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Effect of Protein Hydrolysates on Yield and Chemical Parameters of Oregano Cultivated Under Rainfed Conditions in Mediterranean Environments

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Abstract

A critical challenge for modern agriculture is the adoption of sustainable and effective strategies, also in the medicinal and aromatic plant (MAP) sector. In many areas, MAP cultivation remains dependent on traditional farming systems, with a lack of innovative techniques. This study investigates the effect of the foliar application of two protein hydrolysates, one animal-derived (PH 1) and one plant-derived (PH 2), on oregano (*Origanum vulgare* L.) cultivated under rainfed conditions in a representative area of Sicily (Italy). The aim was to evaluate whether these types of biostimulants could enhance production yield compared to untreated plants. Results showed that both protein hydrolysates induced significant improvement of the agronomic responses compared to the control. Specifically, treatments stimulated a substantial enhancement in fresh biomass (increases from 1.9 to 6.5 t ha^{-1}) and dry biomass (increases from 0.9 to 2.4 t ha^{-1}). Total phenolics and antioxidant activity decreased by 15–24% and 7–15%, respectively, compared to control plants during the two years. However, the aromatic profile of the essential oils was not significantly affected by foliar application of the two protein hydrolysates. The use of these foliar biostimulants represents a sustainable and highly effective strategy to maximize productive parameters while maintaining the chemical stability required by the market, offering a significant contribution to the optimization of oregano cultivation.

Keywords: primary and secondary plant metabolism; vegetable or animal protein matrix; total phenolic; antioxidant activity; rosmarinic acid; aromatic profile

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1. Introduction

Oregano (*Origanum vulgare* spp. *hirtum*) is well-known for its antimicrobial properties, which are primarily due to phenolic compounds such as carvacrol and thymol [1,2]. Oregano is a highly versatile aromatic plant with a wide range of applications in the culinary, pharmaceutical, and cosmetic industries [2–4]. This herb can be utilized in several distinct forms: it is widely consumed fresh or dried as a culinary spice, while its therapeutic

potential is often harnessed through liquid preparations such as infusions (tea) or tinctures [3–5]. Oregano essential oil (EO) is used to treat respiratory infections, gastrointestinal disorders, and skin conditions [2–4]. It is also believed to boost immune function [5–7]. Essential oils (EOs) extracted from these plants are used for their pleasant scents and therapeutic benefits, influencing mood, cognition, and overall well-being [8,9]. Demand for EOs is primarily driven by the following markets: household (16%), pharmaceutical (15%), food and beverage (35%), perfumes, cosmetics, and aromatherapy (29%) [10]. The biosynthesis of active substances takes place through metabolic pathways using intermediate products of primary metabolism which accumulate in plant cells due to a biochemical imperfection or normal physiological process [11]. Secondary metabolism products are multifunctional substances that, following events that cause an alteration in the balance of the plant organism, participate in the processes of restoration of cellular characteristics [1,5,9]. For this reason, the induction of abiotic and biotic stresses can cause an increase in the production of secondary metabolites and active compounds [12,13]. This metabolic shift can be strategically exploited to enhance the phytochemical profile of the plant, where a higher concentration of specific bioactive molecules represents a significant qualitative advantage, depending on the intended industrial or therapeutic application [9,11,13,14]. Yield and quality parameters of medicinal and aromatic plants (MAPs) are influenced by a variety of agronomic factors, including soil management, irrigation, fertilisation, plant density, and harvest time [14,15]. Crop management is crucial for enhancing agronomic performances and buffering crops against abiotic stressors [16,17]. Recent studies have shown that the use of biostimulants can improve the agronomic and productive response of various herbaceous and horticultural crops grown under stress conditions [18–20]. It has been demonstrated that foliar and/or root application of these products can improve physiological processes, water and nutrient absorption [20,21]. Protein hydrolysates (PHs) represent a type of biostimulant whose application can improve primary and secondary plant metabolism [22,23]. PHs are mixtures of polyprotein hydrolysate, oligo-protein hydrolysate and amino acids, and are obtained by chemical or enzymatic hydrolysis of vegetable or animal protein matrix [24,25]. Peptides and amino acids are primarily responsible for the biostimulating action of protein hydrolysates [26,27]. After being absorbed by the plant, amino acids are exploited to make proteins, to generate energy, to manufacture high-bioactivity molecules, or as building blocks for other substances that affect the final product's quality [28,29]. Di Miceli et al. [23] observed an increase in growth, yield parameters and quality parameters in eggplant (*Solanum melongena* L.) treated with protein hydrolysate in open-field conditions. Similar findings were obtained by Sabatino et al. [30] in lettuce (*Lactuca sativa* L.) treated with protein hydrolysate. The authors observed a significant improvement in yield and yield-related features, nutritional and functional traits, as well as nitrogen indices. Paul et al. [31] claimed that protein hydrolysate application on tomato plants can be considered as a sustainable crop enhancement technology for agricultural productivity under water shortage conditions.

To the best of our knowledge, there are no studies on the effect of foliar application of protein hydrolysates on oregano yield and its chemical composition. The positive effects of PH applications are reported by several studies [20–31], and PH can be used as positive tool in the Mediterranean region where rainfed conditions may restrict plant development. With this in mind, the aims of this study evaluates the impact of animal-derived (PH 1) and plant-derived (PH 2) hydrolysates on yield, essential oil composition, and key bioactive compounds—such as phenolic content, antioxidant activity and rosmarinic acid—under organic farming conditions, to optimize both crop productivity and phytochemical quality.

