fortune Journals

Research Article

Ozonated water enhances *Diplotaxis tenuifolia* growth and physiology under field and controlled conditions

Riccardo Greco[1], Davide Danzi[1], Stefano Negri[1], Giorgio Zaffani[2], Flavia Guzzo[1], Elodie Vandelle[1*], Linda Avesani[1*]

Abstract

Ozonated water application holds great potential in agriculture as a novel and eco-friendly approach for enhancing plant growth and boosting the quality of several plant species. Here, we describe the effects of ozonated water-treatments on wild rocket (*Diplotaxis tenuifolia*), the most commonly grown rocket species in Europe, North America, and Australia. We evaluated the impact of ozonated water on plants grown under a shaded plastic tunnel in the field during a growing season as well as in controlled conditions. During rocket harvesting period, we observed an increase in chlorophyll content and leaf fresh weight following ozonated water-treatment in the field, and an increase in the germination rate and hypocotyl elongation following ozonated water-treatment in vitro. Moreover, untargeted metabolomics of plants grown in the field revealed no major effects of ozonated water on the main rocket secondary metabolites (e.g. glucosinolates and flavonoids), thus ensuring a substantial equivalence of the food quality traits. In parallel, ozonated water-treatment increased the expression of marker genes (e.g. MYC2) of the jasmonate-mediated defense pathway. Overall, these findings suggest that ozonated water application could result in a better productivity as well as a major resistance to some pathogens, without affecting product quality.

Keywords: Rocket; Ozonization; Oxidative stress; Plant defence inducer; Molecular responses

Introduction

Ozone is a triatomic allotropic form of oxygen, widely used, under specific conditions, for food treatment due to its antimicrobial properties and favorable safety profile [1]. When added to water, it decomposes naturally into hydrogen peroxide (H_2O_2) and hydroxide (OH) radicals [2-4] at a rate influenced by several environmental factors. The reactive oxygen species (ROS) formed in ozonated water (OW) display antimicrobial activity, with an efficiency sometimes higher than gaseous O_3 [5] and with a wider applicability [6-8]. In this framework, OW's mechanism of action involves damaging microbial membranes through intense oxidation processes, targeting lipoproteins and lipopolysaccharides, ultimately disrupting membrane permeability and resulting in the death of microorganisms [9]. These mechanisms are common to both fungi and bacteria, although bacteria exhibit greater sensitivity to ozone treatment [10,11]. In addition, fungal conidia are particularly affected by OW, with a severe reduction in their germination reported for *Botrytis cinerea, Venturia inaequalis and Neofabrea alba* [11].

Among the reported significant effects of OW on plants, at low doses, it could contribute to plant resistance/tolerance to environmental stresses through

Affiliation:
[1]Department of Biotechnology, University of Verona, Verona, Italy
[2]Storti Spa, Belfiore, Verona, Italy

***Corresponding author:**
Valéria Carvalho and Elodie Vandelle. Department of Biotechnology, University of Verona, Verona, Italy

Citation: Riccardo Greco, Davide Danzi, Stefano Negri, Giorgio Zaffani, Flavia Guzzo, Elodie Vandelle, Linda Avesani. Ozonated water enhances *Diplotaxis tenuifolia* growth and physiology under field and controlled conditions. Journal of Food Science and Nutrition Research. 9 (2026): 01-13.

Received: December 11, 2025
Accepted: December 18, 2025
Published: January 28, 2026

fortune Journals
Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

the activation of the plant antioxidant system [12]. Indeed, to protect themselves from possible oxidative damages caused by ROS produced after OW-treatment, plants may respond through the production of antioxidant molecules, including alkaloids, phenolic compounds, essential oils, tannins, sterols, amongst others [13-15]. OW-treatments do not raise antioxidant compound levels in *Capsicum spp*. seedlings, even with an increased catalase activity. While they help in maintaining the homeostatic balance between ROS and antioxidant compounds in grafted watermelon seedlings, leading to a higher antioxidant and vitamin C content and reducing boron accumulation [15]. At the same time, in OW-treated *Rubus idaeus*, reduced degradation of vitamin C leads to higher levels of soluble solids and phenolic compounds, which ultimately increase the antioxidant capacity of plants [16]. Seed soaking or foliar spraying of seedlings with OW also seems to improve germination efficiency and seedling growth by increasing the antioxidant efficiency of pepper plants, with a maximum effect using OW at 30 and 40 ppm [17]. Thus, though OW can display different effects on the final quality of a product depending on its origin, dry matter content or fat content, overall, OW-treatment could be used as a method to induce the antioxidant system during the seeding, planting, or growth stages, thereby supporting the improvement of plant growth and development.

However, despite the number of reports regarding the effect of ozone on plants, there are still debates about the potential of OW to elicit plant abiotic stress responses [18]. The effectiveness of OW-treatment is indeed determined by several factors, among which the most important are the species and varieties of treated plants, the form of ozone used for the treatment (gaseous vs. ozonated water), the dosage and the number of treatments. High doses and/or number of treatments may lead to a deleterious oxidative stress, while the acute exposure of plants to gaseous O_3 triggers a programmed cell death similar to the pathogen-induced hypersensitive response [19]. Nonetheless, numerous studies have been carried out to investigate the potential of OW on various plant species in diverse environments, including growth chamber, greenhouse, shaded plastic tunnel, or open field [20-24]. It is worth mentioning that some efforts are still needed to figure out the effect of OW since the data obtained, particularly in less-controlled conditions (i.e semi-field and field) are mostly contradictory or inconclusive. This is likely due to the lack of OW application uniformity in terms of method, doses and timing [12,17,18,25]. In this context, we collected anecdotal evidences from commercial rocket producers located in the North of Italy regarding the positive effect of OW on plant growth and development, as well as plant health. *Diplotaxis tenuifolia* (L.) DC (Brassicaceae), commonly known as wild rocket, is a perennial leafy green vegetable highly valued for its peppery taste, nutritional properties, and wide culinary use. It is extensively cultivated in Europe, North America, and Australia, both for fresh consumption and as a component of mixed salads. Wild rocket is primarily grown as either an adult or baby leaf crop, depending on market demand, and is subject to multiple harvests (cuts) throughout its growing cycle [26]. The producers reported that OW-treatment during irrigation led to an increase in the number of cuts during the season and a reduction of symptoms related to pathogen infection, thus suggesting a major fitness, and likely a major defense capacity, in presence of the treatment. The aim of this study was to investigate the effects of OW application on *D. tenuifolia* plants grown under shaded plastic tunnels and controlled conditions, providing scientific evidence through phenotypic and molecular analyses to explain field observations.

Materials and Methods

Plant material and experimental design

Plant material: Plants of the commercial variety Jolizia were used to evaluate the impact of ozonated water (OW) on *Diplotaxis tenuifo* under both field and controlled conditions.

Preparation of ozonated water: OW was prepared by infusing ozone into water by using an ozone generator (Storti Ecofarm Srl) and the concentration of ozone was estimated by measuring the redox potential of the solution. Initially, a spark generates ozone from oxygen in a controlled environment. Subsequently, a porous stone injects ozone microbubbles into the water. Because ozone is insoluble in water, it saturates the solution and reacts with H_2O molecules, resulting in the formation of ROS. The system is set for treatment at 600 mV, corresponding to 0.48 mg/L of O_3 [42] equivalent to 0.48 ppm.

Experiments under controlled conditions: For in vitro germination, seeds of *D. tenuifolia* were sterilized in a 20% commercial bleach solution for 5 minutes. Residual traces of bleach were removed with 4 washes of 5 minutes each in sterile H_2O. The growth medium consisted of 2.15 g/L MS, 10 g/L sucrose and 0.7% plant-agar, adjusted to a final pH of 5.8. To ensure the medium solidified on one side of the plate, it was poured while the plates were tilted. Rooting was induced vertically, mimicking field conditions, by keeping the plates upright throughout the 10-day growth period at 25°C. Two groups of seven seeds each were placed on square plates, with sufficient spacing between plants. The control group seeds were soaked once daily for 2 days in tap water for 20 minutes, while the treated group underwent the same procedure with OW. Root and hypocotyl lengths were measured for all plants using the ImageJ software, and the data were analyzed using Excel.

