1 + 0.18 a
1:100	0.74 + 0.15 ab	1.01 + 0.14 a
1:500	1.12 + 0.24 a	0.92 + 0.17 a
1:1000	1.10 + 0.28 a	0.94 + 0.14 a
<i>Pseudomonas savastanoi</i> pv. <i>phaseolicola</i>		
Control	1 + 0.14 a	1 + 0.10 a
1:100	0.24 + 0.04 c	0.74 + 0.19 ab
1:500	1.13 + 0.13 a	0.83 + 0.11 ab
1:1000	1.14 + 0.15 a	0.82 + 0.12 ab
<i>Pseudomonas savastanoi</i> pv. <i>nerii</i>		
Control	1 + 0.20 a	1 + 0.14 a
1:100	0.14 + 0.03 c	0.85 + 0.10 ab
1:500	0.93 + 0.14 a	0.98 + 0.11 a
1:1000	0.92 + 0.14 a	1.02 + 0.12 a
<i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i>		
Control	1 + 0.11 a	1 + 0.09 a
1:100	0.68 + 0.06 b	1.08 + 0.08 a
1:500	0.97 + 0.09 a	1.03 + 0.08 a
1:1000	0.97 + 0.09 a	1.03 + 0.09 a
<i>Sinorhizobium meliloti</i>		
Control	1 + 0.07 a	1 + 0.06 a
1:100	0.15 + 0.02 c	0.90 + 0.06 a
1:500	0.94 + 0.07 a	1.09 + 0.06 a
1:1000	0.97 + 0.07 a	1.02 + 0.07 a
<i>Bacillus amyloliquefaciens</i>		
Control	1 + 0.14 a	1 + 0.17 a
1:100	0.68 + 0.10 b	0.96 + 0.16 a
1:500	0.94 + 0.11 a	0.97 + 0.16 a
1:1000	0.96 + 0.11 a	1.07 + 0.27 a

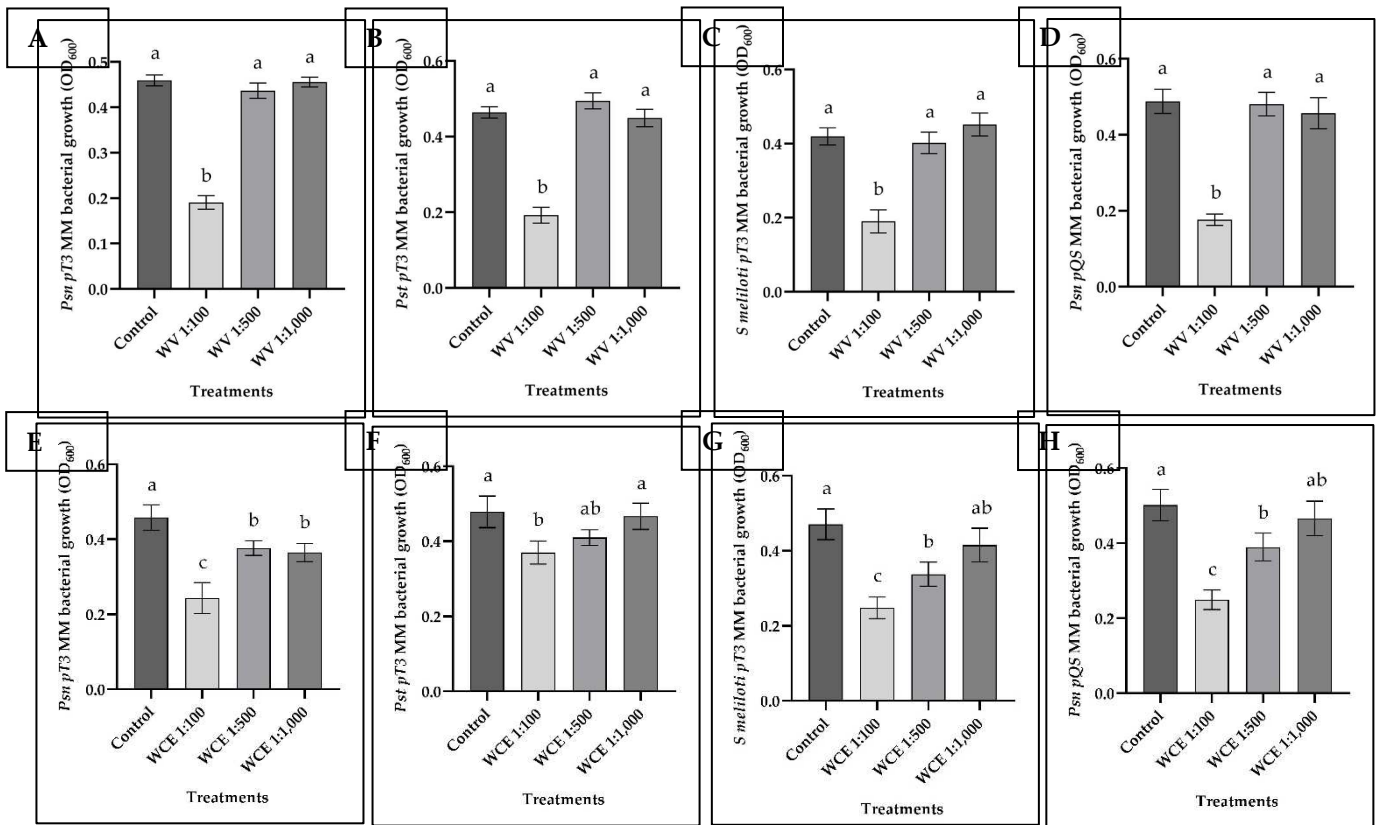


Figure S1. Bacterial growth in MM after 24h incubation. A-D: *Psn*, *Pst* and *Sm* with *hrpA* promoter, *Psn* with *psnI* promoter treated with WV; E-H: *Psn*, *Pst* and *Sm* with *hrpA* promoter, *Psn* with *psnI* promoter treated with WCE. Error bars represent the standard deviation (SD). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's post hoc tests (p -value < 0.05).

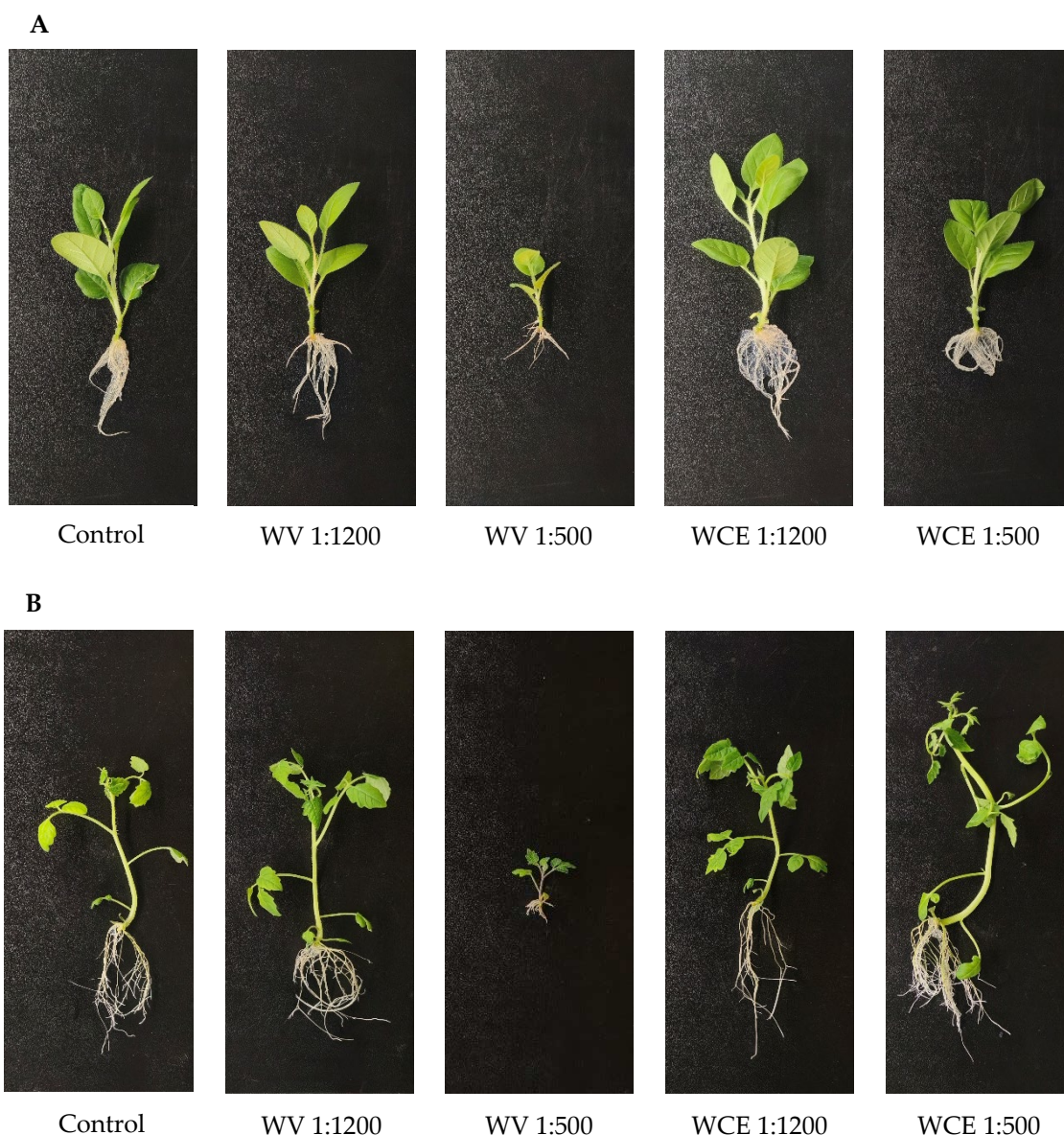


Figure S2. Most representative plant of each treatment of tobacco (A) and tomato (B)

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2. Methodologies and Results

2.2. Effects on plant-soil interaction

Article

Marketed and research-derived chestnut extracts: help or hype for soil health and plant resilience under stress?

Marketed and Research-Derived Chestnut Extracts: Help or Hype for Soil Health and Plant Resilience under Stress?

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Abstract

The increasing occurrence of extreme climatic events, including droughts, floods, and heatwaves, together with adverse soil conditions such as salinity and water scarcity, represents a major constraint to agricultural productivity and plant development. Consequently, there is an urgent need to identify sustainable and environmentally sound strategies to support crop production under changing environmental conditions. In this context, agroforestry by-products can be valorized as bio-based extracts with biostimulant potential, contributing to circular and sustainable agricultural practices. Chestnut biomass is particularly promising due to its high content of polyphenolic compounds, especially tannins. This study investigates the effects of two chestnut-derived bioproducts, namely wood vinegar and an extract obtained from chestnut woodchips, on the growth of baby lettuce (*Lactuca sativa* var. baby) and on soil physicochemical properties under drought and salinity stress conditions. Both bioproducts exhibited biostimulant activity, particularly in plants exposed to abiotic stress, without negatively affecting soil quality. Wood vinegar, in particular, has attracted considerable attention as a potential agricultural input due to its reported ability to enhance crop yield, quality, and plant health. Under optimal growth conditions, no substantial benefits were observed; however, under water stress, treated plants achieved yields comparable to those of non-stressed controls. Under saline conditions, the most pronounced effects were observed at the soil level, notably through enhanced enzymatic activity, suggesting a positive modulation of soil biological functioning.

Keywords: wood vinegar; woodchip extract; chestnut biomass; biostimulants; abiotic stress; salt stress; drought stress; soil enzymatic activities; sustainable agriculture

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1. Introduction

Human-driven climate change, largely caused by rising greenhouse gas (GHG) emissions, represents a significant threat to global agriculture and food security. The steady increase in global temperatures is leading to more frequent and severe extreme weather events, including droughts, floods, and heatwaves [1,2], which negatively affect plant development and reduce crop productivity [3]. At the same time, agriculture itself is a major

contributor to GHG emissions, releasing around 16.2 billion tons of CO₂ equivalent in 2022. Key sources include excessive agrochemical use, unsustainable livestock systems, overexploitation of natural resources, and land-use changes such as deforestation [4,5].

Additionally, approximately 200 billion tons of lignocellulosic biomass are generated annually worldwide, primarily originating from agricultural and forestry residues [6]. A large proportion of this biomass is classified as waste and, in some cases, is burned, resulting in the emission of GHGs. Such practices exacerbate climate change and pose risks to human health and environment quality [7]. Currently, this biomass is only partially reused, for example as construction material, in fiber production or as energy source [6]. Therefore, the development of efficient management strategies represents a promising approach for a green and circular economy.

It should be noted that plant biomasses differ markedly in their chemical composition, and some exhibit specific properties that can be strategically exploited in agriculture. Indeed, certain species, such as chestnut, acacia, pine and oak, are extremely rich in polyphenols, especially tannins [8]. The antimicrobial and insect-repellent properties of tannins have been widely documented [9]. Consequently, the development of efficient extraction methods and suitable formulations is essential to fully exploit these bioactive properties and enhance their application in sustainable agriculture.

In this context, the production of wood vinegar represents a feasible and sustainable approach to valorizing these lignocellulosic biomasses. Wood vinegar, also defined as pyroligneous acid or wood distillate, is a by-product of plant biomass pyrolysis, resulting from the condensation of volatile organic compounds produced during this process [10,11]. It is a dark liquid made up mainly of water (80%-90%) and contains more than 200 water-soluble organic compounds. The organic phase contains many chemical compounds such as acids, alcohols, aldehydes, ketones, ethers, esters, phenols, sugars, alkenes, furans, and various oxygen compounds [10].

Wood vinegar has long been used in agriculture for its ability to enhance plant growth, protect plant against pests and diseases, and improve soil health, showing potential as a natural fertilizer, pesticide, as well as herbicide [13]. Its chemical composition of pyrolysis products is strongly influenced by the characteristics of the raw materials, as well as by process parameters such as residence time, heating rate, temperature, particle size, and feedstock origin, also significantly affect the final wood vinegar composition [11,14].

Besides wood vinegar, chestnut extracts have also been extensively investigated due to their particularly high tannin content [15]. The majority of these studies investigated leaves, galls, bark, and wood components as primary source of tannins [8,16]. Tannins are polyphenolic secondary metabolites commonly present in vascular plants, typically accounting for 5–10% of plant dry biomass. These compounds play a central role in plant defense, exerting direct antimicrobial, insecticidal, and anti-herbivory effects [17]. Chestnut wood water extracts are mainly abundant in hydrolysable tannins (HTs), consisting of a D-glucose molecule esterified partially or fully with phenolic compounds like gallic and ellagic acids [18]. In addition to their established use in animal feed, HTs have gained attention in agronomic studies for their effects on plant growth, soil quality, and irrigation water parameters [19]. HTs have been reported to enhance plant tolerance to abiotic stresses and to provide protection against pests [20].

In this context, the present study aimed to investigate the effects of two commercial wood vinegars derived from the pyrolysis of chestnut wood residues, as well as of a laboratory-scale extract obtained from chestnut woodchips. Specifically, the study evaluated their direct impact on soil physicochemical properties and microbial enzymatic activity. Additionally, the macro- and micronutrient composition of *Lactuca sativa* var. baby

was analyzed under abiotic stress conditions. To this aim, parallel experiments were con- 92
ducted under both drought and salt stress scenarios. 93

2. Materials and Methods

2.1. Tested Products

In this study, two commercial wood vinegars and a chestnut wood chip extract were characterized and evaluated. The commercial products were Biodea® (Arezzo, Italy) wood vinegar (WV) and Nerabiochar® (Turin, Italy) wood distillate (NB), both produced using chestnut wood as starting material. The wood chip extract (WCE), on the other hand, was obtained by infusing 2 L of chestnut woodchips (approximately 1.2–1.3 kg) in 4 L of tap water. The extraction process lasted 3 hours at 80–85°C [21]. At the end, the liquid was filtered and centrifuged to remove macroscopic residues and contaminants.

The pH and electrical conductivity (EC) were evaluated. Total phenolic content was determined and expressed as gallic acid equivalent (GAE) [22]. Carbon (C) and nitrogen (N) contents were quantified, as well as the quantity of heavy metals, macro- and micro-nutrients, using respectively Multi N/C 3100 (Analytic Jena, Germany) and Thermo iCAP 6500 Duo ICP-AES Spectrometer (ThermoFisher Scientific, Waltham, USA). In addition, the phenolic profile of the WCE was further characterized by high-performance liquid chromatography (HPLC).

2.2. Soil Experiment Setup

The products of interest were first applied exclusively to the soil to evaluate their effects on its physicochemical properties. The experiment was carried out in controlled conditions in the laboratory of the Department of Soil and Water Conservation and Organic Waste Management (CEBAS-CSIC, Murcia, Spain). 80 g of agricultural soil collected from the experimental field of the CEBAS-CSIC located in Santomera (Murcia, Spain, 38°06'33.9"N 1°02'16.5"W) was placed in Petri dishes (Ø 92 mm). The soil consisted of 23.2% clay, 27.2% silt, 49.6% sand, and had a water retention capacity of 33%. Only at the beginning of the trial, the soil was treated with WV and NB, each diluted 1:25 in distilled water, and with WCE, diluted 1:10. For each treatment, five replicates were prepared. The soil plates were incubated for 2 weeks in a growth chamber with the following conditions: 18°C night/23°C day, a photoperiod of 16h light/8h dark, and humidity of 60–75%. During the incubation period, soil moisture was maintained at 60% of water holding capacity through regular irrigation. At the end of the experiment, the soil was collected to determine the effects on soil physicochemical properties.

2.3. Lettuce Experiment Setup

Subsequently, the effects of WV, NB and WCE were evaluated in a more complex plant-soil system. Specifically, the experiment was carried out in controlled conditions in the laboratory of the Department of Soil and Water Conservation and Organic Waste Management (CEBAS-CSIC, Murcia, Spain). Briefly, 1-month-old seedlings of lettuce var. baby (*L. sativa* var. baby) were transplanted into plastic pots (13 × 10 cm, h = 12 cm) containing 780 g of the same soil used in the previous experiment.

The experimental design consisted of three main groups: a non-stressed control, drought stress, and salt stress. Each group included four treatments, as shown in Table 1. For each treatment, five replicates were prepared. After transplantation, lettuce plants were irrigated and fertilized once with 1 g per pot of 8:24:8 NPK granular fertilizer diluted in distilled water.

Drought stress was imposed by supplying plants with 70% of the optimal irrigation volume throughout the experiment, whereas salt stress was induced by adding 4.5 g/L NaCl to the irrigation water (electrical conductivity EC = 4.94 mS/cm). Wood vinegars and WCE were also applied immediately after transplantation by diluting them directly in the irrigation water, at a concentration of 1:400 for WV and NB and 1:300 for WCE. Treatments were repeated every 7 days. Lettuce plants were incubated for 23 days in a growth

chamber with the following conditions: 18°C night/23°C day, a photoperiod of 16h light/8h dark, and humidity of 60-75% (Figure 1). They were irrigated three times per week, maintaining the soil at 60% of water holding capacity. Once a week, the chlorophyll content was measured with a chlorophyll meter SPAD-502Plus (Konica Minolta, Chiyoda, Japan) to evaluate variation in the photosynthetic activity.



Figure 1. Lettuce experiment setup in the growth chamber

Table 1. Scheme of all treatments under different conditions

Normal conditions	
N-C	Control
N-WV	Wood vinegar Biodea® 1:400
N-NB	Wood vinegar NeraBiochar® 1 :400
N-WCE	Woodchip Extract 1:300
Drought stress conditions	
D-C	Control
D-WV	Wood vinegar Biodea® 1:400
D-NB	Wood vinegar NeraBiochar® 1 :400
D-WCE	Woodchip Extract 1:300
Salt stress conditions	
S-C	Control
S-WV	Wood vinegar Biodea® 1:400
S-NB	Wood vinegar NeraBiochar® 1:400
S-WCE	Woodchip Extract 1:300

2.4. Morphology Analysis

For each treatment, lettuce leaves were harvested by cutting them at the base of the stem, and the fresh weight was determined. Roots were carefully washed with tap water to completely remove soil particles. Leaves and roots were then incubated at 60°C, and after 2-3 days the dry weight was measured, once a constant weight was reached. Subsequently, both tissues were pulverized with a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) with steel beads for 30s at 30 Hz and analyzed to determine the total C and N content with an ELEMENTAL LECO TruSpec CN (LECO Corporation, St. Joseph, Michigan, USA). The analyses were carried out by the Ionomics Service of the CEBAS-CSIC

(Murcia, Spain). In addition, macro- and micronutrient composition was determined with a Thermo iCAP 6500 Duo ICP-AES Spectrometer (ThermoFisher Scientific, Waltham, USA).

2.5. Analysis of Soil Physicochemical Properties

The soil was air-dried at room temperature and passed through a <2 mm sieve to assess various physicochemical properties and enzymatic activities. Soil respiration was evaluated by placing 15 g of soil into airtight flasks and incubating them at 28°C for 20 days in the dark. The CO₂ produced was measured weekly using a Dansensor® Check-Mate3 gas analyzer (AMETEK MOCON, Ringsted, Denmark). The total CO₂ emission was obtained by adding the weekly values, and basal soil respiration was then calculated using the following equation:

$$\text{mg C-CO}_2\text{kg}^{-1} \text{ soil day}^{-1} = \frac{4.85 * \text{Total CO}_2 * (\text{flask volume} - \text{soil volume})}{\text{Soil dry weight} * n^\circ \text{ total days}}$$

Soil pH was measured in a 1/5 (w/v) soil–water suspension using a LAQUA PH1100 pH meter (HORIBA, Ltd., Tokyo, Japan). Moreover, soil EC was determined in a 1/5 (w/v) aqueous extract with a LAQUA twin EC-33 meter (HORIBA, Ltd., Tokyo, Japan). The same extract was prepared to determine water-soluble carbon (WSC) measured with an analyzer for liquid samples (Multi N/C 3100, Analytic Jena, Germany). Both non-soluble and water-soluble macro- and micronutrients in soil were analyzed by the Ionics Service at CE-BAS-CSIC (Murcia, Spain).

2.6. Analysis of Soil Enzymatic Activity

β-glucosidase activity was determined using 0.5 g of soil and *p*-nitrophenyl-β-D-glucopyranoside as substrate as described by Eivazi and Tabatabai [23]. Alkaline phosphatase activity was evaluated with the method of Tabatabai and Bremner [24], using 0.5 g of soil and *p*-nitrophenyl phosphate (disodium salt, hexahydrate) as substrate. The method of Kandeler and Gerber [25] was followed to assess the urease activity, using 1 g of soil and urea as substrate. Dehydrogenase activity was determined according to García *et al.* [26], using 1 g of soil. To assess glycine-aminopeptidase activity, 0.5 g of soil were combined with 2 mL of 50 mM Tris-HCl buffer (pH 7) and 2 mL of 2 mM glycine *p*-nitroanilide. The mixtures were incubated in a water bath at 40°C for 2 hours. The reaction was halted by placing the samples on ice (approximately 2°C) for 15 minutes. Subsequently, 1 mL of 0.75% (v/v) acetic acid was added, followed by mixing and centrifugating at 3,400 rpm for 10 min. The amount of released *p*-nitroaniline (PNA) was quantified spectrophotometrically at 410 nm. Control samples were prepared in the same way, except that the glycine *p*-nitroanilide substrate was added only after the acetic acid.

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Boston, MA, USA). After checking data for normal distribution, either a one-way ANOVA or a Kruskal–Wallis test was applied, using a significance threshold of $p < 0.05$. *Post hoc* tests were conducted to compare the mean of each treatment group with the means of all other groups.

3. Results

3.1. Product Chemical Analysis

The products used in this study exhibited comparable pH values and total phenolic contents, characterized by high acidity (pH ≈ 2.5–3.5) and elevated phenolic concentrations (7–8 mg/mL). On the contrary, greater variability was observed in EC, with values ranging

from approximately 600 $\mu\text{S}/\text{cm}$ in WCE to 1600 $\mu\text{S}/\text{cm}$ in NB (Table 2). Significant differences were observed in the C and N contents, with wood vinegars showing higher soluble C concentrations, whereas WCE exhibited a higher N content. No substantial differences were observed in macro- and micronutrient composition among treatments, except for NB, which showed markedly higher concentrations of Fe and Ca (Tables S1–S2–S3). The characterization of the phenolic fraction contained in WCE showed the presence of ellagic acid, tannins and their derivatives (Table S4).

Table 2. Analysis of pH, EC, GAE, TOC and TN of all studied products

Product	pH	EC	GAE (mg/mL)	TOC (g/L)	TN (g/L)
WV	2.29	1084 $\mu\text{S}/\text{cm}$	8.31	42.61	0.12
NB	3.37	1596 $\mu\text{S}/\text{cm}$	8.12	25.20	0.49
WCE	3.31	618 $\mu\text{S}/\text{cm}$	7.06	2.74	7.47

3.2. Effects of Bioproducts on Soil

The analysis of soluble total organic carbon (TOC) and N showed a variability in soil nutrient availability after the treatment with different bioproducts. Specifically, all three products significantly increased soluble C compared to the control. The treatment with NB also raised the N available quantity (Table S5). In contrast, no significant differences were detected in soluble macro- and micronutrient concentrations between control and treated soils (Tables S6–S8).

Soil enzymatic activities responded differently to the applied bioproducts (Figure 2). Activity of β -glucosidase, dehydrogenase and glycine-aminopeptidase showed no variation between treated and control samples. By contrast, urease activity was strongly reduced with both WV and NB, while behavior comparable to the control was observed with WCE. Lastly, alkaline phosphatase activity was reduced only with WV.

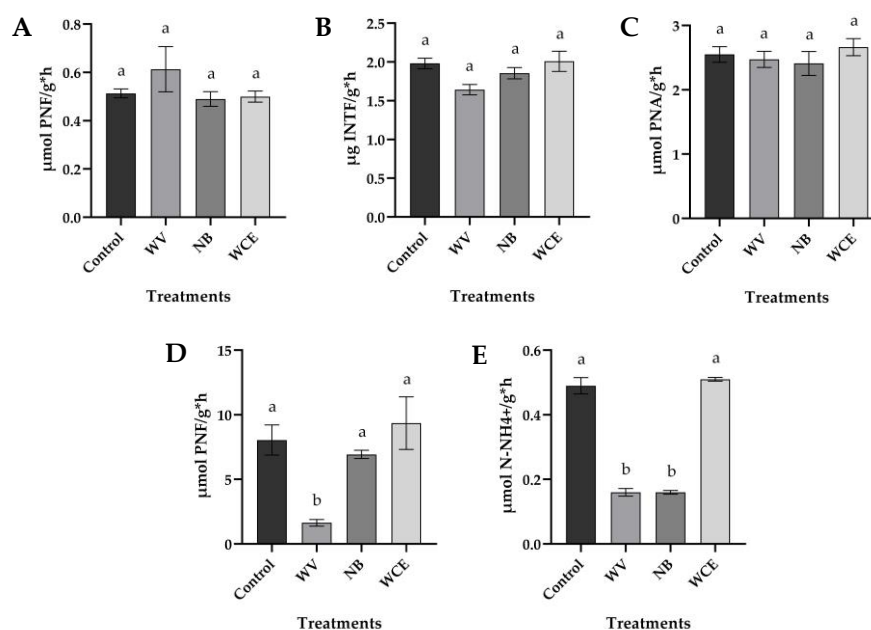


Figure 2. Soil enzymatic activity. Specifically, the activity of β -glucosidase (A), dehydrogenase (B), glycine-aminopeptidase (C), alkaline phosphatase (D), and urease (E) were evaluated. Error bars represent the standard error of the mean (SEM). Significance (p -value < 0.05) was shown by letters.

3.3. Effects of Bioproducts on Lettuce under Normal Conditions

Under non-stress conditions, plant morphology was only marginally affected by the different treatments. Indeed, both leaf and root weights were comparable between control

and treated plants, except for those treated with WV, in which a slight decrease was observed (Figure 3A–B). Chlorophyll, instead, was not affected by any treatment (Figure 3C).

Regarding the amount of C and N, no significant variations were detected between control and treated plants, either in leaves or roots (Table S9). The analyses of macro- and micronutrient composition showed slight variations. Among the macronutrients, an increase in Ca and a decrease in K were observed in lettuce treated with WCE compared to the control (Table S10). A higher accumulation of Ca was also observed in the roots after all treatments, mainly with WV (Table S11). Among the micronutrients, there was only a higher presence of Fe in lettuce leaves treated with WCE and an increase in Mn in the roots of all treated samples (Tables S12–S13). The concentration of heavy metals in leaves remained unchanged, whereas treated samples exhibited increased accumulation of Cu and Zn in the roots (Tables S14–S15).

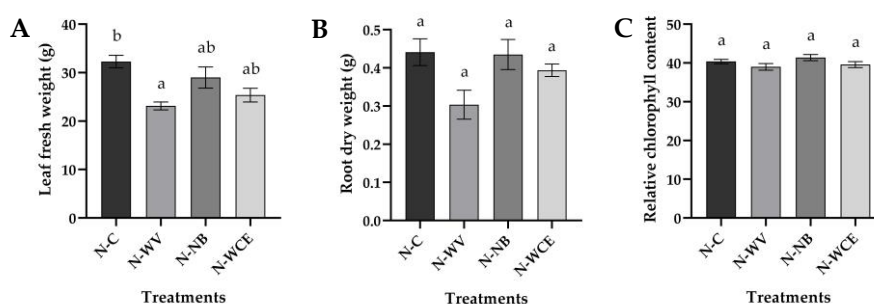


Figure 3. Analysis of leaf fresh weight (A), roots dry weight (B) and chlorophyll content (C) after treatments of lettuce plants under no stress conditions. Error bars represent the SEM. Significance (p -value < 0.05) was shown by letters.

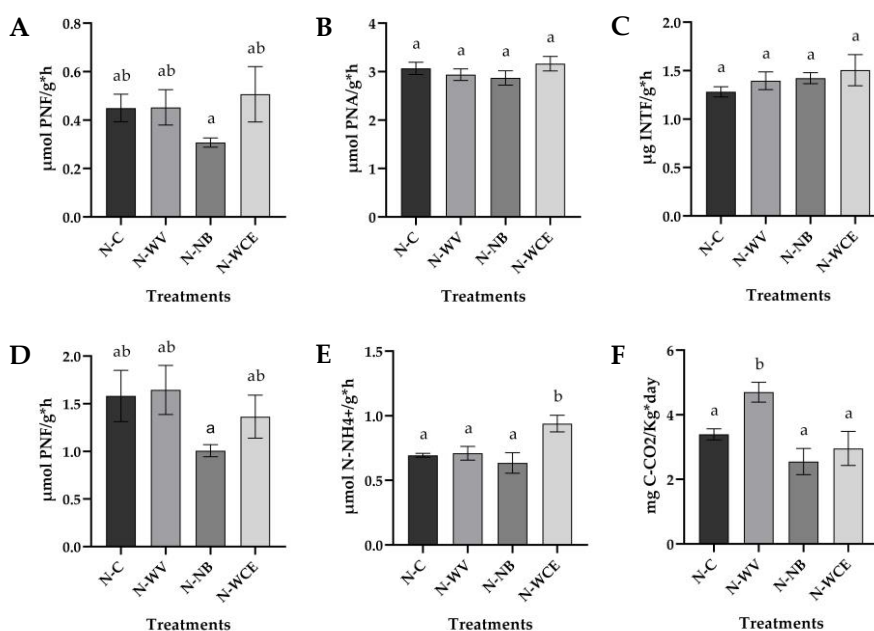


Figure 4. Soil enzymatic activity under normal condition. Specifically, the activity of β -glucosidase (A), glycine-aminopeptidase (B), dehydrogenase (C), alkaline phosphatase (D), and urease (E) were evaluated. Also soil respiration (F) was reported. Error bars represent the SEM. Significance (p -value < 0.05) was shown by letters.

Concerning soil properties, none of the treatments significantly affected pH and EC (Figure S1). In contrast, total C content increased under all treatments, whereas a reduction in soluble nitrogen was observed following NB application (Table S16). Moreover,

the treatments with WV and WCE showed an increase in Ca, P, and S, while a decrease of Na and K with all treatments was observed (Table S17). Concerning soluble macronutrients, both WCE and WV determined an increased availability of K, while there was a decrease in Ca and P after the treatment with NB (Table S18). A slight decrease in soil micronutrients was also observed after all treatments (Table S19), while no significant differences were observed in the soluble fraction (Table S20). Among heavy metals, a decrease was observed exclusively in the amount of total Cr and Cu (Table S21–S22). Soil enzyme activity was not significantly affected by treatments under normal conditions (Figure 4), with the exception of alkaline phosphatase activity, which increased following WCE treatment (Figure 4D). Conversely, soil respiration showed a significant increase in samples treated with WV (Figure 4F).

