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# Animal and plant-derived protein hydrolysates positively affect yield traits but produce contrasting response on chemicals of organic rosemary (*Salvia rosmarinus* Spenn.) grown under rainfed conditions

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ABSTRACT

Rosemary (*Salvia rosmarinus* Spenn.) is a perennial crop which is well known for its antibacterial, anti-inflammatory, and antioxidant properties. It is widely documented that the biosynthesis of bioactive compounds in this plant is strongly influenced by endogenous and exogenous factors. To enhance yield production and reduce the effects of these factors on open field crops, certain agronomic practices can be optimized through the use of innovative products. The foliar application of protein hydrolysates is recognized as a good practice to achieve this. A two-year study was carried out to investigate the impacts of two different protein hydrolysates (animal-based and plant based) on the yield traits and chemical parameters of rosemary. A randomized complete block design was used with three replicates. The results highlighted that both biostimulants produced a substantial increase in fresh biomass between 1.4 and 3.9 Mg ha$^{-1}$, and in dry biomass between 0.5 and 1.3 Mg ha$^{-1}$, in comparison with the control plants. It was found that the lowest dose of plant-based protein hydrolysates significantly increased essential oil yields, producing yields of over twenty kilograms per hectare. It is worth noting that the application of both protein hydrolysates did not modify the aromatic profile of the essential oil. In addition to this, contrasting responses were observed when considering the effect of these biostimulants on the antioxidant activity, phenolic content, and rosmarinic acid. The results of this study demonstrate that protein hydrolysates improve the yield of rosemary plants cultivated under organic agriculture conditions.

## 1. Introduction

Rosemary (*Salvia rosmarinus* Spenn.) is an evergreen perennial shrub, native to the Mediterranean region (Mwithiga et al., 2022). Similar to other medicinal and aromatic plants (MAPs), this species is used in various culinary traditions all over the world due to its many health benefits and intense aroma (Aziz et al., 2022). Rosemary has numerous bioactive compounds that can enhance the digestive process, reduce inflammation and provide antioxidant benefits (Veenstra and Johnson, 2021). Furthermore, it is rich in carnosic and rosmarinic acid, both of which have strong antioxidant properties. These chemicals strengthen the health-promoting properties of the species by helping to scavenge free radicals and provide defense against oxidative stresses (Topal and Gulcin, 2022; Li Pomi et al., 2023). The essential oils (EO) are used for their pleasant aroma and therapeutic benefits, influencing mood, cognition, and overall well-being (Pirzad and Mohammadzadeh, 2018; Fierascu et al., 2021; Tuttolomondo et al., 2021). The main compounds, such as cineole and camphor, contribute to its preservative properties, making it valuable for food preservation and as a natural disinfectant (Jafarı-sales and Pashazadeh, 2020; Günther et al., 2022). The biosynthesis of bioactive compounds takes place through metabolic pathways using intermediate products of primary metabolism. These compounds accumulate in plant cells either due to biochemical inefficiencies or as part of a normal physiological process, (Julsing et al., 2006). The products of secondary metabolism are multifunctional and help restore cellular properties when specific events disrupt the balance of the plant organism (Borges et al., 2017). These substances are commonly stored in various parts of the plant, such as the flowers, leaves, roots, and seeds,

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and their concentration levels can vary depending on environmental conditions, genetic factors, and phenological stages (Julsing et al., 2006; Virga et al., 2020; Tuttolomondo et al., 2021; Huang et al., 2022). Yield and qualitative parameters of the crop are influenced by agronomic factors, including fertilization and irrigation practices, plant density, soil management, and harvest time (Sabatino et al., 2013; Chrysargyris et al., 2022; Nurzyńska-Wierdak et al., 2023; Farruggia et al., 2024a; Di Miceli et al., 2022). Understanding the impact of these practices on the plant could help optimize the yield traits of rosemary, promoting sustainability and maximizing economic value.

The availability of micro- and macro elements in the soil is widely considered to be fundamental in medicinal and aromatic plants (MAPs), affecting plant growth and increasing the synthesis of secondary metabolites (Farruggia et al., 2023; Nurzyńska-Wierdak et al., 2023). The application of biostimulants can influence both primary and secondary metabolism in plants and improve tolerance to adverse climate and soil conditions, as confirmed by previous studies (Ertani et al., 2013; Lucini et al., 2015; Amato et al., 2024; Farruggia et al., 2024a). Protein hydrolysates (PH) are a type of biostimulant obtained from the chemical or enzymatic hydrolysis of animal or plant-based proteins (Colla et al., 2015). Collagen derived from tanning-industry waste is the most frequently used source of animal protein hydrolysates, while legumes are currently used to generate plant protein hydrolysates (Colla and Rouphael, 2015; Du Jardin, 2015). Protein hydrolysates are a mixture of polyprotein hydrolysates, oligo-protein hydrolysates and amino acids (Rouphael et al., 2017), and can be applied to leaves or roots to induce specific plant physiological response, increase crop yields, and improve plant tolerance to stress conditions (Rouphael et al., 2022; Di Miceli et al., 2023). Di Miceli et al. (2023) observed increases in growth, yield and quality parameters in eggplant (*Solanum melongena* L.) treated with plant-derived PH under open field conditions. Similar results were obtained by Sabatino et al. (2021) treating lettuce (*Lactuca sativa* L.) with plant PH. These authors observed significant improvements in yield and yield-related features, nutritional characteristics, functional traits, and nitrogen indices. There seem to be controversial findings in the literature regarding the use of animal/plant PH, and, in some cases, negative effects have been observed (Ertani et al., 2009; De Lucia and Vecchietti, 2012; Moe, 2013; Corte et al., 2014; Colla et al., 2015; Visconti et al., 2015; Wilson et al., 2015; Colla et al., 2017; Pérez-Aguilar et al., 2024).