For the germinability study in soil under controlled conditions (2 weeks at 24°C with light-dark cycles of 8 and 16 hours, respectively), 60 pots were placed in a growth chamber with ten seeds sown in each pot. Half of the seeds were treated with sprayed OW, while the other half received

fortune Journals
Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

tap water using the same method. Starting from the day of sowing, data collection was performed every two days during the growth period.

In field experiments: *D. tenuifolia* seedlings were germinated in pots and transplanted to a shaded plastic tunnel in the field (45°16'39.8"N, 11°00'47.6"E, Isola della Scala, Verona) in October 2020. The plants were cultivated under two agricultural sheds (Figure S1): one served as a control, where plants were periodically irrigated by spraying with water, and the other as the treatment group, where plants were periodically irrigated by spraying with OW. The plants were fertilized weekly from March to April and underwent multiple harvests for commercial purposes until June 2021. Between March and June, plants were sampled four timepoints (T1–T4), aligning with commercial harvests, to measure SPAD value, dry weight and to perform metabolite analysis. Additionally, in March plants were sampled at four timepoints (0, 1, 3 and 5 days) following OW treatment to perform gene expression analysis.

SPAD and dry weight measurement

The chlorophyll content in leaves was measured using the SPAD-502Plus® chlorophyll meter (Konica Minolta), which calculates the SPAD value as the ratio of absorption in the red wavelength to that in the infrared wavelength [42]. SPAD measurements were taken from three leaves on six different plants per treatment. The reported value for each leaf represents the average of three measurements.

To assess differences in weight between treated and untreated plants, bunches of rocket leaves were dried overnight at 80°C. Each bunch consisted of 60 randomly selected leaves, encompassing a range of sizes and shapes.

Untargeted metabolomics analysis

An untargeted metabolomics analysis was conducted to compare the metabolomes of OW-treated and untreated plants grown in the field. For each group, three biological replicates were analysed, each consisting of a pool of about 15 two-weeks-old plants harvested randomly from the field. The samples were ground in liquid nitrogen and 100 mg were used to extract medium polar metabolites with 2 mL of LC-MS grade methanol (Honeywell, Seezle, Germany). The samples were then mixed for 30 s, sonicated for 15 min in an ice-water bath at 40 kHz in the Sonica Ultrasonic Cleaner (SOLTEC, Milan, Italy) and centrifuged at 14000x g for 10 min at 4°C. The supernatants were diluted 1:80 (v/v) with LC-MS grade water, filtered through Minisart RC4 filters with 0.2 µm pores (Sartorius, Göttingen, Germany), and then 2 µl was injected into the UPLC. The latter consisted of an ACQUITY I-Class system (Waters, Milford, MA, USA) equipped with a BEH C18 reversed-phase column (2.1 mm x 100 mm, 1.7 µm) kept at 30°C. The solvents used were water acidified with 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B). The chromatographic method was set as follows: (i) initial condition at 1% B; (ii) isocratic condition at 1% B for 1 min; (iii) gradient from 40% B for 10 min; (iv) gradient to 70% of B at 13.5 min; (v) gradient to 90% of B at 15 min; (vi) gradient at 100% of B at 16.5 min; (vii) 100% isocratic condition of B up to 20 min; (viii) return to 1% B and isocratic condition up to 25 min. The flow rate was set at 0.350 mL/min. Samples were placed in an FTN Autosampler system kept at 8°C. The UPLC system was connected to an Acquity PDA detector (Waters) followed by a Xevo G2-XS QToF mass spectrometer (Waters). The mass spectrometer was equipped with an electrospray ionisation source (ESI) operating in negative ionization mode. The scan range was set at 50-2000 m/z and the scan time at 0.3 s. Argon was used as fragmentation gas and the collision energy was set at 35 V.

The raw chromatograms were processed with Progenesis QI software (Waters) to produce a dataset to be submitted in SIMCA® software (Sartorius) for multivariate statistical analysis.

Metabolites were putatively identified by comparing their m/z value, retention time, and fragmentation pattern (obtained from FAST-DDA analysis) with the data available in our in-house library of authentic standard compounds and in public metabolomics databases (e.g., MassBank, Human Metabolome Database, Pubchem, etc.) or in the literature.

Gene expression analysis

The plants for gene expression analysis were sampled following an ozonated water (OW) treatment conducted in March. The day of the treatment was considered as time zero, and further samples were collected at 1, 3, and 5 days post treatment. For each shed, five leaves from three different plants were harvested in biological triplicate. The collected samples were immediately placed in dry ice and then, stored at -80°C.

We selected three genes as markers of the main defence hormone-mediated pathways, i.e., *pathogenesis-related 1 (PR-1)* as a marker of the salicylic acid (SA)-mediated pathway, *MYC2* and *PDF1.2* as markers of the jasmonate (JA) and JA end ethylene-mediated pathways, respectively (Table 1). The levels of transcripts were analyzed by real-time qPCR.

Total RNA from plant leaves was extracted using TRIzol reagent (Invitrogen), following the protocol provided by the manufacturer with minor modifications [43]. The purity of the RNA was determined using a NanoDrop spectrophotometer (ThermoFisher scientific), and samples that had a 260/230 ratio of less than 1.8 were subjected to a purification step by precipitating RNA with LiCl. RNA integrity was assessed by electrophoresis on a 1% agarose gel. Finally, RNA samples were treated with TurboDNase following manufacturer's instructions [44].

Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

cDNA was synthesised using SuperScript III (ThermoFisher) and used as a template for the gene expression analysis among selected defence genes (*PR-1, MYC2 and PDF1.2*). The analysis was conducted by Real Time RT-PCR using the template and Universal qPCR Master Mix (New England Biolabs). For this study, tubulin (TUB6) was used as reference gene. The *D. tenuifolia* gene sequences were obtained aligning contigs assembled via RNA-Seq by Cavaiuolo 45 and their orthologues in *Arabidopsis thaliana*. Subsequently, primers for the housekeeping genes were added to these mixes in each plate. Thus, by comparing the Cqs obtained for each mix, it could be stated that all samples were similarly amplified and that, therefore, all the results are comparable.

Tag	Sequence	GC%	Amplicon
TUB6_f	CACAAAGGAAGTAGACGAGCAG	50	90
TUB6_r	CTTGACTTCACGTTGTTCGGT	47.62	
PR1_f	ATATTTGCTGGCCGCTCAAG	50	127
PR1_r	CCGTTGCACTTGTAGCTGTT	50	
MYC2_f	TAGGTCCAGCCTTCGTTTGT	50	145
MYC2_r	CCTCCGACATCTCAGCCTTA	55	
PDF1.2_f	ATGCACTTGTGAGCTGGGAA	50	109
PDF1.2_r	CGTGGTCAGGAGTATGTGGA	55	

Results

OW-treatment anticipates in vitro germination of *Diplotaxis tenuifolia* and promotes hypocotyl elongation

The spraying of OW on *D. tenuifolia* seeds promoted their germination in vitro, anticipating this process, with 20% of the treated seeds sprouting 19 days after sowing (Figure 1).

Conversely, control seeds treated with tap water reached the same germination rate 4 days later. This positive effect was observed for the first 23 days following sowing and was progressively lost once the germination rate exceeded 60%.