3.4. Effects of Bioproducts on Lettuce under Drought Conditions

Under drought stress conditions, all tested products, particularly WCE, induced a significant increase in lettuce biomass, both in leaves and roots (Figure 5A–B). Chlorophyll content, on the other hand, increased only after treatment with WCE (Figure 5C). Concerning the C and N content, even in the presence of drought stress, the various treatments did not lead to significant differences compared to the controls, neither in leaves nor in roots (Table S23). Among the macronutrients, a greater accumulation of Na was observed in lettuce leaves treated with WV and an increase in Ca in all the roots of the treated samples (Tables S24–S25). Regarding micronutrients, a reduced accumulation of Mn and Mg was observed in lettuce leaves treated with NB and WCE compared to the control plants (Table S26). Similarly, the accumulation of these nutrients was evident in roots of all treated plants compared to the control (Table S27). Finally, no differences in heavy metal content were found in leaves, while all products led to a greater accumulation of Cu and Zn in roots (Tables S28–S29).

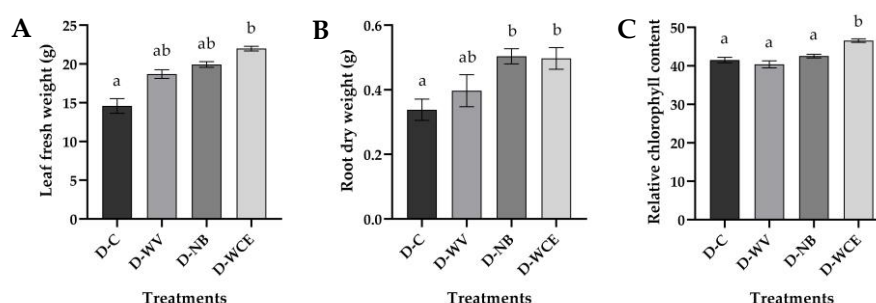


Figure 5. Analysis of leaf fresh weight (A), roots dry weight (B) and chlorophyll content (C) after treatments to lettuce plants under drought stress condition. Error bars represent the SEM. Significance (p -value < 0.05) was shown by letters.

Concerning soil properties, treatments did not significantly affect pH or EC (Figure S2). The analysis of C and N showed only a reduction in soluble N after treatment with NB, and no other significant changes were observed (Table S30). Overall, soil analysis showed slight differences between control and treated samples. Specifically, an increase in soluble K was observed after NB treatment, while a decrease in Cu content was detected across all treatments, particularly with WCE application (Tables S31–S36).

Under drought stress, soil enzyme activities were significantly affected by the various treatments. In particular, NB increased glucosidase and alkaline activity, while aminopeptidase activity increased after the treatment with both NB and WV. Conversely, urease activity showed a slight increase after WCE application

but a marginal decrease following NB treatment. Analysis of soil respiration did not reveal any significant changes, but a trending increment was noted after both wood vinegar treatments (Figure 6).

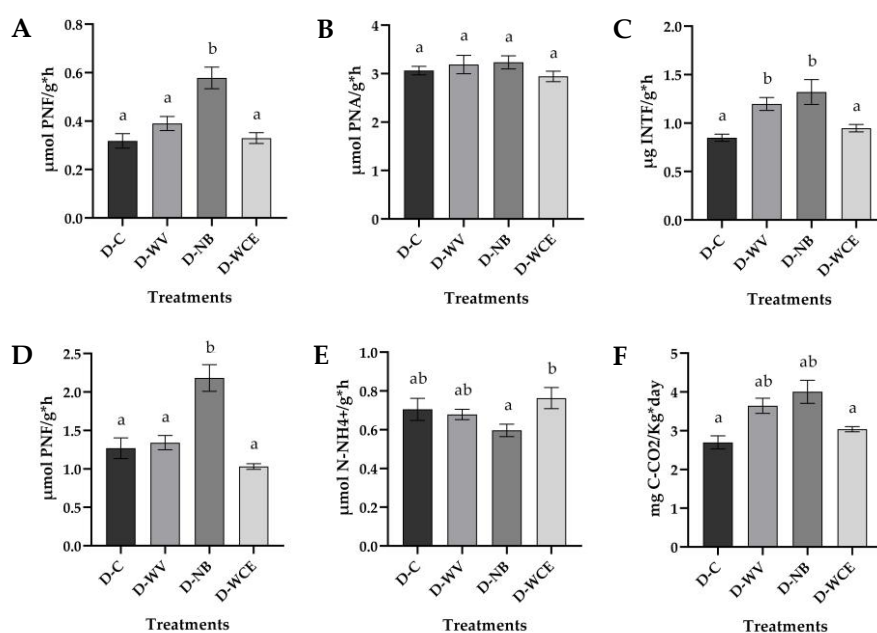


Figure 6. Soil enzymatic activity under drought stress. Specifically, the activity of β -glucosidase (A), glycine-aminopeptidase (B), dehydrogenase (C), alkaline phosphatase (D), and urease (E) were evaluated. Also soil respiration (F) was reported. Error bars represent the SEM. Significance (p -value < 0.05) was shown by letters.

3.5. Effects of Bioproducts on Lettuce under Salt Stress

Under saline stress conditions, plant biomass was not influenced by any of the tested treatments compared to the control (Figure 7A). Only a slight increase in root biomass in plants treated with the two commercial wood vinegars was observed (Figure 7B). Moreover, chlorophyll content increased exclusively in plants treated with WV (Figure 7C). The content of C and N in leaves and roots was not affected by any treatment, also under salt stress conditions (Table S37).

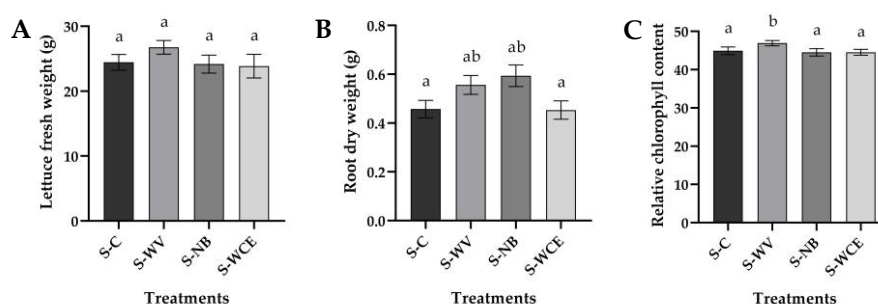


Figure 7. Analysis of leaf fresh weight (A), roots dry wight (B), and chlorophyll content (C) after treatments to lettuce plants under drought stress condition. Error bars represent the SEM. Significance (p -value < 0.05) was shown by letters.

In lettuce leaves, an increase in Ca was observed among the macronutrients after administration of WCE and a decrease in K after the treatment with both WCE and NB (Table S38). Regarding micronutrients, a slight reduction in Mg was reported in plant leaves treated with NB and WCE, and an accumulation of Fe, Mn and Mo in the roots after

treatment with all the products tested (Tables S40–S41). As reported for the other growth conditions (i.e., no-stress and drought stress), the roots showed higher quantities of Cu and Zn when treated with the products compared to the control (Table S43). Concerning soil properties, treatments did not significantly affect pH and EC (Figure S3). No significant variations were observed in macro- and micronutrients composition between treated and untreated soil (Tables S44, S45, S47, S49). Conversely, for the soluble fraction, the treatments with both wood vinegars and WCE partially influenced the concentrations of soluble macro- and micronutrients. Specifically, soluble C content was lower in all treated samples compared to the control, while N decreased only after the application of WV (Table S44). Soil treated with WV showed also a decrease in soluble Ca, K and Mg, while the concentration of soluble K increased with both NB and WCE (Tables S46, S48). Furthermore, Soluble Na and S declined in all treated soils compared to the control (Tables S46, S48).

Various enzymatic activities were affected by the treatments. Commercial wood vinegars increased glucosidase activity, while all the products tested increased aminopeptidase, phosphatase and urease activity to a greater or lesser degree. Soil respiration also increased significantly after treatment with NB and WCE (Figure 8).

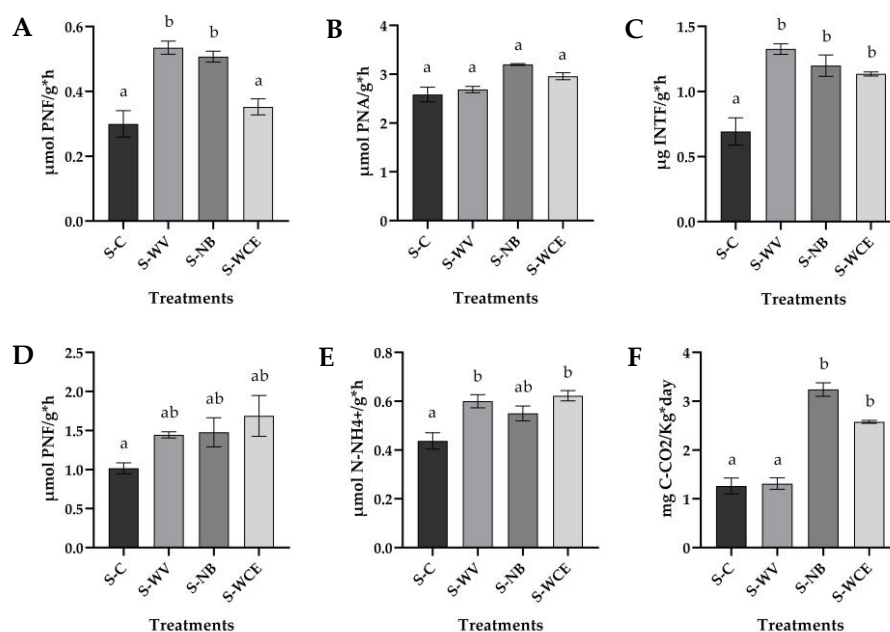


Figure 8. Soil enzymatic activity under salt stress. Specifically, the activity of β -glucosidase (A), glycine-aminopeptidase (B), dehydrogenase (C), alkaline phosphatase (D), and urease (E) were evaluated. Also soil respiration (F) was reported. Error bars represent the SEM. Significance (p -value < 0.05) was shown by letters.

4. Discussion

Increasing extreme weather events are progressively reducing crop productivity and frequently lead to soil degradation[1,2]. In this context, plant-based products are being explored as sustainable tools to improve plant growth and health [3]. Chestnut-based compounds are extremely rich in polyphenols, which can be fundamental for soil health and to protect plant from both biotic and abiotic stress [10,20]. In this study, two sweet chestnut-derived wood vinegars and a laboratory-scale chestnut woodchip extract were tested on lettuce under drought and salt stress conditions, assessing soil properties and activities, as well as their impact on nutrient content in soil and plants. Although some

evidence of their effectiveness is already available, their impact on the soil and, in particular, their benefits in abiotic stress remain among the least explored aspects.

Preliminary soil analyses were conducted using high product concentrations to simulate long-term application scenarios. These analyses showed slight variations in nutrient availability. Specifically, a significant increase in soluble C was observed in treated soils, a trend consistent with the elevated concentrations of this compound in the applied products. Concerning soil enzymatic activities, they were only partially affected when these products were applied. Notably, dehydrogenase, β -glucosidase and glycine-aminopeptidase showed no differences between control and treatments (Figures 2A–2C). On the contrary, a significant decrease in urease activity was observed after the treatment with both WV and NB (Figures 2E). Since urease, released into the soil by microorganisms, catalyzes the hydrolysis of urea into ammonium and CO₂ [27] its inhibition may result in reduced ammonium availability, potentially allowing for lower nitrogen fertilizer inputs. Similarly, alkaline phosphatase activity underwent a significant reduction, but only with the application of WV (Figure 2D). Alkaline phosphatase is an extracellular enzyme which hydrolyzes the phosphor-ester bonds in substrates containing organic P and produces inorganic P, which can be absorbed by plants and soil microorganisms [27,28]. However, no differences in available P content were observed in the samples analyzed.

Considering the soil-plant system, the results highlighted a greater effectiveness of the tested products under stress conditions compared to optimal growth conditions. This observation is consistent with previous studies on lettuce demonstrating the ability of wood vinegar to mitigate both drought and salinity stress, specifically when WV was applied [29,30]. Chestnut extracts and tannin hydrolysates have always been barely investigated, and knowledge about their effects on plants under abiotic stress conditions is still scarce. Our results showed that lettuce biomass was not influenced by the application of the tested wood vinegars and wood extracts when the plants were under no stress conditions (Figures 3). Notably, it was significantly increased when lettuce was subjected to drought stress, especially with WCE (Figure 5). A similar trend was also observed for root biomass, which increased in both drought and salt stress conditions. Unlike previous studies where WV was applied by foliar spray [31–34], in this case the products were administered by fertigation, which may have influenced their uptake and physiological effects.

According to the literature [30,35], chlorophyll content is often positively influenced by WV application. However, in this study, a significant increase in chlorophyll content was observed only following WCE treatment under drought stress (Figure 5C) and WV treatment under saline stress (Figure 6C), indicating a stress-dependent and product-specific response.

In terms of macro- and micronutrients, the tested treatments did not produce significant differences in lettuce. Only WCE promote a higher Ca accumulation under normal and salt stress conditions (Table S10, S38). In roots, increased Ca and Mg concentrations were observed with all the applied treatments. Moreover, the accumulation of heavy metals such as Cu and Zn was observed under all experimental conditions and across all treatments (Tables S15, S29, S43), indicating a higher sequestration from the soil.

Under both drought and salt stress conditions, soil pH and EC were not altered by the tested products (Figures S2–S3), as also reported by Becagli *et al.* [35] about WV in normal condition. A significant increase in EC was observed only in soils of plants under salt stress, as a direct consequence of saltwater irrigation [36].

The effects of the products on soil macro- and micronutrients were generally limited and became more evident only under abiotic stress conditions. Preliminary chemical analyses carried out directly on them have revealed high concentrations of C in wood vinegars, due to their elevated content in organic acids [10,35], whereas WCE exhibited high

levels of N. Consistently, the elevated C content of wood vinegars was reflected in increased soil C under no-stress conditions (e.g. N-WV, N-NB and N-WCE) (Table S16). However, this trend was lost under both drought and salt stresses (Tables S30, S44). Under both normal and drought stress conditions, only minimally significant differences were observed. The stress condition itself did not alter the nutrient content compared to the non-stressed samples.

Notably, an increase in soluble K with N-WV and N-WCE, as well as with D-NB was reported (Tables S18, S32). Soluble potassium plays a fundamental role in plant metabolism, water regulation and sugar transport [37]. Conversely, salt stress conditions exerted the greater influence on nutrient availability and uptake. In particular, soil levels of Na and Ca were markedly higher compared to both normal and drought stress conditions (Tables S18, S32, S46). Excessive bioaccumulation of these elements within the plant can be toxic and compromise its health [38,39]. However, all tested products maintained nutrient levels comparable to the salt-stressed control and, in several cases—particularly with WV—reduced the soluble fractions, likely promoting their sequestration in roots. Tested products maintained nutrient levels comparable to the salt-stressed control and, in several cases—particularly with WV—reduced the soluble fractions, likely promoting their sequestration in roots.

Concerning soil enzyme activities, they were only partially affected by the application of the products, showing positive effects when applied to lettuce plants under stress conditions. In particular, a slight increase in β -glucosidase activity was observed when plants were subjected to both saline and drought stress and treated with WV and NB compared to untreated plants and those treated with WCE (Figure 6A, 8A). β -glucosidase is an extracellular enzyme that hydrolyzes cellobiose to produce glucose, thereby influencing soil microbial activity, given that glucose represent the preferred carbon source for soil microorganisms [27,28]. The slight increase in β -glucosidase activity observed in the soil suggests that commercial wood vinegars may partially stimulate soil microbial activity under abiotic stress conditions.

Similarly, alkaline phosphatase activity increased slightly after application of all products under salt stress, while a more marked increase was observed after NB application under drought stress (Figure 6B, 8B). Enhanced alkaline phosphatase activity is often associated with phosphorus limitation, as low P availability typically stimulates the production of this enzyme [40]. However, no differences in available P content were observed in the samples analyzed under these conditions.

Dehydrogenase activity, an indicator of overall microbial oxidative activity [27,41]. showed a slight increase under salt stress following application of all products (Figure 8C). Comparable results were observed under drought stress, particularly after WV and NB treatments. These findings further support a moderate stimulation of soil microbial activity [42], which was corroborated by the increased soil respiration measured under both drought and saline stress conditions. Urease activity also exhibited a slight increase following treatments under stress conditions; however, this was not accompanied by changes in available N levels (Figure 6D, 8D). Aminopeptidases are extracellular enzymes that catalyze the depolymerization of peptides [43], thus playing a crucial role in the N cycle [44]. However, no condition or treatment has led to significant changes in the activity of glycine-aminopeptidase.

Overall, the results of this study indicate that chestnut-derived wood vinegars and woodchip extracts exerted limited effects under optimal growth conditions but showed a more pronounced and beneficial impact when lettuce plants were exposed to abiotic stress. Their application contributed to maintaining soil functionality, modulating nutrient availability, and supporting plant performance under drought and saline stress, without inducing detrimental alterations in soil physicochemical properties. These findings

highlight the potential of chestnut-based products as stress-mitigating inputs in sustainable cropping systems and provide a basis for further investigation into their long-term effects and practical applicability under field conditions.

5. Conclusions

In conclusion, this study demonstrated that both wood vinegars and the woodchip extract (WCE) exhibit biostimulant properties, particularly when applied to plants subjected to abiotic stress, without adversely affecting soil quality. Under optimal growth conditions, their application appeared to provide limited benefits; however, under drought stress, treated plants achieved yields comparable to those of non-stressed controls. Under saline stress, the most pronounced effects were observed at the soil level, notably through enhanced enzymatic activities, suggesting an improvement in soil biological functioning. These findings further support the potential of wood vinegars as effective tools for enhancing plant resilience under adverse conditions. Moreover, preliminary results obtained with the woodchip extract indicate that it may represent a promising biostimulant, especially in stress-prone agricultural systems.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1. Macronutrient composition of WV, NB and WCE; Table S2. Micronutrient composition of WV, NB and WCE; Table S3. Heavy metal composition of WV, NB and WCE; Table S4. WCE phenol characterization; Table S5. C and N of on plate soil (soluble fraction); Table S6. On plate soil macronutrient (soluble fraction); Table S7. On plate soil micronutrient (soluble fraction); Table S8. On plate soil heavy metals (soluble fraction); Table S9. Lettuce C and N composition under normal condition; Table S10. Lettuce leaf macronutrients under normal condition; Table S11. Lettuce root macronutrients under normal condition; Table S12. Lettuce leaf micronutrients under normal condition; Table S13. Lettuce root micronutrients under normal condition; Table S14. Lettuce leaf heavy metals under normal condition; Table S15. Lettuce root heavy metals under normal condition; Figure S1. Soil pH (A) and EC (B) after treatments under normal condition; Table S16. Lettuce C and N composition under normal condition; Table S17. Soil macronutrients under normal condition; Table S18. Soil macronutrients under normal condition (soluble fraction); Table S19. Soil micronutrients under normal condition; Table S20. Soil micronutrients under normal condition (soluble fraction); Table S21. Soil heavy metals under normal condition; Table S22. Soil heavy metals under normal condition (soluble fraction); Table S23. Lettuce C and N composition under drought condition; Table S24. Lettuce leaf macronutrients under drought condition; Table S25. Lettuce root macronutrients under drought condition; Table S26. Lettuce leaf micronutrients under drought condition; Table S27. Lettuce root micronutrients under drought condition; Table S28. Lettuce leaf heavy metals under drought condition; Table S29. Lettuce root heavy metals under drought condition; Figure S2. Soil pH (A) and EC (B) after treatments under drought condition; Table S30. Soil C and N composition under drought condition; Table S31. Soil macronutrients under drought condition; Table S32. Soil macronutrients under drought condition (soluble fraction); Table S33. Soil micronutrients under drought condition; Table S34. Soil micronutrients under drought condition (soluble fraction); Table S35. Soil heavy metals under drought condition; Table S36. Soil heavy metals under drought condition (soluble fraction); Table S37. Lettuce C and N composition under salt condition; Table S38. Lettuce leaf macronutrients under salt condition; Table S39. Lettuce root macronutrients under salt condition; Table S40. Lettuce leaf micronutrients under salt condition; Table S41. Lettuce root micronutrients under salt condition; Table S42. Lettuce leaf heavy metals under salt condition; Table S43. Lettuce root heavy metals under salt condition; Figure S3. Soil pH (A) and EC (B) after treatments under salt condition; Table S44. Soil C and N composition under salt condition; Table S45. Soil macronutrients under salt condition; Table S46. Soil macronutrients under salt condition (soluble fraction); Table S47. Soil micronutrients under salt

condition; Table S48. Soil micronutrients under salt condition (soluble fraction); Table S49. Soil heavy metals under salt condition; Table S50. Soil heavy metals under salt condition (soluble fraction)

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Marketed and Research-Derived Chestnut Extracts: Help or Hype for Soil Health and Plant Resilience under Stress?

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Table S1. Macronutrient composition of WV, NB and WCE

Treatment	Ca (mg/L)	K (mg/L)	Na (mg/L)	P (mg/L)	S (mg/L)
WV	56.56	5.85	18.26	0.19	6.89
NB	368.42	47.42	4.68	1.58	20.77
WCE	73.21	38.06	49.82	2.40	18.02

Table S2. Micronutrient composition of WV, NB and WCE

Treatment	B (mg/L)	Fe (mg/L)	Mg (mg/L)	Mn (mg/L)	Mo (mg/L)
WV	0.07	12.15	1.82	1.33	<0.01 a
NB	<0.01 a	273.80	24.52	6.96	<0.01 a
WCE	0.37	2.08	23.97	3.59	<0.01 a

Table S3. Heavy metal composition of WV, NB and WCE

Treatment	As (mg/L)	Cd (mg/L)	Cr (mg/L)	Cu (mg/L)	Ni (mg/L)	Pb (mg/L)	Zn (mg/L)
WV	0.02	0.02	0.01	0.17	0.03	0.16	3.87
NB	0.02	0.04	0.12	0.07	0.12	0.23	1.84
WCE	0.01	<0.01 a	0.01	0.06	0.03	0.04	0.15

Table S4. WCE phenol characterization

Classification	Compounds	PPM
Galloyl glucose Derivatives and Ellagitannins	HHDP-tergalloylhexose (I)	2122.0
Galloyl glucose Derivatives and Ellagitannins	HHDP-tergalloylhexose (II)	2398.4
Galloyl glucose Derivatives and Ellagitannins	Digalloylhexose	1062.8
Galloyl glucose Derivatives and Ellagitannins	HHDP-galloylhexose	865.5
Galloyl glucose Derivatives and Ellagitannins	HHDP-digalloylhexose (I)	794.4
Galloyl glucose Derivatives and Ellagitannins	HHDP-digalloylhexose (II)	1629.2
Galloyl glucose Derivatives and Ellagitannins	Trigalloylhexose (I)	1030.0
Galloyl glucose Derivatives and Ellagitannins	Trigalloylhexose (II)	1132.8
Galloyl glucose Derivatives and Ellagitannins	Tetragalloylhexose	1002.1
ORGANICS, Gallic acid deriv.	Ellagic acid	428.7

Table S5. C and N of on plate soil (soluble fraction)

Treatments	C (mg/Kg)	N (mg/Kg)
C	63.15 ± 3.09 a	18.42 ± 2.08 a
WV	82.04 ± 1.00 b	18.78 ± 1.51 a
NB	82.54 ± 17.35 b	23.95 ± 1.35 b
WCE	78.33 ± 5.58 b	18.94 ± 4.42 ab

Table S6. On plate soil macronutrient (soluble fraction)

Treatments	Ca (mg/L)	K (mg/L)	Na (mg/L)	P (mg/L)	S (mg/L)
C	64.04 ± 2.88 a	38.89 ± 0.54 a	13.29 ± 0.24 a	<0.01 a	4.79 ± 0.35 a
WV	66.31 ± 1.92 a	39.23 ± 0.98 a	16.85 ± 0.85 ab	<0.01 a	4.96 ± 0.22 a
NB	81.83 ± 15.80 a	41.55 ± 1.92 a	18.64 ± 2.69 b	<0.01 a	5.71 ± 0.32 a
WCE	85.07 ± 13.99 a	40.39 ± 3.59 a	17.91 ± 1.55 b	<0.01 a	5.91 ± 1.00 a

Table S7. On plate soil micronutrient (soluble fraction)

Treatments	B (mg/L)	Fe (mg/L)	Mg (mg/L)	Mn (mg/L)	Mo (mg/L)
C	0.99 ± 0.01 a	4.04 ± 1.00 a	21.99 ± 0.52 a	0.13 ± 0.01 a	<0.01 a
WV	1.01 ± 0.02 a	4.54 ± 0.47 a	22.31 ± 0.56 a	0.19 ± 0.02 a	<0.01 a
NB	1.00 ± 0.06 a	3.90 ± 1.42 a	23.52 ± 0.73 a	0.17 ± 0.03 a	<0.01 a
WCE	1.02 ± 0.03 a	5.04 ± 0.45 a	22.89 ± 2.26 a	0.16 ± 0.02 a	<0.01 a

Table S8. On plate soil heavy metals (soluble fraction)

Treatments	As (mg/L)	Cd (mg/L)	Cr (mg/L)	Cu (mg/L)	Ni (mg/L)	Pb (mg/L)	Zn (mg/L)
C	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
WV	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
NB	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
WCE	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a

Table S9. Lettuce C and N composition under normal condition

Treatments	Lettuce		Roots	
	C	N	C	N
N-C	37.37 ± 0.50 a	3.61 ± 0.11 a	37.98 ± 0.21 a	2.03 ± 0.07 a
N-WV	37.10 ± 0.79 a	3.80 ± 0.34 a	37.73 ± 0.60 a	2.24 ± 0.07 a
N-NB	37.13 ± 0.64 a	3.56 ± 0.04 a	38.33 ± 0.18 a	2.07 ± 0.17 a
N-WCE	36.36 ± 0.82 a	3.99 ± 0.23 a	37.00 ± 0.28 a	2.22 ± 0.09 a

Table S10. Lettuce leaf macronutrients under normal condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
N-C	9.41 ± 0.74 a	55.25 ± 2.13 b	1.15 ± 0.20 a	2.35 ± 0.21 a	2.30 ± 0.16 a
N-WV	9.58 ± 0.72 a	52.61 ± 2.10 ab	1.11 ± 0.13 a	2.31 ± 0.33 a	2.41 ± 0.19 a
N-NB	10.80 ± 2.04 ab	50.55 ± 3.06 ab	1.07 ± 0.08 a	2.30 ± 0.32 a	2.29 ± 0.08 a
N-WCE	12.57 ± 0.92 b	48.98 ± 2.29 a	0.97 ± 0.23 a	2.19 ± 0.22 a	2.30 ± 0.29 a

Table S11. Lettuce root macronutrients under normal condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
N-C	6.92 ± 0.68 a	33.31 ± 4.08 a	2.93 ± 0.22 a	1.20 ± 0.08 a	2.04 ± 0.37 a
N-WV	8.72 ± 0.33 b	35.94 ± 2.60 a	3.97 ± 1.09 a	1.26 ± 0.07 a	2.08 ± 0.15 a
N-NB	7.81 ± 0.85 ab	36.59 ± 2.44 a	4.16 ± 0.53 a	1.26 ± 0.11 a	2.17 ± 0.11 a
N-WCE	7.43 ± 0.91 ab	34.09 ± 2.58 a	4.56 ± 0.40 a	1.10 ± 0.05 a	1.88 ± 0.12 a

Table S12. Lettuce leaf micronutrients under normal condition

Treatments	B (mg/Kg)	Fe (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
N-C	20.52 ± 0.77 a	336.40 ± 50.57 a	0.40 ± 0.03 a	106.28 ± 9.29 a	<0.01 a
N-WV	20.67 ± 0.98 a	261.32 ± 57.23 a	0.43 ± 0.04 a	122.79 ± 11.44 a	<0.01 a
N-NB	19.21 ± 0.90 a	463.52 ± 126.40 ab	0.33 ± 0.07 a	104.56 ± 11.88 a	<0.01 a
N-WCE	19.82 ± 1.41 a	797.76 ± 167.35 b	0.30 ± 0.04 a	109.31 ± 16.10 a	<0.01 a

Table S13. Lettuce root micronutrients under normal condition

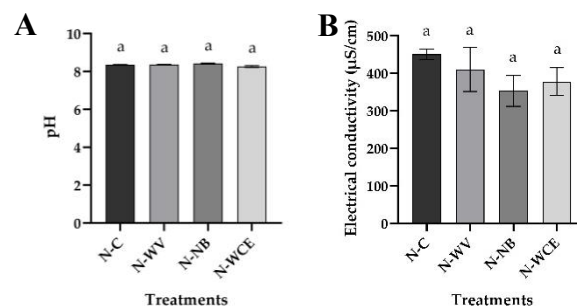
Treatments	B (mg/Kg)	Fe (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
N-C	11.75 ± 1.25 a	673.21 ± 71.45 a	0.30 ± 0.03 a	49.87 ± 4.37 a	1.10 ± 0.11 a
N-WV	10.83 ± 0.33 a	662.24 ± 105.70 a	0.30 ± 0.03 a	62.22 ± 3.65 b	1.92 ± 0.34 b
N-NB	11.23 ± 0.87 a	594.20 ± 190.48 a	0.34 ± 0.02 a	59.08 ± 2.55 b	1.21 ± 0.13 ab
N-WCE	11.30 ± 2.00 a	535.69 ± 118.68 a	0.33 ± 0.03 a	60.16 ± 1.88 b	1.22 ± 0.17 ab

Table S14. Lettuce leaf heavy metals under normal condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
N-C	0.18 ± 0.07 a	0.57 ± 0.08 a	0.93 ± 0.33 a	4.53 ± 0.43 a	1.19 ± 0.70 a	0.83 ± 0.28 a	15.52 ± 4.12 a
N-WV	0.17 ± 0.16 a	0.63 ± 0.05 a	0.84 ± 0.56 a	4.86 ± 0.75 a	0.92 ± 0.29 a	0.70 ± 0.30 a	14.43 ± 2.92 a
N-NB	0.30 ± 0.17 a	0.50 ± 0.04 a	1.34 ± 0.59 a	4.68 ± 0.62 a	1.22 ± 0.15 a	1.15 ± 0.55 a	16.26 ± 2.53 a
N-WCE	0.36 ± 0.22 a	0.70 ± 0.32 a	2.15 ± 0.67 a	5.40 ± 0.59 a	1.50 ± 0.32 a	1.22 ± 0.67 a	17.50 ± 2.30 a

Table S15. Lettuce root heavy metals under normal condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
N-C	2.41 ± 0.81 a	0.30 ± 0.04 a	5.35 ± 1.10 a	7.47 ± 0.73 a	2.10 ± 0.38 a	1.32 ± 0.49 a	13.41 ± 3.41 a
N-WV	2.66 ± 0.69 a	0.39 ± 0.04 a	5.72 ± 1.97 a	10.60 ± 1.15 b	3.43 ± 1.10 a	1.54 ± 0.37 a	21.26 ± 3.38 b
N-NB	3.04 ± 1.28 a	0.31 ± 0.05 a	5.19 ± 1.18 a	9.54 ± 0.79 b	2.19 ± 0.31 a	1.12 ± 0.60 a	24.72 ± 4.33 b
N-WCE	2.64 ± 0.48 a	0.38 ± 0.05 a	4.42 ± 2.63 a	9.81 ± 1.17 b	2.47 ± 0.57 a	1.07 ± 0.29 a	17.63 ± 3.05 ab

**Figure S1.** Soil pH (A) and EC (B) after treatments under normal condition**Table S16.** Lettuce C and N composition under normal condition