To the best of our knowledge there are no studies on the effects of foliar application of PH on rosemary yields and its chemical composition, and studies which compare the effects of different types of PH on rosemary are notably lacking. With this in mind, the aims of this study were: (i) to evaluate the effect of two different PHs types (animal-derived and plant-derived) on morphological, and yield parameters of rosemary plants grown under organic farming conditions; (ii) to investigate the impact of PH application on EO characteristics, and; (iii) to determine how antioxidant activity, rosmarinic acid (RA), and total phenolic (TP) varied depending on the PH dose. 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 positive effects on other chemicals in rosemary plants grown under rainfed conditions.

## 2. Materials and methods

### *2.1. Test site, experimental field and cultivation practices*

The trials were carried out at an organic farm (37°22′32.71″N, 13°38′33.59″E), situated in the countryside of Aragona, South-Western Sicily (Italy) during the growing seasons 2021–2022 and 2022–2023. The soil has a sandy clay loam texture (48 % sand, 26 % silt, and 26 % clay) and shows 1.1 % organic matter, 1.3 % total nitrogen, 22.4 ppm assimilable phosphate, 330.0 ppm assimilable potassium, with a pH of 7.2. It is classified as Regosols (typic xerorthents), in accordance with the United States Department of Agriculture (USDA). The climate of the site is warm temperate with dry and hot summers and mild winters, following the classification provided by Köppen–Geiger (Kottek et al., 2006).

The experimental field of rosemary was established in February 2019 by transplanting herbaceous cuttings obtained from mother plants. The distance between rows and between plants on the same row was 2.5 and 1.0 m, respectively, ensuring a density of 4000 plants ha$^{-1}$. Fertilization was carried out by applying 2.0 Mg ha$^{-1}$ of cattle manure (0.5 % of N, 0.2 % of $P_2O_5$, 0.7 % $K_2O$) prior to transplantation. Rosemary plants were grown under rainfed conditions and weed control was mechanical. In both years, harvesting occurred once per year by cutting the plant to 0.30 m above ground level. Plants were manually harvested during the third 10-day period of June.

### *2.2. Weather data*

During the two-year study, average maximum and minimum air temperatures and total rainfall were monitored from a weather station, owned by the Sicilian Agro-Meteorological Information Service (SIAS, 2024), and situated close to the farm. Fig. 1 (A and B) shows the temperature and rainfall trends (10-day periods).

A total rainfall of 611 mm (2021–2022) and 538 mm (2022–2023) was recorded during the growth period; however, the distribution of the rainfall differed considerably over the two years. During the 1st growing season, 80 % of rainfall (480 mm) was mainly concentrated in October to January, whilst only 15 % (92 mm) was recorded at the onset of vegetative growth through to the harvest date. During the 2nd growing season, rainfall was spread over a wider period from September to March. In spring, May was the wettest month with well-distributed rainy days. Temperature trends influenced the phenological stages of rosemary and were largely consistent with the average temperature of the study area. In both growing seasons, an increase in minimum and maximum temperatures was detected from pre-flowering stage to harvest. When comparing the two growing seasons, the highest maximum temperatures were recorded in the 2nd growing season.

### *2.3. Treatments*

Two protein hydrolysates were used for foliar applications:

- Animal protein hydrolysate (APH), derived from hydrolyzed animal epithelium, with organic nitrogen 8.0 %, organic carbon 27.0 %, amino acids 50.0 % and free amino acids 15 %.
- Plant protein hydrolysate (PPH), obtained from *Fabaceae* with amino acids and plant peptides (31 %), organic nitrogen (5 %) and organic carbon (25 %).

For each biostimulant, two doses were applied. Taking into consideration the nitrogen (N) content of each protein hydrolysate, each biostimulant dose was formulated to ensure all treatments provided.

the same total quantity of N (Table 1).

A total of 4 foliar applications were performed during the vegetative growth stage. Control (C) treatment plants were sprayed with only water. 400.0 L of water ha$^{-1}$ were used for each application and the first event was carried out in the first 10-day period of April. Foliar treatments were made using a portable hand-sprayer 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 foliar sprays to ensure uniform application and consistent dosage, and treatments were applied early in the morning to guarantee optimal foliar absorption (Ruiz-Navarro et al., 2019). The plot size was 50 m$^2$ (2.5 m × 20.0 m) with 20 plants per plot. These 20 plants were taken into consideration for the subsequent measurements. A randomized complete block design with three replications was adopted as the experimental design.