The following experimental hypothesis was taken into consideration: the foliar application of two different protein hydrolysates leads to increases in biomass and EO yields,

enhances the aromatic profile of the EO, and produces a positive effect on other chemicals in oregano plants grown under rainfed conditions.

2. Materials and Methods

2.1. Test Site and Cultivation Practices

The trials were carried out in the growing seasons 2022–2023 and 2023–2024, at an organic farm located in the south-west of Sicily, Italy (37°37′12.30″, 13°67′48.45″). The experimental field was prepared in February 2020, and the agamic propagation was applied. Prior to the transplantation, the field was fertilised using 2.0 t ha^{-1} of cattle manure (0.5% of N, 0.2% of P_2O_5, 0.7% K_2O, approximately). The soil was classified as Regosol (United States Department of Agriculture (USDA) classification: typic xerorthents). Soil parameters are showed in Table 1.

Table 1. Soil parameters of the experimental field.

Parameter	Value	Unit
Sand	48.0	%
Silt	26.0	%
Clay	26.0	%
Organic matter	1.1	%
Total nitrogen	1.3	%
Assimilable phosphate	22.4	ppm
Assimilable potassium	333.0	ppm
pH	7.2	

Vegetative multiplication, specifically through the splitting of existing oregano bushes, was used to set up the experimental site in early 2019. A plant density of 10,000 plants ha^{-1} was obtained, adopting 2.0 m between rows and 0.5 m within rows. Oregano plants were organically grown under rainfed conditions, without irrigation, and no pesticides were used. The weeds were mechanically controlled. In both years, plants were manually harvested during the first 10-day period of June.

In the two-year study, 10-day average maximum and minimum air temperatures and 10-day total rainfall were monitored from a weather station owned by the Sicilian Agro-Meteorological Information Service [32], situated close to the farm. Additionally, the evapotranspiration values were obtained from the Sicilian Agro-Meteorological Information Service database. The experimental site is characterized by a typical Mediterranean climate. During the oregano vegetative period (April to June), environmental conditions are marked by a steady increase in solar radiation and temperatures. This period is also characterized by a progressive reduction in rainfall, leading to the typical early-summer semi-arid conditions of Southern Italy.

Figure 1 shows temperature, rainfall trends and evapotranspiration averages (10-day periods) in the study period.

2.2. Bisotimulant Application

Two protein hydrolysates were used for foliar applications:

- PH 1 (Aswell®, Mugavero fertilizers, Palermo, Italy) at doses of 2 mL L^{-1}, protein hydrolysate derived from hydrolyzed animal epithelium, with organic nitrogen 8.0 %, organic carbon 27.0 %, amino acids 50.0 % and free amino acids 15 %.
- PH 2 (Tyson®, Mugavero fertilizers, Palermo, Italy) at doses of 3 mL L^{-1}, protein hydrolysate obtained from *Fabaceae* with amino acids and plant peptides (31 %), organic nitrogen (5 %), and organic carbon (25 %).

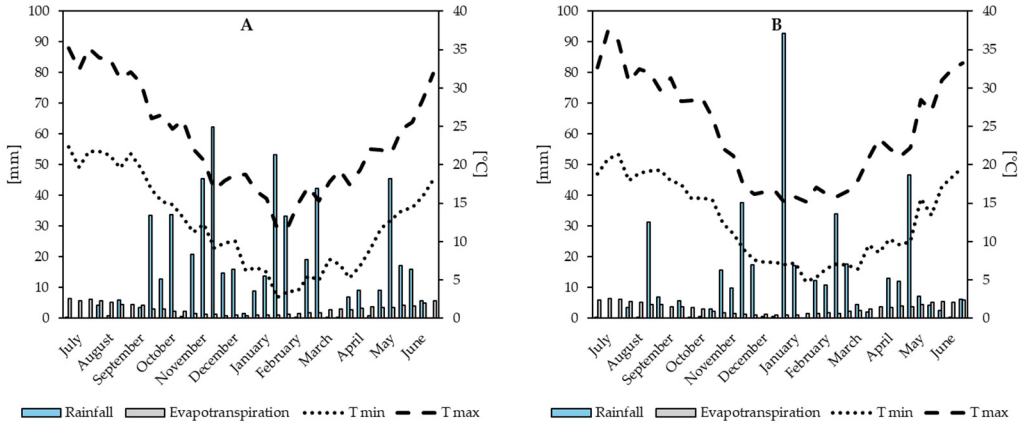

Figure 1. Meteorological trends during the experimental period, first year (**A**) and second year (**B**). Bars represent 10-day total rainfall and evapotranspiration averages, while lines indicate 10-day average maximum and minimum air temperatures.

For each protein hydrolysate, the dose was formulated to provide the same total quantity of N. A total of four foliar applications were performed during the vegetative growth stage, from the first 10-day period of April of each year. For the control (C) treatment, plants were sprayed exclusively with water. A total of 400.0 L of water ha^{-1} was used for each application, using a portable hand-sprayer (Hyundai Corporation, Seoul, South Koreav) equipped with a flat fan nozzle and with an operating pressure of 250.0 kPa. To avoid drift and contamination of nearby plots, plastic panels were used to delineate each plot during application. A single operator managed the foliar sprays to ensure uniform application and consistent dosage, and treatments were applied early in the morning to guarantee optimal foliar absorption [21]. The plot size was 40 m^2 (2.0 m × 20.0 m) with 40 plants per plot. A randomised complete block design with three replicates was used for the experiment.