Treatments	Solid		Soluble	
	C (g/100g)	N (g/100 g)	C (mg/L)	N (mg/L)
N-C	8.05 ± 0.13 a	0.08 ± 0.01 a	51.00 ± 4.14 a	90.08 ± 19.07 b
N-WV	9.03 ± 0.37 b	0.15 ± 0.08 a	56.61 ± 8.57 a	99.42 ± 18.72 b
N-NB	8.62 ± 0.55 ab	0.08 ± 0.00 a	61.99 ± 9.51 a	32.44 ± 18.55 a
N-WCE	8.91 ± 0.44 ab	0.09 ± 0.03 a	62.32 ± 14.18 a	96.08 ± 16.95 b

Table S17. Soil macronutrients under normal condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
N-C	210.58 ± 3.70 a	5.30 ± 0.15 b	0.34 ± 0.02 b	0.62 ± 0.05 a	1.49 ± 0.03 a
N-WV	233.99 ± 11.02 ab	4.31 ± 0.34 a	0.27 ± 0.01 a	0.93 ± 0.16 b	1.61 ± 0.06 b
N-NB	211.90 ± 7.30 a	4.18 ± 0.43 a	0.27 ± 0.03 a	0.64 ± 0.07 a	1.42 ± 0.05 a
N-WCE	244.07 ± 9.65 b	4.29 ± 0.58 a	0.28 ± 0.06 ab	0.79 ± 0.18 ab	1.55 ± 0.08 ab

Table S18. Soil macronutrients under normal condition (soluble fraction)

Treatments	Ca (g/L)	K (g/L)	Na (g/L)	P (g/L)	S (g/L)
N-C	227.14 ± 41.68 ab	14.96 ± 2.76 a	63.13 ± 21.96 a	6.12 ± 1.27 b	65.03 ± 13.55 a
N-WV	281.91 ± 83.62 b	23.23 ± 2.29 b	52.20 ± 27.98 a	4.79 ± 2.34 ab	63.35 ± 17.93 a
N-NB	167.82 ± 37.64 a	15.88 ± 2.93 a	44.80 ± 18.36 a	3.22 ± 0.96 a	59.69 ± 12.26 a
N-WCE	234.42 ± 33.02 ab	21.86 ± 2.87 b	69.49 ± 27.35 a	6.24 ± 1.01 b	60.20 ± 10.82 a

Table S19. Soil micronutrients under normal condition

Treatments	B (mg/Kg)	Fe (g/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
N-C	16.18 ± 0.41 b	12.92 ± 0.55 b	0.61 ± 0.02 b	277.99 ± 8.56 b	0.16 ± 0.06 a
N-WV	12.58 ± 1.12 a	10.54 ± 1.01 a	0.41 ± 0.03 a	251.26 ± 11.97 a	0.14 ± 0.09 a
N-NB	13.37 ± 2.67 ab	11.25 ± 2.04 ab	0.54 ± 0.12 ab	263.15 ± 33.40 ab	0.13 ± 0.08 a
N-WCE	12.61 ± 2.62 ab	10.21 ± 2.29 ab	0.47 ± 0.14 ab	263.08 ± 43.08 ab	0.28 ± 0.04 a

Table S20. Soil micronutrients under normal condition (soluble fraction)

Treatments	B (mg/L)	Fe (mg/L)	Mg (mg/L)	Mn (mg/L)	Mo (mg/L)
N-C	1.28 ± 0.04 a	0.50 ± 0.15 a	37.73 ± 8.20 a	0.30 ± 0.07 a	<0.01 a
N-WV	1.32 ± 0.06 a	0.39 ± 0.12 a	41.97 ± 12.60 a	0.34 ± 0.17 a	<0.01 a
N-NB	1.29 ± 0.03 a	0.78 ± 0.36 a	26.89 ± 7.99 a	0.23 ± 0.06 a	<0.01 a
N-WCE	1.32 ± 0.01 a	0.55 ± 0.24 a	39.13 ± 9.35 a	0.35 ± 0.13 a	<0.01 a

Table S21. Soil heavy metals under normal condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
N-C	6.36 ± 0.41 a	0.28 ± 0.02 a	28.24 ± 0.51 b	24.05 ± 2.00 b	13.45 ± 0.84 a	20.94 ± 0.85 a	30.88 ± 1.22 a
N-WV	5.93 ± 0.66 a	0.25 ± 0.03 a	22.19 ± 1.51 a	21.63 ± 1.60 ab	12.09 ± 1.54 a	18.34 ± 2.14 a	31.76 ± 1.12 a
N-NB	5.72 ± 0.57 a	0.27 ± 0.04 a	24.30 ± 3.70 ab	22.01 ± 3.59 ab	12.81 ± 2.13 a	18.75 ± 2.47 a	29.24 ± 2.90 a
N-WCE	5.77 ± 0.37 a	0.23 ± 0.03 a	22.48 ± 1.97 a	18.71 ± 1.70 a	11.95 ± 2.58 a	19.01 ± 1.88 a	31.95 ± 5.47 a

Table S22. Soil heavy metals under normal condition (soluble fraction)

Treatments	As (mg/L)	Cd (mg/L)	Cr (mg/L)	Cu (mg/L)	Ni (mg/L)	Pb (mg/L)	Zn (mg/L)
N-C	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
N-WV	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
N-NB	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
N-WCE	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a

Table S23. Lettuce C and N composition under drought condition

Treatments	Lettuce		Roots	
	C	N	C	N
D-C	38.09 ± 0.59 a	3.07 ± 0.21 a	37.95 ± 0.17 a	2.00 ± 0.06 a
D-WV	38.14 ± 0.28 a	3.18 ± 0.15 a	37.66 ± 0.31 a	2.35 ± 0.15 a
D-NB	37.76 ± 0.72 a	3.08 ± 0.15 a	37.67 ± 0.48 a	2.21 ± 0.15 a
D-WCE	38.55 ± 0.23 a	3.36 ± 0.19 a	37.13 ± 0.31 a	2.10 ± 0.17 a

Table S24. Lettuce leaf macronutrients under drought condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
D-C	8.72 ± 0.38 a	52.73 ± 2.89 a	1.39 ± 0.24 a	1.88 ± 0.08 a	2.04 ± 0.06 a
D-WV	8.72 ± 0.52 a	52.01 ± 2.92 a	3.88 ± 0.21 b	2.11 ± 0.10 a	2.17 ± 0.12 a
D-NB	9.49 ± 1.58 a	50.06 ± 3.50 a	1.08 ± 0.20 a	1.85 ± 0.13 a	1.90 ± 0.10 a
D--WCE	10.04 ± 0.76 a	48.62 ± 2.74 a	1.12 ± 0.39 a	2.13 ± 0.10 a	2.19 ± 0.18 a

Table S25. Lettuce root macronutrients under drought condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
D-C	6.48 ± 0.60 a	31.84 ± 3.59 a	3.11 ± 0.42 a	1.00 ± 0.12 a	1.53 ± 0.15 a
D-WV	7.94 ± 0.91 ab	36.41 ± 3.07 a	4.10 ± 0.58 a	1.11 ± 0.09 a	1.78 ± 0.14 a
D-NB	8.88 ± 0.59 b	33.39 ± 4.32 a	3.40 ± 0.71 a	1.02 ± 0.12 a	1.77 ± 0.11 a
D-WCE	9.30 ± 1.44 b	35.00 ± 2.73 a	3.96 ± 0.72 a	1.14 ± 0.12 a	1.88 ± 0.15 a

Table S26. Lettuce leaf micronutrients under drought condition

Treatments	B (mg/Kg)	Fe (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
D-C	17.21 ± 0.98 a	255.37 ± 87.79 ab	0.40 ± 0.04 b	108.60 ± 6.56 b	<0.01 a
D-WV	17.84 ± 1.21 a	185.29 ± 60.81 a	0.43 ± 0.04 b	121.37 ± 4.51 b	<0.01 a
D-NB	16.20 ± 3.41 a	339.99 ± 70.28 b	0.24 ± 0.03 a	87.90 ± 12.59 a	<0.01 a
D--WCE	16.95 ± 1.26 a	161.34 ± 27.42 a	0.28 ± 0.02 a	99.79 ± 8.68 ab	<0.01 a

Table S27. Lettuce root micronutrients under drought condition

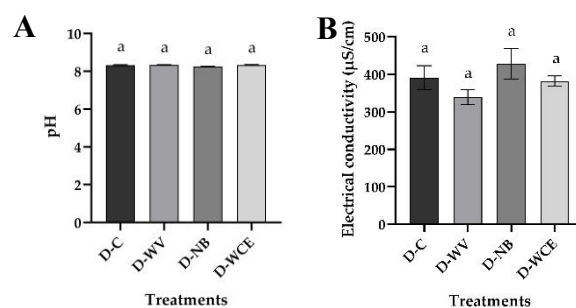
Treatments	B (mg/Kg)	Fe (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
D-C	9.73 ± 1.15 a	590.48 ± 75.99 a	0.33 ± 0.04 a	50.39 ± 3.51 a	0.67 ± 0.05 a
D-WV	10.41 ± 0.85 a	686.98 ± 47.63 a	0.36 ± 0.06 a	60.13 ± 0.75 b	0.91 ± 0.10 ab
D-NB	10.13 ± 1.17 a	649.79 ± 113.28 a	0.37 ± 0.06 a	60.54 ± 3.70 b	0.85 ± 0.14 ab
D-WCE	10.30 ± 1.04 a	955.68 ± 116.45 b	0.39 ± 0.04 a	64.08 ± 4.36 b	1.08 ± 0.11 b

Table S28. Lettuce leaf heavy metals under drought condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
D-C	0.12 ± 0.07 a	0.44 ± 0.03 a	0.73 ± 0.20 a	3.77 ± 0.18 a	0.99 ± 0.60 a	0.61 ± 0.07 a	15.85 ± 1.86 a
D-WV	0.20 ± 0.08 a	0.46 ± 0.05 a	0.59 ± 0.34 a	3.67 ± 0.44 a	0.71 ± 0.17 a	0.69 ± 0.39 a	13.55 ± 1.83 a
D-NB	0.17 ± 0.11 a	0.37 ± 0.08 a	0.87 ± 0.45 a	3.36 ± 0.57 a	1.00 ± 0.37 a	0.79 ± 0.48 a	15.11 ± 1.86 a
D-WCE	0.11 ± 0.07 a	0.41 ± 0.03 a	0.65 ± 0.30 a	3.84 ± 0.54 a	0.76 ± 0.30 a	0.77 ± 0.63 a	13.33 ± 2.56 a

Table S29. Lettuce root heavy metals under drought condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
D-C	1.65 ± 0.42 a	0.42 ± 0.05 a	4.09 ± 0.63 a	6.86 ± 1.66 a	1.76 ± 0.34 a	1.02 ± 0.26 a	10.36 ± 1.56 a
D-WV	2.26 ± 0.29 a	0.31 ± 0.05 a	4.51 ± 0.49 a	10.86 ± 2.72 b	2.87 ± 0.37 a	1.27 ± 0.15 a	17.71 ± 4.08 ab
D-NB	2.33 ± 0.47 a	0.29 ± 0.04 a	4.92 ± 0.80 a	10.50 ± 1.60 b	2.22 ± 0.23 a	1.61 ± 0.41 a	24.57 ± 3.70 b
D-WCE	2.55 ± 0.92 a	0.29 ± 0.06 a	4.14 ± 0.62 a	8.50 ± 1.08 ab	2.22 ± 0.48 a	2.04 ± 0.51 a	15.38 ± 1.89 ab

**Figure S2.** Soil pH (A) and EC (B) after treatments under drought condition**Table S30.** Soil C and N composition under drought condition

Treatments	Solid		Soluble	
	C (g/100g)	N (g/100 g)	C (mg/L)	N (mg/L)
D-C	8.53 ± 0.73 a	0.10 ± 0.00 a	53.45 ± 9.20 a	103.23 ± 12.70 b
D-WV	8.22 ± 0.55 a	0.09 ± 0.02 a	41.74 ± 0.56 a	64.83 ± 7.35 a
D-NB	7.69 ± 1.09 a	0.11 ± 0.03 a	51.59 ± 5.22 a	132.30 ± 18.08 b
D-WCE	7.63 ± 0.44 a	0.08 ± 0.00 a	42.95 ± 3.10 a	109.53 ± 21.08 b

Table S31. Soil macronutrients under drought condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
D-C	245.39 ± 18.81 a	4.96 ± 0.39 a	0.33 ± 0.07 a	1.01 ± 0.38 a	1.70 ± 0.12 a
D-WV	242.87 ± 16.33 a	4.72 ± 0.23 a	0.32 ± 0.03 a	0.92 ± 0.22 a	1.59 ± 0.18 a
D-NB	254.86 ± 14.79 a	4.71 ± 0.49 a	0.33 ± 0.04 a	1.04 ± 0.33 a	1.70 ± 0.08 a
D-WCE	252.61 ± 26.99 a	4.69 ± 0.66 a	0.30 ± 0.08 a	0.74 ± 0.09 a	1.61 ± 0.11 a

Table S32. Soil macronutrients under drought condition (soluble fraction)

Treatments	Ca (g/L)	K (g/L)	Na (g/L)	P (g/L)	S (g/L)
D-C	237.43 ± 37.60 a	15.73 ± 3.30 a	80.16 ± 20.11 a	5.59 ± 2.22 a	75.44 ± 5.82 a
D-WV	225.11 ± 29.92 a	15.61 ± 2.91 a	57.05 ± 14.90 a	4.14 ± 0.68 a	57.71 ± 4.27 a
D-NB	215.97 ± 43.05 a	23.25 ± 3.85 b	67.96 ± 21.17 a	4.89 ± 1.45 a	65.92 ± 16.40 a
D-WCE	221.74 ± 30.13 a	15.20 ± 2.12 a	49.75 ± 18.21 a	5.79 ± 2.68 a	59.09 ± 12.54 a

Table S33. Soil micronutrients under drought condition

Treatments	B (mg/Kg)	Fe (g/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
D-C	14.82 ± 2.60 a	12.17 ± 1.12 a	0.56 ± 0.08 a	307.96 ± 38.84 a	0.30 ± 0.07 a
D-WV	14.53 ± 0.46 a	12.83 ± 1.08 a	0.60 ± 0.06 a	282.45 ± 12.10 a	0.22 ± 0.02 a
D-NB	14.42 ± 1.77 a	11.79 ± 1.26 a	0.56 ± 0.09 a	286.74 ± 21.81 a	0.20 ± 0.09 a
D-WCE	13.89 ± 3.14 a	11.34 ± 2.76 a	0.52 ± 0.17 a	258.64 ± 30.83 a	0.22 ± 0.02 a

Table S34. Soil micronutrients under drought condition (soluble fraction)

Treatments	B (mg/L)	Fe (mg/L)	Mg (mg/L)	Mn (mg/L)	Mo (mg/L)
D-C	1.23 ± 0.03 a	0.40 ± 0.15 a	46.32 ± 9.23 a	0.36 ± 0.07 a	<0.01 a
D-WV	1.30 ± 0.06 a	0.60 ± 0.06 a	35.06 ± 0.62 a	0.28 ± 0.01 a	<0.01 a
D-NB	1.28 ± 0.02 a	0.41 ± 0.13 a	40.90 ± 13.12 a	0.33 ± 0.10 a	<0.01 a
D-WCE	1.32 ± 0.02 a	0.70 ± 0.17 a	33.06 ± 8.30 a	0.28 ± 0.06 a	<0.01 a

Table S35. Soil heavy metals under drought condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
D-C	6.44 ± 0.67 a	0.28 ± 0.02 a	26.75 ± 3.37 a	24.15 ± 0.74 b	13.59 ± 0.95 a	22.46 ± 3.40 a	29.16 ± 4.88 a
D-WV	6.18 ± 0.31 a	0.28 ± 0.02 a	26.16 ± 0.70 a	22.55 ± 2.61 ab	13.63 ± 0.76 a	20.17 ± 1.10 a	29.61 ± 3.67 a
D-NB	6.59 ± 0.14 a	0.28 ± 0.03 a	25.78 ± 3.61 a	22.31 ± 1.65 ab	14.27 ± 1.93 a	20.06 ± 2.32 a	30.95 ± 2.88 a
D-WCE	6.15 ± 0.57 a	0.25 ± 0.04 a	25.06 ± 5.52 a	20.18 ± 2.25 a	13.38 ± 2.73 a	19.37 ± 2.47 a	30.99 ± 2.41 a

Table S36. Soil heavy metals under drought condition (soluble fraction)

Treatments	As (mg/L)	Cd (mg/L)	Cr (mg/L)	Cu (mg/L)	Ni (mg/L)	Pb (mg/L)	Zn (mg/L)
D-C	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
D-WV	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
D-NB	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a
D-WCE	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a	<0.01 a

Table S37. Lettuce C and N composition under salt condition

Treatments	Lettuce		Roots	
	C	N	C	N
S-C	36.82 ± 0.45 a	3.41 ± 0.08 a	37.14 ± 0.29 a	1.92 ± 0.13 a
S-WV	37.07 ± 0.92 a	3.36 ± 0.17 a	37.27 ± 0.14 a	1.92 ± 0.16 a
S-NB	37.74 ± 0.24 a	3.52 ± 0.06 a	36.63 ± 0.36 a	1.99 ± 0.09 a
S-WCE	36.99 ± 0.66 a	3.58 ± 0.23 a	36.22 ± 0.47 a	1.92 ± 0.08 a

Table S38. Lettuce leaf macronutrients under salt condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
S-C	10.31 ± 0.53 a	54.48 ± 1.85 b	7.70 ± 0.50 a	2.44 ± 0.11 a	2.35 ± 0.09 a
S-WV	9.55 ± 1.07 a	50.42 ± 2.89 b	5.86 ± 0.91 a	2.45 ± 0.36 a	2.40 ± 0.21 a
S-NB	11.39 ± 1.02 a	43.95 ± 2.46 a	6.26 ± 0.63 a	2.37 ± 0.37 a	2.29 ± 0.19 a
S-WCE	12.58 ± 0.69 b	44.84 ± 2.02 a	6.64 ± 0.50 a	2.40 ± 0.35 a	2.37 ± 0.16 a

Table S39. Lettuce root macronutrients under salt condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
S-C	5.68 ± 0.67 a	34.03 ± 2.96 a	8.29 ± 0.77 a	1.23 ± 0.10 a	2.03 ± 0.12 a
S-WV	7.02 ± 0.48 a	32.80 ± 6.76 a	8.68 ± 1.85 a	1.22 ± 0.29 a	2.05 ± 0.47 a
S-NB	8.13 ± 1.51 a	34.18 ± 4.14 a	9.23 ± 1.05 a	1.26 ± 0.14 a	2.15 ± 0.06 a
S-WCE	7.32 ± 0.87 a	35.46 ± 2.16 a	9.19 ± 1.10 a	1.23 ± 0.08 a	1.99 ± 0.12 a

Table S40. Lettuce leaf micronutrients under salt condition

Treatments	B (mg/Kg)	Fe (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
S-C	20.79 ± 0.47 a	251.75 ± 37.45 a	0.45 ± 0.03 b	147.35 ± 8.99 a	<0.01 a
S-WV	21.36 ± 2.19 a	304.88 ± 73.25 a	0.46 ± 0.07 b	147.98 ± 9.96 a	<0.01 a
S-NB	18.95 ± 1.89 a	372.06 ± 157.93 a	0.30 ± 0.03 a	130.20 ± 11.86 a	<0.01 a
S-WCE	19.07 ± 1.63 a	847.35 ± 258.95 b	0.32 ± 0.02 ab	139.15 ± 8.93 a	<0.01 a

Table S41. Lettuce root micronutrients under salt condition

Treatments	B (mg/Kg)	Fe (mg/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
S-C	9.75 ± 0.94 a	486.57 ± 69.14 a	0.29 ± 0.03 a	51.88 ± 3.11 a	0.43 ± 0.07 a
S-WV	9.24 ± 1.39 a	529.54 ± 67.42 ab	0.32 ± 0.04 a	59.61 ± 3.79 ab	0.90 ± 0.18 b
S-NB	10.21 ± 1.39 a	864.09 ± 111.36 b	0.32 ± 0.05 a	70.96 ± 6.41 b	1.00 ± 0.13 b
S-WCE	9.53 ± 0.62 a	707.73 ± 172.87 b	0.35 ± 0.06 a	62.96 ± 5.26 b	0.94 ± 0.15 b

Table S42. Lettuce leaf heavy metals under salt condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
S-C	0.12 ± 0.08 a	0.86 ± 0.13 a	0.63 ± 0.15 a	4.93 ± 0.52 a	0.87 ± 0.04 a	0.73 ± 0.24 a	16.20 ± 2.31 a
S-WV	0.11 ± 0.06 a	0.93 ± 0.20 a	0.75 ± 0.29 a	5.20 ± 0.68 a	1.04 ± 0.25 a	0.84 ± 0.22 a	13.74 ± 2.76 a
S-NB	0.13 ± 0.09 a	0.78 ± 0.06 a	0.84 ± 0.36 a	4.80 ± 0.57 a	0.93 ± 0.22 a	0.77 ± 0.17 a	12.86 ± 1.96 a
S-WCE	0.22 ± 0.14 a	0.80 ± 0.07 a	0.79 ± 0.27 a	5.24 ± 0.59 a	1.24 ± 0.43 a	0.81 ± 0.26 a	15.49 ± 2.56 a

Table S43. Lettuce root heavy metals under salt condition

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
S-C	1.73 ± 0.27 a	0.26 ± 0.06 a	2.56 ± 0.92 a	6.26 ± 0.38 a	2.13 ± 1.04 a	0.75 ± 0.28 a	11.03 ± 1.50 a
S-WV	2.11 ± 0.62 a	0.40 ± 0.06 a	2.97 ± 0.67 a	8.05 ± 0.61 ab	2.63 ± 0.96 a	1.02 ± 0.13 a	13.85 ± 2.00 ab
S-NB	2.31 ± 0.28 a	0.42 ± 0.05 a	3.47 ± 0.97 a	10.04 ± 1.82 b	2.87 ± 0.69 a	1.91 ± 0.60 b	18.61 ± 2.86 b
S-WCE	1.69 ± 0.67 a	0.41 ± 0.05 a	4.74 ± 1.58 a	7.89 ± 0.83 ab	2.08 ± 0.34 a	1.23 ± 0.36 ab	12.65 ± 1.72 ab

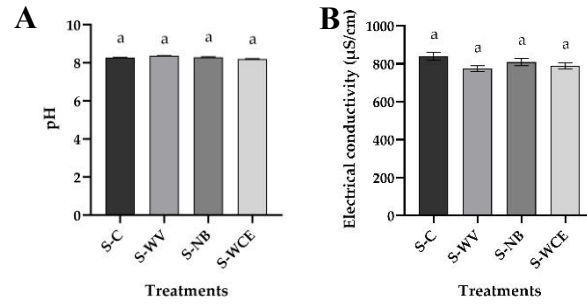


Figure S3. Soil pH (A) and EC (B) after treatments under salt condition

Table S44. Soil C and N composition under salt condition

Treatments	Total		Soluble	
	C (g/100g)	N (g/100 g)	C (mg/L)	N (mg/L)
S-C	8.11 ± 0.64 a	0.09 ± 0.02 a	56.25 ± 0.58 b	114.70 ± 20.41 b
S-WV	8.15 ± 0.24 a	0.08 ± 0.01 a	44.02 ± 1.18 a	80.70 ± 8.12 a
S-NB	8.22 ± 0.44 a	0.09 ± 0.01 a	41.74 ± 4.15 a	144.84 ± 5.36 b
S-WCE	7.69 ± 0.51 a	0.09 ± 0.01 a	44.00 ± 3.83 a	162.04 ± 15.25 b

Table S45. Soil macronutrients under salt condition

Treatments	Ca (g/Kg)	K (g/Kg)	Na (g/Kg)	P (g/Kg)	S (g/Kg)
S-C	234.84 ± 23.88 a	5.03 ± 0.67 a	0.34 ± 0.06 a	0.65 ± 0.07 a	1.62 ± 0.19 a
S-WV	243.55 ± 14.86 a	4.87 ± 0.49 a	0.32 ± 0.04 a	0.95 ± 0.27 a	1.63 ± 0.12 a
S-NB	229.34 ± 6.72 a	4.53 ± 0.47 a	0.30 ± 0.01 a	0.70 ± 0.07 a	1.53 ± 0.21 a
S-WCE	239.84 ± 12.50 a	4.80 ± 0.84 a	0.30 ± 0.08 a	0.79 ± 0.09 a	1.58 ± 0.03 a

Table S46. Soil macronutrients under salt condition (soluble fraction)

Treatments	Ca (g/L)	K (g/L)	Na (g/L)	P (g/L)	S (g/L)
S-C	365.01 ± 30.17 b	11.05 ± 1.04 ab	644.74 ± 26.80 b	5.56 ± 0.49 ab	95.99 ± 11.53 b
S-WV	241.99 ± 27.71 a	2.17 ± 1.31 a	492.12 ± 29.20 a	5.20 ± 0.46 ab	62.45 ± 11.11 a
S-NB	326.74 ± 22.42 b	20.07 ± 2.22 b	502.61 ± 67.50 a	6.11 ± 0.58 b	77.08 ± 8.54 ab
S-WCE	339.39 ± 41.35 b	19.48 ± 4.45 b	500.75 ± 80.27 a	4.31 ± 0.53 a	64.95 ± 10.49 a

Treatments	B (mg/Kg)	Fe (g/Kg)	Mg (mg/Kg)	Mn (mg/Kg)	Mo (mg/Kg)
S-C	14.86 ± 3.18 a	11.97 ± 2.36 a	0.55 ± 0.12 a	269.03 ± 21.83 a	0.24 ± 0.10 a
S-WV	14.25 ± 1.72 a	12.92 ± 1.78 a	0.56 ± 0.14 a	275.64 ± 26.12 a	0.28 ± 0.02 a
S-NB	14.48 ± 2.92 a	12.18 ± 2.23 a	0.58 ± 0.13 a	284.74 ± 34.94 a	0.17 ± 0.08 a
S-WCE	14.88 ± 2.88 a	12.74 ± 1.54 a	0.63 ± 0.16 a	280.98 ± 32.38 a	0.17 ± 0.06 a

Treatments	B (mg/L)	Fe (mg/L)	Mg (mg/L)	Mn (mg/L)	Mo (mg/L)
S-C	1.15 ± 0.07 a	<0.01 a	59.66 ± 8.32 b	0.40 ± 0.05 a	<0.01 a
S-WV	0.91 ± 0.05 a	<0.01 a	36.04 ± 3.50 a	0.22 ± 0.02 a	<0.01 a
S-NB	0.98 ± 0.04 a	<0.01 a	54.76 ± 3.82 b	0.37 ± 0.03 a	<0.01 a
S-WCE	0.89 ± 0.05 a	<0.01 a	49.16 ± 5.87 ab	0.31 ± 0.08 a	<0.01 a

Treatments	As (mg/Kg)	Cd (mg/Kg)	Cr (mg/Kg)	Cu (mg/Kg)	Ni (mg/Kg)	Pb (mg/Kg)	Zn (mg/Kg)
S-C	6.33 ± 0.46 a	0.33 ± 0.12 a	26.11 ± 5.13 a	20.50 ± 2.46 a	13.73 ± 2.37 a	20.19 ± 2.88 a	30.96 ± 3.59 a
S-WV	6.27 ± 0.48 a	0.27 ± 0.03 a	25.29 ± 3.43 a	20.33 ± 1.77 a	13.23 ± 1.84 a	20.11 ± 1.99 a	32.61 ± 4.75 a
S-NB	6.19 ± 0.60 a	0.29 ± 0.05 a	26.31 ± 4.04 a	21.42 ± 1.59 a	13.87 ± 2.31 a	20.30 ± 2.69 a	31.65 ± 3.13 a
S-WCE	6.43 ± 0.42 a	0.28 ± 0.04 a	27.71 ± 5.01 a	21.66 ± 1.98 a	15.16 ± 2.88 a	20.79 ± 1.54 a	32.52 ± 2.43 a

[illegible]

2. Methodologies and Results

2.3. Plant defence induction under abiotic stress

Article

Wood to wellness: from chestnut residues to bio-extracts to boost plant growth and defense under salt stress

Wood to Wellness: from Chestnut Residues to Bio-Extracts to Boost Plant Growth and Defense under Salt Stress

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Abstract

Soil salinity is a rapidly intensifying constraint on agricultural productivity and food security, further aggravated by climate change and the progressive degradation of soil physical and biological properties. In this context, the identification of effective, environmentally friendly and economically sustainable agrochemical alternatives, enhancing crop resilience, represents a major research priority. Natural products derived from agroforestry residues offer a promising solution, particularly within circular economy frameworks. Chestnut (*Castanea sativa*) biomass is of special interest due to its high abundance of bioactive polyphenols, notably tannins. However, despite their growing presence on the market in the last couple of years as well as their agronomic use, the actual agrochemical potential and molecular modes of action of chestnut-derived extracts remain largely unresolved. Here, we present an integrated agrochemical and biological characterization and comparison of two commercially available chestnut-derived wood vinegars and an aqueous chestnut wood chip extract, aimed at elucidating their functional roles in supporting plant growth and defense. A field-based experimental approach was adopted using *Nicotiana tabacum* as a model crop, combining physiological, biochemical, and molecular analyses under both non-salt and salt stress conditions. Plant growth and development, photosynthetic performances, and stomatal regulation exhibited clear and predominantly dose-dependent responses, demonstrating the capacity of these natural products to modulate key physiological processes under contrasting environmental regimes. At the molecular level, gene expression profiling revealed a pronounced activation of hormone-mediated and defense-related signaling networks. In particular, the consistent induction of pathogenesis-related proteins and associated immune markers indicate that both chestnut-derived formulations act as effective elicitors of plant innate immunity, mimicking pathogen-triggered defense responses. Importantly, these responses were preserved under salt stress, highlighting the robustness of the induced defense priming and its relevance under abiotic stress conditions. Collectively, this study advances the agrochemical understanding of chestnut-derived natural products by linking their bioactive composition to defined physiological and molecular responses in plants. The results provide a strong scientific basis for the development of chestnut-based biostimulants and defense elicitors as sustainable agrochemical tools to enhance crop performance and stress resilience in salinity-affected agroecosystems.

Keywords: wood vinegar; woodchip extract; chestnut biomass; biostimulants; tobacco; abiotic stress; salt stress; gene expression; plant defence; sustainable agriculture

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1. Introduction

Climate change, largely driven by human activities, poses a serious threat to agriculture and food security by intensifying extreme weather and increasing soil salinization, thereby reducing crop growth, yield, and quality [1,2].

Increasing abiotic pressures — particularly soil salinity resulting from expanded irrigation practices and the use of low-quality water — further disrupt plant physiological and metabolic processes, thereby compromising productivity and crop quality [3]. Soil salinity represents a major constraint for irrigated agriculture, particularly in hot, arid, and semi-arid regions, where inadequate irrigation management and insufficient precipitation lead to the accumulation of salts in the soil [4]. Salts originate from natural processes such as mineral weathering, as well as human activities including the use of irrigation water and fertilizers, and they can also migrate upward from shallow groundwater. When salts are not adequately leached from the soil profile, they accumulate and severely affect soil structure, fertility, and microbial activity, while suppressing plant growth [4]. Although plants can activate adaptive responses to salinity, these are often associated with reduced productivity. Given the limited availability of salt-tolerant cultivars, improving knowledge of salinity-tolerance mechanisms and developing effective adaptation and mitigation strategies are essential to ensure sustainable agriculture and global food security.

Drought and high temperatures further constrain plant growth and development. Under these conditions, water scarcity disrupts their biochemical and metabolic functions. These disruptions lead to excessive accumulation of reactive oxygen species (ROS), resulting in oxidative stress [5]. Although plants possess natural defense systems to counter oxidative damage, there remains a strong need for innovative and sustainable strategies to mitigate the negative effects of drought stress on plant performance [6].

At the same time, meeting global food demand cannot rely on the continued intensive exploitation of natural resources, deforestation or the heavy use of fertilizers and pesticides. These practices contribute substantially to greenhouse gas emissions and cause severe environmental and health damage. The increasing use of pesticides has led to soil, water, and air contamination, loss of biodiversity, accumulation in the food chain and the emergence of resistant strains. Considering these interconnected challenges, there is an urgent need to develop sustainable, environmentally friendly, and innovative approaches to protect crops, reduce the environmental impact of agriculture and ensure resilient food systems in the long term.