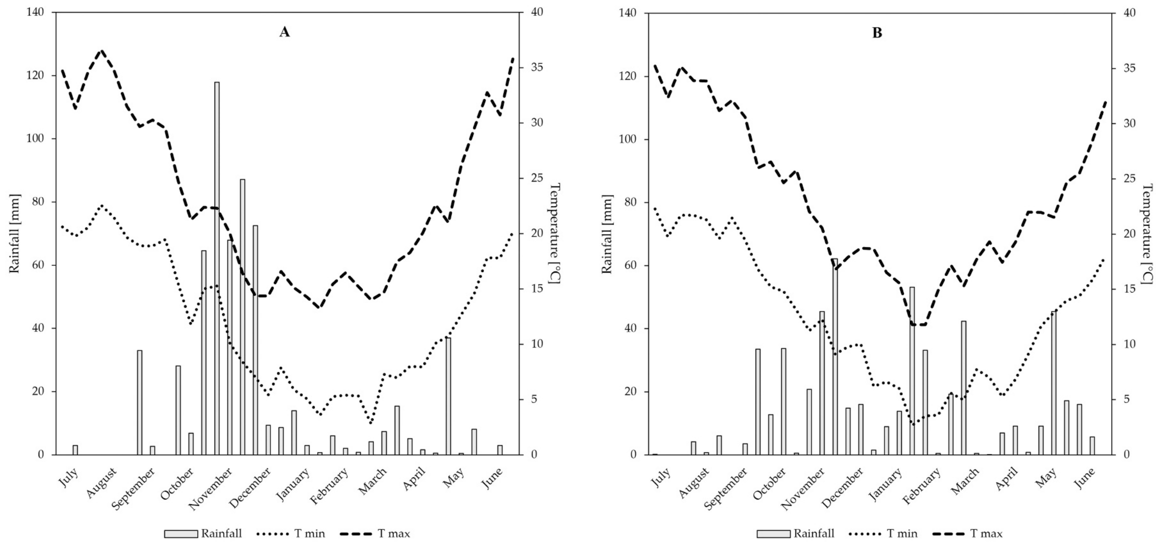


**Fig. 1.** Temperature and rainfall trends during the first (A) and the second (B) year of study.

**Table 1**
The protein hydrolysates doses.

| Biostimulant | Doses for each application [L hL$^{-1}$] | Abbreviation |
|---|---|---|
| Animal protein hydrolysates | 0.100 | APH−100 |
| | 0.200 | APH−200 |
| Plant protein hydrolysates | 0.150 | PPH−150 |
| | 0.300 | PPH−300 |

### 2.4. Morphological traits and biomass yield

When the plants were harvested, plant height, relative water content (RWC), chlorophyll content (CC), and total fresh yield (FY) were recorded. Harvested plants were dried in a shaded and ventilated environment for approximately 10 days at a temperature of 25–30 °C and total dry yield (DY) was determined. Chlorophyll content was measured using a Dualex Scientific (Force A, Orsay, France) portable chlorophyll meter, and the device was used on 40 leaves per plot. Relative water content was evaluated by applying the method described by Farruggia et al. (2024b) and the following equation by Alyemeni et al. (2018):

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

where FW is the fresh weight, DW is the dry weight, and TW is the turgid weight.

### 2.5. Essential oil content, composition and yield

For each plot, 500.0 g of air-dried plant material was hydro-distilled for 3 h to obtain essential oils, in accordance with the Ph. Eur. 7.0, 20812 (European Pharmacopoeia, 2008). For each treatment, 3 EO extractions were performed. Essential oil concentration was calculated by dividing EO volume for each sample by biomass weight. Essential oil yield was calculated by multiplying EO content by the total dry yield. EO samples were stored at −18 °C. Methods for determination of the EO components were described in detail by Farruggia et al. (2024c).

### 2.6. Determination of chemical parameters

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:

- antioxidant activity (AA), expressed as milligram trolox equivalents per gram of dry weight (mg t.e. g$^{-1}$ dw), using the method described by Chizzola et al. (2008);
- rosmarinic acid (RA) content, as described Farruggia et al. (2024c);
- total phenolic (TP) content, expressed as milligram caffeic acid equivalents per gram of dry weight (mg c.a.e. g$^{-1}$ dw), adopting the method provided by Lamien-Meda et al. (2010).

### 2.7. Statistical analyses

Data for each year were subjected to one-way analysis of variance (ANOVA). Differences between means were compared using Tukey's test ($p \leq 0.05$). In the linear model/ANOVA, foliar treatments were used as fixed effects. Before ANOVA, the RWC data were subjected to arcsine transformation. 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

### 3.1. Morphological traits and biomass yield

Data on morphological traits of rosemary plants subjected to foliar applications with animal and plant-based protein hydrolysates are shown in Table 2. ANOVA revealed that, in both years, all morphological parameters were significantly affected ($p \leq 0.01$) by foliar treatments with both biostimulants, with the exception of RWC (Table 2). During the 1st year, no significant differences in plant height were found between APH- and PPH-treated plants at any dose of application. However, substantial variations were observed when APH- and PPH-treated plants were compared to control plants, highlighting the effect of biostimulants on plant growth. In general, plant height ranged from 46.5 cm to 71.8 cm, depending on the use or not of biostimulant. The highest average value (71.8 cm) was recorded in the 2nd year for plants treated with PPH-150 every two weeks. In the 2nd year, it is worth noting that, for each protein hydrolysate, the best performance was achieved by applying of the lowest dose of biostimulant.

Concerning chlorophyll content, significant differences were found

**Table 2**
Influence of foliar treatment on rosemary plant height, chlorophyll content and relative water content (RWC) during the two-year research period.

| Foliar treatment | Plant height [cm] | | Chlorophyll content [μg cm$^{-2}$] | | RWC [%] | |
|---|---|---|---|---|---|---|
| | I year | II year | I year | II year | I year | II year |
| C | 48.3 b | 46.5 d | 29.6 b | 28.4c | 79.3 ab | 77.2 a |
| APH−100 | 60.6 a | 68.2 bc | 29.8 b | 30.0c | 76.9 b | 82.6 a |
| APH−200 | 60.2 a | 66.0c | 31.9 a | 33.4 ab | 79.8 a | 80.8 a |
| PPH−150 | 60.4 a | 71.8 a | 31.2 ab | 32.7 b | 79.6 a | 79.4 a |
| PPH−300 | 60.3 a | 67.0 b | 30.8 ab | 34.6 a | 79.2 ab | 74.0 a |
| *p*-value | 0.000 | 0.000 | 0.007 | 0.000 | 0.022 | 0.149 |

Means are shown. The values followed by different letters are significantly different for $p \leq 0.05$ according to Tukey's test. C = control (only water); APH-100 = 100 mL hL$^{-1}$ of animal protein hydrolysate; APH-200 = 200 mL hL$^{-1}$ of animal protein hydrolysate; PPH-150 = 150 mL hL$^{-1}$ of plant protein hydrolysate; PPH-300 = 300 mL hL$^{-1}$ of plant protein hydrolysate.

in both years between control plants and treated-plants, and between APH- and PPH-treated plants. In the 2nd year, regardless of the dose, foliar applications of APH and PPH produced higher chlorophyll content in rosemary plants compared to the 1st year. The highest chlorophyll content (34.6 μg cm$^{-2}$) was obtained with the application of PPH-300 during the 2nd year, confirming the effectiveness of the biostimulants. The lowest content (28.4 μg cm$^{-2}$) was detected on average in control plants. When comparing the two years, no significant differences were found between control plants and APH-100-treated plants, whilst differences were observed with the slightly higher application of APH-200, highlighting the importance of the dose in this type of biostimulant.