2.3. Yield Parameters

In both years, harvesting was conducted during the blooming stage. Plants were cut at 5 cm above ground level and dried in a shaded, ventilated environment at 25–30 °C for approximately 10 days. At harvest, plant height, chlorophyll content, and total fresh yield were recorded. Chlorophyll content was measured using a Dualex Scientific (Force A, Orsay, France) portable chlorophyll meter, and the device was used on 40 young apical leaves per plot. To assess dry yield, a sample of 100 g of fresh biomass for each treatment was oven-dried at 105 °C for 24 h. The plant material was manually separated into stems, leaves, and flowers. Due to their negligible EO content, stems were excluded from chemical analysis [3,21]; therefore, analysis of EO content and yield, EO profile, antioxidant activity, rosmarinic acid content, and total phenolic content was performed exclusively on flowers and leaves. For each plot, 500.0 g of air-dried plant material (in a shaded and ventilated environment for approx. 10 days at a temperature of 25–30 °C) was hydro-distilled for 3 h to obtain EO, in accordance with the Ph. Eur. 7.0, 20812 [33]. For each treatment, three extractions of EO were performed. EO concentration was calculated by dividing EO volume for each sample by biomass weight. EO yield was calculated by multiplying EO content by the total dry yield. EO samples were stored at −18 °C.

2.4. Chemical Parameters

Methods for determination of the EO components are described in detail by Farruggia et al. [21]. Chemical profiling of the EO was conducted via GC-MS using an HP 6890/5972 system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a DB5-MS column

(30 m × 0.25 mm × 0.25 µm). Chromatography was performed using Helium (42 cm s^{-1}) as the mobile phase, a 100:1 split ratio, and an injector set at 250 °C. The thermal gradient involved an initial 4 min hold at 60 °C, a 5 °C min^{-1} ramp to 100 °C, and a final 9 °C min^{-1} rise to 280 °C. Components were identified by matching their mass spectra and retention indices (determined using n-alkane standards, Sigma-Aldrich, Vienna, Austria) against published databases. Relative concentrations were calculated by peak-area normalization without the use of correction factors (response factor = 1). Only the data for the three major constituents of oregano EO (γ-terpinene, carvacrol methyl ether, and thymol) are presented in the results.

Using 25.0 mL of 70.0% aqueous methanol, 0.15 g of finely ground dry biomass was extracted in an ultrasonic bath DU-32 (Argo Lab, Carpi, Italy) operating at 40 kHz at 120 W for 30 min, at room temperature. After being filtered, the extracts were stored at − 20 °C for further examination. The extracts were used to determine the following:

The determination of total phenolics was performed using the Folin-Ciocalteu assay as described by Farruggia et al. [21]. In brief, 5 µL of extract were mixed in a microplate with distilled water (105 µL), Folin–Ciocalteu reagent (5 µL), sodium carbonate (10 µL, 35% solution), and a final volume of 125 µL distilled water. A calibration curve was constructed using caffeic acid (Sigma-Aldrich, Vienna, Austria) as the standard, with volumes ranging from 0 to 25 µL. Samples and standards were analyzed in four replicates. After incubating the plate for one hour in the dark, absorbance was recorded at 750 nm (i-mark microplate reader, Bio-Rad, Hercules, CA, USA). Results are reported as mg caffeic acid equivalents per gram of dry weight (mg CAE g^{-1} dw).

The DPPH radical scavenging assay was performed according to the method of Chizzola et al. [34]. Briefly, 5 µL of sample extract wascombined with 95 µL of methanol and 100 µL of DPPH working solution (0.0038 g/25 mL methanol; Sigma-Aldrich, Darmstadt, Germany). Trolox (0.62 mg mL^{-1}) served as the reference standard (0–8 µL range, brought to 100 µL with methanol). A specific blank containing fully reduced DPPH (50 µL Trolox + 50 µL water + 100 µL reagent) was used for background subtraction. Measurements were taken at 490 nm (i-mark microplate reader, Bio-Rad, Hercules, CA, USA) in four replicates. The final antioxidant activity was calculated as mg Trolox equivalents (TE) g^{-1} dry weight.

HPLC analysis was performed to quantify rosmarinic acid, based on the method described by Farruggia et al. [21]. The system (Waters S.A.S, Saint-Quentin, France) comprised a 626 pump, 600 S controller, 717 plus autosampler, and a 996-DAD detector. A Symmetry C18 column (4.6 × 150 mm, 5.0 µm particle size) kept at 25 °C was used for separation. The elution program employed two solvents: Solvent A (1% acetic acid in acetonitrile, 85:15) and Solvent B (methanol). The gradient started at 90% A and 10% B, reaching 100% B linearly within 30 min, with a flow rate of 1.0 mL min^{-1} and a 20 µL sample injection. Calibration was performed at 330 nm using external standards ranging from 3.9 to 500 µg mL^{-1}. Average retention time was found to be 7.27 ± 0.13 min.