Plant biostimulants are defined as substances or microorganisms applied to plants with the aim of improving nutrient use efficiency, enhancing tolerance to abiotic stress, and/or increasing crop quality [7]. They have gained interest because of their ability to stimulate growth and mitigate stress-induced limitations. Furthermore, biostimulants are increasingly regarded as an eco-friendly tool to help plants withstand environmental stresses.

In this context, wood vinegar could be a feasible and sustainable alternative to common chemical products. Wood vinegar, also known as pyroligneous acid, is a sustainable by-product of biomass pyrolysis, produced from condensed volatile compounds [8,9]. It has long been used in agriculture as a natural amendment to promote plant growth, control pests, and improve soil properties, serving as an eco-friendly alternative to conventional agrochemicals [10]. Its chemical composition is highly variable and depends on factors such as feedstock type, pyrolysis temperature, particle size, and residence time [11]. Chestnut biomass is one of the most common materials used, owing to its high tannin content. These polyphenolic compounds, which typically account for 5–10% of a plant dry matter, contribute strongly to plant defense due to their antimicrobial and anti-insect properties. Chestnut wood can also be processed through water extraction, yielding

hydrolysable tannins (HTs), formed by glucose molecules esterified with phenolics like gallic and ellagic acids [12,13]. HTs are increasingly studied for their ability to support plant growth, improve soil characteristics and water quality, and enhance plant tolerance to biotic and abiotic stresses [14,15].

Tobacco (*Nicotiana tabacum* L.) is a globally cultivated broadleaf crop with significant economic importance. It is also widely used today as a model species for research on dicotyledonous plants. Nevertheless, tobacco is highly susceptible to drought stress [16].

The aim of this study was to investigate the effects of two commercial wood vinegars derived from the pyrolysis of chestnut wood residues and a laboratory-scale extract obtained from chestnut wood chips. Specifically, the study focused on their impact on *Nicotiana tabacum* plants, under saline and non-saline stress conditions. Field trials were conducted during the summer season, assessing key morphological traits related to plant yield, followed by a detailed analysis of gene expression. Particular attention was given to pathways involved in hormonal regulation, plant defense responses to biotic stress, and tolerance mechanisms associated with abiotic stresses, including salinity, drought, and heat.

2. Materials and Methods

2.1. Experimental Design

To evaluate the effects of chestnut-derived products on plant growth, development, and defense mechanisms, an open-field experiment was carried out at the Laboratorio di Patologia Vegetale Molecolare (DAGRI, University of Florence, Italy), in Sesto Fiorentino (43°49'12.7" N 11°11'38.1" E), from July to August 2025. Plants were grown in pots (9 x 11 x h 9 cm), containing approximately 250 g of a professional potting mix composed of 23% coconut granules (0–5 mm), 27% Irish peat (0–5 mm), 14% volcanic pumice (3–8 mm), 36% superfine peat (0–10 mm), and slow-release fertilizers (pH [H₂O] 6.0–7.0, and EC 0.30–0.40 dS/m) (VigorPlant Italia srl - professional mix).

In this study, three chestnut-derived products were evaluated: two commercial wood vinegars and a chestnut wood chip extract were analyzed and tested. The commercial products were Biodea® (Arezzo, Italy) wood vinegar (WV) and Nerabiochar® (Turin, Italy) wood distillate (NB), both produced using chestnut wood as the raw material. The chestnut woodchip extract (WCE) was prepared by infusing 2 L of chestnut woodchips (approximately 1.2–1.3 kg) in 4 L of water. The process lasted 3 hours at 80–85°C [17]. After extraction, the liquid was filtered and centrifuged to remove solid residues and contaminants.

All products were acidic and rich in phenolic compounds. Their chemical characteristics were as follows: pH values of 2.29 (WV), 3.37 (NB), and 3.31 (WCE), and total phenolic content expressed as gallic acid equivalents (GAE, mg mL^{−1}) of 8.31 (WV), 8.12 (NB), and 7.06 (WCE).

Tobacco plantlets (*Nicotiana tabacum* var. Burley White) were transplanted into the pots at 2 months of age. The seeds were grown under controlled conditions (24 °C ± 2 °C, 16 h light/8 h dark cycle) until they reached the required developmental stage. The experimental design consisted of two main conditions: non-stressed (control) and salt-stressed plants. Each condition included seven different treatments, as shown in Table 1. Six plants per treatment were used. As control, plants were irrigated with tap water, whereas salt-stressed plants received irrigation water supplemented with NaCl at a concentration 2 g/L. Irrigation was performed four to five times per week, depending on climatic conditions. On the contrary, the treatments with the three products were carried out once a week. Plants were harvested 50 days after potting.

Table 1. Scheme of all treatments under different conditions

Normal conditions		Salt stress conditions	
Control	Control	S-Control	Control
WV 1: 300	Wood vinegar Biodea® 1:300	S-WV 1:300	Wood vinegar Biodea® 1:300
NB 1:300	Wood vinegar NeraBiochar® 1:300	S-NB 1:300	Wood vinegar NeraBiochar® 1:300
WCE 1:300	Woodchip Extract 1:300	S-WCE 1:300	Woodchip Extract 1:300
WV 1:600	Wood vinegar Biodea® 1:600	S-WV 1:600	Wood vinegar Biodea® 1:600
NB 1:600	Wood vinegar NeraBiochar® 1:600	S-NB 1:600	Wood vinegar NeraBiochar® 1:600
WCE 1:600	Woodchip Extract 1:600	S-WCE 1:600	Woodchip Extract 1:600

2.2 Analysis of Plant Physiological Parameters and Sampling

Before plant sampling, stomatal conductance and photosystem II (PSII) activity were measured with a porometer/fluorometer LICOR-600 (Li-Cor, Lincoln, NE, USA). Moreover, the fourth youngest leaf of each plant per treatment was collected to determine chlorophyll (chl) *a* and *b* content, following the method described by Wang *et al.* [18]. Briefly, 0.05 g of leaves (fresh weight, FW) were incubated in 2.5 mL (V) of 80% acetone for 24 h

in the dark at room temperature. The chl content was determined by measuring the optical density at 663 nm (OD_{663}) and 645 nm (OD_{645}) for chl *a* and *b*, respectively, with a UV spectrophotometer and multimode plate reader Infinite® 200 PRO (Tecan, Zürich, Switzerland). Afterward, the chl concentration was calculated using the following formulas:

$$\text{chl } a \text{ (mg/g)} = \frac{(12.72 \times OD_{663} - 2.59 \times OD_{645}) \times V}{1000 \times FW}$$

$$\text{chl } b \text{ (mg/g)} = \frac{(22.88 \times OD_{645} - 4.67 \times OD_{663}) \times V}{1000 \times FW}$$

Furthermore, the third youngest leaves of each plant were collected and immediately frozen in liquid nitrogen (N_2) for subsequent gene expression analysis. Subsequently, plants were carefully removed from the pots, and the aerial part was separated from the roots, which were thoroughly washed to remove all soil. Plant height and internode length were measured for each individual. After determination of fresh weight, roots and aerial parts were oven-dried at 60°C for 2–3 days until constant weight was reached to determine dry biomass.

2.3. RNA Extraction and Plant Gene Expression Analysis

Plant samples were ground with liquid nitrogen, and RNA extraction was performed using the NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Deutschland) on plant samples, following the manufacturer's instructions. The total RNA was eluted in 60 µL of nuclease-free water. RNA concentration and quality were assessed using both a NanoDrop Spectrophotometer ND-100 (Nanodrop Technologies Inc., Wilmington, DE, USA) and gel electrophoresis (1% agarose gel in 1X TAE). To eliminate any genomic DNA (gDNA) contamination, total RNA samples were further purified using the RapidOut DNA Removal Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Then, cDNA was synthesized using the RevertAid RT Kit (ThermoFisher Scientific) following the manufacturer's protocol. PCR primers to be used in gene expression analysis have been designed and optimized for each plant species using the Primer-BLAST tool (National Center for Biotechnology Information, NCBI) [19] and the "PCR Primer Stats" tool of The Sequence Manipulation Suite [20]. The primers were synthesized by Eurofins Genomics (Ebersberg, Germany) and are listed in the Supplementary Materials (Tables S1). The expression profile of the following genes was analyzed: auxin-responsive gene 1 (*ARF1*), transport inhibitor response 1 (*TIR1*), phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), pathogenesis-related proteins (*PR1*), enolase (*ENO*), thaumatin-like protein (*TLP*) and Na^+/H^+ exchanger 1 (*NHX1*). Real-time quantitative PCR (RT-qPCR) was performed with the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific), according to manufacturer's protocol. Gene expression levels were normalized using *L25* as housekeeping gene. Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ method [21]. The data from the treated plants were compared with those from the controls (normal condition and salt stress condition). For each sample, three technical replicates were processed in each of the three independent RT-qPCR runs carried out.

2.4. Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, Boston, MA, USA). After assessing normality, data were analyzed using one-way ANOVA or Kruskal–Wallis tests with a significant threshold of $p < 0.05$. Afterwards, a *post hoc* analysis using either Tukey's or Dunn's test was performed by comparing the mean of each treatment with the mean of any other treatment for each plant species.

3. Results

3.1. Effects of Bio-Products on Plant Development and Physiology

Plant growth and developmental responses to the treatments varied depending on the cultivation conditions (non-stress or salt stress) (Figure 1). To improve the clarity and interpretability of the results, the data were normalized to their respective controls. In the salt-stress analyses, the values of the control sample (non-stress conditions) are also displayed to enable direct comparison between the two experimental conditions.

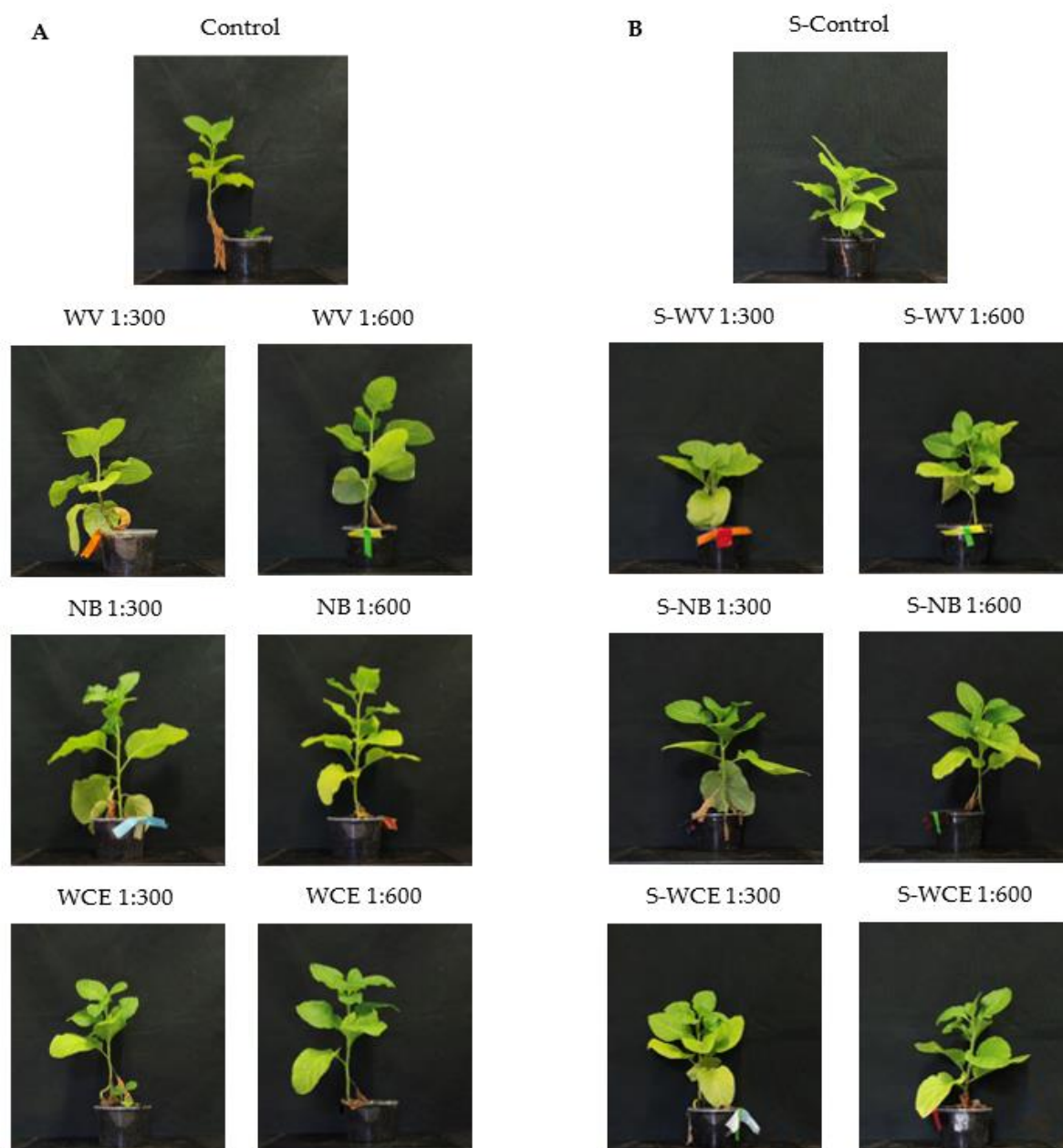


Figure 1. Most representative plant of each treatment under non-stress conditions (A) and salt stress conditions (B)

Under non-stress conditions, plant height of the aerial part was not affected by WV 1:300 compared to the Control, while it significantly increased with WV 1:600 and both concentrations of NB (Figure 2A). In contrast, under salt stress, all treatments significantly increased plant height compared with the salt-stressed control (S-Control) (Figure 2E),

which showed a marked reduction relative to the control grown under non-stress conditions.

Internode number was counted for each individual plant and compared to its height to calculate an average distance between internodes. Analysis of this ratio clearly showed that both WV and NB diluted 1:300 significantly reduced the distance between internodes under non-stress conditions (Figure 2B). Conversely, under salt stress, all treatments promoted plant elongation, reaching values similar to the control (Figure 2F).

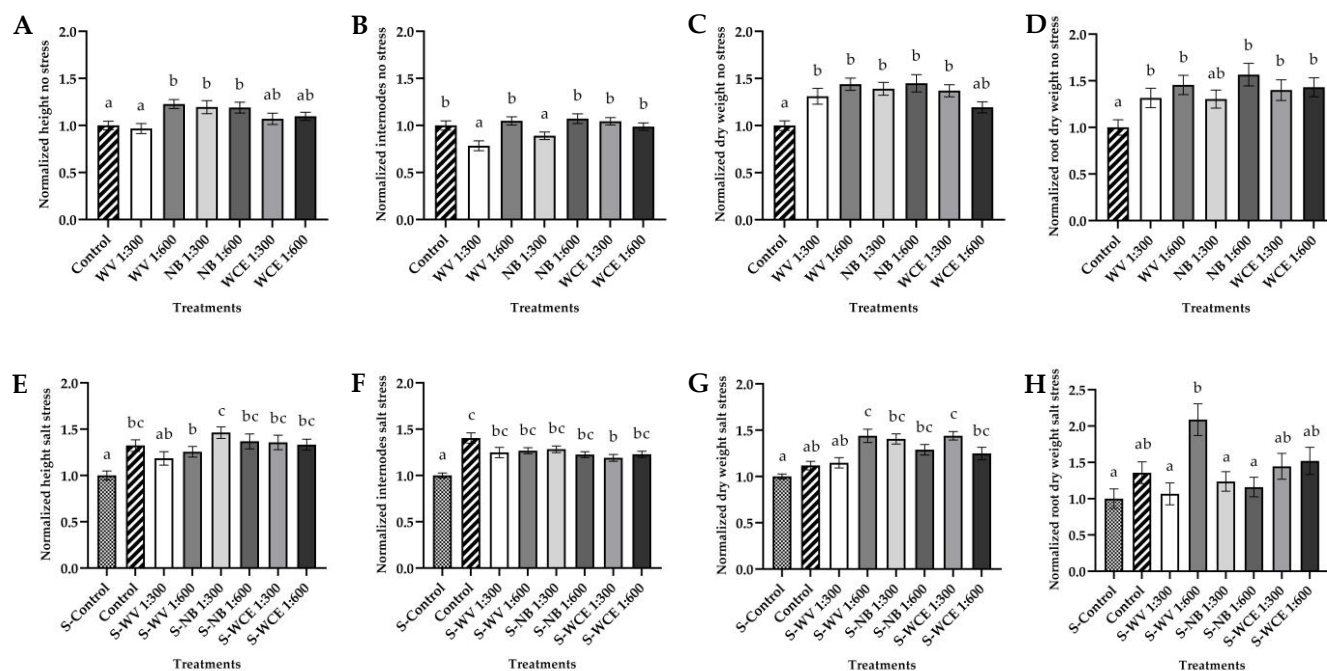


Figure 2. Height (A, E), internodes (B, F), dry weight of the aerial part (C, G), and root dry weight (D, H) of treated *N. tabacum* plants under non-stress conditions (A-D) and salt stress condition (E-H). All values have been normalized by ratioing them to the corresponding control. Error bars represent the standard error of the mean (SEM). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

In terms of dry weight, all treatments determined an increment compared to the controls under both growing conditions. This increment was even more pronounced under salt conditions, after the application of WV 1:600 and WCE 1:300 (Figures 2C-2G).

Root system development was enhanced under non-stress conditions by all treatments except NB 1:300 (Figure 2D). Under salt stress, however, root weight increased significantly only after the application of WV 1:600 (sample S-WV 1:600, Figure 2H)

Photosynthetic parameters were differently affected by both the tested bio-product and the growth condition. Indeed, the chl *a* content, under non-stress conditions, was significantly increased only after the application of NB 1:300, while all the other samples were comparable to the Control. Under salt stress, a trend towards an increase was observed with all treatments, especially after the addition of NB. Data on chl *b* content were comparable to those for chl *a* across all the plant species here tested (data not shown).

Stomatal conductance was slightly influenced by the application of all products. Under non-stress conditions, a general increase was observed with all treatments, with the most pronounced effects with WV 1:300 and WCE 1:600 (Figure 3B). On the contrary, the tested treatments had no effect on this parameter under salt stress conditions (Figure 3E), concerning PSII activity, no significant differences were noted (Figures 3C-3F). Overall, salt stress resulted in a general reduction in chlorophyll content and stomatal conductance.

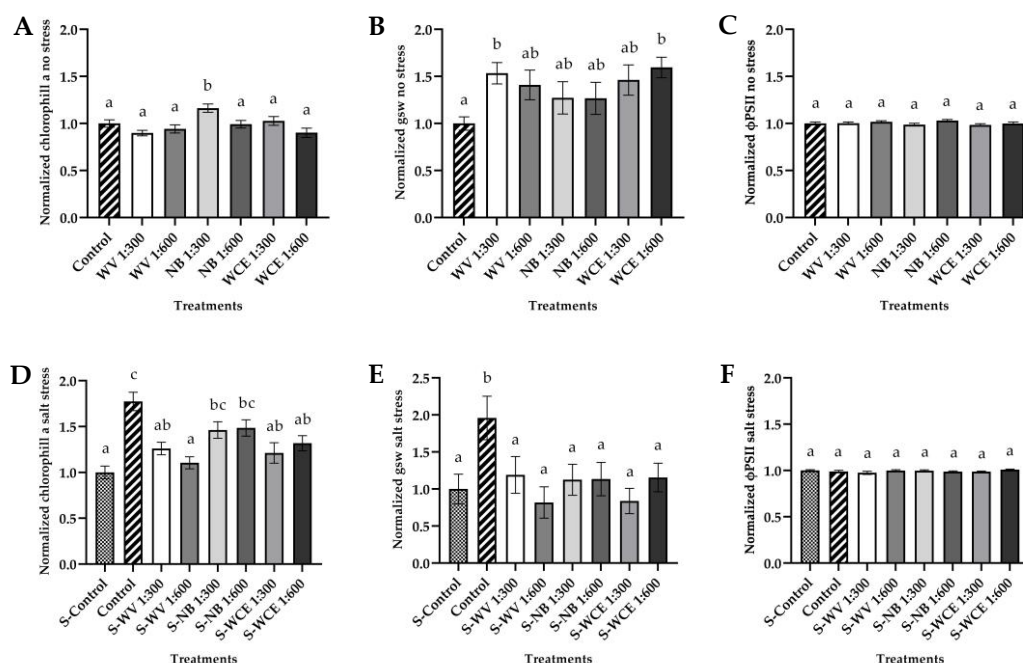


Figure 3. Chlorophyll *a* content (A–D), stomatal conductance (B–E) and photosystem II activity (ϕ PSII) (C–F) of treated *N. tabacum* plants under non-stress conditions (A–C) and salt stress condition (D–F). All values have been normalized by ratioing them to the corresponding control. Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

3.2. Impact of Bio-Products on the Expression of Defense-Related Genes in Plants

Bio-product treatments also modulated the expression of defense-related genes, depending on the different development conditions. Within the auxin signaling pathway, *ARF1* expression was not significantly affected under non-stress conditions, except after the NB 1:300 treatment, which induced a marked upregulation (Figure 4A). Conversely, under salt stress, the *ARF1* gene was overexpressed with all three products at the highest concentration (i.e., 1:300) (Figure 4B). The *TIR1* gene was significantly upregulated under both non-stress and salt stress conditions following treatment with all tested bio-products, with the strongest effects observed for WV 1:300 and NB 1:300 (Figures 4C and 4D). Notably, salt stress itself induced *TIR1* expression, leading to overall higher transcript levels under stress conditions compared with non-stress controls.

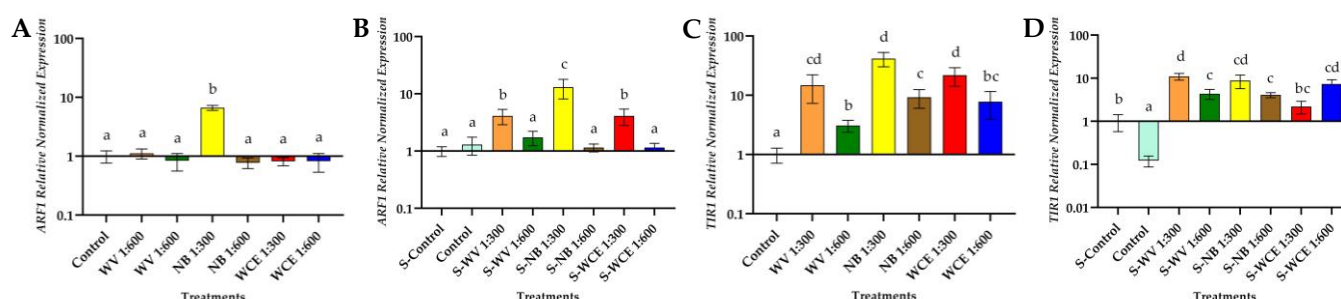


Figure 4. Gene expression analysis of auxine-related genes in treated plants: *ARF1* expression profile under non-stress (A) and salt stress condition (B); *TIR1* expression under non-stress (C) and salt stress condition (D). Error bars represent the standard deviation (SD). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

Concerning the *PAL* gene, a significant overexpression was observed after the application of NB 1:300 and WCE 1:600 under both normal and stress conditions (Figures 5A–5D). Without stress, *PAL* gene was also overexpressed with WCE 1:300 (Figure 5A). Conversely, *CHS* expression exhibited contrasting trends depending on growth conditions. Under non-stress conditions, *CHS* overexpression was detected only after WV 1:600 treatment, while both NB concentrations led to downregulation of the gene (Figure 5B). Under salt stress, the gene was overexpressed after the application of all treatments. Moreover, the presence of salt reduced *CHS* expression levels; however, all treatments restored these levels to values comparable to Control (Figure 5E). Lastly, *PR1* was overexpressed after the application of NB 1:300, as well as WCE 1:300 and 1:600 in non-stress conditions (Figure 5C). Under salt stress, *PR1* expression was significantly induced by all treatments (Figure 5F),

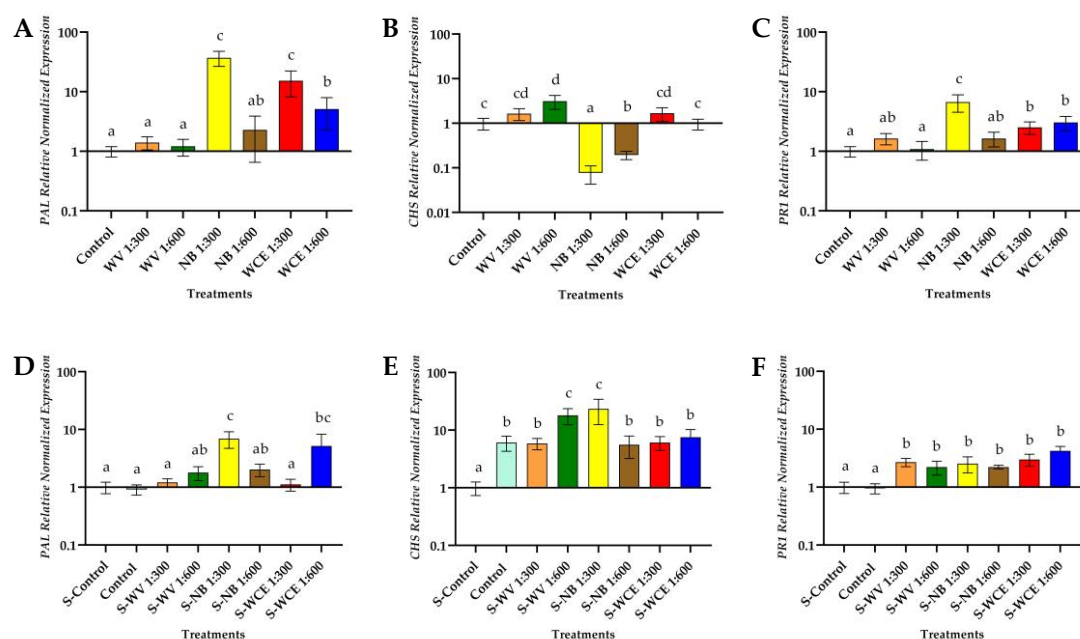


Figure 5. Gene expression analysis of defence-related genes in treated plants: *PAL* expression profile under non-stress (A) and salt stress condition (D); *CHS* expression under non-stress (B) and salt stress condition (E); *PR1* expressions under non-stress (C) and salt stress condition (F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

Concerning abiotic stress-related genes, *ENO* gene was overexpressed in all non-stress samples (Figure 6A), while under salt stress this result was only observed in S-NB 1:300 and S-WCE 1:600 samples compared to S-Control (Figure 6B). The *TLP* gene was significantly upregulated following NB 1:300 treatment under non-stress conditions (Figure 6C). On the contrary, the gene was downregulated in S-WCE 1:300, while it was overexpressed in S-NB 1:300 and S-WV 1:300 under salt stress conditions. (Figure 6D). Both genes were shown to be more highly expressed under stress than under normal conditions.

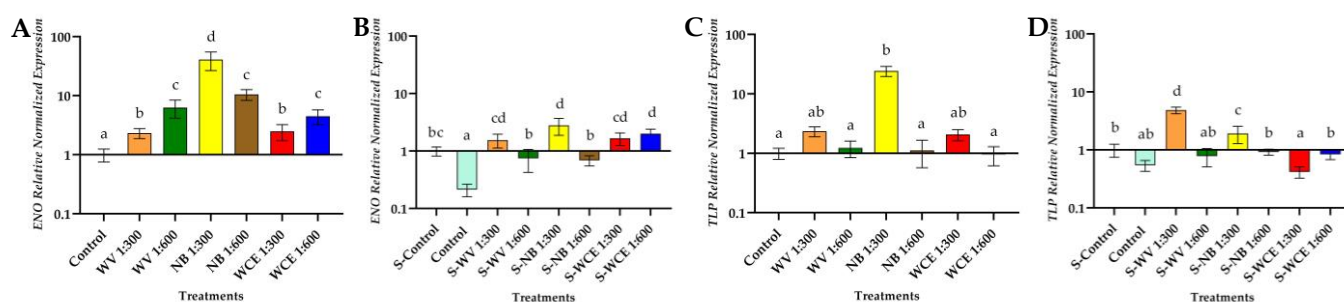


Figure 6. Gene expression analysis of abiotic stress-related genes in treated plants: *ENO* expressions under non-stress (A) and salt stress condition (B); *TLP* expressions under non-stress (C) and salt stress condition (D). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

Lastly, *NHX1*, a gene related to salt stress, was generally overexpressed in all the samples under stress conditions compared to those without stress. Although treated plants consistently showed higher *NHX1* expression levels than their respective controls, these differences were not statistically significant (Figures 7A–C).

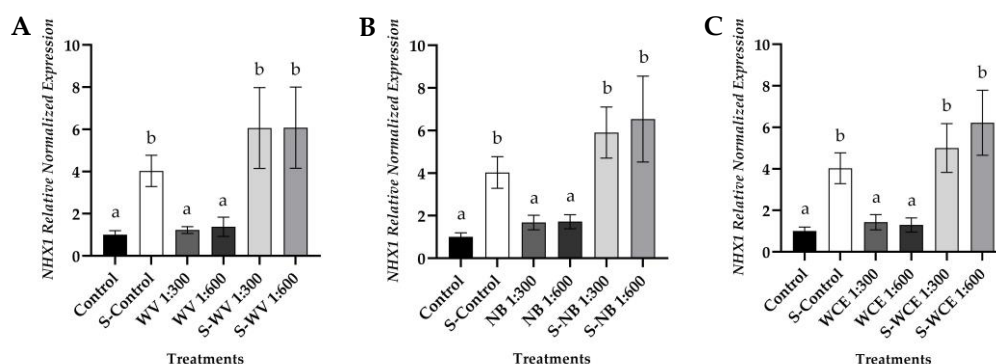


Figure 7. Gene expression analysis of salt stress-related gene *NHX1* in plants treated with WV (A), NB (B) and WCE (C). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

4. Discussion

The development of new environmentally friendly approaches to preserve plant health has become a key priority in agriculture. The sector needs to increase yields, cope with the growing pressure of diseases caused by global changes, and limit the use of conventional agrochemicals that harm both people and ecosystems [22,23]. In addition, the increasing frequency of extreme weather events is further constraining agricultural production worldwide [1,24]. To address these challenges, researchers are investigating plant-derived substances as sustainable options for supporting plant growth and vitality [2]. Among these, chestnut-based products, rich in polyphenols, can be promising tools for helping plants resist biotic and abiotic stresses [9,10,15].

This study evaluated the effects of sweet chestnut-derived wood vinegars and a laboratory-scale chestnut woodchip extract on tobacco under non-stress and salt stress conditions. Multiple concentrations were tested to assess dose-dependent responses and to compare the effects of different chestnut-derived products on the development of a model plant species. In addition, a comprehensive gene expression study was carried out to

investigate whether these treatments could activate key molecular pathways involved in defense mechanisms.

Wood vinegar, in particular, has been extensively studied in recent years, but it has always been clear that different variables involved in its production influence its actual effect [25–27]. Furthermore, it is well established that its biological activity strongly depends on production parameters, including feedstock and pyrolysis conditions. Moreover, the optimal dose varies widely depending on plant species and experimental settings. Among the commercial products tested, WV has been the subject of numerous studies, whereas NB remains largely unexplored. A comparative evaluation of these two products, derived from the same raw material, is therefore essential to better understand their relative efficacy. Furthermore, information on chestnut wood extracts is very limited, even more when considering woodchips as the starting matrix.

The results of this study showed that the development of the tobacco plants was influenced by both growth conditions and applied treatments. Under non-stress conditions, the highest tested concentrations of both wood vinegars (i.e., WV and NB 1:300) determined a reduction of plant aerial development (Figures 2A–B). High concentrations of wood vinegar have already been observed to have a potential herbicidal effect [28–30]. Consequently, it is possible that the use of inappropriate doses may partially compromise the correct development of the plant, simulating an auxin-like effect. However, this is not observed in plants under salt stress (Figures 2E–F). Considering that salt stress reduces plant growth [31], the application of the products of interest determined an increase in plant height when they were subjected to salt stress.

Consistent with these observations, dry biomass accumulation increased in treated plants under both non-stress and salt stress conditions, with particularly pronounced effects observed in salt-stressed plants treated with WV 1:300 and WCE 1:600 (Figures 2C–G). At the root level, the greatest improvements were observed without stress, while in the presence of salt, only WV 1:600 promoted a significant increase (Figures 2D–H). These results suggest that both dose and environmental context play critical roles in determining the effectiveness of chestnut-derived bio-products.