In the case of RWC, ANOVA showed a significant effect ($p \leq 0.01$) for the foliar application of APH and PPH on rosemary plants in the 1st year alone. The highest RWC values were determined in APH-200- (79.8 %) and PPH-150-treated plants (79.6 %). It should be noted that control plants showed higher RWC than APH-100-treated plants, highlighting the limited effect of this dose regarding APH. In the 2nd year, no increases in RWC values were observed in treated plants compared to control plants.

The ANOVA results indicated that foliar treatment with APH and PPH had significant effects ($p \leq 0.01$) on FY and DY in both years (Fig. 2). Fresh yield results differed based on the year, type of biostimulant and application dose. In the 1st year, APH-100- (6.0 Mg ha$^{-1}$), PPH-150- (6.5 Mg ha$^{-1}$) and PPH-300-treated plants (6.0 Mg ha$^{-1}$) obtained the highest biomass yields on average. This trend changed for the 2nd year and the best biomass yield performance was observed in the PPH-150-treated plants (6.8 Mg ha$^{-1}$). When comparing DY over the two years, applications of APH and PPH produced similar trends of biomass yield both with the lowest and highest dose. This confirms that treatments with biostimulants increased the crop yield compared to control plants. In particular, PPH-150-treated plants showed the highest crop yields both in the 1st (2.5 Mg ha$^{-1}$) and 2nd year (2.4 Mg ha$^{-1}$). It is worth mentioning the fact that the lowest dose of both biostimulants produced the highest biomass yields in rosemary plants.

### 3.2. Essential oil content, composition and yield

Table 3 and Fig. 3 summarize the results of ANOVA for essential oil content and yield. The statistical test revealed that foliar treatments with APH and PPH significantly affected ($p \leq 0.01$) EO content and yield in both years. The effects of biostimulant application on EO content differed over the two years. During the 1st year, significant differences were found between control plants and treated plants. However, when comparing the various treatments, no significant variations were detected. It is worth noting that control plants produced the highest EO content (2.1 %). By contrast in the 2nd year, the highest EO content

**Table 3**
Influence of foliar treatment on rosemary essential oil (EO) content during the two-years research.

| Foliar treatment | EO content [%] | |
|---|---|---|
| | I year | II year |
| C | 2.1 a | 2.2c |
| APH−100 | 1.6 b | 2.4 ab |
| APH−200 | 1.6 b | 2.3 b |
| PPH−150 | 1.5 b | 2.5 a |
| PPH−300 | 1.5 b | 2.3 bc |
| *p*-value | 0.000 | 0.000 |

Means are shown. The values followed by different letters are significantly different for $p \leq 0.05$ according to Tukey's test. C = control (only water); APH-100 = 100 mL hL$^{-1}$ of animal protein hydrolysate; APH-200 = 200 mL hL$^{-1}$ of animal protein hydrolysate; PPH-150 = 150 mL hL$^{-1}$ of plant protein hydrolysate; PPH-300 = 300 mL hL$^{-1}$of plant protein hydrolysate

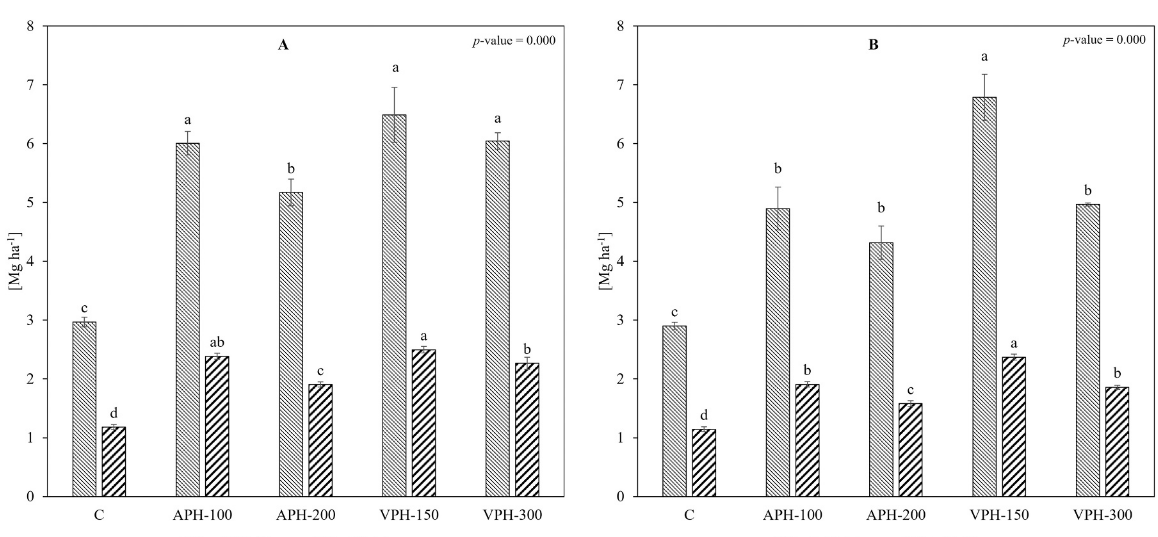


**Fig. 2.** Influence of foliar treatment on fresh and dry yield (DY) of rosemary. Bars indicate mean ± SD. The values with different letters are significantly different for $p \leq 0.05$ according to Tukey's test. C = control (only water); APH-100 = 100 mL hL$^{-1}$ of animal protein hydrolysate; APH-200 = 200 mL hL$^{-1}$ of animal protein hydrolysate; PPH-150 = 150 mL hL$^{-1}$ of plant protein hydrolysate; PPH-300 = 300 mL hL$^{-1}$ of plant protein hydrolysate.