2.5. Statistical Analysis

Data for each year were subjected to two-way analysis of variance (ANOVA). Differences between means were compared using Tukey's test ($p \leq 0.05$). In the mixed model/ANOVA, foliar treatments and year were used as fixed effects, and block as the random factor. However, block factors did not significantly affect ($p > 0.05$) any of the evaluated parameters. Prior to applying the ANOVA test, homogeneity of variance and normality of all data were checked using Levene's test and the Shapiro–Wilk test. Statistical analyses were performed using the software MINITAB 19 (State College, PA, USA) for Windows.

3. Results

Statistical analysis revealed a significant effect (p-value < 0.05) of year (Y), foliar treatments (F) and their interaction (Y × F) on morphological and physiological parameters of oregano plants. The highest plant height and stem percentage have been observed during the first year, with the highest chlorophyll content and inflorescence and leaf percentage during the second year (Table 2). The application of PH 1 generated the highest plant height and inflorescence and leaf percentage. PH 2 produced the highest chlorophyll content and stem percentage (Table 2).

Table 2. Morphological and physiological parameters in response to treatments during the two-year study.

Source of Variation	Plant Height [cm]	Chlorophyll Content [μg cm^{-2}]	Inflorescences and Leaves [%]	Stems [%]
Year (Y)				
I	39.7 b	32.8 a	69.2 a	30.8 b
II	55.8 a	25.7 b	65.8 b	34.2 a
Foliar treatments (F)				
Control	39.3 c	26.1 c	67.3 ab	32.7 ab
PH 1	53.3 a	30.1 b	69.0 a	31.0 b
PH 2	50.8 b	31.4 a	66.3 b	33.7 a
Significance				
Y	**	**	**	**
F	**	**	**	**
Y × F	**	**	*	*

Means and standard deviations are shown. Values with different letters are significantly different for $p \leq 0.05$ according to the Fisher LSD test. ** = significant at 0.01 probability level; * = significant at 0.05 probability level. Control: only water; PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

Considering the interaction Y × F, the highest plant heights have been recorded during the second year in plants treated with both PHs (Figure 2). Untreated plants generated the lowest plant height during the first year.

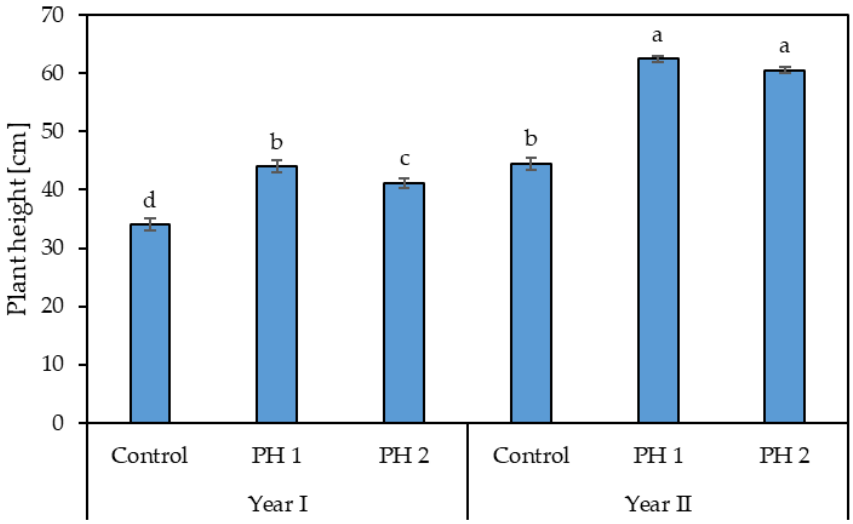

Figure 2. Influence of the interaction Y × F on plant height. Means and SDs are reported. The values followed by the same letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

The application of PH 2 generated the highest chlorophyll content during the second year. The lowest value has been observed during the first year in control plants (Figure 3).

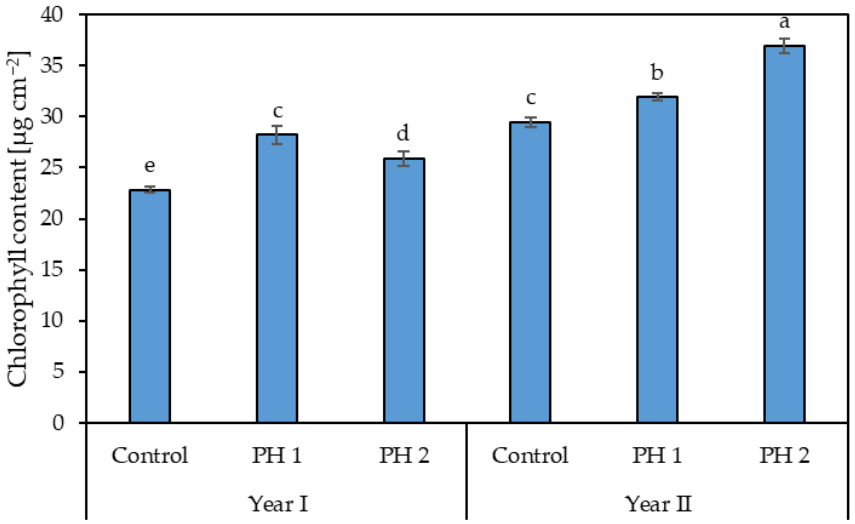

Figure 3. Influence of the interaction Y × F on chlorophyll content. Means and SDs are reported. The values followed by the same letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

Considering the distribution among the various plant parts, PH 1-treated plants produced the highest inflorescence and leaf percentages and the lowest stem percentages during the first year, while PH 2-treated plants produced the highest stem percentage and the highest inflorescence and leaf percentages during the second year (Figure 4). The lowest value has been recorded in control plants.