Concerning photosynthetic parameters, numerous studies indicate that wood vinegar can enhance chlorophyll levels [32,33]; however, chl *a* content increased only in NB-treated plants (Figures 3A–D). PSII activity, which is used as an indicator of the functional state of the photosynthetic apparatus [34], did not differ significantly among treatments. Stomatal conductance—reflecting the rate of gas exchange and transpiration through the stomata and thus providing an estimate of stomatal opening—suggested that WV 1:300 and WCE 1:600 promoted a wider stomatal aperture (Figures 3B–E) [35].

Overall, at a macroscopic level, the application of wood vinegar at a higher dilution (i.e., 1:600) appeared to be more suitable when plants are cultivated in small pots. Higher concentrations seemed to compromise the aerial development of the plant under normal conditions. However, plants exhibiting reduced vegetative growth without a corresponding decline in yields may offer advantages in some agricultural systems. It is important to emphasize that all treatments help the plant to grow under salt stress. This results further confirms the effectiveness of wood vinegar already observed in previous studies [3,36,37], and highlighting a promising role for chestnut wood extracts.

Once absorbed by plant tissues, wood vinegar is known to trigger defense-related signaling processes. Previous molecular studies have mainly focused on the activity of antioxidant enzymes [6,38–40]. Here, we analyzed the expression profiles of defense-related genes involved in various pathways, such as the signaling pathways for auxin (i.e., *ARF1*, *TIR1*), secondary metabolites synthesis (i.e., *PAL*, *CHS*), and salicylic acid (SA) (i.e., *PR1*). Genes related to abiotic stress tolerance were also analyzed, both those involved in drought and heat stress (i.e., *ENO*, *TLP*) and those involved in salt stress (i.e., *NHX1*). The

results revealed differential regulation of these genes depending on growth conditions and treatments, providing new insights into the molecular responses triggered by chestnut-derived bio-products. To investigate the auxin signaling cascade, the expression profile of *ARF1* and *TIR1* was assessed. ARF proteins act as transcription factors that associate with Aux/IAA proteins and recognize auxin-responsive elements (Aux-REs) within the promoters of auxin-regulated genes under low-auxin conditions [41]. *ARF1* is an auxin-inducible gene and has been characterized as a transcriptional repressor of auxin-responsive targets [42,43]. *TIR1/AFB* proteins play crucial roles in plant responses to diverse abiotic and biotic stress [44]. In this manner, these proteins positively regulate downstream auxin-responsive pathways once auxin is detected [45,46]. In the present work, *ARF1* was upregulated in plants treated with the highest concentration of all the tested products (Figures 4A-B), which could suggest an increase in auxin levels. Moreover, *TIR1* was upregulated in all the treated plants and in both conditions, suggesting an increased auxin abundance. (Figures 4C-D). Although sometimes an opposite trend is reported between the presence of auxins and the induction of SA pathways [47], a slight overexpression of *PR1* was also reported. *PR1* proteins are abundantly produced during plant defense responses, where they help restrict pathogen spread and enhance resistance to biotic and abiotic stresses [48]. Beyond defense, *PR1* and *PR1*-like proteins also participate in plant development, including flowering and seed growth [49,50]. Moreover, the increased *PAL* expression observed in plants treated with NB 1:300 and WCE could be also associated with a rise in salicylic acid (SA) (Figures 5A-D). Indeed, *PAL* is a key enzyme in the phenylpropanoid biosynthesis pathway [51], and it is also involved in SA biosynthesis. The increased expression levels observed could also suggest an increment in the biosynthesis of secondary metabolites, such as flavonoids and lignin, which are known to contribute to pathogen resistance. Furthermore, *CHS* is a key enzyme of the flavonoid/isoflavonoid biosynthesis pathway, representing an important molecular marker. Beyond its role in plant development, *CHS* expression is induced under stress conditions and is involved in SA-mediated defense responses [52]. An upregulation of this gene was observed in treated plants subjected to saline stress (Figures 5B-E), further supporting its role in stress adaptation and defense activation.

Enolase (*ENO*) is a conserved enzyme in plants that also participates in stress responses, development, and other metabolic pathways [53]. *ENO* is involved in heat stress resistance as it is linked to the 70-kDa heat shock protein [54]. In this study, an upregulation of this gene was observed under stress-free conditions, while few differences were observed under salt stress (Figures 6A-B). TLPs are a highly complex protein family associated with host defense and developmental processes in plants, animals, and fungi. They are mostly associated with responses to biotic stresses, in addition to some predicted activities under drought and osmotic stresses [55]. An upregulation of this gene was observed after the application NB and WV 1:300 in both tested conditions (Figures 6C-D). These findings suggested that wood vinegars, in particular, could modulate these drought-responsive genes to improve plant drought tolerance, as also reported also Mohd Amnan *et al.* [6].

The *NHX1* gene encodes a Na^+/H^+ antiporter localized to the tonoplast, which contributes to salt tolerance by sequestering Na^+ into vacuoles, thereby limiting its harmful effects on cellular and physiological processes [56]. As expected, all samples under salt stress showed greater expression levels than their non-stressed counterparts (Figure 7). Although the application of chestnut-derived treatments tended to further increase *NHX1* expression, these effects were not statistically significant. Nevertheless, the upregulation of *NHX1* in response to wood vinegar has been previously reported in rapeseed [39], supporting the consistency of these observations.

In summary, the present findings confirm the effects of wood vinegar reported in previous studies, particularly its capacity to stimulate hormonal signaling and defense-related pathways, with pronounced benefits under abiotic stress conditions. On the other hand, investigations on woodchip extract provides a solid foundation for its potential applications in agriculture, revealing multiple beneficial effects under both stress and non-stress conditions.

5. Conclusions

In conclusion, the results of this study demonstrate that these chestnut-based bioproducts have a significant impact on plant growth, development and physiology, particularly under stress conditions. The applied dose proved to be a critical factor: for wood vinegar, the highest dilution (1:600) did not negatively affect plant traits, whereas higher concentrations occasionally impaired aerial growth under non-stress conditions. In contrast, WCE consistently exhibited stimulatory effects, with the 1:300 dilution appearing to be the most effective.

Gene expression analyses further indicated that both wood vinegars and WCE actively modulate key molecular pathways involved in plant defense responses against abiotic and biotic stresses. These findings suggest that chestnut-derived products can enhance plant resilience by activating hormonal signaling and stress-related defense mechanisms.

Overall, wood vinegar and WCE represent promising ecofriendly alternatives to conventional agrochemicals. However, further investigations are required to fully elucidate their molecular modes of action and to optimize application strategies. Such knowledge will be essential for their effective and sustainable integration into crop protection and stress management practices.

Supplementary Materials: The following supporting information can be downloaded at: [https://www.mdpi.com/article/doi/s1, Table S1. Primer information for *Nicotiana tabacum*.](https://www.mdpi.com/article/doi/s1, Table S1. Primer information for <i>Nicotiana tabacum</i>)

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Wood to Wellness: from Chestnut Residues to Bio-Extracts to Boost Plant Growth and Defense under Salt Stress

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Table S1. Primer information for *Nicotiana tabacum*.

Gene		Primer sequence 5' – 3'	Tm (°C)	Designed by
<i>L25</i>	Forward	TATCCAAATGGCTCCAGC	59	[1]
	Reverse	TCTTCAAAGTCTTAGGTCGG		
<i>ARF1</i>	Forward	CGGCAAATCACTTCGGTGG	60	[1]
	Reverse	AATCCCTGATGTGTCGATGC		
<i>TIR1</i>	Forward	GCTCCAGACTCACCTCTT	57	[1]
	Reverse	GGCAGCAAGTTCCTCAAG		
<i>PAL</i>	Forward	TAGACAAACCAACCCCTTTACACTT	60	[1]
	Reverse	GCCACCATTTTCTTCACTTCATC		
<i>CHS</i>	Forward	ATGTGGATAGTATGGTTGGAC	57	[1]
	Reverse	CAAGTGAAATGTTAGCCCGA		
<i>PR1</i>	Forward	CCTCGTACATTCTCATGGTCAAT	57	[1]
	Reverse	CCATTGTTACACTGAACCCTAGC		
<i>ENO</i>	Forward	AGGAACTTGGATCAGACGCT	60	This study
	Reverse	TCGCAGGGTCACATACTACA		
<i>TLP</i>	Forward	TGTCAGCCTTCGAGCTACT	60	This study
	Reverse	AAGGGAGAGGGCAGAAGAT		
<i>NHX1</i>	Forward	ACATCGGTGGTGCTTTTCA	60	This study
	Reverse	TTGCTGCTCCCAGTAAAGTG		

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2. Methodologies and Results

2.3. Plant defence induction under abiotic stress

Article

Bridging the gap: evaluating commercial and research chestnut extracts for tomato resilience under stress

Bridging the Gap: Evaluating Commercial and Research Chestnut Extracts for Tomato Resilience under Stress

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Abstract

This study presents advanced and original research on the use of chestnut (*Castanea sativa*)–derived natural products to enhance plant resilience under abiotic stress. Developing sustainable and innovative strategies to maintain crop health under increasing environmental pressures, such as soil salinity and climate change, is a pressing challenge for modern agriculture. Within a circular economy framework, agroforestry residues like chestnut biomass represent a valuable source of polyphenol-rich bioactive compounds with potential agrochemical applications. Despite growing interest, the physiological and molecular mechanisms by which chestnut-derived extracts modulate plant defense remain largely unexplored in horticultural crops, particularly under field conditions. Here, we evaluated and compared the effects of commercially available chestnut-derived wood vinegars and an aqueous extract from chestnut woodchips on tomato (*Solanum lycopersicum*), integrating field trials, physiological measurements, and gene-expression analyses. Experiments were conducted during the summer under both optimal and saline conditions to assess plant responses under realistic abiotic stress. Key physiological traits, including biomass accumulation, chlorophyll content, and stomatal conductance, were dose-dependently modulated, demonstrating the capacity of these extracts to improve plant performance under stress. Molecular analyses revealed activation of defense-related pathways, particularly via induction of pathogenesis-related proteins and hormone-mediated signaling networks, effectively priming pathogen-like defense responses. Notably, these effects persisted under salinity stress, indicating robust potential for enhancing crop resilience. Overall, this paper provides mechanistic insights into the bioactivity of chestnut-derived natural products and highlights their applicability as sustainable biostimulants to support plant growth and defense in horticultural systems. By linking agrochemical valorization of biomass with field-relevant physiological and molecular responses, this work underscores a practical approach for applying natural products in modern agriculture.

Keywords: wood vinegar; woodchip extract; chestnut biomass; biostimulants; abiotic stress; salt stress; gene expression; plant defence; sustainable agriculture

1. Introduction

The planet is currently facing a global climate crisis primarily driven by human activities that raise atmospheric greenhouse gas levels, including fossil fuel burning and widespread deforestation [1]. These long-term shifts in temperature, rainfall, and other

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atmospheric patterns are intensifying extreme weather events and raising the salinity of soil and groundwater [2,3]. As a result, agriculture is increasingly threatened by coastal erosion, more frequent and severe flooding, and the loss of arable land due to seawater intrusion, all of which undermine crop cultivation and global food security [4,5]. Soil salinity, exacerbated by intensive irrigation practices and the use of low-quality water, disrupts plant physiology and significantly reduces crop yields, while the availability of salt-tolerant cultivars remains limited [6]. Drought and high temperatures further impair essential processes such as photosynthesis, nutrient uptake, and mineral transport, leading to oxidative stress [7]. Although plants have natural defenses to mitigate these environmental stresses, a deeper understanding of these responses and the development of sustainable strategies are urgently required to improve tolerance to salinity, heat, and drought [8]. Moreover, to meet growing global food demand, agriculture cannot continue depending on resource-intensive practices such as deforestation and heavy fertilizer and pesticide use. These approaches contribute significantly to greenhouse-gas emissions and cause environmental and health problems, including pollution, biodiversity loss, food-chain contamination, and the rise of resistant pest strains [9,10]. Therefore, sustainable and innovative solutions are urgently needed to protect crops and reduce agriculture's environmental impact.

Various strategies exist to improve crop tolerance to salt stress, including traditional breeding, gene cloning, and genetic engineering, but these methods are costly and time-consuming [6]. As a result, increasing attention has been directed toward low-cost biological alternatives, such as the application of exogenous compounds functioning as biostimulants, which can mitigate salt stress and promote plant growth [11].

Wood vinegar, or pyroligneous acid, is a sustainable by-product of biomass pyrolysis and offers an eco-friendly alternative to conventional agrochemicals [12–14]. Numerous studies have reported its beneficial effects on soil health, metal toxicity alleviation, and growth promotion in crops such as eggplant, pepper, tomato, cucumber, rice, and wheat [15–18]. Highly diluted wood vinegar exhibits antioxidant and antibacterial properties and may act as a complex growth regulator [18,19]. Its benefits are likely due to its composition, which includes water, organic acids (especially acetic acid), and over 200 organic compounds, such as phenols, ketones, and nitrogenous compounds, which stimulate physiological responses in plants [13].

Chestnut biomass is commonly used due to its high tannin content, which provides antimicrobial and insect-repellent properties [20]. Hydrolysable tannins extracted from chestnut wood also enhance plant growth, soil quality, water management, and tolerance to pests and environmental stresses, making them a promising tool for sustainable agriculture [21,22].

Tomato (*Solanum lycopersicum*) is one of the world's most widely cultivated vegetable crops, originally from western South America and later domesticated in Central America. Over time, selective breeding has enhanced its yield, fruit quality, and resilience to environmental and biological stresses. Beyond its role as a staple food, tomato has become a valuable model organism for plant research. It possesses distinctive features—such as fleshy fruits, sympodial branching, and compound leaves—that are not found in other common model species like rice or *Arabidopsis*, making it especially useful for studying unique aspects of plant biology [23].

This study explored how chestnut-derived wood vinegars and laboratory-prepared chestnut extracts affect tomato (*Solanum lycopersicum*) growth under both normal and saline conditions. Experiments were conducted outdoors during the summer, simulating realistic field environments. Key agronomic traits related to productivity were first evaluated, followed by gene expression analyses focusing on pathways involved in hormone signaling and plant responses to abiotic and biotic stresses, including salinity, drought,

heat, and pathogen challenge. This work provides new insights into the potential of natural chestnut-based products to modulate plant growth and activate defense mechanisms under stress conditions.

2. Materials and Methods

2.1. Experimental Design

To evaluate the effects of some chestnut-derived products on plant growth, development, and defense mechanisms triggering, an open-field experiment was carried out at the Laboratorio di Patologia Vegetale Molecolare (DAGRI, University of Florence, Italy), in Sesto Fiorentino (43°49'12.7" N 11°11'38.1" E), from July to September 2025. Plants were grown in pots, each containing approximately 300 kg of sandy soil having the following characteristics: pH 8.3, electrical conductivity (EC) 66 μ S/cm, and water retention capacity of 34%. Three chestnut-derived products were evaluated: two commercial wood vinegars and a chestnut wood chip extract were analyzed and tested. The commercial products were Biodea® (Arezzo, Italy) wood vinegar (WV) and Nerabiochar® (Turin, Italy) wood distillate (NB), both produced using chestnut wood as the raw material. The chestnut woodchip extract (WCE) was prepared by infusing 2 L of chestnut woodchips (approximately 1.2–1.3 kg) in 4 L of water. The process lasted 3 hours at 80–85°C [24]. After extraction, the liquid was filtered and centrifuged to remove solid residues and contaminants.

All products were acidic and rich in phenolic compounds. Their chemical characteristics were as follows: pH values of 2.29 (WV), 3.37 (NB), and 3.31 (WCE), and total phenolic content expressed as gallic acid equivalents (GAE, mg mL^{−1}) of 8.31 (WV), 8.12 (NB), and 7.06 (WCE).

Tomato plantlets (*Solanum lycopersicum* var. *cerasiforme*) were transplanted into pots at 6 weeks of age. Seeds were germinated and grown under controlled conditions (24°C ± 2°C, 16 h light/8 h dark cycle) until they reached the required developmental stage. The experimental design included two macro-groups: plants grown under normal conditions and plants subjected to salt stress. Each macro-group consisted of seven different treatments (Table 1). Five plants were placed in each pot, and three pots were used as biological replicates per treatment.

Control plants were irrigated with tap water, while salt-stressed plants received irrigation water supplemented with 2 g/L NaCl twice per week. All pots were irrigated with 9 L of water per pot two to three times per week, depending on climatic conditions. Treatments with chestnut-derived products were applied once per week. Plants were harvested two months after transplanting.

Table 1. Scheme of all treatments under different conditions

Normal conditions		Salt stress conditions	
Control	Control	S-Control	Control
WV 1: 300	Wood vinegar Biodea® 1:300	S-WV 1:300	Wood vinegar Biodea® 1:300
NB 1:300	Wood vinegar NeraBiochar® 1:300	S-NB 1:300	Wood vinegar NeraBiochar® 1:300
WCE 1:300	Woodchip Extract 1:300	S-WCE 1:300	Woodchip Extract 1:300
WV 1:600	Wood vinegar Biodea® 1:600	S-WV 1:600	Wood vinegar Biodea® 1:600
NB 1:600	Wood vinegar NeraBiochar® 1:600	S-NB 1:600	Wood vinegar NeraBiochar® 1:600
WCE 1:600	Woodchip Extract 1:600	S-WCE 1:600	Woodchip Extract 1:600

2.2 Analysis of Plant Physiological Parameters and Sampling

Before harvest, stomatal conductance and photosystem II (PSII) activity were measured with a porometer/fluorometer LICOR-600 (Li-Cor, Lincoln, NE, USA). Moreover, the fourth youngest leaves of each plant per treatment were collected and used to determine chlorophyll (chl) *a* and *b* content, following the method described by Wang *et al.* [25]. Briefly, 0.05 g of leaves (fresh weight, FW) were incubated in 2.5 mL (V) of 80% acetone for 24 h in the dark at room temperature. The chl content was determined by measuring

the optical density at 663 nm (OD₆₆₃) and 645 nm (OD₆₄₅) for chl *a* and *b*, respectively, with a UV spectrophotometer and multimode plate reader Infinite® 200 PRO (Tecan, Zürich, Switzerland). Afterward, the chl concentration was calculated using the following formulas:

$$\text{chl } a \text{ (mg/g)} = \frac{(12.72 \times \text{OD}_{663} - 2.59 \times \text{OD}_{645}) \times V}{1000 \times \text{FW}}$$

$$\text{chl } b \text{ (mg/g)} = \frac{(22.88 \times \text{OD}_{645} - 4.67 \times \text{OD}_{663}) \times V}{1000 \times \text{FW}}$$

At harvest, the third youngest leaves were collected, immediately frozen in liquid nitrogen, and stored at −80°C for subsequent gene expression analysis. The aerial parts were harvested to evaluate physiological and agronomic parameters, while roots were excluded due to extraction constraints. Tomato fruits were collected, counted, and weighed. Plant height, fresh weight, and dry weight were also recorded. For dry weight determination, aerial tissues were dried at 60°C for 2–3 days until constant weight.

2.3. RNA Extraction and Plant Gene Expression Analysis

Plant samples were ground with liquid nitrogen, and RNA extraction was performed using the NucleoSpin RNA Plus kit (Macherey-Nagel, Düren, Deutschland) according to the manufacturer's instructions. The total RNA was eluted in 60 µL of nuclease-free water. RNA concentration and quality were assessed using both a NanoDrop Spectrophotometer ND-100 (Nanodrop Technologies Inc., Wilmington, DE, USA) and gel electrophoresis (1% agarose gel in 1X TAE). To eliminate any genomic DNA (gDNA) contamination, the total RNA samples were further purified using the RapidOut DNA Removal Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Then, cDNA was synthesized using the RevertAid RT Kit (ThermoFisher Scientific) following the manufacturer's protocol.

Gene-specific primers were designed and optimized using Primer-BLAST tool (National Center for Biotechnology Information, NCBI) [26] and the "PCR Primer Stats" tool of The Sequence Manipulation Suite [27]. Primers were synthesized by Eurofins Genomics (Ebersberg, Germany) and are listed in the Supplementary Materials (Tables S1).

The expression profile of the following genes was analyzed: auxin-responsive gene 1 (*ARF1*), transport inhibitor response 1 (*TIR1*), auxin efflux carrier *PIN1*, phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), pathogenesis-related proteins (*PR1*, *PR3* and *PR5*), enolase (*ENO*), thaumatin-like protein (*TLP*) and Salt Overly Sensitive 1 (*SOS1*).

Real-time quantitative PCR (RT-qPCR) was performed with the PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific), according to manufacturer's protocol. The expression of the target genes was normalized using both *Act*, coding for actin, and *Ubi3*, coding for ubiquitin, as housekeeping gene [28]. Relative expression levels were calculated using the 2^{−ΔΔCt} method [29], comparing treated plants with their respective controls under normal and saline conditions. For each sample, three technical replicates were processed in each of the three independent RT-qPCR runs carried out.

2.4. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism 8 (GraphPad Software, Boston, MA, USA). After verifying the normal distribution of the data, either one-way ANOVA or the Kruskal–Wallis test was applied, with significance set at *p* < 0.05. *Post hoc* comparisons were then performed using Tukey's or Dunn's test, evaluating differences between all treatment pairs for each plant species.

3. Results

3.1. Effects of Bio-products on the Development and Physiology of Plants

The effects of both wood vinegars and WCE on plant growth and development varied depending on the condition they grew and the applied treatments (Figures S1-S2). To facilitate comparison, all data were normalized to their respective controls. In salt-stress graphs, Control values were also included to allow comparison between the two conditions.

Analysis of aerial plant growth revealed treatment-dependent effects on plant height. Salt stress did not affect the aerial plant growth. Similarly, NB application did not influence plant height under either control or salt-stress conditions. In contrast, plants treated with both WV 1:300 and WCE (in both tested dilutions) increased significantly in height compared to the Control (Figures 1A-E).

Dry biomass accumulation showed more pronounced differences among treatments (Figures 1B-F). Indeed, a significantly higher biomass was reported in the Control compared to the S-Control. Conversely, NB application resulted in opposite trends depending on the condition: under non-stress conditions, dry biomass was reduced compared to the Control, whereas under salt stress it increased markedly, particularly in the S-NB 1:600 treatment. WCE treatment led to an overall increasing trend in dry weight under both conditions.

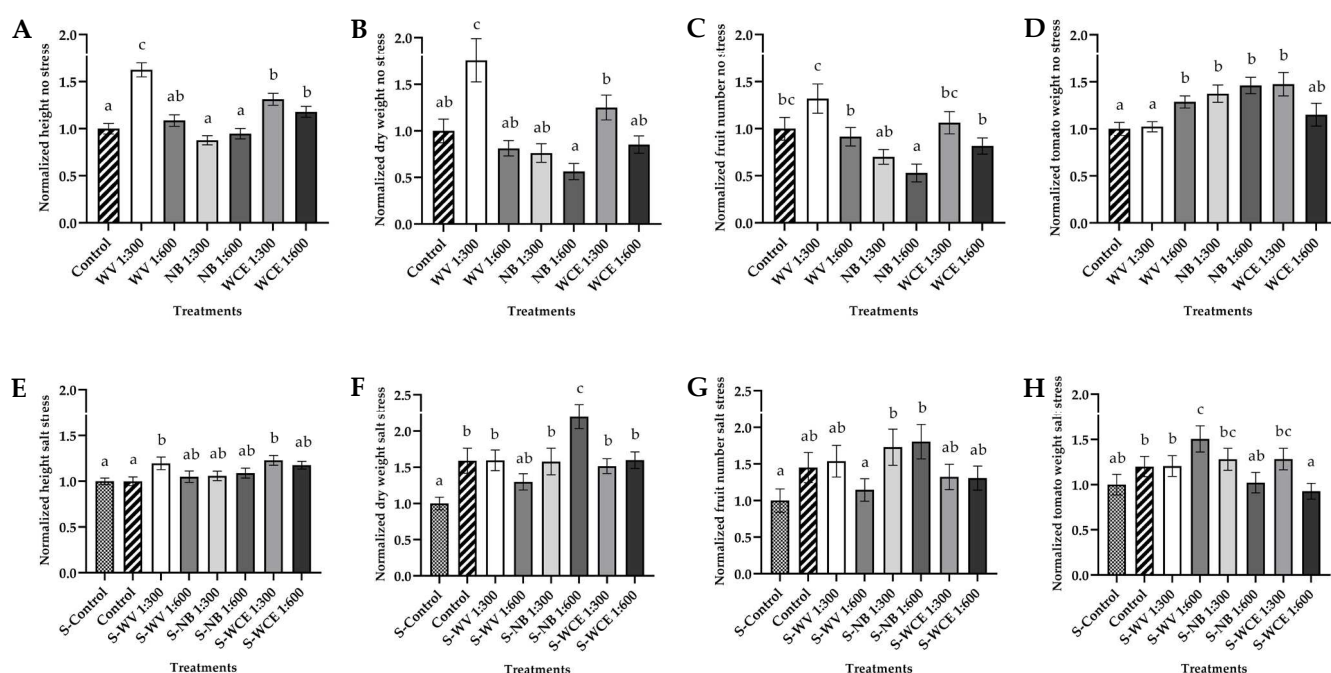


Figure 1. Height, dry weight, fruit number and weight of treated *S. lycopersicum* plants under non-stress conditions (A-D) and salt stress condition (E-H). All values have been normalized by ratioing them to the corresponding control. Error bars represent the standard error of the mean (SEM). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (*p*-value < 0.05).

Fruit production was assessed by counting fruit number per plant. Even in this case, the average level of the S-Control was lower than the Control. Under non-stress conditions, most treatments did not significantly affect fruit number, with the exception of WV 1:300, which showed an increasing trend. In contrast, NB 1:600 caused a significant reduction in fruit number compared to the Control. Under salt-stress conditions, WV treatments

restored fruit production to levels comparable to the Control. Similarly, fruit number increased significantly in S-NB samples compared to the S-Control. WCE treatments did not significantly affect fruit number under either condition.

Average fruit weight, calculated as the total fruit weight divided by the number of fruits per plant, showed an inverse trend compared to fruit number. Treatments associated with lower fruit counts, such as NB applications, generally produced heavier fruits.

Concerning photosynthetic parameters, under non-stress conditions, the chl *a* content increased when WV 1:300 and WCE 1:300 were applied, while it decreased with both NB concentrations tested (Figure 2A). Stomatal conductance increased especially with WV 1:600 and decreased with NB 1:300 (Figure 2B). No differences were, instead, detected for PSII (Figure 2C). Conversely, the photosynthetic parameters were slightly influenced by the application of the bio-products under salt stress conditions (Figures 2D-F).

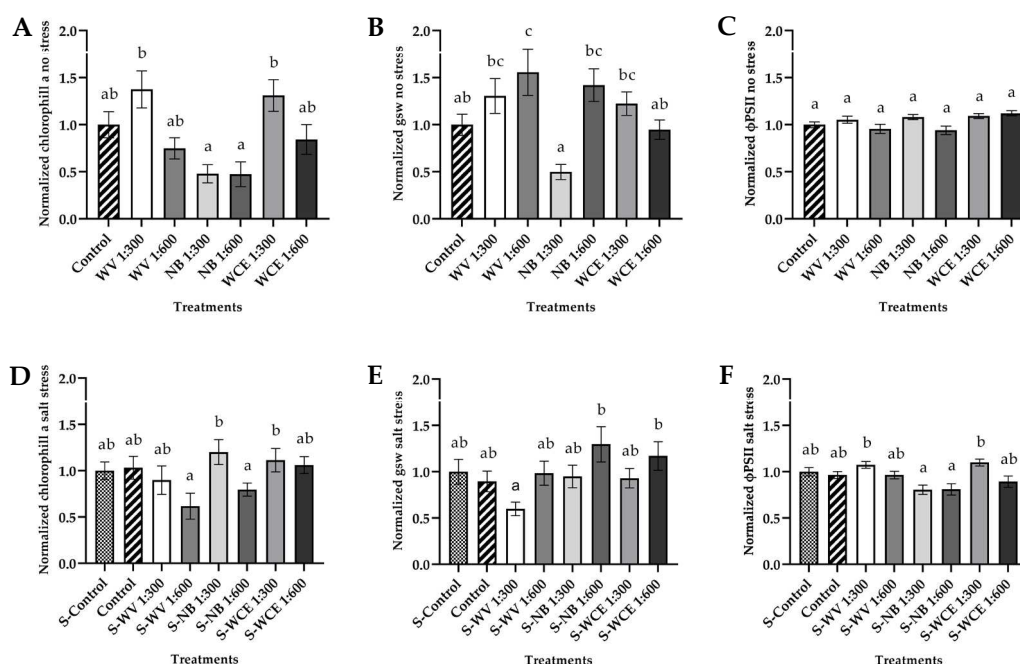


Figure 2. Chlorophyll *a* content, stomatal conductance and photosystem II activity (ϕ PSII) of treated *S. lycopersicum* plants under non-stress (A–C) and salt stress (D–F) conditions. All values have been normalized by ratioing them to the corresponding control. Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey’s or Dunn’s *post hoc* tests (p -value < 0.05).

3.2. Impact of Bio-products on the Expression of Defense-Related Genes in Plants

Bio-product treatments also affected the expression of defense-related genes, depending on the different growth conditions. Concerning the auxin pathway, *ARF1* showed no changes in expression under non-stress conditions, except for its overexpression after NB 1:600 treatment (Figure 3A). In contrast, under salt stress, the gene was overexpressed after the application of all the three products at the highest concentration (Figure 3D). Moreover, in the Control it was overexpressed compared to the S-Control. *TIR1* gene, on the other hand, was slightly overexpressed in normal condition, especially in plants treated with NB 1:600 and WCE 1:600. Under salt stress, the gene was instead downregulated in S-NB 1:300 samples, while it was upregulated in S-WCE 1:300. Moreover, salt stress influenced *TIR1* expression, leading to its upregulation (Figures 3B–E). Lastly, *PIN1* was upregulated only after the treatment with NB 1:300 in non-stress conditions (Figure 3C). Under salt stress, this gene was downregulated in all the treated samples

compared to the S-Control, although a trend toward overexpression was observed compared to the Control (Figure 3F).

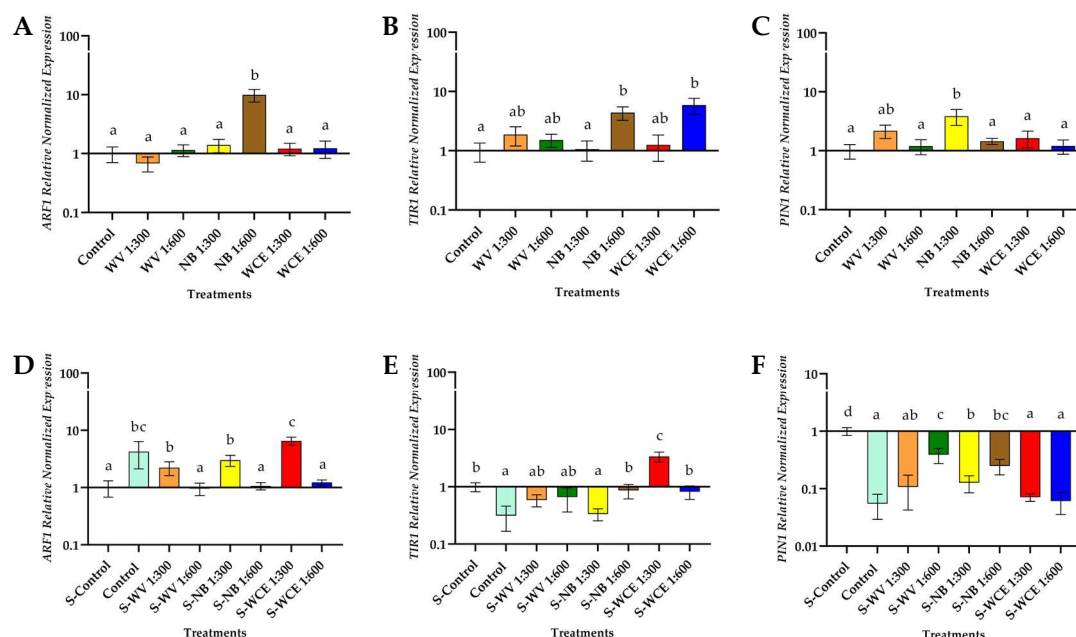


Figure 3. Expression profile of genes involved in the auxin pathway: *ARF1* expressions under non-stress (A) and salt stress condition (D); *TIR1* expressions under non-stress (B) and salt stress condition (E); *PIN1* expressions under non-stress (C) and salt stress condition (F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

Concerning the synthesis of secondary metabolites, all treatments did not significantly stimulate *PAL* expression compared to the Control under normal conditions (Figure 4A). On the contrary, the gene was downregulated with all treatments except in S-WCE 1:300 (Figure 4B). *CHS*, on the other hand, was upregulated in tomato plants treated with the WV 1:300, NB 1:300 and 1:600 compared to the Control, while it was downregulated by WCE 1:600 under normal conditions (Figure 4C). Under salt stress, no significant changes were observed except in S-WV 1:300, in which the *CHS* gene was overexpressed compared to the S-Control (Figure 4D).