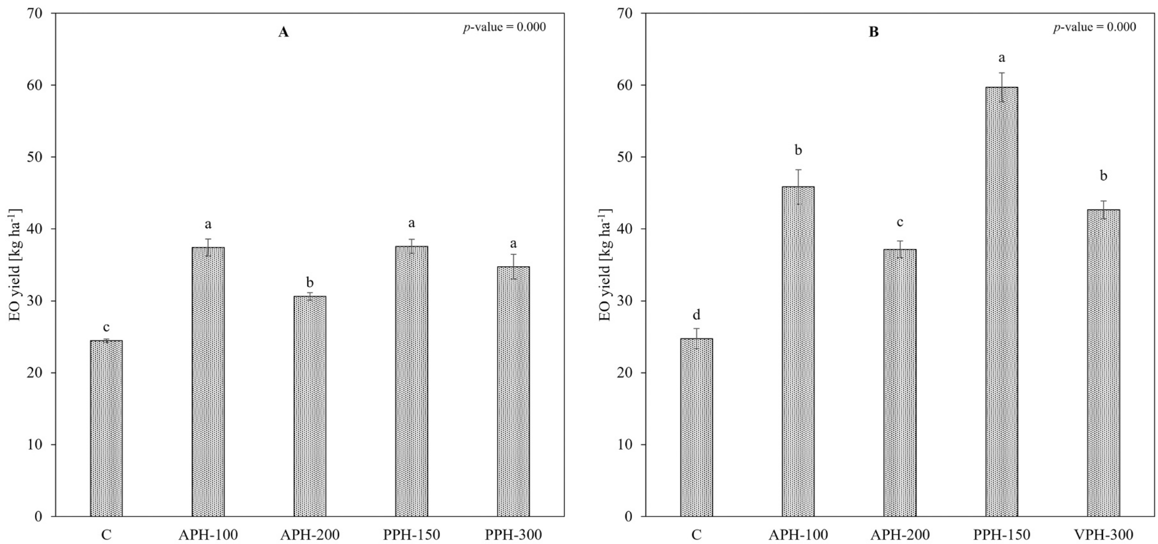


**Fig. 3.** Influence of foliar treatment on rosemary essential oil (EO) yield during the two-years research. Bars indicate mean ± SD. The values with different letter are significantly different for $p \leq 0.05$ according to Tukey's test. C = control (only water); APH-100 = 100 mL $hL^{-1}$ of animal protein hydrolysate; APH-200 = 200 mL $hL^{-1}$ of animal protein hydrolysate; PPH-150 = 150 mL $hL^{-1}$ of plant protein hydrolysate; PPH-300 = 300 mL $hL^{-1}$ of plant protein hydrolysate.

(2.5 %) was recorded in PPH-150-treated plants, whilst the lowest (2.2 %) in control plants. Moreover, greatest EO performance was obtained with the lowest doses of APH and PPH (Table 3).

In the case of EO yield, similar trends were observed in both years (Fig. 3). In general, control plants had similar EO yield values in both years whilst the highest performance for treated-plants were recorded on average in the 2nd year. In particular, application of the lowest doses of APH generated the highest EO yields both in the 1st year (37.4 kg $ha^{-1}$) and 2nd year (45.9 kg $ha^{-1}$). On the contrary, when considering foliar treatment with PPH, similar values of EO yield were obtained regardless of the dose during the 1st year only. In the study period, the highest EO yield performance (59.7 kg $ha^{-1}$) was detected in PPH-150-treated plants in the 2nd year.

Table 4 refers to the main EO compounds. Gas chromatography-mass spectrometry detected a total of 29 compounds. The ANOVA results showed that foliar application with APH and PPH produced significant effects ($p \leq 0.01$) on comparatively few EO compounds, with variations depending on the year. For example, in the 1st year, borneol was the only compound whose percentage was significantly affected by protein hydrolysates. The highest value (4.06 %) was found in APH-200-treated plants. No significant differences were found for other EO compounds when comparing control plants with treated plants. During the 2nd year, the treatment factor had significant effects on the percentages of tricyclene, terpinolene, bornyl acetate, and α-copaene. In the case of tricyclene, the highest percentage value (0.22 %) was obtained in control plants. The best performance for terpinolene was obtained in control plants (1.31 %), APH-100- (1.31 %) and PPH-300-treated plants (1.31 %). Applications of both doses of APH and PPH determined similar percentages for bornyl acetate, ranging from 3.56 % to 3.11 %. Finally, the application of the highest dose of APH resulted in the highest percentage (0.54 %) for α-copaene, whereas no differences were found with varying doses of PPH.

### 3.3. Antioxidant activity, rosmarinic acid and total phenolic contents

The results of the ANOVA revealed that foliar treatment with PH significantly affected ($p \leq 0.05$) antioxidant activity (AA) and phenolic content (PC) of rosemary extracts in both years, as shown in Table 5. During the 1st year, the highest AA values were recorded in control plants and PPH-300-treated plants, 140.8 and 138.8 mg t.e. $g^{-1}$, respectively. Conversely, in the 2nd year, the application of APH-200 generated the highest AA value (144.1 mg t.e. $g^{-1}$). In both years, the best performances were obtained by applying the highest doses of both biostimulants. Regarding PC, in the 1st year, the highest PC (109.2 mg c.a.e. $g^{-1}$), on average, was recorded in control plants. It is interesting to observe that no significant differences were found between the various treatments regarding either biostimulant. On the contrary, during the 2nd year, APH-100- and PPH-150-treated plants produced the highest PC values, with 81.2 and 82.1 mg c.a.e. $g^{-1}$, respectively. It is also worth noting that the best performances were obtained when using both biostimulants at their lowest dose. As regards the rosmarinic acid content, ANOVA showed that the foliar treatment factor had a significant effect during the 2nd year only. The highest percentage (1.6 %) was recorded in control plants and APH-200- and PPH-300-treated plants. By contrast, the highest doses of APH and PPH produced the best results for this parameter.