After germination, hypocotyls developed and grew faster from seeds treated with OW compared with seeds treated with tap water (Figure 2). Indeed, an increase in hypocotyl size was observed at 5 and 7 days post-germination (DPG), while root elongation was reduced, though only at 7 DPG (Figure 2). This suggests a role of OW in boosting the early phases of seed germination and growth of the areal part of the plant.

OW-treatment in field increases the levels of SPAD value by *Diplotaxis tenuifolia* leaves and their fresh weight

Leaf chlorophyll content (LCC) of adult plants of *D. tenuifolia* was evaluated at a field scale during the growing season, i.e. from April to June 2021, using SPAD as a proxy, since routinely used to provide an instantaneous estimation of in situ LCC. The values of absorbance were taken from two different groups of adult plants, sprayed with OW or tap water, as a negative control (Figure 3). Nonetheless, regardless of the season-related effect, OW-treated plants always displayed significantly higher SPAD values compared to the control group, suggesting an increased LCC following and a greater light absorption OW-application.

We also investigated the impact of OW-treatment on plant biomass by evaluating the differences in terms of both fresh (FW) and dry (DW) weights (Figure 3). While treated leaves showed a significantly higher FW compared with the untreated ones, no statistically significant differences could be observed among samples regarding the DW. These data indicate that weight differences observed in the field could be due to a higher water content in the leaves of plants sprayed with OW.

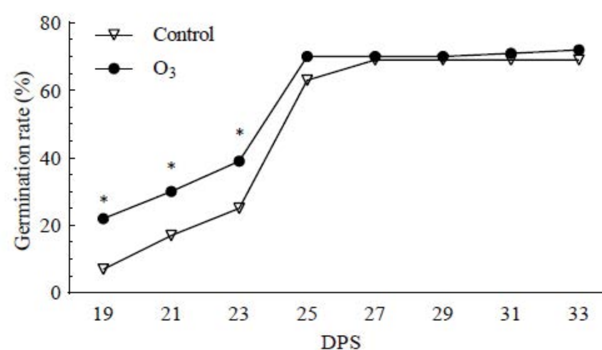


Figure 1: Evaluation of the effect of ozonated water on the germination rate of D. tenuifolia seeds sown in controlled conditions. The germination rate was monitored at different time points following seed sowing in the presence (circle dots) or absence (triangle dots) of ozonated water. The values shown represent the average +/- SE of 30 biological replicates for each condition. Asterisks indicate statistically significant differences between OW-treated and untreated plants according to the t-Student test with a p-value < 0.05, at each time point.

OW-treatment has a negligible impact on secondary metabolome of *Diplotaxis tenuifolia* in the field

Using an untargeted metabolomic approach, we performed a relative comparison of the medium-polar metabolite levels in OW-treated and untreated plants of D. tenuifolia. Data from the negative LC-MS dataset (178 m/z features) were normalized on the different water contents of the samples and subjected to a multivariate statistical analysis. The clustering of the samples in PCA reflected the different harvest timepoints, with a distinct separation between the first two timepoints compared to the third and fourth (Figure S2a). This pattern was supported by a 3-class O2PLS-DA model

Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

and validated through the corresponding PLS-DA (Figure S2b). However, no robust statistical models supported the hypothesis of an impact of OW on *D. tenuifolia* metabolome throughout the harvesting season, even when focusing the analysis on samples collected at the same timepoint. Only a few metabolites were slightly reduced or increased at specific timepoints (Figure 4; LC-MS features are reported in Table S1).

These included two glycosylated flavonols, namely isorhamnetin-O-di-hexoside and quercetin-O-(di-hexoside)-O-(sinapoyl-hexoside), one dihydroxybenzoic acid hexoside, and, within the glucosinolates, a dimeric form of 4-mercaptobutyl glucosinolate and two previously unreported glucosinolates featured by the diagnostic fragments of sulphur-containing metabolites. For all but isorhamnetin-O-di-hexoside, the effect of OW on metabolite accumulation was mostly appreciable at the last time point with a slight reduction in OW-treated plants. When considering the sum of the levels of all glucosinolates and flavonols identified, no significant differences were observed among the control and OW-treated plants.

OW-treatment modulates the expression of defense marker genes in *Diplotaxis tenuifolia* plants in the field

According to field observations reported by rocket producers employing OW routinely, the treatment seems to reduce the development of disease symptoms. We thus attempted to provide scientific evidences related to the defense mechanisms of *D. tenuifolia* following OW-spraying. Three defense genes were selected namely, PR1, MYC2 and PDF1.2, as markers of salicylic acid (SA)-, jasmonate (JA)- and JA/ethylene (ET)-mediated defense signaling (Figure 5).

While PDF1.2 did not show any variation during the time course of the analysis following OW-application, MYC2 and PR1 were up- or downregulated in OW-treated plants, respectively. On the one hand, this suggests that ethylene is not involved in OW-mediated defenses. Conversely, OW could activate the JA-related pathway after 1 and 3 days of treatment. Such induction could likely be transient since the expression returns to the basal level after 5 days. Moreover, in line with the antagonistic relationship between SA and JA pathways (35), a reduction of the SA-dependent gene PR1 is simultaneously downregulated in OW-treated plants.

Discussion

Despite the large body of literature on OW effects on various plant species, there is still debate concerning the real OW ability to impact plant physiology [18]. Indeed, an effect of OW seems to be correlated with the plant species, with its dosage and finally with the number of treatments performed. The existing research about the effect of OW on crops has been performed on different plant species and culture conditions and the results are often conflicting or equivocal [20-24]. Moreover, there is very poor literature about field trials and thus, drawing conclusions about its effect is cumbersome.

Within the plant species, research aimed at assessing the effects of OW in *D. tenuifolia* is still scarce, even though it is a commercially valuable plant that is widely consumed. Furthermore, numerous researches have been conducted to evaluate the potential of OW for different plants in a variety of growth conditions, but the efficacy of OW in the shaded tunnels and in the field has yet to be proven, as the available data is mostly conflicting or equivocal [20-24]. For these reasons, we tested the OW effects on D. tenuifolia, using both in vitro (for seed germination and seedling growth) and in-field conditions in plastic tunnels, very commonly used for commercial wild rocket production.

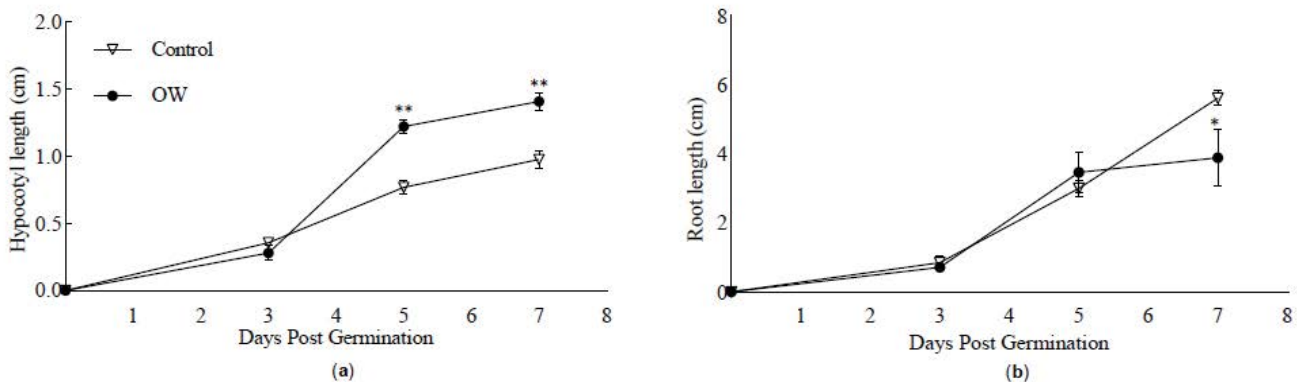


Figure 2: Evaluation of seed treatment with OW on the length of (a) hypocotyls and (b) roots of D. tenuifolia grown in controlled conditions. Hypocotyl and root lengths were measured for 7 days after the germination of seeds treated (circles) or not (triangles) with OW. The values shown represent the average +/- SE of 17 and 21 biological replicates for OW-treated and non-treated seeds, respectively. Asterisks indicate a statistically significant differences according to the t-Student test with * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001. DPG, days post-germination.

Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

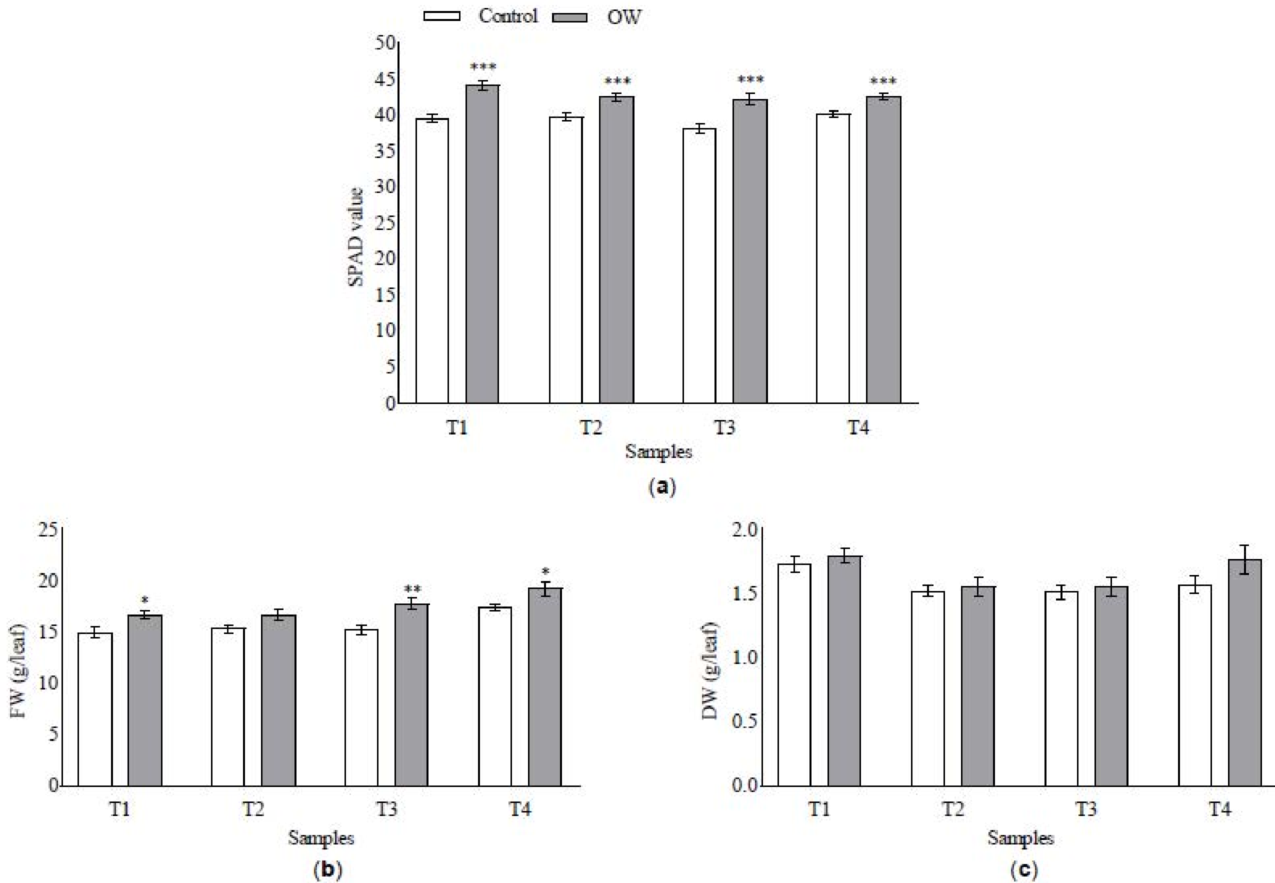


Figure 3: Effect of OW-treatment on the (a) SPAD value, (b) fresh weight (FW) and (c) dry weight (DW) of D. tenuifolia leaves grown in the field. The different parameters were measured in the presence (gray bars) or absence (white bars) of OW. Tap water spraying was used as a negative control. The SPAD values shown represent the average +/- SE of 18 biological replicates for each set of samples. The FW and DW values shown represent the average +/- SE of 9 biological replicates for set of samples. Asterisks indicate a statistically significant differences according to the t-Student test with * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001.

Similar treatments in terms of dosage were performed in *Capsicum annuum* and *Citrullus lanatus* seedlings reporting an overall increase of total phenolics and Vitamin C [15,26].

Although our main objective was to evaluate the effect of OW in real conditions, we performed tests in controlled conditions that demonstrated a faster seed germination. This stage is the first and foremost susceptible stage of plant growth and therefore more prone to stress [28]. During this process, ROS in seeds are involved in the regulation of cellular growth, providing protection against pathogens and controlling the cell redox status, thus highlighting their importance for seed germination and seedling growth. We could assume that OW-treatment on seed may activate ROS scavenging machinery (SOD, CAT, and APX), which may facilitate morphological, physiological and biochemical changes, leading to an enhanced stress tolerance potential of plants [29] and thus a faster germination rate [17]. These results should be confirmed in field conditions since in vitro seedling cultures, used to evaluate seed germination, may impact plant physiology due to root exposure to light [30]. Moreover, following seed treatment with OW, we observed a reduction of radicle length, thus providing complementary information to previous reports in which the application of OW on tomato plantlets caused an increase of root mass or no effect on this parameter [31]. These results are not in contradiction considering the timing of OW application (seed vs plantlets) and the dosage of ozone. Finally, we observed longer hypocotyls following OW-seed treatment, not previously reported in the literature, that may be correlated to the earlier germination.

In field experiments, we detected a significant increase in SPAD value in OW-treated plants, suggesting a higher content in total chlorophyll [32]. This may be due to an increase in the expression of chlorophyll biosynthesis-related genes, as previously reported in tomato [33]. Nevertheless, higher SPAD values did not lead to an increase in dry weight, as an indicator of a boosted primary metabolism. By contrast, OW did increase the fresh weight of the aerial part of the treated plants, indicating a higher water content. A similar increase has been previously reported in sweet peppers, likely

Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

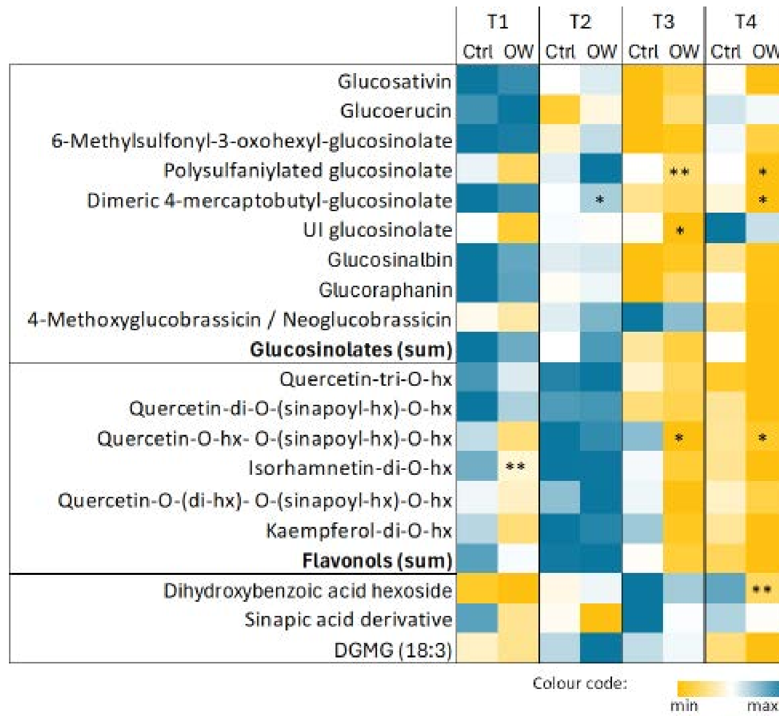


Figure 4: Main secondary metabolites characterizing D. tenuifolia leaf metabolome. Metabolites in each group are listed in order of decreasing abundance. Normalized peak are values are reported as average (n=3) with a colour code. Differences among control and OW-treated plants were evaluated by Student's t-test: *, p<0.05; **, p<0.01. Abbreviations: hx, hexoside.