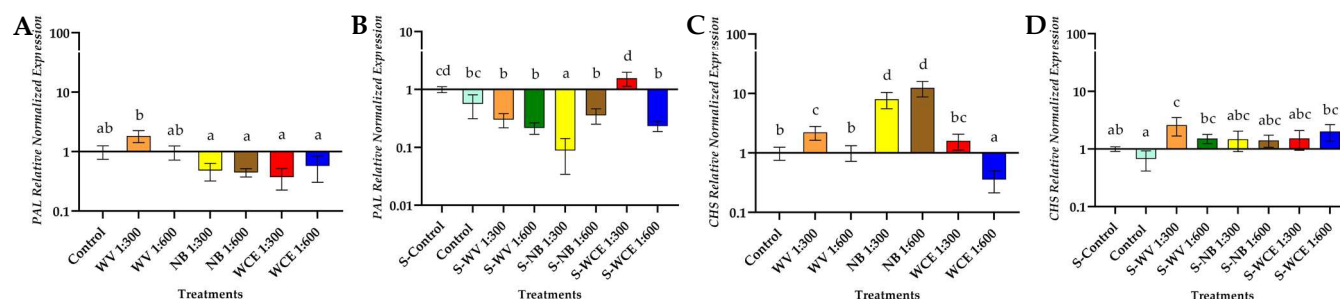


Figure 4. Expression profile of genes involved in secondary metabolites synthesis: *PAL* expressions under non-stress (A) and salt stress condition (B); *CHS* expressions under non-stress (C) and salt stress condition (D). Error bars represent the standard deviation (SD). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

Expression of pathogenesis-related (PR) genes showed marked condition-dependent patterns. Under non-stress conditions, *PR1* expression was upregulated by WCE 1:300 and downregulated by WCE 1:600 (Figure 5A). Under salt stress, *PR1* was significantly downregulated in the S-Control compared to the Control. However, this variation was balanced out in all treated samples, in which the expression profiles were comparable to the Control and significantly higher than to the S-Control. (Figure 5D). On the other hand, *PR3* was upregulated when WV 1:300, NB 1:300, WCE 1:300 and 1:600 were applied compared to the Control (Figure 5B). Conversely, under salt stress conditions, *PR3* was downregulated with all treatments at the lowest concentration and overexpressed only in the presence of WV 1:300 (Figure 5E). *PR5* displayed an expression pattern opposite to that of *PR1*. Under non-stress conditions, *PR5* was significantly overexpressed in all treated plants (Figure 5C). Under salt stress, no significant differences were observed between treated plants and the S-Control, although *PR5* expression was higher in the S-Control than in the Control (Figure 5F).

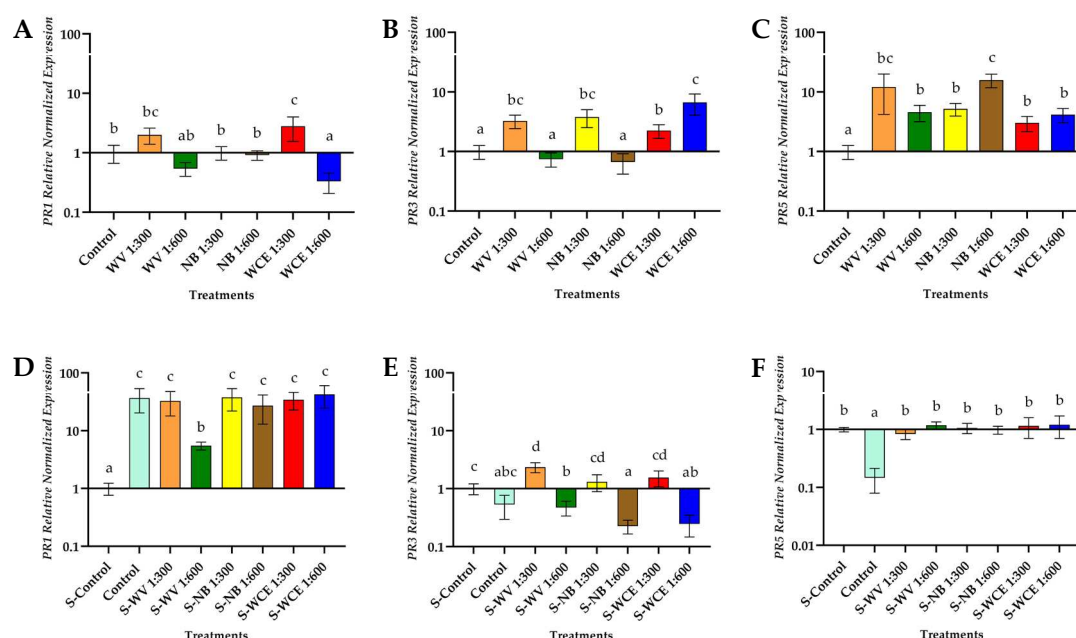


Figure 5. Expression profile of genes coding for PR proteins: PR1 expressions under non-stress (A) and salt stress condition (D); PR3 expressions under non-stress (B) and salt stress condition (E); PR5 expressions under non-stress (C) and salt stress condition (F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (*p*-value < 0.05).

About abiotic stress-related genes, *ENO* was slightly overexpressed in both conditions tested. A significant increase was observed in WV 1:300, WCE 1:300 and 1:600, S-WV 1:600 and S-WCE 1:600 samples (Figures 6A-D). In contrast, some differences were observed in *TLP* expression levels. Under normal conditions, all treatments determined an overexpression compared to the Control (Figure 6B). Interestingly, in the Control the gene was significantly downregulated compared to the S-Control. Under stress conditions, *TLP* was downregulated in most treated samples compared to the S-Control, except in S-WV 1:600, which showed significant upregulation (Figure 6E). *NAC4* was consistently overexpressed in all treated plants under both conditions compared to their respective controls (Figures 6C-F).

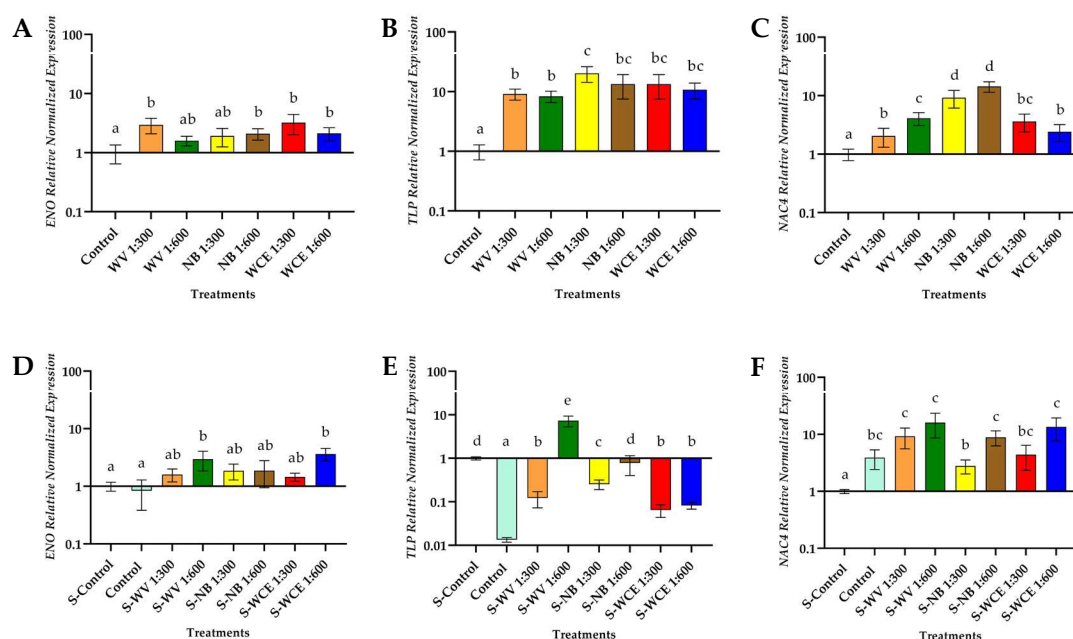


Figure 6. Gene expression analysis of genes related to abiotic stresses: *ENO* expressions under non-stress (A) and salt stress condition (D); *TLP* expressions under non-stress (B) and salt stress condition (E); *NAC4* expressions under non-stress (C) and salt stress condition (F). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

Finally, the salt-stress-related gene *SOS1* showed a consistent response to salinity. Under stress conditions, the gene was overexpressed in all samples compared to those grown under non-stress conditions. Although treated plants generally exhibited higher expression levels than their respective controls, these differences were not statistically significant (Figures 7A-C).

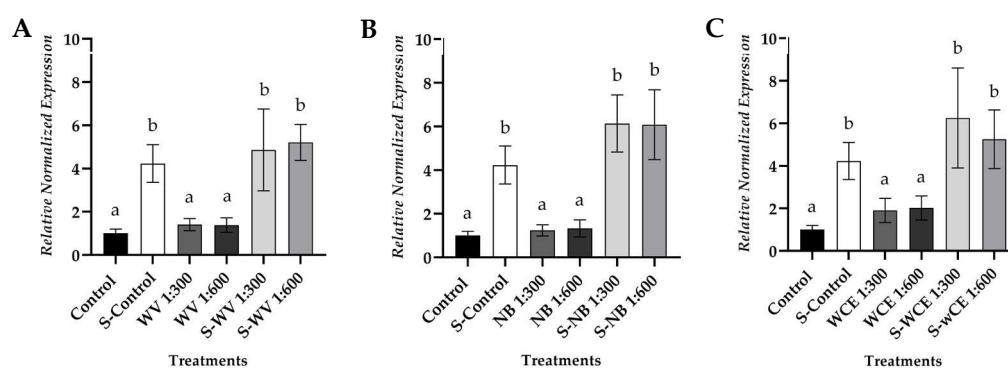


Figure 7. Gene expression analysis of salt stress-related gene *SOS1* in plants treated with WV (A), NB (B) and WCE (C). Error bars represent the SD. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's *post hoc* tests (p -value < 0.05).

4. Discussion

The present study contributes to the growing body of research aimed at identifying plant-derived compounds as sustainable tools to support plant growth and vigor [5]. These searches have become increasingly urgent, as currently agriculture has to face declining yields caused by frequent extreme weather events [4,30] and rising disease pressure driven by global environmental change [31]. At the same time, the need to reduce the

use of traditional agrochemicals—which negatively affect both ecosystems and human health—has become a central concern for the sector [29,32]. In this context, chestnut-derived products rich in polyphenols have recently attracted attention for their potential to enhance plant tolerance to both biotic and abiotic stresses [13,14].

Building on this framework, the present work investigated the effects of sweet chestnut wood vinegars and a laboratory-made extract from chestnut woodchips affect tomato plants grown either under normal conditions or exposed to salt stress. To evaluate the influence of each formulation, multiple concentrations were applied, in order to understand how dosage and product type shape the development of *S. lycopersicum*, one of the most agronomically important crops. A comprehensive gene expression analysis was also conducted to determine whether these treatments activate key molecular pathways involved in plant defense.

In recent years, wood vinegar has been widely studied, and its biological activity has been shown to depend strongly on production parameters such as feedstock type, pyrolysis temperature, and purification processes [33–35]. Moreover, the effective concentration varies considerably depending on plant species and environmental conditions. Although WV has been frequently analyzed as a commercial product, NB has received far less attention. Evaluating two commercial products derived from the same raw material therefore provides valuable insight into how formulation-specific characteristics influence biological efficacy. In parallel, the literature on chestnut wood extracts—particularly those derived from woodchips—remains scarce. Tannins, which are abundant in chestnut biomass, play a crucial role in mitigating stress effects and reinforcing plant defensive capacity, particularly by preserving organs essential for survival and reproduction. Nevertheless, even with their numerous functions, knowledge of how defense-associated phytohormones control tannin metabolism remains quite limited [36].

Salt stress is known to severely impair plant growth by inducing osmotic stress and ionic toxicity caused by excessive Na^+ and Cl^- accumulation, ultimately leading to reduced water uptake, leaf area, and biomass. In addition, salinity negatively affects photosynthetic efficiency and yield, triggering hormonal responses—such as abscisic acid (ABA) signaling—that allow plants to partially adapt to adverse conditions [37].

Consistent with these well-established effects, salt stress in the present study caused clear morphological deterioration, including reduced total biomass and fruit number. Among the tested treatments, WV 1:300 and WCE 1:300 proved to be the most effective. Both under normal and stress conditions, they showed improvements in almost all the parameters analyzed, with an increase in height, biomass and fruit weight. An interesting trend was observed after the treatment with NB, which appeared to worsen plant growth and development under normal conditions but significantly improved them under salt stress (Figure 1).

Regarding photosynthetic traits, several studies report that wood vinegar can increase chlorophyll content [38,39]. The activity of PSII—an indicator of the overall performance of the photosynthetic machinery [40], showed no notable differences among the treatments, similar to the results for chlorophyll *a* content (Figure 2). Finally, measurements of stomatal conductance—which reflect gas exchange and transpiration rates and provide insight into stomatal regulation—revealed increased stomatal aperture in plants treated with WV 1:600 respect to Control [41]. This response may indicate improved physiological plasticity, potentially contributing to enhanced stress tolerance.

In a similar study carried out on *Nicotiana tabacum*, a model plant belonging to the same family (i.e., Solanaceae) of *S. lycopersicum*, it was observed that high concentrations of wood vinegar (i.e., 1:300) could be partially toxic to the plant, while a concentration of 1:600 could be more effective [42]. However, the experiment was carried out by growing plants in smaller pots compared to this study. The larger soil volume used in the present

research facilitated a more uniform diffusion of the applied treatments throughout the soil. This observation further confirms that the growing conditions substantially influence the optimal dosage to be applied.

Importantly, all tested treatments improved plant performance under saline stress, reinforcing previous reports on the effectiveness of wood vinegar as a biostimulant [6,43,44]. In addition, the present study provides novel evidence supporting the potential of chestnut woodchip extracts, offering greater specificity regarding their physiological and molecular effects.

Once absorbed by plant tissues, wood vinegar is known to trigger defense-related signaling processes. Previous molecular studies have mainly focused on the activity of antioxidant enzymes [6,8,25,45]. Tannins also play a key role in regulating plant life during interactions between plants and their environment [46]. As polyphenolic compounds, they help protect against various diseases through their antimicrobial activity and by reducing oxidative stress [36]. Here, we analyzed the expression profiles of defense-related genes involved in various pathways, such as the signaling pathways for auxin (i.e., *ARF1*, *TIR1*, *PIN1*), secondary metabolites synthesis (i.e., *PAL*, *CHS*), jasmonic acid (JA) (i.e., *PR3*) and salicylic acid (SA) (i.e., *PR1*, *PR5*). Genes related to abiotic stress tolerance were also analyzed, focusing on those involved in drought and heat stress (i.e., *ENO*, *TLP*, *NAC4*) and those involved in salt stress (i.e., *SOS1*).

To study the auxin signaling pathway, the expression profiles of *ARF1*, *TIR1* and *PIN1* were evaluated (Figure 3). ARF proteins serve as transcription factors that connect with Aux/IAA proteins and target auxin-responsive elements (Aux-REs) in the promoters of genes regulated by auxin under conditions of low auxin levels [47]. *ARF1*, an auxin-inducible gene, has been identified as a transcriptional repressor of genes responsive to auxins [48,49]. *TIR1*/AFB proteins play crucial roles in plant responses to diverse abiotic and biotic stress [50]. In this manner, these proteins positively regulate downstream auxin-responsive pathways once auxin is detected [51,52]. *PIN1* in plants encodes a crucial auxin efflux carrier protein, essential for directing the flow of the hormone auxin to establish patterns for organ formation, like leaves and flowers, and root development [53]. At the hormonal level, no statistically significant differences or repeated trends were observed, with the exception of a general downregulation of *PIN1* under salt stress (Figure 3F). The effects of salt stress on auxin-related genes warrant further investigation, as *ARF1* exhibited trends opposite to those reported in some studies [54], whereas *TIR1* and *PIN1* showed expression patterns consistent with previous findings under salinity (Figure 3D–F) [55,56].

PR proteins play a crucial role in plant signal transduction pathways. PR1 proteins are abundantly produced during plant defense responses, where they help restrict pathogen spread and enhance resistance to biotic and abiotic stresses [57]. PR-3 plant genes encode chitinases, a type of pathogenesis-related (PR) protein that breaks down chitin in fungal cell walls to defend against pathogens [58]. PR5 genes are a crucial component of plant defense mechanisms, that provide resistance against fungal and bacterial pathogens [59]. Interestingly, the *PR1* and *PR5* were significantly overexpressed under salt stress and normal conditions, respectively, across all treatments tested, suggesting the activation of the SA pathway (Figure 5). This suggests that chestnut-derived products may prime plant defenses by mimicking pathogen-related signals, even in the absence of biotic stress.

Concerning the synthesis of secondary metabolites, *PAL* gene was generally down-regulated in the treated samples under saline stress (Figure 4). These results could suggest a reduced accumulation of phenylpropanoids, as *PAL* is a key enzyme in the phenylpropanoid biosynthesis pathway [60]. In contrast, *CHS*, a central enzyme in flavonoid/isoflavonoid biosynthesis pathway. Besides being part of the plant developmental program,

CHS is induced by stress and participates in SA-mediated defense responses [61], supporting its involvement in stress adaptation observed here.

Concerning PR proteins, the *PR3* gene was generally overexpressed in plants treated with the highest concentration of the products tested under both non-stress and salt-stress con, suggesting the activation of the JA signaling pathway.

In plants, enolase is a highly conserved enzyme in the glycolytic pathway, with additional roles in development, stress responses, and other metabolic functions [62]. It contributes to heat stress tolerance through its interaction with the 70-kDa heat shock protein [63]. In this study, enolase expression was enhanced by the bio-products of interest under both growth conditions (Figure 6).

TLPs consist of a complex protein family that participates in defense mechanisms and developmental processes across plants, animals, and fungi. While mainly associated with responses to biotic stress, some TLPs are also predicted to respond to drought and osmotic stresses [64]. TLPs are closely linked to the *PR5* gene [65]. In the present study, the *TLP* gene exhibited an expression pattern comparable to *PR5*, showing a significant upregulation under non-stress conditions (Figures 5C and 6C). These observations suggested that wood vinegar may regulate drought-responsive genes, thereby potentially enhancing plant tolerance to drought, consistent with findings by Mohd Amnan *et al.* [8]. Additionally, *NAC4*, was a stress-responsive transcription factor, was significantly overexpressed in treated plants under both control and saline conditions, supporting its role in enhancing tolerance to salt and drought stress [66].

Finally, *SOS1*, encoding a plasma membrane Na^+/H^+ antiporter essential for salt tolerance [67], was upregulated in all salt-stressed samples, as expected. Although treatment-induced increases in *SOS1* expression were not statistically significant, a consistent upward trend was observed, aligning with previous findings in rapeseed treated with wood vinegar (Figure 7) [6]. Many of the genes analyzed here have also been previously studied in tobacco [42], showing similar expression trends, further supporting the hypothesis that chestnut-derived products can trigger conserved defense mechanisms within the Solanaceae family.

In summary, the present work confirms and extends existing literature on wood vinegar, demonstrating its ability to modulate hormonal signaling and activate plant defense responses, particularly under abiotic stress conditions. Furthermore, it provides the first solid experimental basis for the agricultural application of chestnut woodchip extracts, which exhibited multiple beneficial effects under both optimal and saline conditions. Together, these findings highlight the potential of chestnut-derived bio-products as sustainable tools for improving crop resilience in the context of climate change.

5. Conclusions

In conclusion, this study indicates that chestnut-derived bioproducts can markedly influence plant growth, development, and physiological responses, particularly under stress conditions. Dosage emerged as a key factor determining efficacy: for wood vinegar, the highest concentration tested did not negatively affect plant traits and frequently enhanced plant performance. WCE also exhibited stimulatory effects, with the 1:300 dilution appearing most effective.

Gene-expression analyses further revealed that both products modulate molecular pathways associated with abiotic and biotic stress responses, supporting their role as plant defense inducers. Although wood vinegar and WCE represent promising, eco-friendly alternatives to conventional pesticides, additional research is required to fully elucidate their molecular mechanisms of action. A deeper understanding of these processes will be essential to support their reliable and sustainable integration into crop protection and stress management strategies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/doi/s1, Table S1>. Primer information for *Solanum lycopersicum*; **Figure S1**. Most representative plant of each treatment under no-stress conditions; **Figure S2**. Most representative plant of each treatment under salt stress conditions

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Bridging the Gap: Evaluating Commercial and Research Chestnut Extracts for Tomato Resilience under Stress

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Table S1. Primer information for *Solanum lycopersicum*.

Gene		Primer sequence 5' – 3'	Tm (°C)	Designed by
<i>Ubi3</i>	Forward	TCGTAAGGAGTGCCCTAATGCTGA	60	[1]
	Reverse	CAATCGCCTCCAGCCTTGTGTAA		
<i>Act</i>	Forward	AGGCAGGATTTGCTGGTGATGATGCT	60	[1]
	Reverse	ATACGCATCCTTCTGTCCCATTCCGA		
<i>ARF1</i>	Forward	CGGCAAATCACTTCGGTGG	54	[2]
	Reverse	AATCCCTGATGTGTCTCGATGC		
<i>TIR1</i>	Forward	AGGGGTCCTCCAGATACAAG	60	[3]
	Reverse	CGCTAATACCTGCCCATCTTT		
<i>PIN1</i>	Forward	CTCAAGAAGAGACCAAGGCAA	58	[4]
	Reverse	AGAGACCAAGGACCAAGTTAG		
<i>PAL</i>	Forward	ATGGAACACATTTTGGATGG	58	[2]
	Reverse	TCTCGATCATCTTAGTTGC		
<i>CHS</i>	Forward	ATGTGGATAGTATGGTTGGAC	58	[2]
	Reverse	CAAGTGAAATGTTAGCCCGA		
<i>PR1</i>	Forward	GGCATCCCGAGCACAAAA	60	[5]
	Reverse	CCCAGCACCAGAATGAATCA		
<i>PR3</i>	Forward	ACCCGTTGCACTGTCTTGTCT	60	[5]
	Reverse	ATCCTTTGGCAGGACATGC		
<i>PR5</i>	Forward	TTGAGGTCCATAACAACCTGC	60	[2]
	Reverse	AACCTCTTCCAGCACCATC		
<i>ENO</i>	Forward	AATGACCAGTCACCGCAGT	60	This study
	Reverse	AGCTGGTTGTACTTGGCGA		
<i>TLP</i>	Forward	ATTGAACGGCAACGGAAAG	60	This study
	Reverse	TGTGATGAGTCCCTGTCA		
<i>NAC4</i>	Forward	TCTTCCGACTTCGTTACCTG	60	[6]
	Reverse	CTGATGTCCGTAATTTTCAAGCA		
<i>SOS1</i>	Forward	TCCTCAGGTTCTCCGAGTT	60	This study
	Reverse	AGTCTGGACAGCATCGTGA		

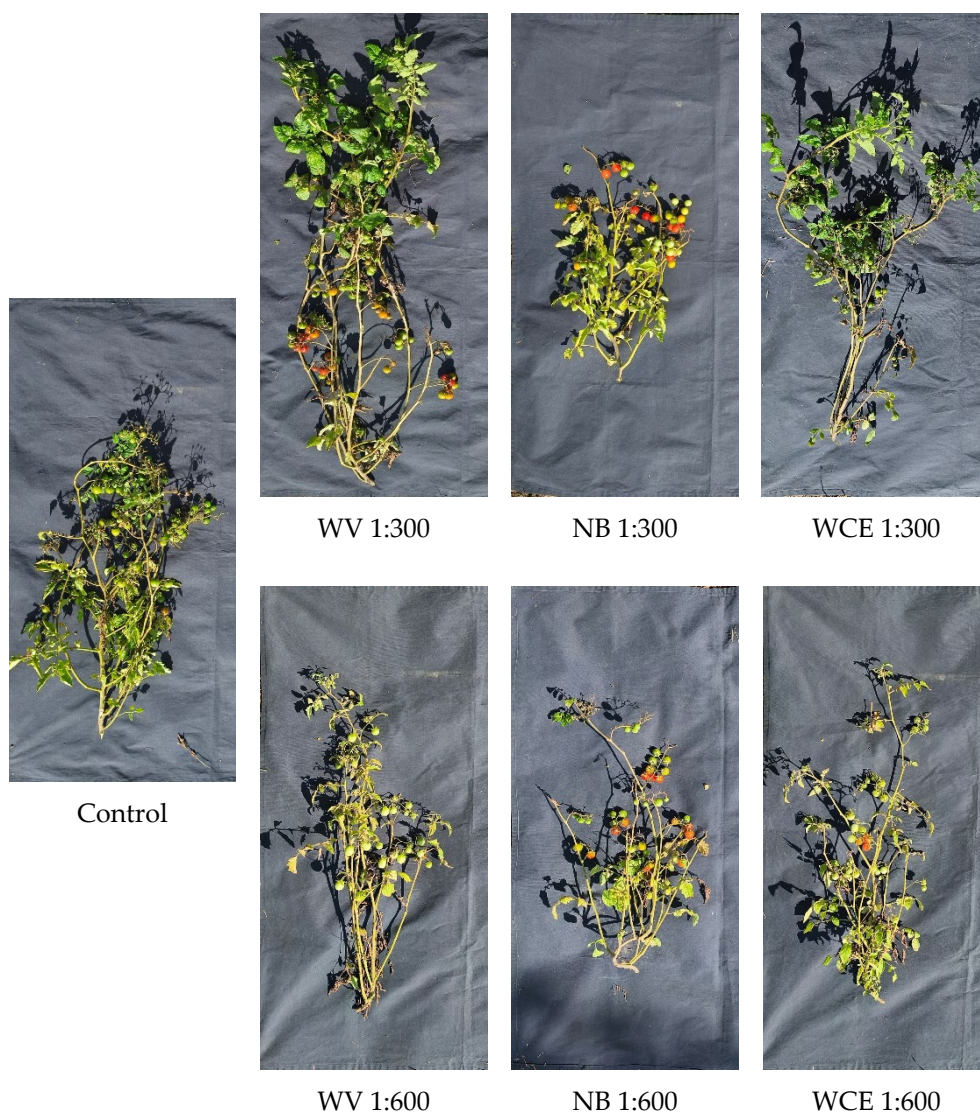


Figure S1. Most representative plant of each treatment under no-stress conditions

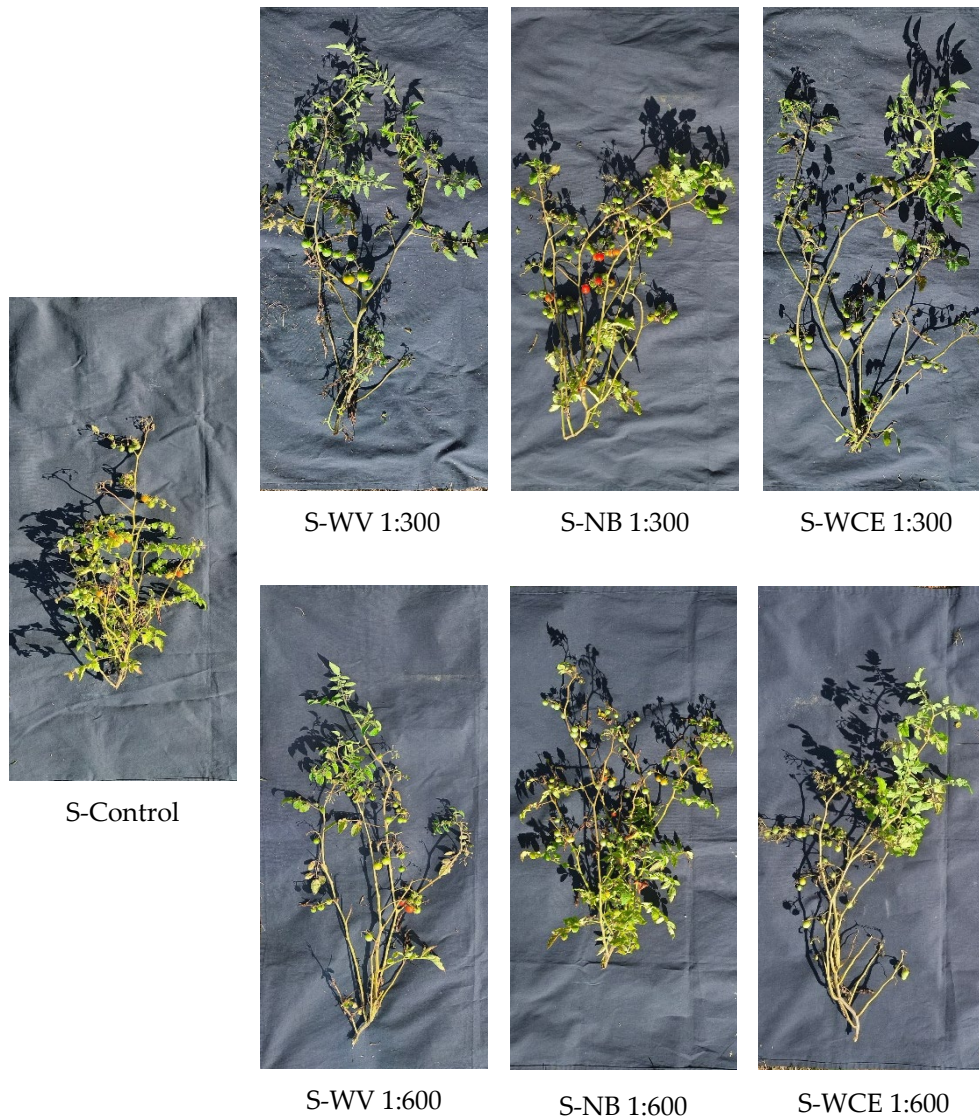


Figure S2. Most representative plant of each treatment under salt stress conditions

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Conclusions and perspectives

This comprehensive study demonstrates the significant potential of chestnut-derived bioproducts, including wood vinegars (WV and NB) and woodchip extract (WCE), as multifunctional tools for sustainable agriculture within the framework of the circular economy. Across multiple experimental scales—from controlled *in vitro* assays to soil-plant systems and field trials—both products exhibited biological activities that enhance plant growth, modulate defense responses, and support soil functionality, particularly under abiotic stress conditions such as drought and salinity.

In vitro assays highlighted the dose-dependent effects of the two products. WV showed strong antibacterial and anti-virulence activity, particularly under conditions simulating plant infection, suggesting potential applications in integrated plant protection strategies such as endotherapy. In plants, WV exhibited inhibitory effects at high concentrations and growth-promoting, hormonal-stimulating effects at lower doses, confirming its dual functionality as both a phytotoxic agent and a corroborant. Therefore, at high concentrations, it could be tested for weed control. WCE primarily acted as a biostimulant, promoting seed germination, early seedling growth, and activation of hormonal and defense-related pathways, while exhibiting limited antimicrobial effects. Even at very high concentrations, it showed no phytotoxic effects; considering that both products have an extremely acidic pH, it is reasonable to assume that the inhibitory effect does not depend purely on acidity.

Experiments on soil-plant interaction under abiotic stress conditions further demonstrated the benefits of these products. Lettuce exposed to drought or salinity stress achieved yields comparable to non-stressed controls when treated with WV, NB or WCE, while soil enzymatic activity improved especially under saline conditions. These findings indicate that chestnut-derived products can enhance plant resilience and maintain soil health even under challenging environmental conditions. Field studies confirmed these trends in tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*). Physiological measurements, including biomass accumulation, chlorophyll content, and stomatal conductance, showed dose-dependent responses, while gene expression analyses revealed activation of defense-related pathways, including pathogenesis-related proteins. These results indicate that WV, NB and WCE can elicit defense mechanisms like those triggered by pathogen attack, reinforcing their role as eco-friendly elicitors of plant immunity.