## 4. Discussion

### 4.1. Morphological and yield traits

The management of available resources and the correct choice of agronomic practices are of fundamental importance for the agricultural sector to ensure high quality standards for products. Nutrition thus plays a key role in the growth and development of crops, and the application of low doses of PHs can increase the nutritional availability of substrates, nutrient absorption and improve nutrient usage efficiency also in MAPs (Nardi et al., 2016; Paul et al., 2019).

The application of the two PHs over both years led to increases in plant height of between 12 and 25 cm compared to untreated plants. These values were consistent with those reported by Singh and Wasnik (2013) and Mostafa (2019) who assessed the effect of organic and inorganic fertilizers on growth, and chemical composition of rosemary plants. Some authors observed similar plant heights and chlorophyll content in rosemary plants when using different biostimulants (Waly et al., 2019; Al-Fraihat et al., 2023; Farruggia et al., 2024b). Carillo et al. (2022) report that root devlovement can be improved by the application of bioactive peptides contained in plant-derived protein hydrolysates, which can elicit auxin and/or gibberellin-like activities. This indirectly enhances the efficiency of nutrient uptake and exploitation, plant growth, and yield, as described by Colla et al. (2017). In addition, PHs have been demonstrated to positively affect chloroplast size and

**Table 4**
Components of rosemary EO and p-value in response to foliar treatment during the two-year research period.

| Compounds | $RI_{calc}$ | $RI_{lit}$ | I year | | | | | | II year | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Foliar treatment | | | | | | Foliar treatment | | | | | |
| | | | C | APH-100 | APH-200 | PPH-150 | PPH-300 | *Significance* | C | APH-100 | APH-200 | PPH-150 | PPH-300 | *Significance* |
| tricyclene | 919 | 921 | 0.11 | 0.11 | 0.22 | 0.26 | 0.16 | n.s. | 0.22 a | 0.18 ab | 0.15 b | 0.18 ab | 0.19 ab | * |
| a-thujene | 923 | 924 | 0.18 | 0.20 | 0.18 | 0.20 | 0.22 | n.s. | 0.24 | 0.22 | 0.18 | 0.22 | 0.21 | n.s. |
| a-pinene | 931 | 932 | 35.52 | 36.75 | 35.44 | 38.59 | 42.93 | n.s. | 36.89 | 32.92 | 33.60 | 35.29 | 33.25 | n.s. |
| camphene | 943 | 946 | 3.93 | 4.36 | 3.96 | 4.36 | 4.59 | n.s. | 4.18 | 3.66 | 3.51 | 3.85 | 3.79 | n.s. |
| thuja−2,4(10)-diene | 949 | 953 | 1.02 | 1.01 | 0.86 | 1.04 | 1.03 | n.s. | 0.90 | 0.93 | 0.89 | 0.89 | 0.88 | n.s. |
| sabinene | 972 | 969 | 1.08 | 1.20 | 1.16 | 1.21 | 1.27 | n.s. | 2.50 | 2.18 | 2.16 | 2.34 | 2.19 | n.s. |
| b-myrcene | 990 | 988 | 1.33 | 1.32 | 1.26 | 1.17 | 1.35 | n.s. | 1.19 | 1.08 | 1.08 | 1.10 | 1.09 | n.s. |
| a-phellandrene | 1002 | 1002 | 0.32 | 0.33 | 0.29 | 0.31 | 0.34 | n.s. | 0.39 | 0.38 | 0.34 | 0.37 | 0.38 | n.s. |
| a-terpinene | 1014 | 1014 | 0.79 | 0.83 | 0.77 | 0.81 | 0.88 | n.s. | 1.02 | 1.00 | 0.93 | 1.00 | 1.01 | n.s. |
| p-cymene | 1022 | 1024 | 1.55 | 1.55 | 1.43 | 1.41 | 1.52 | n.s. | 0.59 | 0.53 | 0.51 | 0.49 | 0.56 | n.s. |
| 1.8 cineol | 1029 | 1026 | 27.00 | 26.83 | 27.01 | 27.79 | 25.45 | n.s. | 24.43 | 24.20 | 24.92 | 25.08 | 24.44 | n.s. |
| γ-terpinene | 1055 | 1054 | 0.96 | 1.01 | 1.01 | 1.20 | 1.29 | n.s. | 1.73 | 1.75 | 1.68 | 1.72 | 1.80 | n.s. |
| terpinolene | 1085 | 1088 | 0.75 | 0.81 | 0.73 | 0.63 | 0.73 | n.s. | 1.31 a | 1.31 a | 1.22 b | 1.27 ab | 1.31 a | * |
| linalool | 1099 | 1098 | 1.93 | 1.65 | 1.90 | 1.39 | 1.21 | n.s. | 1.61 | 1.86 | 1.92 | 1.72 | 1.83 | n.s. |
| chrysanthenone | 1121 | 1124 | 0.47 | 0.46 | 0.52 | 0.39 | 0.37 | n.s. | 0.79 | 0.83 | 0.71 | 0.71 | 0.77 | n.s. |
| camphor | 1138 | 1141 | 2.36 | 2.26 | 2.34 | 2.11 | 1.85 | n.s. | 1.96 | 2.07 | 2.12 | 1.96 | 2.08 | n.s. |
| pinocarvone | 1155 | 1160 | 0.48 | 0.39 | 0.48 | 0.36 | 0.27 | n.s. | 0.33 | 0.36 | 0.36 | 0.34 | 0.36 | n.s. |
| borneol | 1160 | 1165 | 3.83 ab | 3.29 ab | 4.06 a | 3.05 ab | 2.41 b | * | 3.40 | 4.03 | 4.04 | 3.60 | 3.97 | n.s. |
| terpinen−4-ol | 1172 | 1174 | 0.63 | 0.50 | 0.65 | 0.39 | 0.28 | n.s. | 0.44 | 0.52 | 0.50 | 0.46 | 0.49 | n.s. |
| α-terpineol | 1186 | 1186 | 2.03 | 1.64 | 2.03 | 1.30 | 1.11 | n.s. | 1.21 | 1.49 | 1.50 | 1.31 | 1.45 | n.s. |
| myrtenol | 1192 | 1194 | 0.22 | 0.28 | 0.31 | 0.17 | 0.10 | n.s. | 0.23 | 0.28 | 0.27 | 0.24 | 0.27 | n.s. |
| verbenone | 1205 | 1204 | 6.10 | 5.12 | 5.64 | 4.75 | 3.75 | n.s. | 5.52 | 6.22 | 6.28 | 5.62 | 6.44 | n.s. |
| carvacrol methylether | 1243 | 1241 | 0.08 | 0.14 | 0.13 | 0.06 | 0.05 | n.s. | 0.29 | 0.37 | 0.27 | 0.29 | 0.34 | n.s. |
| geranial | 1262 | 1264 | 0.61 | 0.21 | 0.68 | 0.14 | 0.14 | n.s. | 1.38 | 1.91 | 1.92 | 1.57 | 1.75 | n.s. |
| bornyl acetate | 1287 | 1285 | 1.96 | 2.06 | 2.00 | 1.83 | 1.51 | n.s. | 2.54 b | 3.56 a | 3.48 a | 3.11 a | 3.40 a | * |
| α-copaene | 1371 | 1374 | 0.19 | 0.11 | 0.35 | 0.19 | 0.37 | n.s. | 0.35 b | 0.50 ab | 0.54 a | 0.39 ab | 0.44 ab | * |
| β-caryophyllene | 1419 | 1417 | 4.07 | 4.70 | 3.93 | 4.16 | 4.50 | n.s. | 3.65 | 4.48 | 4.29 | 4.08 | 4.36 | n.s. |
| α-humulene | 1455 | 1452 | 0.28 | 0.23 | 0.29 | 0.19 | 0.17 | n.s. | 0.45 | 0.58 | 0.52 | 0.50 | 0.54 | n.s. |
| caryophyllene oxide | 1589 | 1582 | 0.08 | 0.05 | 0.08 | 0.04 | 0.04 | n.s. | 0.08 | 0.13 | 0.09 | 0.08 | 0.09 | n.s. |