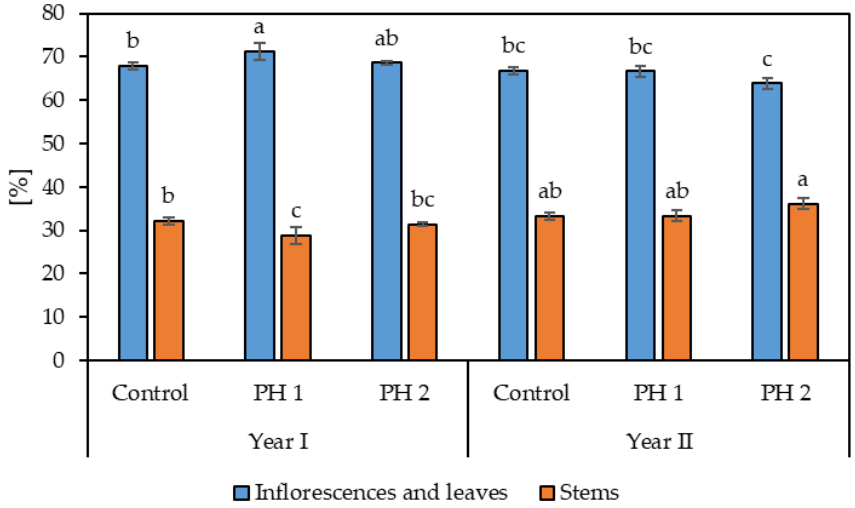

Figure 4. Influence of the interaction Y × F on inflorescence and leaf, and stem percentages. Means and SDs are reported. The values followed by the same letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

As reported in Table 3, crop yields and EO parameters have been significantly influenced (p-value < 0.01) by the effect year (Y) factor, foliar treatment (F) factor, and their interaction (Y × F). Only EO yield showed no significant differences in response to the interaction factors (Table 3). During the second year, the highest fresh yield, dry yield and

EO yield have been observed. The highest EO content has been measured during the first year. PH 1-treated plants produced the highest fresh and dry yield, while PH 2-treated plants showed the highest EO content. The application of both PHs generated the best response in terms of EO yield (Table 3).

Table 3. Crop yield and essential oil parameters in response to treatments during the two-year study.

Source of Variance	Fresh Yield [t ha^{-1}]	Dry Yield [t ha^{-1}]	Essential Oil Content [%]	Essential Oil Yield [kg ha^{-1}]
Year (Y)				
I	4.5 b	2.2 b	3.4 a	49.3 b
II	9.1 a	3.4 a	3.0 b	52.5 a
Foliar treatments (F)				
Control	4.1 c	1.7 c	2.8 c	39.9 b
PH 1	8.6 a	3.5 a	3.3 b	56.9 a
PH 2	7.7 b	3.2 b	3.5 a	56.0 a
Significance				
Y	**	**	**	**
F	**	**	**	**
Y × F	**	**	**	n.s.

Means and standard deviations are shown. Values with different letters are significantly different for $p \leq 0.05$, according to the Fisher LSD test. ** = significant at 0.01 probability level; n.s. = no significant. Control: only water; PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

Considering the interaction Y × F, PH 1-treated plants produced the highest fresh and dry yields during the second year, with 11.6 t ha^{-1} and 4.3 t ha^{-1}, respectively (Figure 5). The lowest values have been measured in untreated plants during the first year (3.1 t ha^{-1} for fresh yield and 1.5 t ha^{-1} for dry yield).

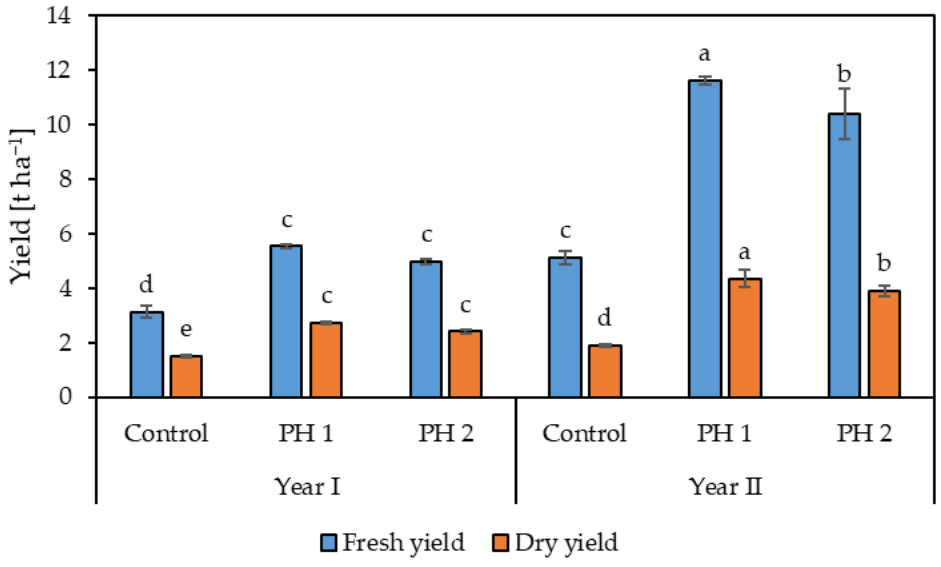

Figure 5. Influence of the interaction Y × F on fresh yield and dry yield. Means and SDs are reported. The values followed by the same letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

The application of PH 2 during the first year allowed to obtain the highest EO content. The lowest EO percentages have been recorded in untreated plants during both years (Figure 6).

