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Morphological traits, yield, antioxidant activity and essential oil composition of oregano as affected by biostimulant foliar applications

Giuseppe Amato [a], Loriana Cardone [b,*], Nunzia Cicco [c], Michele Denora [b], Michele Perniola [b], Donato Casiello [b], Laura De Martino [a], Vincenzo De Feo [a], Vincenzo Candido [b]

[a] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 132, Fisciano 84084, Italy
[b] Department of European and Mediterranean Cultures, Environment, and Cultural Heritage, University of Basilicata, Via Lanera, 20, Matera 75100, Italy
[c] Institute of Methodologies for Environmental Analysis-National Research Council (IMAA-CNR), c.da Santa Loja, Tito Scalo, PZ 85050, Italy

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ABSTRACT

Biostimulants are interesting products which can promote the plant growth and improve its qualitative traits. In this research, the effects of foliar applications of four biostimulants on oregano (*Origanum vulgare* L.) crop have been investigated by evaluating morphological parameters, fresh and dry yield, antioxidant activity, total phenolic content, and chemical composition of essential oil. The highest plant height (78 cm), inflorescence height (22.2 cm), inflorescences+leaves dry yield (0.5 t ha^{-1}), dry matter yield (38.3 %) and essential oil yield (3.4 %) were obtained in plants treated with biostimulant BlueN®, composted of *Methylobacterium symbioticum* SB23. Plants treated with Acadian MPE®, containing vitamins, amino acids and proteins, and betaines, showed the highest total phenolic content (60.3 GAE mg mL^{-1}). The combination of 21 traits with principal component analysis (PCA) allowed to discriminate the biostimulant Biostimol Plus plus Peptamin-V Plus®, based on *Ascophyllum nodosum* extract and other elements as nitrogen, carbon, manganese, zinc and iron chelates, in a correlated way with *p*-cymene, α-thujene, α-terpinene, β-myrcene contents, showing values significantly higher. The heat-map identified two main clusters where the treatments with biostimulants were clearly separated from the control. Biostimulant treatments influenced the chemical composition of essential oil by increasing the number of components, monoterpene hydrocarbons and sesquiterpene hydrocarbons contents. Therefore, biostimulants can play an important role with a view to sustainable oregano cultivation by representing a safe source of nitrogen and especially for organic farming.

1. Introduction

Between 2010 and 2050 the worldwide demand of food will enhance by 35–56 % (van Dijk et al., 2021). With the main aim of satisfying the food demands of the world population, the traditional agriculture has based its strategies on deforestation, more intensive use of cropland, and extreme application of pesticides and chemical fertilizers which has generated serious impacts on population health and consequences such as disruption of marine, freshwater, and terrestrial ecosystems, biodiversity loss, environmental pollution and (Dirzo & Raven, 2003; Gitau et al., 2022; Tilman et al., 2011). To obtain more eco-sustainable, healthier, and quality crop production, it is increasing the attention towards the application of plant biostimulants (Colla et al., 2020). They are defined as '*fertilizing products able to stimulate plant nutrition process independently of the products nutrient content to improve nutrient uptake and efficiency, tolerance to abiotic stress and to modulate qualitative traits of the product*', according to the recent European Union regulation (2019/1009) (Colla et al., 2015, 2020). Positive responses of horticultural crops to biostimulant application have been widely reported by scientific literature (Bulgari et al., 2019; Du Jardin, 2015; Kisvarga et al., 2022; Parađiković et al., 2019). As far as it concerns the seaweeds, brown ones, comprising the class Phaeophyceae, i.e. *Ascophyllum nodosum*, are the most commonly used by the industries involved in extraction, due to the bioactive compounds such as minerals (nitrogen, phosphorous, potassium, calcium, iron, magnesium, zinc, sodium and sulphur), polysaccharides (alginate, cellulose), phytohormones (cytokinins, auxins, gibberellins and abscisic acid), osmolites (betaines), sterols, carotenoid and protein, peptides, and amino acid (Ali et al., 2021; Battacharyya et al., 2015). Positive effects of brown seaweeds on vegetable crops growth (shoot and root), development, yield, resistance

* Corresponding author.
E-mail address: loriana.cardone@unibas.it (L. Cardone).