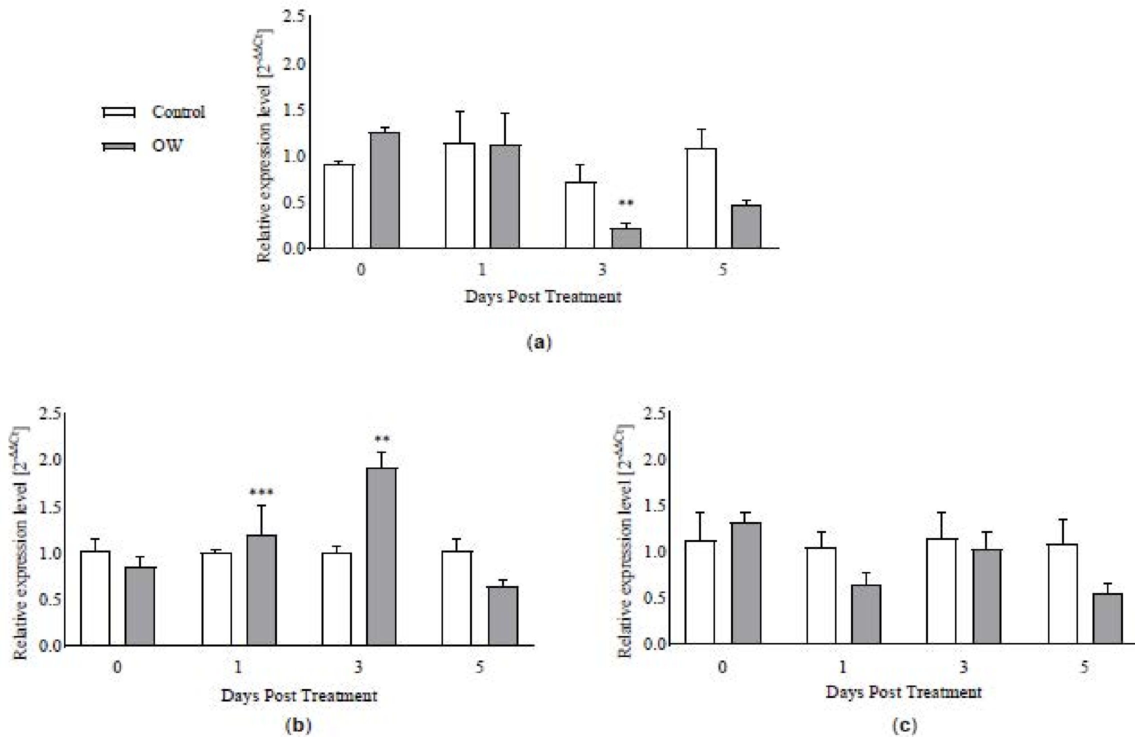


Figure 5: Evaluation of the effect of OW-treatment on the expression of selected defense marker genes in D. tenuifolia in the field. The expression of (a) PR1 (Pathogenesis-related protein 1), (b) MYC2 (MYC-related transcriptional activator 2) and (c) PDF1.2 (Plant Defensin 1.2) was assessed by real-time PCR in the presence (gray bars) or absence (white bars) of OW. Plants were collected at different time points following OW-application after the last cut. The values shown represent the average +/- SE of three biological replicates including three technical replicates each. Asterisks indicate a statistically significant differences according to the t-Student test with * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001.

related to a reduced stomatal aperture [17]. Such higher water content in the leaves may positively affect crop shelf-life, thus optimizing plant processability and marketability [34].

The molecular analysis revealed that MYC2 is induced at 1 and 3 days after OW-treatment, while it reduced PR1 transcript level at 3 days post treatment, thus suggesting an activation of the jasmonate pathway, which may in turn repress SA-related defenses. These data are in contrast with previous works showing an activation of SA-dependent defense responses in tomato, without affecting JA-mediated pathway [35]. This could likely be due to the differences in the experimental design, including plant species and development stage as well as controlled vs. field conditions. This highlights the importance of evaluating the effects of OW in real cultivation conditions to better evidence the impact of the treatment. In our study, the induction of JA pathway may represent a strategy of the plants to cope with the oxidative stress caused by OW. Indeed, under cyanide-induced oxidative stress, exogenous JA reduces ROS content, NADPH oxidase expression and activity in rice plants [36], while in wheat JA increases the activity of antioxidant enzymes [36,37]. Interestingly, among the observations following OW application on rocket salad, farmers noticed a decrease of insect or fungal attacks, in particular *Fusarium oxysporum*. The transient induction of the JA-related defenses may contribute to such resistance increase, since that pathway is known to be efficient against necrotrophic pathogens as well as herbivorous insects [39]. Overall, we may assume that rocket plants induce JA-mediated response as a resistance mechanism against oxidative stress, which turns to be efficient against pathogens and pests. Though still speculative, further studies could deserve attention to better understand the mechanism responsible for OW-mediated JA activation and confirm the increased resistance of *D. tenuifolia* to pathogens and pests that may contribute to rocket plant fitness increase observed in the field. Of note, besides a direct effect on the plant, OW could also influence the phytobiota of both leaves and rhizosphere. Thus, the positive effect observed in the field could be the result of a combined effect [40].

The untargeted metabolomics approach revealed major differences in the *D. tenuifolia* leaf metabolome based on the harvest periods, thus suggesting that environmental parameters play a major role. On the other hand, the effect of the OW-treatment was negligible, with no impact on the total levels of glucosinolates and flavonols accumulated in the leaves. Metabolites belonging to these classes, such as glucosativin, are known to contribute to flavor, bitterness and pungency of rocket [41], and provide numerous positive health benefits with regular consumption. These findings support the application of OW which improves physiological parameters still maintaining a substantial bioequivalence with the original product.

Conclusions

This work demonstrates that the effect of treatment with ozonated water is pleiotropic and albeit having a minimal impact on the parameters we evaluated at the molecular level, it shows significant physiological effects in field-grown plants improving their physiological characteristics and stress tolerance thus supporting the use of ozone in agriculture.

Acknowledgments

We thank Stefano Aldà for providing the field for the experimental design and Diego Bujazha for his assistance with sampling.

Funding

The Department of Biotechnology contributes to the funding of this project through Fondo Unico per la Ricerca.

Conflict of Interest

The authors RG, DD, SN, FG, EV, LA declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author GZ collaborates with the Company that provided the water ozonation system

Author Contributions

RG: acquisition and analysis of data for the work. Drafting the Material and Methods and the Result sections of the manuscript. DD and SN: contributes respectively in the analysis of transcriptomic and metabolomic data. GZ: provided the machine for OW and co-planned the experimental design. FG: revised the manuscript. EV: co-planned the experimental design and revised the work for plan-pathology-relevant sections. LA: plan the work, interpreted data, draft the work globally and wrote the introduction and discussion sections.