From a circular economic perspective, the valorization of chestnut residues through the production of wood vinegars and WCE exemplifies sustainable biomass use. These products transform underutilized forestry and agricultural residues into high-value inputs, reducing waste, minimizing reliance on synthetic chemicals, and generating local economic opportunities. WV and WCE thus represent both an agricultural and environmental innovation, integrating plant protection, soil enhancement, and biostimulant effects with sustainable resource management.

Future research should focus on optimizing dosing strategies, clarifying molecular mechanisms, and validating efficacy across diverse crops and environmental conditions. Long-term field trials will be essential to establish reproducibility and best-practice guidelines.

As regards wood vinegar, future innovations could be sought in its application. For example, micro- and nano-encapsulation of wood vinegar in natural matrices could allow controlled release of the active compounds, reducing phytotoxicity and volatility and ensuring greater persistence and effectiveness in the soil. Its co-application should also be studied. Many studies have already been carried out with biochar, but it could be tested together with other plant extracts or beneficial microorganisms. For wood chip extract, on the other hand, there is much less background information, but the potential that has emerged is nevertheless significant. It should be further evaluated in all aspects relevant to agriculture and the extraction method optimized so that it can then be scaled up to industrial levels and an extract produced and used on site.

Further research works

Article

On the effectiveness of ozone treatments: a silver bullet for plant health?

Article

On the Effectiveness of Ozone Treatments: A Silver Bullet for Plant Health?

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Abstract: The development of innovative and eco-friendly strategies to protect plant health is one of the main challenges for the agricultural sector to respond to the increasing global food demand. In this contest, ozone (O₃) could be a promising sustainable alternative to current pesticides, since it is a powerful oxidizing agent and does not leave residues in the environment. However, the molecular mechanisms involved in its potential bioactivity as a plant defense inducer are still poorly known. Thus, this study aimed to understand the role of O₃ in plant defenses, as well as in plant growth, development and physiology, by a multidisciplinary approach. Here, O₃ was applied as ozonated water to the soil in field experiments or to the nutrient solution of hydroponically grown plants. Experiments were carried out on different plant species, including the model plant *Nicotiana tabacum* and agronomically important crops such as lettuce, bean, and tomato. The analysis of several physiological parameters, such as plant weight, chlorophyll content, and stomatal conductance, indicated that O₃ effects are species-specific. Moreover, the expression analysis of specific defense-related genes showed that O₃ induced substantial changes in key hormonal and defense signaling pathways. Overall, O₃ was demonstrated to trigger plant defenses, mainly mediated by pathogenesis-related proteins, mimicking a pathogen attack.

Keywords: ozone; plant health; defense-related genes; sustainable agriculture; ozonated water; hydroponic cultivation

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1. Introduction

Climate change is one of the main threats to contemporary agriculture, which can, in turn, affect global food security. The world population is expected to rise to approximately 10 billion by 2050 [1], driving a significant increase in food demand. Consequently, the agricultural sector must face several challenges to meet this demand by increasing food production while minimizing environmental impacts [2,3]. Therefore, it is mandatory to avoid the pressure on the agricultural sector to sustain the growing food demand leading to an increased use of agrochemicals, the overexploitation of natural resources, and an unchecked deforestation for livestock and crop production [3].

Pesticides are chemical substances, either single or in mixtures, used in agriculture to protect plants from harmful pests, thereby ensuring crop production as well as food quality and quantity [4,5]. In 2022, the global pesticide market reached nearly 4 million

tons, doubling since 1990 [6]. However, these products have several well-known negative effects on human health and the environment, including soil, water, air, and ecosystems, where they can also impact non-target species, leading to a reduction in biodiversity. Additionally, pesticides can bioaccumulate in animal fatty tissues, remaining persistent in the food chain for long periods [5,7]. Lastly, the extensive use of these chemicals has led to the development of pesticide-resistant strains in many pathogens, making their application ineffective [5]. Thus, the development of innovative, eco-friendly, and sustainable strategies to protect plant health is urgently needed. In this context, ozone (O_3) could be a feasible alternative.

O_3 is a gas that is naturally part of the atmosphere, and it is made up of three atoms of oxygen. O_3 is a very reactive and unstable molecule that rapidly decomposes in oxygen and OH radicals when dissolved in water (i.e., half-life of 20 min at 20 °C) [8,9] without leaving any harmful by-product in the environment. Moreover, it is a powerful oxidizer that targets several biological processes in microorganisms [10]. Consequently, it is widely used as a broad-spectrum and eco-friendly disinfectant agent in wastewater treatment and to sanitize the surface of fruits and vegetables [10–12]. In recent years, O_3 has also gained increasing interest in the agricultural sector as a suitable alternative to traditional agrochemicals. Preliminary studies suggested that at low concentrations, O_3 can trigger plant defense mechanisms and stimulate the production of secondary metabolites [13]. Moreover, when applied to soil or nutrient solutions, it increases the amount of oxygen, which in turn enhances root respiration [10,14]. At these concentrations, soil enzymatic activities and microbial communities were not affected by O_3 application, suggesting that this molecule does not compromise soil health or fertility [15,16]. Conversely, at higher concentrations, O_3 has been shown to negatively affect plant growth and development, primarily by reducing photosynthesis and stomatal conductance [13,17,18]. Therefore, understanding the optimal O_3 doses that benefit plants is crucial for developing innovative and eco-friendly pesticides.

The positive effects of O_3 can be achieved by treating plants with ozonated water, which can be applied directly to the soil (e.g., through soil drench), or sprayed on plant organs [13], or by fumigating gaseous O_3 directly onto plants, seeds, or soil [10,13].

To date, ozonated water has gained the most interest as a potential sustainable strategy to improve crop yield and reduce the use of conventional pesticides. Indeed, treatment with ozonated water has been shown to increase biomass, yield, and fruit quality in tomatoes [19], as well as when plants were cultivated in nutrient-deficient soils [20]. Similarly, fruit yield in cucumber was improved under drought stress after ozonated water treatment [21]. Irrigation with ozonated water could induce the expression of genes involved in antioxidant activity, nitrogen uptake, and chlorophyll synthesis in tomato seedlings, thereby improving their physiology [22]. Concerning plant protection, ozonated water was shown to efficiently control *Ralstonia solanacearum* and *Alternaria solani* infections in tomato [23]. Moreover, O_3 has been shown to modulate plant defense mechanisms, protecting tomato plants against the root-knot nematode *Meloidogyne incognita* [24,25]. Lastly, O_3 has also been shown to induce callose deposition in tomato plants through the activation of the ABA signaling pathway, leading to a reduction in the fitness of *Bemisia tabaci* [26].

However, the molecular pathways underlying the beneficial effects of O_3 are still poorly understood. The present study aimed to uncover the biological mechanisms triggered by O_3 on plant growth, development, and defense. Here, O_3 was applied as ozonated water to the soil of plants grown in pots in field experiments and directly to the nutrient solution of hydroponically grown lettuce. The results indicate that O_3 affects plant morphology, physiology and defense in a species-specific manner with the effects also depending on the mode of O_3 application. Furthermore, gene expression analysis suggests

that O₃ may be involved in defense mechanisms mediated by salicylic acid (SA), as evidenced by the upregulation of genes encoding pathogenesis-related (PR) proteins.

2. Materials and Methods

2.1. Experimental Design and O₃ Treatments

To evaluate the effects of ozonated water on plant growth, development, and defense mechanisms triggering, open-field experiments were carried out at the Laboratorio di Patologia Vegetale Molecolare (DAGRI, University of Florence, Italy), in Sesto Fiorentino (43°49'12.7" N 11°11'38.1" E), from June to August 2024. Plants were grown in pots containing approximately 300 kg of sandy soil per pot with the following characteristics: pH 8.3, electrical conductivity (EC) 66 µS/cm, and water retention capacity of 34%. Plants were irrigated (9 L/pot) two to three times per week, depending on the season.

The plant species analyzed in this study include *Nicotiana tabacum* var. White Burley, *Lactuca sativa* var. Canasta, *Solanum lycopersicum* var. *cerasiforme*, and *Phaseolus vulgaris* var. Supernano Giallo. Tomato and tobacco plantlets were transplanted into the pots at 6 weeks of age, lettuce at 4 weeks, and beans at 2 weeks. The tomato, tobacco, and bean seedlings were propagated from seeds, which were sown in a high-quality professional potting mix composed of 23% coconut granules (0–5 mm), 27% Irish peat (0–5 mm), 14% volcanic pumice (3–8 mm), 36% superfine peat (0–10 mm), and slow-release fertilizers (pH [H₂O] 6.0–7.0, and EC 0.30–0.40 dS/m). The seeds were grown under controlled conditions (24 °C ± 2 °C, 16 h light/8 h dark cycle) until they reached the required developmental stage. Conversely, lettuce plantlets were sourced from the market at 4 weeks of age. All seeds and plantlets were purchased from the Agricultural Consortium of Florence.

Ozonated water was prepared by insufflating O₃ directly into a tank containing 9 L of tap water. O₃ was diffused in water through an air stone to create microbubbles and increase mass transfer. In addition, tap water was kept in agitation by a magnetic stirrer during O₃ treatment to ensure solution homogeneity. For all experiments, O₃ was generated using a prototype of the Defender 1000™ system, developed by TEA Group srl (Signa, Florence, Italy), which transforms ambient oxygen into O₃ through corona discharge capable of producing 20 g of O₃ per hour, which is equivalent to approximately 9400 ppm/h. Two different ozonated water treatments were applied by insufflating O₃ for 30 min (T1) and 1 h (T2). Immediately after, the concentration of dissolved oxygen (DO) was measured with a dissolved oxygen meter (DO-9100, Hedao, Fujian, China). The T1 treatment resulted in 0.3 mg/L DO, while the T2 treatment resulted in 0.5 mg/L DO. The ozonated water was applied within 5 min of preparation. As a control, plants were irrigated with tap water. Twelve plants per treatment were used for each species. Plants were harvested 28 days after potting for lettuce and 45 days for the other species. Specifically, the third youngest leaves of three plants per treatment were collected for each species and immediately frozen in liquid nitrogen (N₂) for subsequent gene expression analysis. Furthermore, both the aerial parts and the roots were collected and analyzed separately to assess several physiological parameters.

Regarding the hydroponic experiment, two separate trials with *L. sativa* var. Canasta under nutritional stress were conducted in controlled conditions. Specifically, 4-week-old lettuce plantlets were cultivated in tanks containing 4.5 L of Hoagland nutrient solution [27] (Table 1), continuously oxygenated for 14 h a day with specific air pumps, and renewed every 2 weeks. In each tank, three plantlets were placed after their roots were washed with tap water to remove any soil. They were then allowed to acclimate to the hydroponic conditions for one week before starting the O₃ treatments. The experiment was conducted into a growth chamber, set to 24 °C ± 2 °C and with a photoperiod of 14 h light/10 h dark.

Table 1. Composition of Hoagland solution.

Components	Volume (mL)
1 M KH ₂ PO ₄	1.0
1 M KNO ₃	6.0
1 M Ca(NO ₃) ₂ ·4H ₂ O	4.0
1 M MgSO ₄ ·7H ₂ O	2.0
Micronutrient solution *	1.0
Fe-EDDHA 60 g/L	1.0
Distilled H ₂ O	Up to 1000

* 2.86 g H₃BO₃; 1.81 g MnCl₂·4H₂O; 0.22 g ZnSO₄·7H₂O; 0.08 g CuSO₄·5H₂O; 0.02 g H₂MoO₄·H₂O.

Nutritional stress was induced by cultivating plants in a 20% Hoagland nutrient solution (pH 7.5, EC ca. 260 µS/cm) (hereafter, C20%). Then, O₃ was applied by insufflating the gas directly into the nutrient solution for either 15 or 30 min (T1 and T2 treatment, respectively), with each treatment repeated twice a week. As a positive control, lettuce plants were cultivated in full-strength Hoagland solution (pH 7, EC ca. 1200 µS/cm) (hereafter, C100%). After 28 days, lettuce plants were harvested and analyzed as described above. Additionally, half of the roots of each plant were immediately frozen in liquid N₂ for subsequent gene expression analysis.

In both the experiments conducted on plants grown in soil and those in hydroponics, each sample consisted of six biological replicates, and two independent experiments were carried out. Specifically, for each independent experiment, four plants per pot were sampled (i.e., pseudoreplicates), with three pots per plant species per treatment. To avoid data loss, more than four plants were planted per pot (i.e., an average of 5–6, depending on the species), but only four were randomly sampled and considered as one replicate. Similarly, for hydroponically grown lettuce plants, three tanks per plant species per treatment were used in each experiment, with three plants per tank randomly sampled, giving a total of six replicates.

2.2. Analysis of Plant Physiological Parameters

Before sampling the potted plant, stomatal conductance and photosystem II (PSII) activity were measured with a porometer/fluorometer LICOR-600 (Li-Cor, Lincoln, NE, USA). Moreover, the fourth and fifth youngest leaves of three plants per treatment were collected and used to determine chlorophyll (chl) *a* and *b* content, following the method described by Wang et al. [28]. Briefly, 0.2 g of leaves (fresh weight, FW) were incubated in 10 mL (V) of 80% acetone for 24 h in the dark at room temperature. The chl content was determined by measuring the optical density at 663 nm (OD₆₆₃) and 645 nm (OD₆₄₅) for chl *a* and *b*, respectively, with a UV spectrophotometer and multimode plate reader Infinite® 200 PRO (Tecan, Zürich, Switzerland). Afterward, the chl concentration was calculated using the following formulas:

$$\text{chl } a \text{ (mg/g)} = \frac{(12.72 \times \text{OD}_{663} - 2.59 \times \text{OD}_{645}) \times V}{1000 \times \text{FW}}$$

$$\text{chl } b \text{ (mg/g)} = \frac{(22.88 \times \text{OD}_{645} - 4.67 \times \text{OD}_{663}) \times V}{1000 \times \text{FW}}$$

Subsequently, the plants were removed from the pots, and the aerial part (hereafter referred to as ‘leaf’) was separated from the roots, which were thoroughly washed to remove all soil. After measuring fresh weight, the roots and aerial parts were incubated at 60 °C for 2–3 days to determine the dry weight.

The same analyses have been performed on hydroponically grown lettuce plants.

2.3. RNA Extraction and Plant Gene Expression Analysis

Plant samples were ground with liquid N₂, and RNA extraction was performed using the NucleoZOL reagent (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions. The total RNA was eluted in 30–60 µL of nuclease-free water. RNA concentration and quality were assessed using both a NanoDrop Spectrophotometer ND-100 (Nanodrop Technologies Inc., Wilmington, DE, USA) and gel electrophoresis (1% agarose gel in 1X TAE). To eliminate any genomic DNA (gDNA) contamination, the total RNA samples were further purified using the RapidOut DNA Removal Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Then, cDNA was synthesized using the RevertAid RT Kit (ThermoFisher Scientific) following the manufacturer's protocol.

PCR primers to be used in gene expression analysis have been designed and optimized for each plant species using the Primer-BLAST tool (National Center for Biotechnology Information, NCBI) [29] and the “PCR Primer Stats” tool of The Sequence Manipulation Suite. The primers were synthesized by Eurofins Genomics (Ebersberg, Germany) and are listed in the Supplementary Materials (Tables S1–S4). The expression profile of the following genes was analyzed: auxin-responsive genes 1 (*ARF1*), transport inhibitor response 1 (*TIR1*), auxin efflux carrier *PIN1*, phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), jasmonoyl–L-amino acid synthetase (*JAR1*), and pathogenesis-related proteins (*PR1*, *PR3* and *PR5*).

Real-time quantitative PCR (RT-qPCR) was performed with the SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA), according to Tegli et al. [30]. The expression of the target genes was normalized using two housekeeping genes for each plant species analyzed. In particular, *EF1α* and *L25* were used for tobacco, *Ubi3* and *Act* for tomato, *IDE* and *Act11* for common bean, and *APT1* and *TIP41* for lettuce.

The RT-qPCR data were analyzed using CFX Maestro 1.0 software (Bio-Rad). Relative gene expression was determined using the 2^{−ΔΔCt} method [31]. The data from the treated plants were compared with those from the controls. CFX Maestro 1.0 was also used to perform statistical analysis with a significance threshold set at a *p*-value < 0.05. For each sample, three technical replicates were processed in each of the three independent RT-qPCR runs carried out.

2.4. Statistical Analysis

The statistical analysis was performed using GraphPad Prism 8 software (GraphPad Software, Boston, MA, USA). After assessing normality, data were analyzed using either one-way ANOVA or Kruskal–Wallis tests with a significant threshold of *p* < 0.05. Afterwards, a post hoc analysis using either Tukey's or Dunn's test was performed by comparing the mean of each treatment with the mean of any other treatment for each plant species.

3. Results

3.1. Effects of Ozonated Water on the Development and Physiology of Potted Plants

The effects of ozonated water treatments on plant growth and development varied depending on the plant species tested with lettuce being demonstrated as the most sensitive to the O₃ treatments (Figure 1). Concerning the aerial part, a statistically significant increase in leaf dry weight compared to the control was observed only in lettuce, and not in the other plant species, following the T1 treatment, while no differences were observed with the T2 treatment (Figure 1B). Similarly, in tobacco, the leaf's dry weight was slightly increased after ozonated water treatments, being more pronounced after the T2 treatment, as shown in Figure 1E. Conversely, root development was

negatively affected by ozonated water in lettuce, with both T1 and T2 treatments causing a statistically significant reduction in root dry weight compared to the control (Figure 1C). In contrast, root development was positively influenced by ozonated water treatments in tobacco plants, showing a statistically significant increase following the T1 treatment (Figure 1F). In tomato, plant development was barely influenced by the treatment of ozonated water. Indeed, the leaf dry weight was slightly increased after both T1 and T2 treatments, while a trending reduction in root dry weight was observed compared to the control (Figure 1H,I). No statistically significant differences in leaf and root dry weight were observed between treated and control plants in bean (Figures 1K,L).

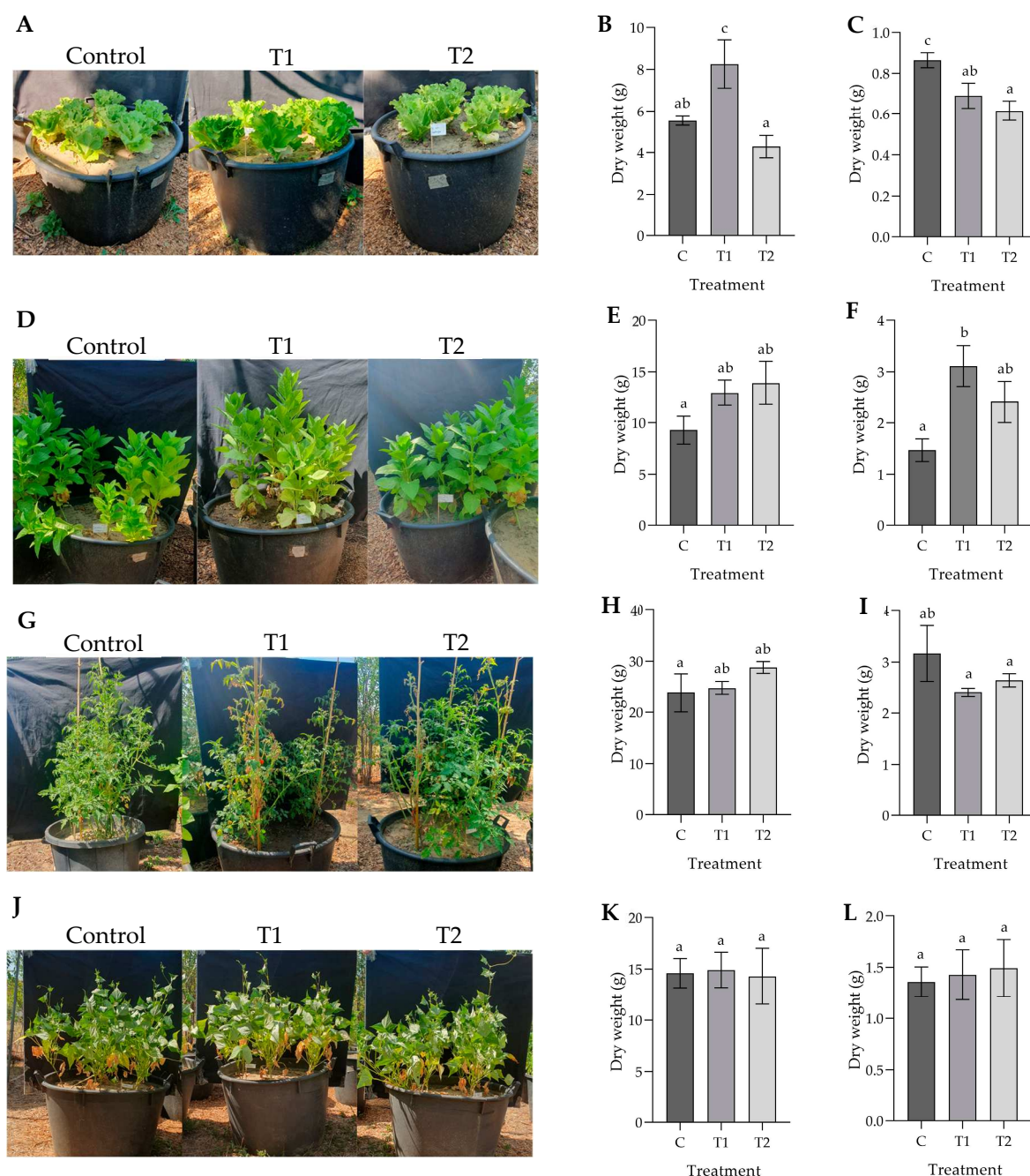


Figure 1. Plants of *L. sativa* (A), *N. tabacum* (D), *S. lycopersicum* (G), and *P. vulgaris* (J), cultivated in pots in an open-field experiment, and irrigated with water ozonated for 30 min (T1 treatment) or for 1 h (T2 treatment). Dry weights of treated and control plants: *L. sativa* leaves (B) and roots (C), *N. tabacum* leaves (E) and roots (F), *S. lycopersicum* leaves (H) and roots (I), *P. vulgaris* leaves (K) and

roots (L). Error bars represent the standard error of the mean (SEM). Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's post hoc tests (p -value < 0.05).

Photosynthetic parameters were also affected differently by ozonated water treatments, depending on the plant species. Regarding chl a content, the values found in the untreated controls were comparable among the plant species tested (Figure 2). An increase in chl a content was observed in both lettuce and tomato following O_3 treatments compared to the control. In lettuce, both T1 and T2 treatments were effective, while in tomato, the increase in chl a content was primarily observed with the T2 treatment (Figures 2A and 2C, respectively).

A statistically significant decrease in chl a content was observed in both tobacco and bean plants after the T1 treatment (Figures 2B and 2D, respectively). Conversely, the chl a content in T2-treated plants was significantly increased compared to T1-treated plants and was comparable to the control. Data on chl b content were comparable to those for chl a across all the plant species here tested.

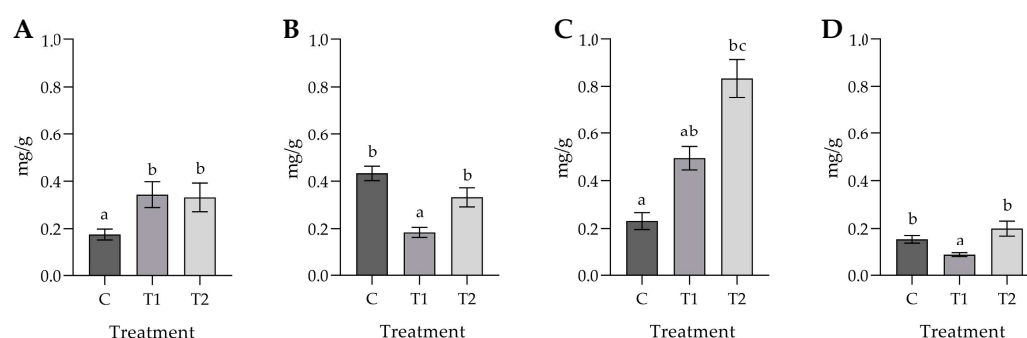


Figure 2. Chlorophyll a content in plant leaves after irrigation with ozonated water (30 min, T1 treatment; 1 h, T2 treatment) in (A) *L. sativa*, (B) *N. tabacum*, (C) *S. lycopersicum*, and (D) *P. vulgaris*. Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's post hoc tests (p -value < 0.05).

Regarding stomatal conductance and PSII activity, these photosynthetic parameters were not affected by O_3 treatments on potted lettuce and bean plants. Conversely, stomatal conductance was significantly affected in both tobacco and tomato plants, albeit differently. In particular, a statistically significant reduction in stomatal conductance was found in tobacco after irrigation with ozonated water, being more pronounced following the T1 treatment (Figure 3A). In contrast, a significant increase was observed in tomato following the T2 treatment, while it was slightly reduced in T1-treated plants (Figure 3B).

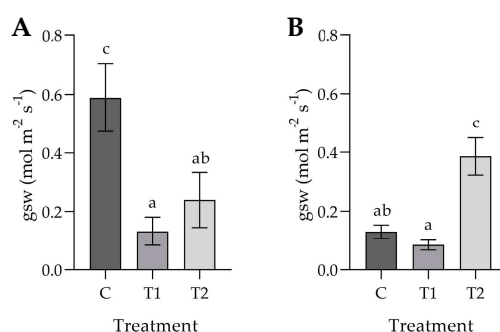


Figure 3. Stomatal conductance of plants irrigated with ozonated water T1 (30 min treatment) and T2 (1 h treatment) at the end of the open-field experiment measured with a porometer/fluorometer LICOR-600: (A) *N. tabacum*; (B) *S. lycopersicum*. Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's post hoc tests (p -value < 0.05).

As far as PSII activity was concerned, a significant increase was observed in tobacco after the T2 treatment compared to the control (Figure 4A). Similarly, in tomatoes, a slight increase was observed after the T2 treatment, whose results were statistically significant compared to the T1-treated plants (Figure 4B).

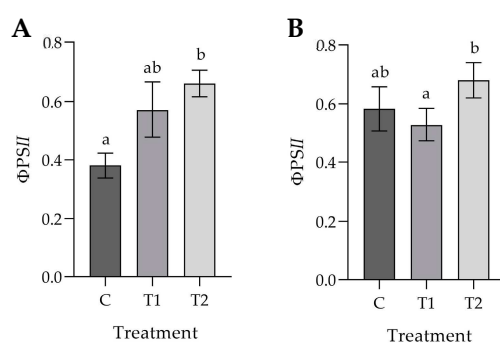


Figure 4. Photosystem II activity (ϕ PSII) of plants irrigated with ozonated water T1 (30 min treatment) and T2 (1 h treatment) at the end of the open-field experiment measured with a porometer/fluorometer LICOR-600: (A) *N. tabacum*; (B) *S. lycopersicum*. Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's or Dunn's post hoc tests (p -value < 0.05).

3.2. Impact of Ozonated Water on the Expression of Defense-Related Genes in Potted Plants

Ozonated water treatments also affected the expression of defense-related genes, depending on the plant species tested. Concerning the auxin pathway, *ARF1* and *TIR1* genes were upregulated in common bean after the T1 treatment but downregulated in tomato and common bean after the T1 and T2 treatments, respectively (Figure 5C,D). Moreover, the relative expression of the *PIN1* gene was slightly increased in tomato and common bean after the T2 treatment, while an opposite result was observed in tobacco (Figure S1).

The relative expression levels of *PAL* were significantly increased in tobacco and common bean after the T1 and T2 treatments, respectively (Figure 5B,D).

Interestingly, the *PR1* gene was upregulated in all the plants tested after ozonated water treatments (Figure 5). Notably, the increased expression of *PR1* was significant after T1 treatment in tobacco and lettuce (Figure 5A,B) and after the T2 treatment in common bean (Figure 5D). Moreover, the *PR3* was significantly upregulated in tomato and in lettuce after T1 treatment and in common bean after T2 treatment, as shown in Figure S1. On the contrary, the T1 treatment induced the downregulation of the gene in common bean (Figure S1).

Lastly, *JAR1* expression was significantly induced in tobacco after ozonated water treatments (Figure 5B). A similar trend was also observed in lettuce, as shown in Figure 5A. In contrast, *JAR1* was significantly downregulated in tomato plants after the treatments (Figure 5C). No variations in the expression of this gene were observed in the treated plants compared to control in common bean (Figure 5D).

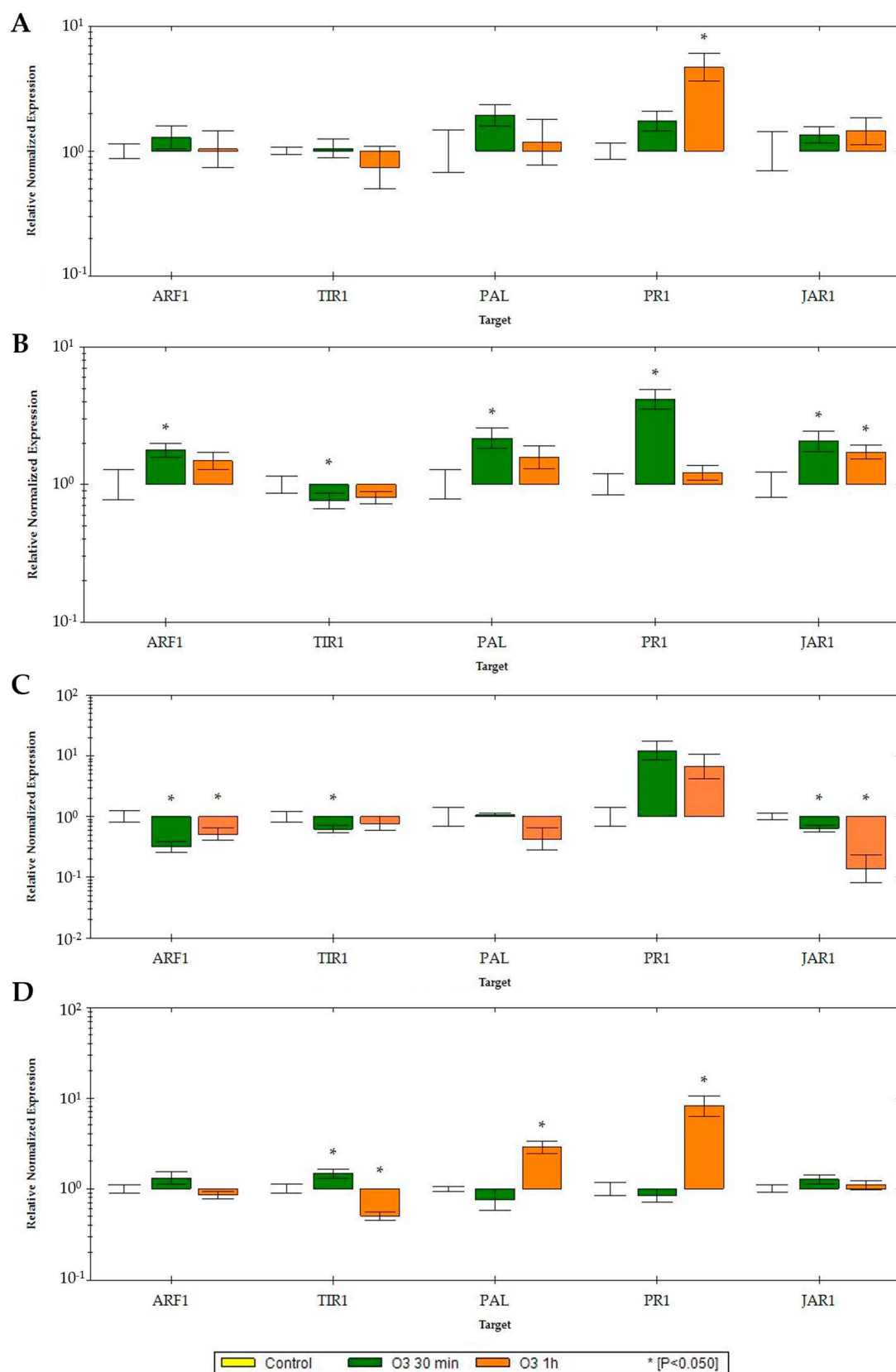


Figure 5. Gene expression analysis of defense-related genes in potted plants irrigated with ozonated water (30 min, T1 treatment; 1 h, T2 treatment): (A) *L. sativa*; (B) *N. tabacum*; (C) *S. lycopersicum*; (D) *P. vulgaris*. Error bars represent the SEM. * p -value < 0.05.