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to drought stress, and quality are confirmed by many authors; indeed, interesting studies have been conducted on tomato and sweet pepper (Ali et al., 2019), soybean (Martynenko et al., 2016), lettuce (Yusuf et al., 2019), strawberry (Soltaniband et al., 2022), potato (Dziugieł et al., 2020), onion (Hidangmayum et al., 2017), spinach (Rouphael et al., 2018; Castronuovo et al., 2023) and wild rocket (Candido et al., 2023; Schiattone et al., 2023).

Wide attention was also paid to the application of bacterial plant biostimulants that are based on plant growth-promoting rhizobacteria, including nitrogen-fixing *Rhizobium* spp., *Azotobacter* spp., *Azospirillum* spp., *Pseudomonas* spp., *Bacillus* spp. and *Methylobacterium* spp. (Hamid et al., 2021; Pellegrini et al., 2020). Its direct effects concern the availability of macronutrients and micronutrients as nitrogen, phosphorus (solubilization of phosphate) and iron (production of chelating molecules), and the synthesis of plant growth regulators (Novello et al., 2021). An interesting correlation between bacteria and food functional quality has been found in many crops as in bell pepper, broccoli, lettuce and strawberry. The plants treated with some bacterial strains are characterized by high content of ascorbic acid, polyphenols and terpenoids (Ganugi et al., 2021). Also, biostimulants containing amino acids, betaines, vitamins, are interesting because improve the growth and development, and reduce the transplantation stress in basil, increase the fruit yield parameters in pepper, tomato and garlic, and the antioxidant activity in strawberry (Parađiković et al., 2019).

Few studies about the effect of biostimulants on medicinal and aromatic plants have been reported in literature (Farruggia et al., 2024a; Rafiee et al., 2016; Sun et al., 2023). Promising effects of biostimulants have been highlighted on the synthesis and accumulation of secondary metabolites of peppermint (Al-Ghezi and Obaid., 2021), rosemary (Farruggia et al., 2024a), marigold (Machado et al., 2014), majoram (Hanafy et al., 2018), dill, basil, savory and chamomile (Rathore and Kumar, 2022). However, influence of the application of biostimulants on the agronomic and qualitative parameters of oregano (*Origanum vulgare* L.) are less reported in the literature (Abdali et al., 2023).

Oregano is a perennial plant of the Lamiaceae family used as spicy to flavor food due to strong aroma and pungent taste (Ilić et al., 2022). As well as being known as essential ingredient of Mediterranean cooking which could replace the salt (Zuzarte et al., 2023), it is used for beneficial properties (antifungal, antibacterial, antiviral, and anticancer) thanks to its high content of bioactive compounds as polyphenols, flavonoids and triterpenoids (Boroski et al., 2012; Dambolena et al., 2010; Kokkini et al., 2004). Dominant components of the essential oil (EO) of oregano are carvacrol and/or thymol, followed by γ-terpinene, *p*-cymene, linal-ool, terpinen-4-ol and sabinene hydrate (Azizi et al., 2009). EO content and composition determined the quality of oregano and are influenced by different factors as genotype, climate conditions and nutrient availability (Azizi et al., 2009). Oregano plants under moisture shortage stress conditions and treated with Acadian® extract of *Ascophyllum nodosum*, showed higher production of soluble sugar and EO yield compared to no-treated plants (Abdali et al., 2023). Aytac et al. (2022) evaluated three doses of humic acid on plant yield, EO content and composition of *Origanum vulgare* L. subsp. *hirtum* (Link.) cv 'Tinmaz'. The results highlighted that carvacrol and γ-terpinene content increased when high dose of the humic acid was used. All the studies reported to date evaluated only one type of biostimulant; therefore, the literature lacks data on the response of oregano to the foliar application of various plant biostimulants from different origins, as well as on the potential to increase the sustainable yield without resorting to synthetic fertilizers. In addition, the organic oregano has been found to have a high content of bioactive compounds, making it an important raw material for herbal products compared to conventionally cultivated oregano (Matłok et al., 2020).