References

1. Karaca H, Velioglu YS. Ozone applications in fruit and vegetable processing. Food Rev Int 23 (2007): 91–106.
2. Shezi S, Magwaza LS, Mditshwa A, Tesfay SZ. Changes in biochemistry of fresh produce in response to ozone postharvest treatment. Sci Hortic 269 (2020): 109397.
3. Zheng L, Liu C, Song W. Effect of ozonated nutrient solution on the growth and root antioxidant capacity of substrate and hydroponically cultivated lettuce (Lactuca sativa). Ozone Sci Eng 42 (2020): 286–292.
4. Üner Öztürk K, Koyuncu MA. Effects of ozone and salicylic acid on post-harvest quality of parsley during storage. Biol Agric Hortic 37 (2021): 183–196.
5. Miller FA, Silva CLM, Brandão TRS. A review on ozone-

fortune Journals
Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

based treatments for fruit and vegetables preservation. Food Eng Rev 5 (2013): 77–106.

6. Kim JG, Yousef AE, Dave S. Application of ozone for enhancing the microbiological safety and quality of foods: A review. J Food Prot 62 (1999): 1071–1087.
7. Kobayashi F, Ikeura H, Ohsato S, Goto T, Tamaki M. Disinfection using ozone microbubbles to inactivate Fusarium oxysporum and Pectobacterium carotovorum. Crop Prot 30 (2011): 1514–1518.
8. Najarian M, Mohammadi-Ghehsareh A, Fallahzade J, Peykanpour E. Responses of cucumber (Cucumis sativus L.) to ozonated water under drought stress. J Plant Nutr 41 (2018): 1–9.
9. Khadre MA, Yousef AE, Kim JG. Microbiological aspects of ozone applications in food: A review. J Food Sci 66 (2001): 1242–1252.
10. Brodowska A, Nowak A, Kondratiuk-Janyska A, Piątkowski M, Śmigielski K. Modelling ozone-based treatments for inactivation of microorganisms. Int J Environ Res Public Health 14 (2017): 1196.
11. Pagès M, Kleiber D, Violleau F. Ozonation of fungal conidia associated with apple disease. Food Sci Nutr 8 (2020): 5292–5297.
12. Guo Z, Wang Z, Li Y, Wang Q. Effect of ozone on plant pathogens, tomato yield, and enzymatic activities. Ozone Sci Eng 41 (2019): 531–540.
13. Piechowiak T, Balawejder M. Impact of ozonation on oxidative stress markers in raspberries. Food Chem 298 (2019): 125093.
14. Sachadyn-Król M, Materska M, Chilczuk B. Ozonation of hot red pepper fruits increases antioxidant activity. Antioxidants 8 (2019): 356.
15. Martínez-Sánchez A, Aguayo E. Effects of ozonated water irrigation on grafted watermelon seedlings. Sci Hortic 261 (2020): 109047.
16. Onopiuk A, Półtorak A, Moczkowska M, Szpicer A, Wierzbicka A. Impact of ozone on quality and microbiological properties of raspberries. CyTA J Food 15 (2017): 563–573.
17. Sharaf-Eldin MA, et al. Influence of ozonated water on sweet pepper under cold stress. Sustainability 14 (2022): 13453.
18. Sachadyn-Król M, Agriopoulou S. Ozonation as abiotic elicitation improving plant health properties. Molecules 25 (2020): 2416.
19. Vainonen JP, Kangasjärvi J. Plant signalling in acute ozone exposure. Plant Cell Environ 38 (2015): 240–252.
20. Flores P, Hernández V, Fenoll J, Hellín P. Pre-harvest ozonated water application in broccoli. J Food Compos Anal 83 (2019): 103260.
21. Campayo A, de la Hoz KS, García-Martínez MM, Salinas MR, Alonso GL. Spraying ozonated water on grapevines and wine quality. Biomolecules 10 (2020): 213.
22. García-Martínez MM, Campayo A, Moratalla-López N, et al. Ozonated water affects wine chemical quality. Eur Food Res Technol 247 (2021): 1869–1882.
23. Campayo A, et al. Ozonated water rearranges grapevine transcriptomes. Sci Rep 11 (2021): 87542.
24. Modesti M, et al. Effects of ozonated water in vineyards on fruit quality. BIO Web Conf 13 (2019): 04011.
25. Thakur M, Bhattacharya S, Khosla PK, Puri S. Improving plant secondary metabolites via elicitation. J Appl Res Med Aromat Plants 12 (2019): 1–12.
26. Nicoletti R, Raimo F, Miccio G. *Diplotaxis tenuifolia*: Biology, production and properties. (2019).
27. Martínez-Sánchez A, Aguayo E. Ozonated irrigation and capsicum seedling quality. Agric Water Manag 221 (2019): 547–555.
28. Caarls L, Pieterse CMJ, Van Wees SCM. Salicylic acid control of jasmonic acid signaling. Front Plant Sci 6 (2015): 170.
29. Bussotti F, et al. Ozone stress in woody plants assessed by chlorophyll fluorescence. Environ Exp Bot 73 (2011): 19–30.
30. Vitha S, Zhao L, Sack FD. Root gravitropism and phototropism interaction. Plant Physiol 122 (2000): 453–462.
31. Labair H, et al. Ozonated water and organic fertilization in tomato. Emir J Food Agric 36 (2024): 1–9.
32. Pinzón-Sandoval EH, et al. SPAD and chlorophyll correlation in blueberry leaves. Rev Colomb Cienc Hortic 16 (2022): 1–10.
33. Xu JP, Yu YC, Zhang T, Ma Q, Yang HB. Ozone water effects on tomato physiology and genes. Hortic Res 8 (2021): 180.
34. Clarkson GJJ, O'Byrne EE, Rothwell SD, Taylor G. Traits improving postharvest salad processing. Postharvest Biol Technol 30 (2003): 287–298.
35. Prigigallo MI, et al. Ozone activates defense responses in tomato. Pest Manag Sci 75 (2019): 2251–2263.
36. Yu XZ, Chu YP, Zhang H, Lin YJ, Tian P. Jasmonic acid and oxidative stress in rice. Ecotoxicology 30 (2021): 1511–1520.

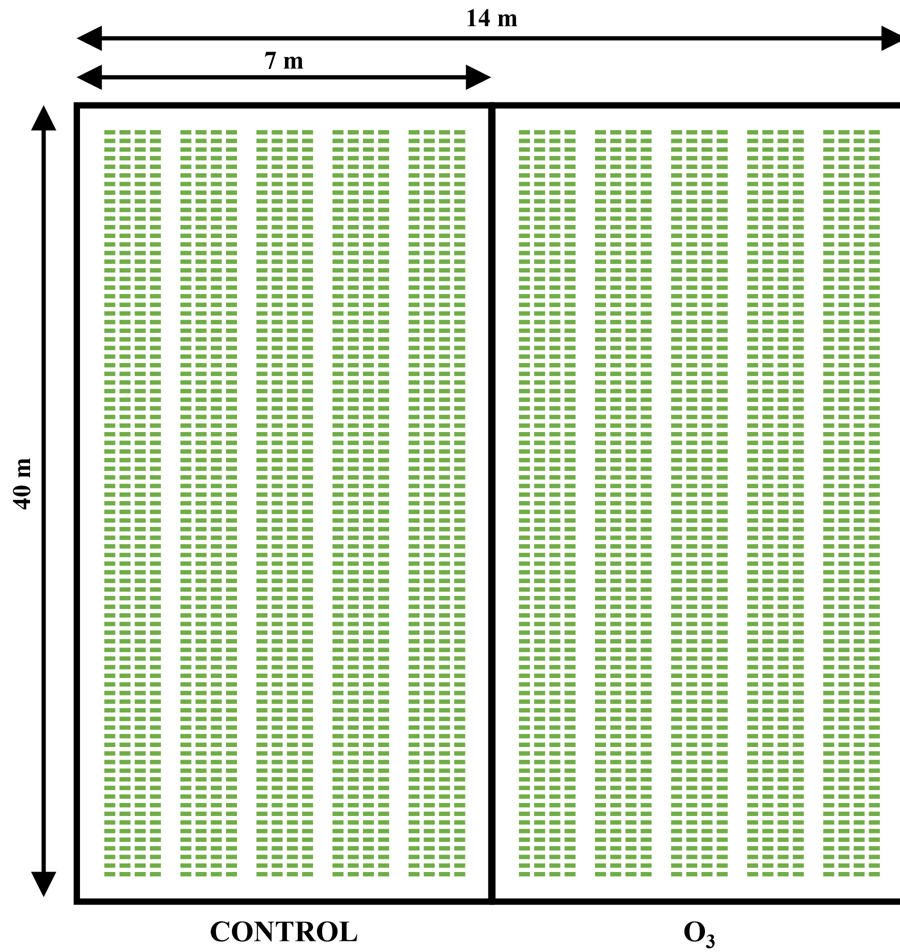
Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