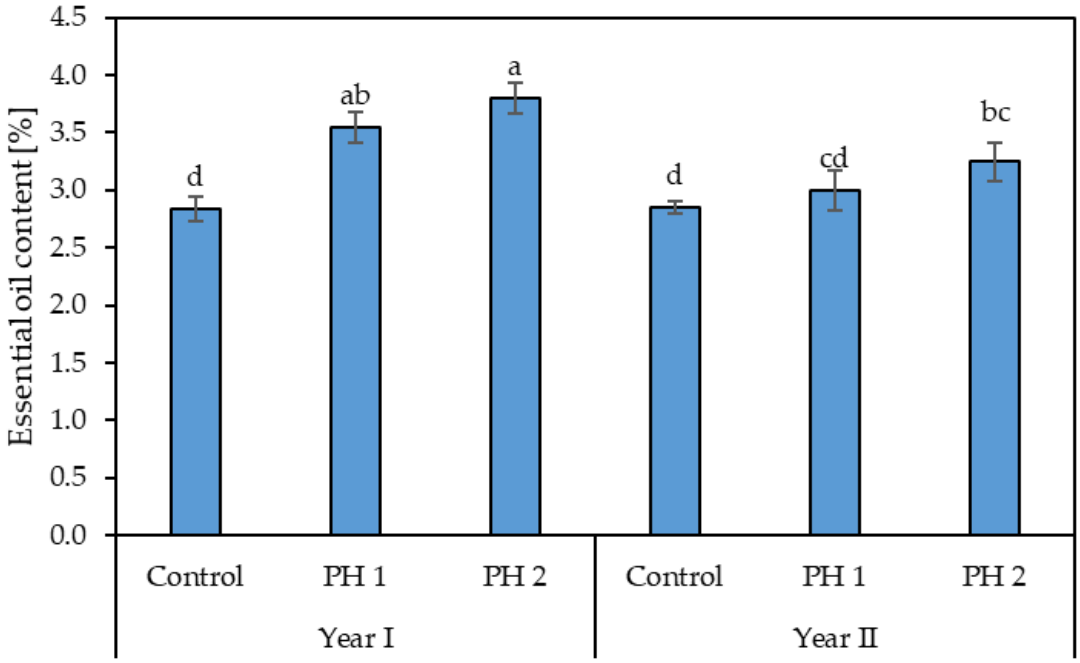

Figure 6. Influence of the interaction Y × F on essential oil content. Means and SDs are reported. The values followed by the same letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

The factors under investigation showed significant effect (p-value < 0.01) on total phenolic content, antioxidant activity and rosmarinic acid (Table 4). During the first year, the highest values have been obtained. Control plants produced the highest values for all parameters. A statistically similar rosmarinic acid value has been recorded in PH 2-treated plants (Table 4).

Table 4. Phenolic content, antioxidant activity and rosmarinic acid content of oregano biomass in response to treatments during the two-year study.

Source of Variance	Total Phenolic Content [mg c.a.e. g^{-1}]	Antioxidant Activity [mg t.e. g^{-1}]	Rosmarinic Acid Content [%]
Year (Y)			
I	117.6 a	150.1 a	2.7 a
II	105.1 b	125.6 b	2.3 b
Foliar treatments (F)			
Control	123.7 a	145.8 a	2.7 a
PH 1	111.3 b	130.0 c	2.2 b
PH 2	99.0 c	137.7 b	2.6 a
Significance			
Y	**	**	**
F	**	**	**
Y × F	**	**	**

Means and standard deviations are shown. Values with different letters are significantly different for $p \leq 0.05$, according to the Fisher LSD test. ** = significant at 0.01 probability level. Control: only water; PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

Considering the interaction factor, during the first year the control plants always generated the highest total phenolic content, antioxidant activity and rosmarinic acid content (Figures 7–9).

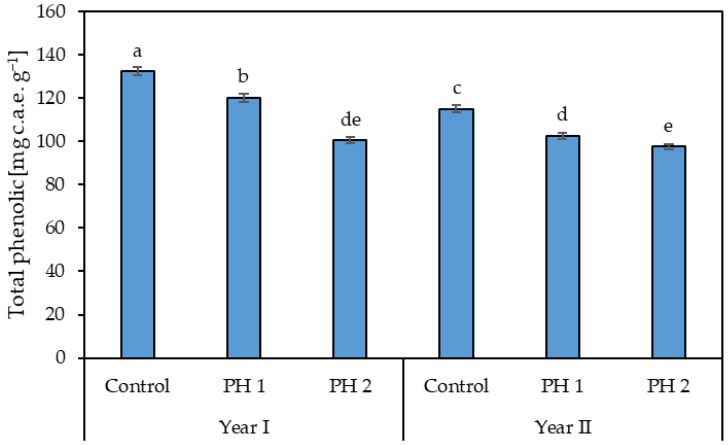

Figure 7. Influence of the interaction Y × F on total phenolic content. Means and SDs are reported. The values followed by the same letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