$RI_{calc}$: Retention indices relative to C9-C27 n-alkenes from a HP-5MS-column; $RI_{lit}$ = Retention Indices based on Adams (2017). * * = significant at 0.01 probability level; * = significant at 0.05 probability level; n.s. = not significant. Means are shown. The values followed by different letters are significantly different for $p \leq 0.05$ according to Tukey's test. C = control (only water); APH-100 = 100 mL $hL^{-1}$ of animal protein hydrolysate; APH-200 = 200 mL $hL^{-1}$ of animal protein hydrolysate; PPH-150 = 150 mL $hL^{-1}$ of plant protein hydrolysate; PPH-300 = 300 mL $hL^{-1}$ of plant protein hydrolysate.

**Table 5**
Influence of foliar treatment on rosemary phenolic content (PC), antioxidant activity (AA) and rosmarinic acid (RA).

| Foliar treatment | PC [mg c.a.e. $g^{-1}$] | | AA [mg t.e. $g^{-1}$] | | RA [%] | |
|---|---|---|---|---|---|---|
| | I year | II year | I year | II year | I year | II year |
| C | 109.2 a | 78.5 ab | 140.8 a | 136.7 ab | 1.6 a | 1.6 a |
| APH−100 | 97.3 b | 81.2 a | 111.1 b | 124.4 b | 1.4 a | 1.2 b |
| APH−200 | 97.6 b | 69.8c | 121.2 b | 144.1 a | 1.5 a | 1.6 a |
| PPH−150 | 97.3 b | 82.1 a | 111.1 b | 124.9 b | 1.5 a | 1.2 b |
| PPH−300 | 95.3 b | 75.3 b | 138.8 a | 135.6 ab | 1.6 a | 1.6 a |
| *p*-value | 0.000 | 0.000 | 0.000 | 0.013 | 0.300 | 0.000 |

Means are shown. The values followed by different letters are significantly different for $p \leq 0.05$ according to Tukey's test. C = control (only water); APH-100 = 100 mL $hL^{-1}$ of animal protein hydrolysate; APH-200 = 200 mL $hL^{-1}$ of animal protein hydrolysate; PPH-150 = 150 mL $hL^{-1}$ of plant protein hydrolysate; PPH-300 = 300 mL $hL^{-1}$ of plant protein hydrolysate.

abundance within cells, which in turn encourages chlorophyll production (Abdali et al., 2023). Several studies have reported that chlorophyll levels increased following the application of foliar PHs (Omer et al., 2013; Sabatino et al., 2021; Al-Fraihat et al., 2023). Bandurska (2022) reports that photosynthetic activity and stomatal conductance in plant tissues are strongly correlated to leaf relative water content (RWC). Relative water content values found in this study were consistent with those provided by other studies carried out on rosemary plants when grown under different agronomic conditions (Munné-Bosch et al., 1999; Farruggia et al., 2024b). However, contrary to the findings of Elansary et al. (2019) and Rahimi et al. (2022), the present study did not observe any increase in RWC in biostimulated plants compared to control plants.

Paul et al. (2019) highlight that low doses of protein hydrosylates can be considered as a sustainable practice to increase agricultural production under water stress conditions. In our study, foliar application of both protein hydrolysates obtained significant increases in biomass per unit area. A number of direct and indirect physiological processes, such as promoting enzymatic action, prompting hormone-like activity, and modulating the plant root system, improve a plant's uptake capacity of water and nutrients. These processes may be linked to increases in crop yields promoted by protein hydrolysates under favorable or unfavorable growth conditions (Vultaggio et al., 2024). Some authors (Colla et al., 2017; Jiménez-Arias et al., 2022; Iacuzzi et al., 2024) propose that, even under conditions of limited water availability, peptide and amino acid content is essential for protein biosynthesis, energy production, and the generation of molecules with high biological activity. However, in this study, crop yields were lower than those reported by other authors who investigated the effects of different types of fertilizers

(Singh and Wasnik, 2013) and foliar biostimulants (Farruggia et al., 2024b) on rosemary.