37. Dai H, Jia G, Shan C. Jasmonic acid-induced redox regulation in wheat. Acta Physiol Plant 37 (2015): 200.
38. Karpets YV, Kolupaev YE, Lugovaya AA, Oboznyi AI. Jasmonic acid and wheat hyperthermia tolerance. Russ J Plant Physiol 61 (2014): 339–346.
39. Zhang L, Zhang F, Melotto M, Yao J, He SY. Jasmonate signaling manipulation by pathogens. J Exp Bot 68 (2017): 1371–1385.
40. Sangiorgio D, Spinelli F, Vandelle E. Impact of pesticides on phytobiota. Front Agron 4 (2022): 936032.
41. Bell L, Lignou S, Wagstaff C. Glucosinolates and sensory quality in rocket leaves. Foods 9 (2020): 1799.
42. Colunje J, Garcia-Caparros P, Moreira JF, Lao MT. Ozonated fertigation in pepper cultivation. Agronomy 11 (2021): 544.
43. Fisher Scientific. Procedural guidelines. (2012).
44. Life Technologies Corporation. TURBO DNase. (2012).
45. Cavaiuolo M, et al. Transcriptomic analysis of rocket salad stress. PLoS ONE 12 (2017): e0178119.
46. Cataldi TRI, Lelario F, Orlando D, Bufo SA. Glucosinolate structure elucidation by MS. Anal Chem 82 (2010): 5686–5696.
47. Bell L, Kitsopanou E, Oloyede OO, Lignou S. Odorants in Brassicaceae species. Foods 10 (2021): 1055.
48. Bennett RN, et al. Major glucosinolate in Eruca sativa leaves. Phytochemistry 61 (2002): 25–30.
49. Martínez-Sánchez A, et al. Flavonoid profiles of rocket salads. J Agric Food Chem 55 (2007): 1356–1363.
50. Zhou B, et al. Glucosinolates in Chinese cabbage. Food Chem 393 (2022): 133414.
51. Dernovics M, Molnár A, Szalai G. UV-B-induced glucosinolate derivatives. J Food Compos Anal 122 (2023): 105485.
52. Masullo M, Cerulli A, Pizza C, Piacente S. Chemical profile and antioxidant activity of Pouteria lucuma. Molecules 26 (2021): 5236.

This article is an open access article distributed under the terms and conditions of the
Creative Commons Attribution (CC-BY) license 4.0

Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

SUPPLEMENTARY FILES



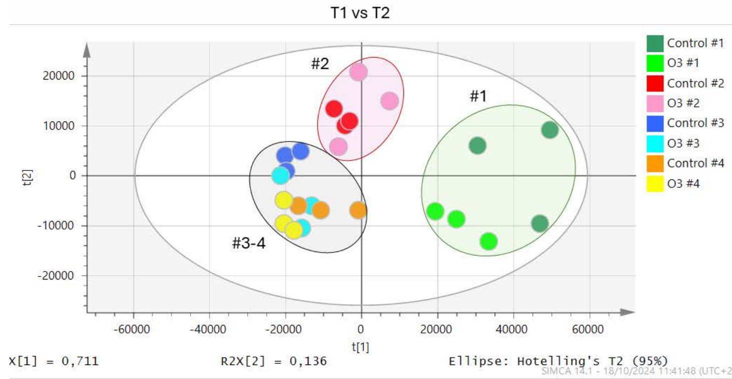
Citation: Riccardo Greco, Davide Danzi, Stefano Negri, Giorgio Zaffani, Flavia Guzzo, Elodie Vandelle, Linda Avesani. Ozonated water enhances *Diplotaxis tenuifolia* growth and physiology under field and controlled conditions. Journal of Food Science and Nutrition Research. 9 (2026): 01-13.

fortune journals
Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

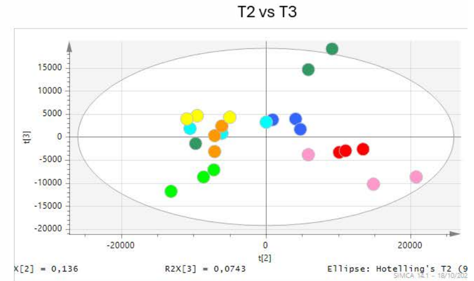
A)

PCA-X
Negative ionization LC-MS dataset
Score plots

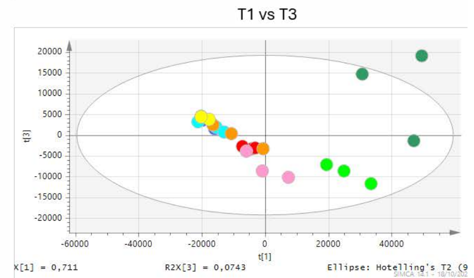
T1 vs T2



T2 vs T3

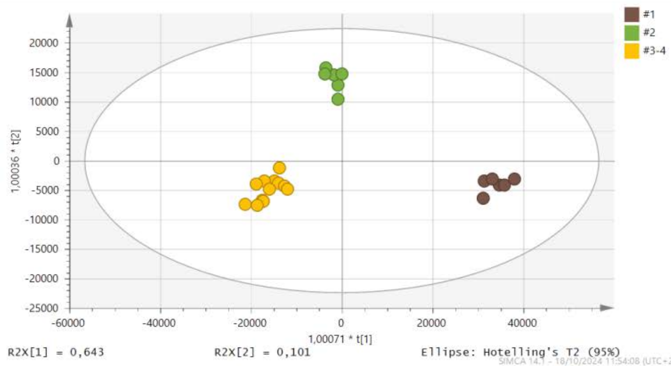


T1 vs T3

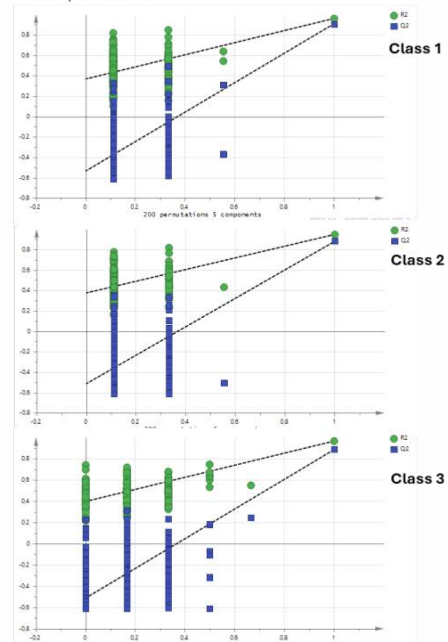


B)

3-classes O2PLS-DA model
Score plot
Class 1: timepoint #1
Class 2: timepoint #2
Classe 3: timepoint #3+#4



Validation: PERMUTATION TEST
200 permuatations



Validation: ANOVA (PLS-DA)

	SS	DF	MS	F	p	SD
Total corr.	46	46	1			1
Regression	40,717	18	2,26207	11,99	8,98E-09	1,50402
Residual	5,2827	28	0,18867			0,43436

Supplementary Figure2 Multivariate statistical analysis of the untargeted metabolomics dataset. A) PCA-X score plots of *Diplotaxis tenuifolia* samples. B) Score plot of a 3-class O2PLS-DA model discriminating the samples according to the indicated timepoints. Validation was performed on the corresponding PLS-DA with 200 permutations and CV-ANOVA.