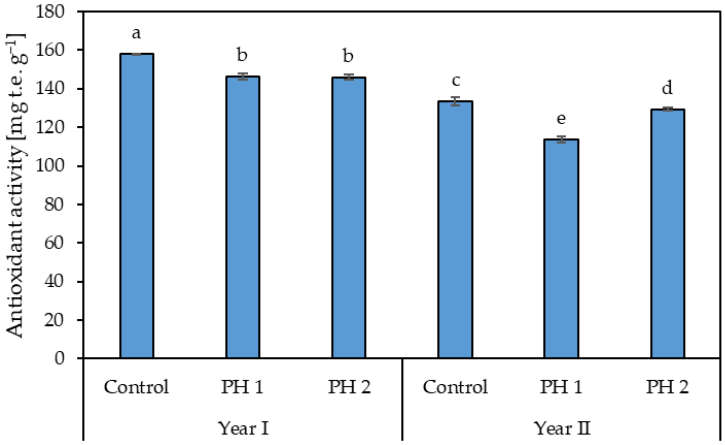

Figure 8. Influence of the interaction Y × F on antioxidant activity. Means and SDs are reported. The values followed by the same letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

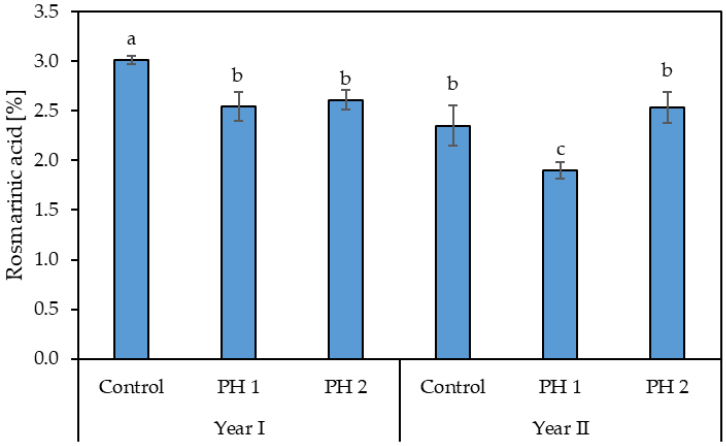

Figure 9. Influence of the interaction Y × F on rosmarinic acid. Means and SDs are reported. The values followed by the equal letter are not significantly different for $p \leq 0.05$, according to Tukey's test. C: control (only water); PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

As reported in Table 5, statistical analysis of the three main compounds of oregano EO (γ-terpinene, car-vacrol methyl ether, and thymol) revealed no significant differences (p-value > 0.01) due to year (Y) factor, foliar treatments (F) factor, and their interaction (Y × F). The percentages of the three compounds were similar across all treatments and years.

Table 5. Percentage of the three main compounds of oregano essential oil in response to treatments during the two-year study.

Source of Variance	γ-Terpinene [%]	Carvacrol Methyl Ether [%]	Thymol [%]
Year (Y)			
I	14.0 a	5.1 a	50.6 a
II	13.7 a	5.1 a	52.7 a
Foliar treatments (F)			
Control	14.6 a	4.9 a	54.6 a
PH 1	14.0 a	5.1 a	50.2 a
PH 2	12.9 a	5.2 a	50.1 a
Significance			
Y	n.s.	n.s.	n.s.
F	n.s.	n.s.	n.s.
Y × F	n.s.	n.s.	n.s.

Means and standard deviations are shown. Values with different letters are significantly different for $p \leq 0.05$, according to the Fisher LSD test. n.s. = no significant. Control: only water; PH 1: protein hydrolysates derived from hydrolyzed animal epithelium; PH 2: protein hydrolysates obtained from *Fabaceae*.

4. Discussion

The global expansion of oregano cultivation reflects its versatile applications in several sectors, such as food, chemical and cosmetic [1,3,6,10]. Developing advanced and effective strategies to optimize yields and qualitative parameters of MAPs is essential to meet the demands of modern sustainable agriculture. Our observations suggest that foliar application of protein hydrolysates represents a valid tool to enhance the resilience of oregano grown under rainfed conditions. In our study, the physiological status of the plants was improved, as highlighted by the chlorophyll content. Plants treated with both protein hydrolysates showed higher photosynthetic pigment concentrations compared to the control, which likely contributed to the enhanced vigour observed. The results indicate that foliar biostimulants are highly effective in maximising crop productivity, in terms of fresh and dry yields. The most striking improvement was observed in biomass accumulation, where PH 1 was the most effective treatment, nearly doubling the fresh and dry yields compared to the control plants in the second year. The bioactive peptides and amino acids contained in PH likely trigger signalling pathways that improve water use efficiency and stomatal regulation, thereby counteracting the physiological strain caused by thermal and water stresses [22,23]. The action promoted by biostimulants effectively creates a buffer against adverse climatic factors, leading to a higher productive response [35]. Furthermore, PH application supports the synthesis of bioactive compounds, thus preserving the qualitative standards of the final product, even under sub-optimal water availability [27,36]. As documented by several studies [21,36,37], foliar biostimulants can enhance oregano's efficiency in CO_2 utilisation, water uptake, root growth, and the synthesis of growth-promoting substances. The application of PH 1 increases fresh biomass from 77.4% to 127.5%, and PH 2 from 61.3% to 103.9%. The significant increase in biomass production in treated plants can be attributed to the action of the bioactive molecules, such as peptides and free amino acids, contained in the protein hydrolysates [22,28]. These compounds stimulated carbon and nitrogen metabolism, leading to vigorous vegetative growth, as confirmed by the literature [26,37]. Peptides and amino acids within PHs act as signalling molecules that upregulate nitrate reductase (NR) and glutamine synthetase (GS), the rate-limiting enzymes in nitrogen assimilation [19,38]. In oregano, this enzymatic

stimulation promotes a more efficient conversion of inorganic nitrogen into organic forms, improving the synthesis of proteins and chlorophyll [37,39]. Furthermore, PH applications can increase the activity of Rubisco and other enzymes involved in the Calvin cycle, thereby optimizing the photosynthetic rate [40,41].