It is reasonable to suggest that the significant increase in biomass yields, mainly observed in plants treated with the lowest dose of PPH, are related to the tryptophan content. Tryptophan serves as a precursor of indole−3-acetic acid, a regulator of shoot and root expansion in plants, as documented by Sabatino et al. (2021) in a study carried out on lettuce. In additional, plant-based PHs contain peptides and amino acids, such as glutamate and aspartate, which can act as signaling molecules for rhizosphere microbiota or function as iron chelators, facilitating iron uptake in plants and enhancing their Fe-nutritional status, improving physiological processes and plant growth (Carillo et al., 2022),.

### 4.2. Essential oil characteristics

The differing trend in EO yield over the two years could be related to factors influencing the synthesis of secondary metabolites (Tawfeeq et al., 2016; Tarasevičienė et al., 2021; Chrysargyris et al., 2022; Medeiros et al., 2024) and biostimulants (Nardi et al., 2016; Colla et al., 2017; Paul et al., 2019). Significant increases in biomass production observed in PPH-treated plants resulted in the best EO yield in both years. This result is particularly attractive for achieving greater yields of bioactive compounds (Napoli et al., 2020; Tarasevičienė et al., 2021; Angane et al., 2022). As several studies confirm (Nardi et al., 2016; Colla et al., 2017; Colla and Rouphael, 2015; Consentino et al., 2020; Di Miceli et al., 2023), the high peptide or amino acid content of protein hydrolysates influences various metabolic processes, including the synthesis of secondary metabolites and overall plant performance. Considering EO composition and the number of EO compounds, several biotic and abiotic factors should be taken into account as they significantly influence these characteristics, as previously reported by many authors (Elansary et al., 2019; Jafari Khorsand et al., 2022; Formica et al., 2024). In this study, consistent with previous studies (Jiang et al., 2011; Micić et al., 2021; Formica et al., 2024), the predominant compounds in rosemary EO were α-Pinene and 1,8-Cineole. However, their percentage content was not influenced by APH and PPH treatments. Vosoughi et al. (2018) propose that foliar application of biostimulants can affect gene regulation and enzyme activity in the secondary metabolic pathway. It is worth noting that, in this study, biostimulants only affected the percentage content of some minor compounds (e.g. borneol, tricyclene, terpinolene, bornyl acetate, and α-copaene). In contrast, the same authors observed a change in the EO profile of MAPs when treated with foliar biostimulants (Truzzi et al., 2021; Amato et al., 2024; El-Hefny and Hussien, 2025).

### 4.3. Chemical parameters

The literature reports that chemical parameters such as antioxidant activity, rosmarinic acid content, and total phenolic content are affected by a number of different variables (Tawfeeq et al., 2016). It has been demonstrated that protein hydrolysates contain a combination of amino acids useful for plant growth and to enhance qualitative characteristics, especially under unfavorable environmental conditions (Fraihat et al. (2023)). Wang et al. (2022) stated that amino acids are recognized as biostimulant molecules that significantly reduces damage caused by abiotic stress and supports plant growth. The ability of MAPs to produce secondary metabolites is well known to be influenced by stressful conditions (Kulak et al., 2020Farruggia et al., 2023). In this study, contrasting responses for chemical parameters were detected, dependent on the type of biostimulant and application dose. It is not easy to explain these phenomena in relation to each chemical parameter analyzed in the study. Consequently, our findings suggest that the impact of biostimulants on secondary metabolites should not be generalized. It is also important to emphasize that stress and/or stress-relief conditions should be thoroughly considered. These statements are further supported by the results of previous studies carried out on various MAPs. Rahimi et al. (2022) and Saia et al. (2021) reported that the application of biostimulants could improve the quantity of secondary metabolites in thyme (*Thymus vulgaris* L.) and basil (*Ocimum basilicum* L.). However, Cardone et al. (2025) found contrasting responses in thyme plants treated with various foliar biostimulants. The authors attributed these results to the possibility that, in the case of thyme, the tested products may not have the same ability to affect antioxidant capacity and total phenolic content.

## 5. Conclusions

For medicinal and aromatic plants, exploring innovative cultivation practices is crucial when attempting to optimize crop yields and quality standards whilst still adhering to sustainability criteria. This study assessed the impact of two different protein hydrolysates (one animal-based and one plant-based) on yield traits and chemical parameters in rosemary. The main findings highlight that foliar applications using the lowest dose of plant protein hydrolysate produce the highest productivity, both in terms of biomass and essential oil yields. Furthermore, the use of animal or plant protein hydrolysates does not induce any significant changes in the aromatic profile of the essential oil. This means that both animal or plant protein hydrolysates can be used whilst maintaining the chemical profile of rosemary stable over time. By exploiting the benefits of biostimulants, essential oil production can meet both consumer needs and those of producers. However, the application of animal and plant protein hydrolysates at different doses differentially affects antioxidant capacity, rosmarinic acid content, and total phenolic content in rosemary. It is worth noting, therefore, that stress events should be evaluated carefully, avoiding any generalization regarding their effects on secondary metabolites. Although further studies are needed to better investigate the effects of variations in the dose of each protein hydrolysate on physiological processes, it can be concluded that they could contribute to making rosemary cultivation more sustainable, thus reducing chemical fertilizer needs and producing significant environmental benefits.

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## CRediT authorship contribution statement

**Johannes Novak:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Data curation. **Francesco Salamone:** Software, Formal analysis, Data curation. **Giovanni Urso:** Software, Formal analysis, Data curation. **Giuseppe Di Miceli:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Conceptualization. **Mario Licata:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Davide Farruggia:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

## Declaration of Competing Interest

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

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### Author contributions statement

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### Additional information

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