3.3. Effects of Ozonated Water on the Development and Physiology of Hydroponically Grown Lettuce Under Nutritional Stress

Concerning hydroponically grown lettuce, the reduction in the Hoagland solution (up to 20%) did not statistically affect its growth compared to the control plants grown in a full-strength nutritional solution (i.e., C100%). Only a slight reduction in leaf dry weight was observed in C20% samples compared to C100%. Similarly, neither the T1 nor the T2 O₃ treatments applied to plants under nutritional stress caused any alterations in root and leaf dry weight data in comparison to the C20% control (Figure 6). Stomatal conductance was slightly increased in the T1-treated plants compared to C20%, while the PSII activity was not affected by nutritional deprivation or O₃ (Figure S2).

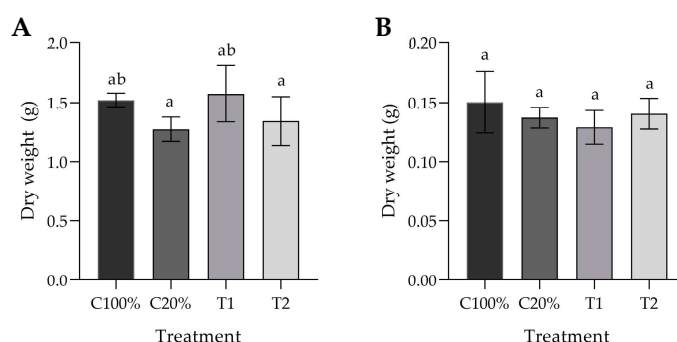


Figure 6. Dry weights of leaves (A) and roots (B) of hydroponically grown *L. sativa* plants after treatment of the C20% nutrient solution with O₃ for 15 min (T1) and 30 min (T2). Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's post hoc tests (p -value < 0.05).

3.4. Impact of Ozonated Water on the Expression of Defense Related Genes in Hydroponically Grown Lettuce

In hydroponic lettuce, defense-related genes were regulated differently in leaves and roots by O₃ treatments. Indeed, most of the genes analyzed were significantly overexpressed in the nutritionally stressed plants (C20%) both with and without O₃ treatment of the 20% nutrient solution for 30 min compared to the C100% control plants except for the *CHS*, *PAL*, *PR3*, and *JAR1* genes (Figure 7A). Interestingly, both the O₃ treatment for 15 or 30 min caused an overexpression of the *PR5* gene in comparison to C20%, comparable to C100%, suggesting a recovery from a potential trade-off in plant fitness (Figure S3). Conversely, the O₃ treatment of the 20% nutrient solution for 15 min appeared to alleviate plants from this abiotic stress, with most of the genes analyzed expressed as in the C100% control plants, with the exception of *PIN1* and *JAR1* genes (Figure 7A).

Surprisingly, the highest overexpression of defense-related genes was observed in the lettuce roots (Figure 7B). Following the treatment of the 20% nutrient solution with O₃ for 15 min, a statistically significant increase was induced in the relative expression levels of *PIN1*, *PR3*, and *PR5* compared to both the C100% control (Figure 7B) and the C20% control (Figure S3). A recovery in the defense response of C20% control plants was observed for *ARF1* and *JAR1* genes in root samples, following treatment with the 20% nutrient solution and O₃ for 30 min (Figure S3).

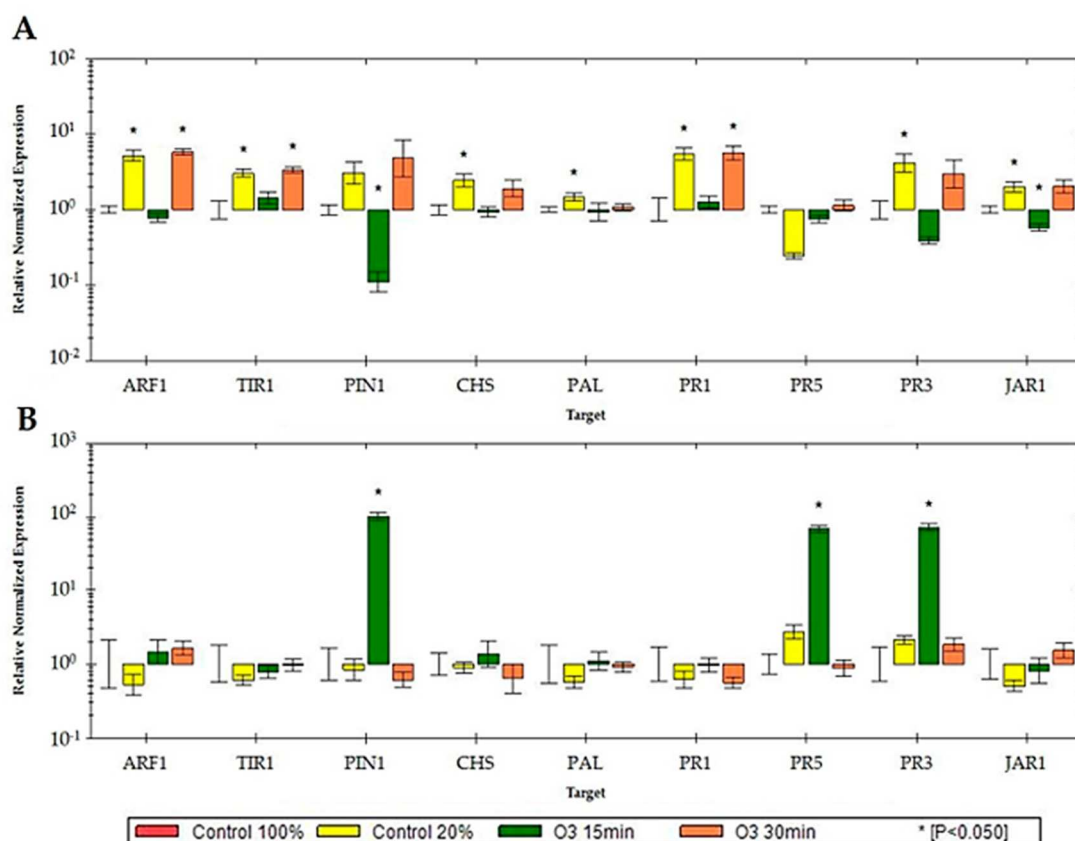


Figure 7. Gene expression analysis of defense-related genes of leaves (A) and roots (B) of *L. sativa* hydroponically grown plants under nutritional stress treated with O₃ for 15 min (T1) and 30 min (T2). As a control for each gene, the C100% samples were used. Error bars represent the SEM. * *p*-value < 0.05.

4. Discussion

The development of innovative and eco-friendly strategies to protect plant health is currently one of the main challenges for the agricultural sector, aiming to integrate several issues, such as boosting crop production and addressing the increased risk of epidemic outbreaks due to ongoing global changes while reducing the use of traditional agrochemicals for their well-known negative impacts on human health and the environment [5,7]. In this general frame, O₃ has emerged as a potential eco-friendly alternative to pollutant conventional plant protection products [10,13]. In recent times, it has been demonstrated that O₃ is an environmentally friendly option, because it rapidly decomposes into active oxygen species, such as hydroxyl radicals (OH⁻) and superoxide anions (O₂⁻), in aqueous solutions, leaving no harmful residues behind [32]. However, the molecular mechanisms involved in its bioactivity are not completely understood.

In this study, we evaluated the effects of O₃ applied as ozonated water to soil cultivated with different plant species as well as insufflated into the nutrient solution of hydroponically grown lettuce. The main variables assessed in this study are related to plant growth and development, photosynthetic parameters, and changes in the expression of genes associated with the activation of plant defense mechanisms. Firstly, our results demonstrate that the effects of O₃ treatments on plants are species-specific and depend on both O₃ concentration and application methods.

Plant growth and development were affected differently by ozonated water treatment across the plant species tested. Soil-grown lettuce has been demonstrated to be particularly sensitive to treatments with ozonated water where leaf biomass was

significantly increased while roots dry weight was reduced after O₃ treatments. In tobacco, irrigation with ozonated water enhanced leaf and root biomass. Overall, these data align with previous studies on tomato, non-heading Chinese cabbage, and pepper [19,23,33]. Moreover, the growth of tomato and bean plants was not negatively affected by ozonated water irrigation, which was consistent with findings by Veronico et al. [24]. Interestingly, the growth and development of hydroponically grown lettuce were not significantly affected when O₃ was directly insufflated into the nutrient solution, as also reported by Zheng et al. [34]. Concerning plant physiology, ozonated water induced an increase in chl content in tomato and lettuce, as also shown by Guo et al. [23], where chl content was slightly enhanced after treatment with 6 mg/L ozonated water. The measurement of PSII activity reflects the status of the photosynthetic machinery [23]. In tobacco, an increase in PSII activity could be correlated with the enhanced plant biomass. Stomatal conductance, which measures the rate of gas exchange and transpiration through leaf stomata, provides a relative indication of stomatal aperture [35]. In tobacco, both ozonated water treatments resulted in a significant reduction in stomatal conductance by the end of the experiment, suggesting increased stomatal closure. In contrast, in tomato plants, the T2 treatment led to an increase in stomatal conductance. In hydroponic lettuce, however, O₃ did not negatively affect plant physiology, as no statistically significant differences in stomatal conductance or PSII activity were observed between the control and treated plants.

Although atmospheric O₃ is one of the major air pollutants that negatively impacts plant growth and development, the results presented here further support the beneficial effects of O₃ when applied as ozonated water to plants. It is well known that long-term exposure to low O₃ concentrations reduces photosynthetic rates and plant growth in addition to causing premature senescence [16,36]. However, in this study, the application of ozonated water did not negatively affect plant growth; in fact, in some plant species, growth was enhanced, as demonstrated for lettuce and tobacco.

Once O₃ enters plant tissues, it begins triggering various defense signaling pathways involving the production of reactive oxygen species (ROS), second messengers (e.g., calcium), and phytohormones [36]. To date, most research on the molecular mechanisms involved in O₃ activity has focused on analyzing antioxidant enzymes, including catalase, superoxide dismutase, ascorbate peroxidase, and peroxidase [22,24,34,37]. Here, we analyzed the expression profiles of defense-related genes involved in various pathways, such as secondary metabolites synthesis (i.e., *PAL*, *CHS*), and the signaling pathways for auxin (i.e., *ARF1*, *TIR1*, *PIN1*), jasmonic acid (JA) (i.e., *JAR1*, *PR3*) and salicylic acid (SA) (i.e., *PR1*, *PR5*). The results indicated that the expression of these genes was differently regulated among the plant species tested. Notably, in hydroponically cultivated *L. sativa*, distinct expression profiles were also observed between leaves and roots. These contrasting expression profiles suggest that O₃ may stimulate defense mechanisms differently in various plant organs [25]. Additionally, in leaves, the expression levels of the defense-related genes showed a comparable pattern between C100% plants and those treated with 15-min O₃ exposure. This suggests that plants under nutritional deficiency (C20%) may experience higher stress levels and that O₃ treatment could have partially alleviated this stress. In particular, in the experiments carried out on hydroponically grown *L. sativa* under nutritional stress, the trigger of the crosstalk existing between the auxin and SA pathways was very well demonstrated with *PIN1* affecting auxin distribution and influencing the plant's ability to activate SA-mediated defense responses, including the expression of defense-related genes like *PR3* and *PR5*. Ozone is able to stimulate plant defenses responses through the SA signaling pathway [25,38,39]. Interestingly, *PR1* and *PR5* genes were significantly upregulated in plants treated with ozone both in soil and hydroponic systems. This can suggest the activation of systemic acquired resistance (SAR) [40]. Moreover, in tobacco, the increase in *PR1* expression after

the T1 treatment, along with elevated SA levels, could be linked to a reduction in stomatal conductance, leading to stomatal closure [40,41]. The rise in SA could be also associated with the increased expression of *PAL* observed in tobacco and lettuce after the T1 treatment and in beans after the T2 treatment. Indeed, *PAL* is a key enzyme in the phenylpropanoid biosynthesis pathway [42], and it is also involved in SA biosynthesis [43]. The *PAL* gene was significantly upregulated in *P. vulgaris* after the T2 treatment, as also reported by Paolacci et al. [44]. A similar trend was also observed in *L. sativa* grown in pots, particularly after the T1 treatment. In hydroponically grown lettuce, the *PAL* gene was also slightly upregulated in the roots. This could suggest an increase in the biosynthesis of secondary metabolites, such as flavonoids and lignin, which are known to promote pathogen resistance [45]. Moreover, the enhanced biosynthesis of secondary metabolites could also improve the content of bioactive compounds, as demonstrated in red-leaf lettuce [46] and *Salvia officinalis* [47]. Regarding the auxin pathway, we evaluated the expression profiles of *ARF1* and *TIR1* genes. ARFs are transcriptional factors that form a complex with the Aux/IAA proteins and bind to auxin-responsive elements (Aux-REs) in the promoter regions of auxin-responsive genes when auxin levels are low [48]. When auxins increase, they bind to the TIR1/AFB receptor and form a complex with the Aux/IAA, leading to its degradation through the proteasome [48]. *ARF1* is an auxin-induced gene [49] and is reported as a repressor of auxin-responsive genes [48,50]. In the present work, *ARF1* was upregulated in tobacco and beans after the T1 treatment as well as in hydroponic lettuce roots after 30 min of O₃ exposure. These results could suggest an increase in auxin levels in these plants. Moreover, the *TIR1* gene was upregulated in bean and hydroponic lettuce roots after the T1 treatment and 30-min O₃ exposure, respectively. These data further support the potential increase in auxin content in the treated plants. Conversely, the *TIR1* gene was significantly downregulated in tobacco and tomato plants after the T1 treatment as well as in T2-treated bean plants. This downregulation of *TIR1* is consistent with the increased expression of *PR1*, suggesting an increased SA content and a consequent inhibition of auxin responses [51,52]. Indeed, other studies have highlighted the antagonistic crosstalk between SA and auxin in plant defense with SA known to repress auxin-related genes, including *TIR1* [51,52].

Concerning the JA signaling pathway, the *JAR1* gene was significantly downregulated in tomato with ozonated water, while it was upregulated in tobacco. A similar trend was also observed in lettuce cultivated in pots. *JAR1* (jasmonate resistant 1) is a key enzyme in the JA signaling pathway, and it is responsible for producing the JA-Ile conjugate [53]. The increase in *JAR1* expression could enhance plant defenses against herbivores, nematodes, and necrotrophic pathogens [54]. Furthermore, the *PR3* gene was upregulated after both ozonated water treatment in tomato and lettuce cultivated in pots. Its expression levels were also significantly increased in T2-treated bean plants and in the roots of hydroponic lettuce when O₃ was applied for 15 min. The *PR3* gene is generally induced by necrotrophic pathogen through the activation of the JA signaling pathway [40]. The *PR3* encodes for chitinases, which hydrolyze chitosan, a major component of fungal cell walls. The upregulation of *PR3* suggests that ozonated water may enhance resistance to fungal pathogens [40,55].

Taken together, these results indicate that ozonated water affects plant growth, development, and physiology as well as defense mechanisms in a species-specific manner. Interestingly, ozonated water triggered plant defenses particularly through the SA signaling pathway and the induction of PR proteins. Therefore, ozone could serve as a potential elicitor of plant defenses. However, further studies are needed, especially in more complex plant-pathogen systems, to fully understand its potential and to successfully tailor O₃ treatment on the different plant species.

5. Conclusions

In conclusion, the results of this study suggest that ozonated water has a significant impact on plant growth, development, physiology, and defense mechanisms, although its effects vary depending on plant species, O₃ concentration, and application method. O₃ was shown to stimulate plant defenses mainly through the induction of PR proteins and the activation of salicylic acid signaling pathway. While ozone shows promise as an eco-friendly alternative to traditional pesticides, further research is necessary to fully understand its molecular mechanisms. This knowledge could enable the application of O₃, mainly as ozonated water, as a sustainable tool in crop protection strategies.

Supplementary Materials: The following supporting information can be downloaded at www.mdpi.com/10.3390/agronomy15030567/s1, Tables S1–S4: Primers designed and used for *L. sativa*, *N. tabacum*, *S. lycopersicum* and *P. vulgaris*; Figure S1: Gene expression analysis of defense-related genes in potted plants irrigated with ozonated water (30 min, T1 treatment; 1 h, T2 treatment): (A) *L. sativa*; (B) *N. tabacum*; (C) *S. lycopersicum*; (D) *P. vulgaris*. Error bars represent the standard error of the mean (SEM). * *p*-value < 0.05. Figure S2: Stomatal conductance (A) and photosystem II activity (ΦPSII) (B) of *L. sativa* plants cultivated in the hydroponic system after treatment of the nutrient solution with O₃ for 15 min (T1) and 30 min (T2). Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with Tukey's post hoc tests (*p*-value < 0.05). Figure S3: Gene expression analysis of defense-related genes in leaves (A) and roots (B) of *L. sativa* hydroponically grown plants under nutritional stress treated with O₃ for 15 min (T1) and 30 min (T2). As a control for each gene, the C20% samples were used. Error bars represent the SEM. * *p*-value < 0.05.

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On the effectiveness of ozone treatments: a silver bullet for plant health?

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Table S1. Primer information for *Lactuca sativa*.

Gene		Primer sequence 5' - 3'	Tm (°C)	Designed by
TIP41	Forward	GAGAGATTGCTGGAGGGAACTA	60	[1]
	Reverse	CCTTTGACTGATGATGTTTGA		
APT1	Forward	AACCTTGTGTGCTGCCATC	60	This study
	Reverse	ACGAGCACATACAGTGGCTT		
ARF1	Forward	ATCTTCATGGCAACGAATGGC	60	This study
	Reverse	AAGCCTCCTTACTCCAACGC		
TIR1	Forward	AGTTTCTCAGCCCTGGAACG	60	This study
	Reverse	TTGCAGCGTGAAAAGGCTTC		
PIN1	Forward	TCATTGGGCTCACATGGTC	58	This study
	Reverse	ATCTTCGGTTGCAATGCC		
PAL	Forward	AAGGGAAGCCGGAGTTTAC	57	This study
	Reverse	ATGTACGGAGAGCATAACG		
CHS	Forward	TTGTCTGAAGTCCCCAAGCTC	60	This study
	Reverse	TGTTCTCAGCGAGGTCCTTGG		
JAR1	Forward	ACGAGCCAAGTTGACCTTTCC	63	This study
	Reverse	ATCAACAAACGCCGAGTCC		
PR1	Forward	ATGGGACAGTCGTGTGGCTAGTTT	58	[2]
	Reverse	TGTTACAGCATCTACACCGGTCA		
PR3	Forward	ACAGGTGGGCGTTCAAAAGA	60	This study
	Reverse	CCAGAACCATAGCGCTGTCT		
PR5	Forward	ATCAGTTACGGGCTCCTG	60	This study
	Reverse	AAGCATCAGGGCATCGAG		

Table S2. Primer information for *Nicotiana tabacum*.

Gene		Primer sequence 5' - 3'	Tm (°C)	Designed by
<i>EF1</i>	Forward	AAGATTGGTGGTATTGGTA	57	This study
	Reverse	GAAGTGCCTCCTGAAG		
<i>L25</i>	Forward	TATCCAAATGGCTCCAGC	59	This study [3]
	Reverse	TCTTCAAAGTCTTAGGTCGG		
<i>ARF1</i>	Forward	CGGCAAATCACTTCGGTGG	63	This study
	Reverse	AATCCCTGATGTGTCGATGC		
<i>TIR1</i>	Forward	GCTCCAGACTCACCTCTT	57	This study
	Reverse	GGCAGCAAGTTCCTCAAG		
<i>PIN1</i>	Forward	GGTGCAAAAGCAGTGGGTT	57	[4]
	Reverse	CTTACATCCTTAGCTGTAT		
<i>PAL</i>	Forward	TAGACAAACCAACCCCTTACACTT	60	[5]
	Reverse	GCCACCATTTTCTTCACTTCATC		
<i>CHS</i>	Forward	ATGTGGATAGTATGGTTGGAC	57	[6]
	Reverse	CAAGTGAAATGTTAGCCCGA		
<i>JAR1</i>	Forward	CTGAAGGATGGGTTGGAGTAAA	60	This study
	Reverse	CACTGGAGAATACGCTTGCT		
<i>PR1a</i>	Forward	CCTCGTACATTCTCATGGTCAAT	57	[7]
	Reverse	CCATTGTTACACTGAACCCTAGC		

Table S3. Primer information for *Solanum lycopersicum*.

Gene		Primer sequence 5' - 3'	Tm (°C)	Designed by
<i>Ubi3</i>	Forward	TCGTAAGGAGTGCCCTAATGCTGA	60	[8]
	Reverse	CAATCGCCTCCAGCCTTGTGTAA		
<i>Act</i>	Forward	AGGCAGGATTGCTGGTGATGATGCT	60	[8]
	Reverse	ATACGCATCCTTCTGTCCCATTCGA		
<i>ARF1</i>	Forward	CGGCAAATCACTTCGGTGG	54	This study
	Reverse	AATCCCTGATGTGTCGATGC		
<i>TIR1</i>	Forward	AGGGGTCTCCAGATACAAG	60	[9]
	Reverse	CGCTAATACCTGCCATCTTT		
<i>PIN1</i>	Forward	CTCAAGAAGAGACCAAGGCAA	58	[10]
	Reverse	AGAGACCAAGGACCAAGTTAG		
<i>PAL</i>	Forward	ATGGAACACATTTTGGATGG	58	This study
	Reverse	TCTCGATCATCTTAGTTGC		
<i>CHS</i>	Forward	ATGTGGATAGTATGGTTGGAC	58	This study
	Reverse	CAAGTGAAATGTTAGCCCGA		
<i>JAR1</i>	Forward	CTGAAGGATGGGTTGGAGTAAA	58	This study
	Reverse	CACTGGAGAATACGCTTGCT		
<i>PR1</i>	Forward	GGCATCCCGAGCACAAAA	60	[11]
	Reverse	CCCAGCACCAGAATGAATCA		
<i>PR3</i>	Forward	ACCCGTTGCACTGTCTTGTCT	60	[11] This study
	Reverse	ATCCTTTGGCAGGACATGC		
<i>PR5</i>	Forward	TTGAGGTCCATAACAACCTGC	60	This study
	Reverse	AACCTCTTCCAGCACCATC		

Table S4. Primer information for *Phaseolus vulgaris*.

Gene		Primer sequence 5' - 3'	Tm (°C)	Designed by
<i>IDE</i>	Forward	GCAACCAACCTTTCATCAGC	58	[12]
	Reverse	AGAAATGCCTCAACCCTTTG		
<i>Act11</i>	Forward	ATACGTTGGTGATGAGGCAC	58	This study [12]
	Reverse	AGCCTTGGGGTTAAGAGGAG		
<i>ARF1</i>	Forward	AGACATCTATCCACCAGC	58	This study
	Reverse	CCTTGCTCTTCTCTCCAG		
<i>TIR1</i>	Forward	TGGAACATCATCGCCAAGTCC	60	This study
	Reverse	GCTTAGCCAGTGCCCACTAA		
<i>PIN1</i>	Forward	TGTCTGGAGTCTAGTGC	58	This study
	Reverse	ATAGTCTTTCGGGTGAGC		
<i>PAL</i>	Forward	TGAGAGAGGAGTTGGGCACT	58	[13]
	Reverse	TTCCAATCTCCAAGGCATTC		
<i>CHS</i>	Forward	TGGTTCTGACCCAATTCAC	58	This study
	Reverse	AGGTGAAACGTGAGTCCAAC		
<i>JAR1</i>	Forward	AAGTTGAGGCACTATGCAG	58	This study
	Reverse	AACCAATGTGAGGAAGCAC		
<i>PR1</i>	Forward	AAGGAGATGCTCCACCCAATG	58	[12]
	Reverse	CAGGGATTGGCCAGAAGGTA		
PR3	Forward	TGGAGTTGGTTATGGCAACAA	58	[13]
	Reverse	ATTCTGATGGGATGGCAGTGT		
PR5	Forward	TCTCGTGGACGGTTACAAC	58	This study
	Reverse	TCTAACCTGAAGCTCCGTC		

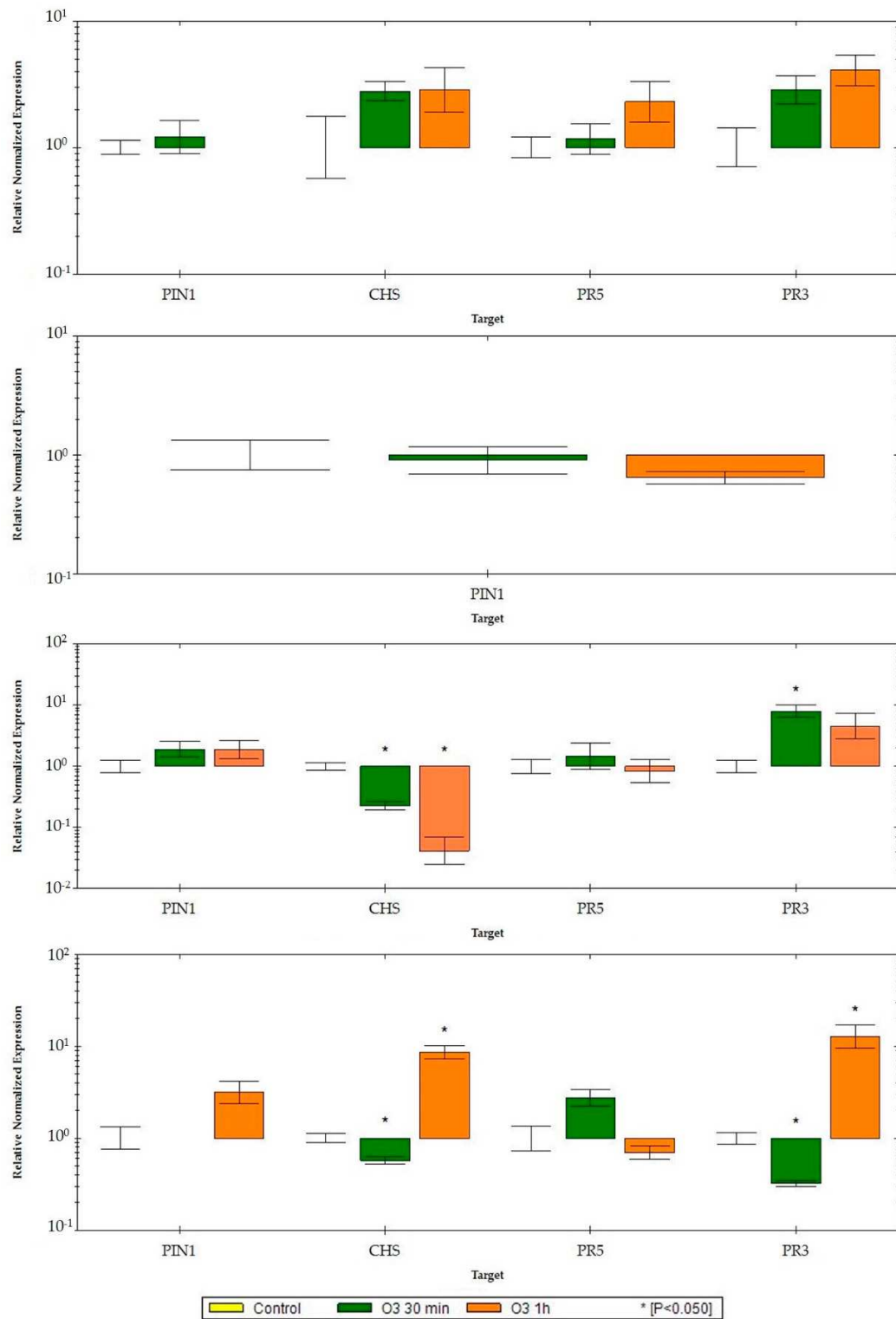


Figure S1. Gene expression analysis of defense-related genes in potted plants irrigated with ozonated water (30 mins, T1 treatment; 1 h, T2 treatment): A) *L. sativa*; B) *N. tabacum*; C) *S. lycopersicum*; D) *P. vulgaris*. Error bars represent the standard error of the mean (SEM). * p -value < 0.05.

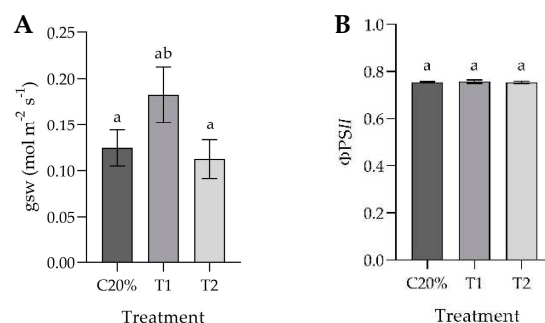


Figure S2. Stomatal conductance (A) and photosystem II activity (Φ PSII) (B) of *L. sativa* plants cultivated in the hydroponic system after treatment of the nutrient solution with O₃ for 15 mins (T1) and 30 mins (T2). Error bars represent the SEM. Letters indicate statistically significant differences between treatments assessed with the Tukey's post hoc tests (p -value < 0.05).

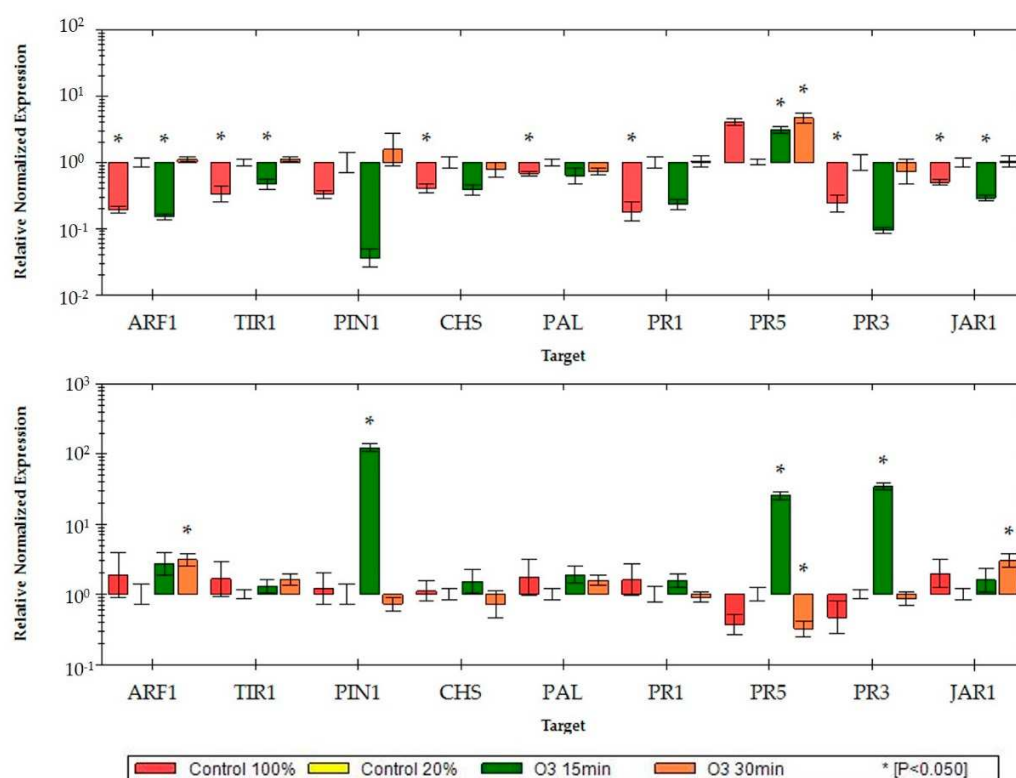


Figure S3. Gene expression analysis of defense-related genes in leaves (A) and roots (B) of *L. sativa* hydroponically grown plants under nutritional stress, treated with O₃ for 15 mins (T1) and 30 mins (T2). As a control for each gene, the C20% samples were used. Error bars represent the SEM. * p -value < 0.05.

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The End.

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