Our data showed a strong correlation between the higher yield and the improvements in both plant height and leaf chlorophyll content, suggesting an optimisation of the photosynthetic apparatus. Improvements in chlorophyll content have been observed in oregano [36,37], but also in other species [27,42], using treatments with foliar protein hydrolysates. Our findings confirm that PH application enhances chlorophyll biosynthesis and cell elongation, even under environmental constraints. The positive effect of foliar application of protein hydrolysates (PHs) on the biomass yield of oregano observed in this study aligns with extensive findings in the literature regarding the use of biostimulants in horticulture [21,26]. In a similar study, the authors revealed that the application of PH produced an enhancement in rosemary productivity, both in terms of biomass and EO yields [27]. This increase in vegetative growth can be attributed to the direct supply of signalling molecules such as soluble peptides and free amino acids contained in both animal- and plant-derived PH [28,43]. Several authors [26,43,44] have reported that these compounds stimulate primary metabolism by upregulating carbon- and nitrogen-assimilation enzymes and improving nutrient uptake efficiency [24,45]. Specifically, the response observed in oregano also results in other leafy crops, such as spinach (*Spinacia oleracea* L.) [46] and lettuce [47], where PHs were shown to elicit auxin- and gibberellin-like activities, promoting cell division and expansion, and ultimately leading to superior fresh- and dry-matter accumulation. PH 2-treated plants occasionally showed a slight benefit in EO content; the overall EO yield per hectare was statistically similar when comparing the two biostimulants treatments. Compared to control plants, the application of both PHs generated an average EO yield increase of approximately 41–42%. This suggests that the increase in total oil production is primarily driven by the substantial increase in vegetative biomass, rather than the concentration of secondary metabolites in the tissue [48,49]. Our findings indicate that the expansion of vegetative biomass is linked to the developmental patterns and spatial distribution of glandular trichomes. In oregano, these specialized epidermal appendages serve as the primary metabolic hubs for the synthesis and storage of volatile compounds [50]. The application of PH may promote the early formation of these structures, suggesting a synergistic relationship where enhanced vegetative growth provides the necessary physiological framework to support a higher concentration of secretory tissues [43,51]. Both formulations represent a great agronomic advantage over untreated plants. It is plausible that PH application can reduce oxidative stress in the plant tissues, allowing for a more efficient allocation of carbon resources toward glandular development [52,53]. No significant effects have been observed considering the three main EO components, but the foliar application of PH affected the total phenolic content, antioxidant activity and rosmarinic acid. Many authors affirm that the biosynthesis of the secondary metabolite in MAPs, including oregano, is influenced by several factors [54–58], with biostimulants acting as potential modulators of gene regulation and enzymatic activity [59]. Despite various studies [60–62] having reported that exposing species to microbial and non-microbial biostimulants typically increases the quantity of secondary metabolites, our findings present an opposite trend. In the present study, the highest accumulation of bioactive compounds has been observed in the untreated plants. Our findings suggest that stress conditions may stimulate the biosynthesis of secondary metabolites in oregano but, at the same time, biostimulant application tends to improve biomass production by alleviating plant stress. The overall positive effect of biostimulants on secondary metabolism is not absolute, but context-dependent [63,64]. Since secondary metabolites often accumulate as

a defence response to stress, the application of the biostimulant likely alleviated physiological constraints, shifting metabolic resources towards vegetative growth, rather than defence [46,65–67]. Therefore, our data suggest avoiding broad generalities: the impact of biostimulants must be interpreted by considering the balance between stress conditions and the plant's physiological well-being. A deeper exploration of the metabolic pathways—both primary and secondary—is required, to better understand how these substances modulate MAP productivity and quality.

5. Conclusions

The present study provides evidence regarding the effectiveness of foliar biostimulants in enhancing the agronomic performance of oregano. From a productivity perspective, the findings point to two distinct, yet equally effective, pathways to attaining high levels of yield. While PH 1 was the superior driver for biomass accumulation, PH 2 induced a marginal increment in the EO percentage; consequently, the overall EO yield per unit area remained similar to that observed in the PH 1-treated plants. This suggests that farmers may choose either biostimulant, based on their main commercial objective, whether maximizing biomass production or enhancing extraction efficiency.

Regarding the phytochemical profile, the data revealed a trade-off between rapid growth and secondary metabolite accumulation. In this case, PH application generated a better growth condition and a decline in the accumulation of some secondary metabolites. However, this metabolic shift did not compromise the qualitative standards of the essential oil. The stability of the chemotype—specifically, the lack of significant variation in thymol, γ-terpinene, and carvacrol methyl ether—confirms that these biostimulants can boost industrial yields without altering the pharmaceutical or aromatic signature of the final product.

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