Citation: Riccardo Greco, Davide Danzi, Stefano Negri, Giorgio Zaffani, Flavia Guzzo, Elodie Vandelle, Linda Avesani. Ozonated water enhances *Diplotaxis tenuifolia* growth and physiology under field and controlled conditions. Journal of Food Science and Nutrition Research. 9 (2026): 01-13.

Greco R, et al., J Food Sci Nutr Res 2026
DOI:10.26502/jfsnr.2642-110000187

Rt (min)	Class	Putative identification	Formula	ESI⁻ ion	*m/z* detected	Fragments	Reference
2.56	Glucosinolates	Glucosinalbin	$C_{14}H_{19}NO_{10}S_2$	$[M-H^+]^-$	424.0369	96.9591; 95.9538; 259.0161	HMDB
3.02	Glucosinolates	Glucoraphanin	$C_{12}H_{23}NO_{10}S_3$	$[M-H^+]^-$	436.0401	372.0444; 178.0189; 96.9591; 95.9518; 79.9556	MassBank
3.12	Glucosinolates	6-Methylsulfonyl-3-oxohexyl-glucosinolate	$C_{14}H_{25}NO_{12}S_3$	$[M-H^+]^-$	494.0458	414.0907; 218.0490; 96.9591; 95.9518; 252.0366; 298.0086	Cataldi et al., 2010
3.47	Benzoic acids	Dihydroxybenzoic acid hexoside	$C_{13}H_{16}O_9$	$[M-H^+]^-$	315.0711	152.0117; 108.0214; 153.0178; 109.0279	MassBank
3.55	Glucosinolates	Glucosativin	$C_{11}H_{21}NO_9S_3$	$[M-H^+]^-$	406.0298	164.0212; 259.0128; 241.0036; 96.9591; 274.9894	Bell et al., 2021
4.19	Glucosinolates	Glucoerucin	$C_{12}H_{23}NO_9S_3$	$[M-H^+]^-$	420.0453	96.9591; 959.9518; 259.0128; 241.0036;	Bell et al., 2021
4.66	Glucosinolates	Dimeric 4-mercaptobutyl-glucosinolate	$C_{22}H_{40}O_{18}N_2S_6$	$[M-2H^+]^{2-}$	405.0211	811.0568; 96.9591; 95.9518	Bennett et al., 2002
5.12	Flavonols	Quercetin-tri-O-hexoside	$C_{33}H_{40}O_{22}$	$[M+FA-H^+]^-$	833.1993	787.1932; 625.1457; 463.0889; 301.0341; 300.0263	manual identification
5.20	Phenylpropanoids	Sinapic acid derivative	-	-	385.1128	205.0515; 190.0273; 175.0056; 223.0620;	manual identification
5.40	Flavonols	Quercetin-O-(di-hexoside)- O-(sinapoyl-hexoside)-O-hexoside	$C_{50}H_{60}O_{31}$	$[M-H^+]^-$	1155.3061	993.2659; 831.2114; 669.1579; 625.1457; 463.0889; 301.0377; 300.0263	Martínez-Sánchez et al., 2007
5.43	Glucosinolates	4-Methoxyglucobrassicin / Neoglucobrassicin	$C_{17}H_{22}N_2O_{10}S_2$	$[M-H^+]^-$	477.0628	96.9611; 95.9518; 259.0095	Zhou et al., 2022
5.72	Flavonols	Kaempferol-di-O-hexoside	$C_{27}H_{30}O_{16}$	$[M-H^+]^-$	609.1459	283.0244; 284.0309; 255.0320; 446.0857; 285.0426; 447.0893	manual identification
5.86	Flavonols	Isorhamnetin-di-O-hexoside	$C_{33}H_{40}O_{22}$	$[M-H^+]^-$	639.1563	313.0352; 314.0429; 476.0991; 477.1046	MassBank
6.25	Flavonols	Quercetin-O-hexoside-O-(sinapoyl-hexoside)-O-hexoside	$C_{33}H_{40}O_{22}$	$[M-H^+]^-$	993.2513	831.2114; 669.1579; 625.1457; 463.0889; 301.0377; 300.0263	manual identification
6.76	Glucosinolates	UI glucosinolate	-	-	321.1003	96.9591; 292.8121; 276.8391	
7.07	Flavonols	Quercetin-di-O-(sinapoyl-hexoside)-O-hexoside	$C_{55}H_{60}O_{30}$	$[M-H^+]^-$	1199.3098	1037.2572; 669.1473; 301.0377; 463.0889; 831.2055;	manual identification
10.24	Glucosinolates	Polysulfaniylated glucosinolate	$C_{16}H_{28}N_2O_9S_5$	$[M-H^+]^-$	551.0320	96.9611; 95.9518; 259.0095; 241.973; 274.989	Dernovics et al., 2023;
13.12	Lipids	DGMG (18:3)	$C_{33}H_{56}O_{14}$	$[M+FA-H^+]^-$	721.3651	675.3624; 397.1351; 415.1452; 277.2173	Masullo et al., 2021

Supplementary Table1 LC-MS features of the main metabolites identified in *Diplotaxis tenuifolia* samples. Manual identifications were supported by the evaluation of neutral losses (-162.05 Da, hexoside; -206.06 Da, sinapoyl moiety) and aglycone fragments compared to those present in a in-house library of authentic standards.

References:

Bell, L., Kitsopanou, E., Oloyede, O. O., & Lignou, S. (2021). Important odorants of four Brassicaceae species, and discrepancies between glucosinolate profiles and observed hydrolysis products. *Foods*, *10*(5), 1055.

Bennett, R. N., Mellon, F. A., Botting, N. P., Eagles, J., Rosa, E. A., & Williamson, G. (2002). Identification of the major glucosinolate (4-mercaptobutyl glucosinolate) in leaves of Eruca sativa L.(salad rocket). Phytochemistry, 61(1), 25-30.

Cataldi, T. R., Lelario, F., Orlando, D., & Bufo, S. A. (2010). Collision-induced dissociation of the A+ 2 isotope ion facilitates glucosinolates structure elucidation by electrospray ionization-tandem mass spectrometry with a linear quadrupole ion trap. Analytical Chemistry, 82(13), 5686-5696.

Dernovics, M., Molnár, A., & Szalai, G. (2023). UV-B-radiation induced di-and polysulfide derivatives of 4-mercaptobutyl glucosinolate from Eruca sativa. Journal of Food Composition and Analysis, 122, 105485.

Martínez-Sánchez, A., Llorach, R., Gil, M. I., & Ferreres, F. (2007). Identification of new flavonoid glycosides and flavonoid profiles to characterize rocket leafy salads (Eruca vesicaria and Diplotaxis tenuifolia). Journal of agricultural and food chemistry, 55(4), 1356-1363.

Masullo, M., Cerulli, A., Pizza, C., & Piacente, S. (2021). Pouteria lucuma pulp and skin: in depth chemical profile and evaluation of antioxidant activity. Molecules, 26(17), 5236.

Zhou, B., Huang, W., Feng, X., Liu, Q., Ibrahim, S. A., & Liu, Y. (2022). Identification and quantification of intact glucosinolates at different vegetative growth periods in Chinese cabbage cultivars by UHPLC-Q-TOF-MS. Food Chemistry, 393, 133414.