Based on this, the objective of this research was to study the effects of four commercial biostimulants on: (1) morphological traits and yield, (2) antioxidant activity and total phenolic content, and (3) the yield and composition of EO of oregano.

2. Materials and methods

2.1. Experimental design and plant material

The trial experiment was carried out at the "Podere Malvarosa" farm situated in the countryside of Melfi, Basilicata region, Southern Italy (41°07' N; 15°60' E; 252 m a.s.l.) on a previously ploughed and fertilized loamy-texture soil during the 2022–2023 period. According to the Köppen–Geiger climate classification, the study location is characterized by a warm temperate climate with dry summers (Csa). The seeds of *O. vulgare* were purchased from 'La Semiorto Sementi' seed company, and the seedlings were produced by a nursery and grown in hole polystyrene containers. The plants were transplanted on July 18th 2022. The distance between rows and within rows was 1.0 m and 0.30 m, respectively, to have a crop density of 3.33 plants m^{-2}. The plants grew until mid-October when they were cut 5 cm from the ground, before the winter rest. The weeds were controlled by hand weeding and no pesticides and chemical fertilizers were used.

The experiment was laid out in a randomized block design with five treatments (control, Biostimol Plus + Peptamin-V Plus®, Acadian MPE®, Megafol®, and BlueN®) with three replications. The experimental plots were 3 m × 6 m (18 m^2) with 3 rows 1 m apart.

2.1.1. Treatments

For the control treatment, plants were sprayed with only water, without any addition of biostimulants. The second treatment is a combination between Biostimol Plus and Peptamin-V Plus®. Biostimol Plus® (Biophyt Agro S.r.l., Andria, BT, Italy) is a product based on algae extract of *Ascophyllum nodosum* and contains total organic carbon (C) of biological origin (1.7 %), mannitol (7 %) and alginic acid (2–3 %). Peptamin-V Plus® (Biophyt Agro S.r.l., Andria, BT, Italy) contains organic nitrogen (N) (3 %) and total organic carbon (C) of biological origin (10 %), and chelates of iron, manganese and zinc.

Acadian® MPE (Acadian Sea plants Lte, Dartmouth, NS, Canada), is a product based on algae extract of *Ascophyllum nodosum* and contains organic nitrogen (N) (1 %), potassium oxide (K_2O) (19 %) betaines (0.1 %), mannitol (4 %), total organic carbon (C) of biological origin (20 %).

Megafol® (Valagro, Atessa, CH, Italy) contains total nitrogen (3 %), organic nitrogen (1 %), urea nitrogen (2 %), potassium oxide (K_2O) (8 %), total organic carbon (C) of biological origin (9 %), amino acids (glycin, glutamic acid), betaines, proteins, vitamins (B5, PP, B1, B6), auxin, gibberellin, cytokine.

BlueN® (Symborg Business Development s.l.u., Cabezo Cortado, Murcia) is based on the natural bacteria, *Methylobacterium symbioticum* (SB23, 3 ×107 UFC/g) which fixes nitrogen from the air and converts it for the plant.

These commercial biostimulants were applied by foliar three times during the growing period of 2023 according to recommended dosage by manufacturers. The first application was at the initiation of stem elongation (May 15th 2023) and the second and the third applications were conducted at 2-week intervals. In particular, 1.35 L of each biostimulant aqueous suspension were distributed for each tesis at following concentrations: 2 g L^{-1} for Biostimol Plus, 3 g L^{-1} for Peptamin dose, 1 g L^{-1} for Acadian® MPE, 2.5 mL L^{-1} for Megafol®, and 0,33 g L^{-1} for BlueN®.

2.2. Climatic data

Daily rainfall and mean temperature were monitored during the evaluation period (Fig. 1), from a meteorological station.

During the time of cultivation (July 2022–October 2023) a total rainfall of 817 mm, mainly concentrated in November, in January, and between May and June was recorded; May was the wettest month ever, with high intensity rainfall mainly concentrated in few days. The lowest temperatures were recorded in February (7.7 °C average monthly

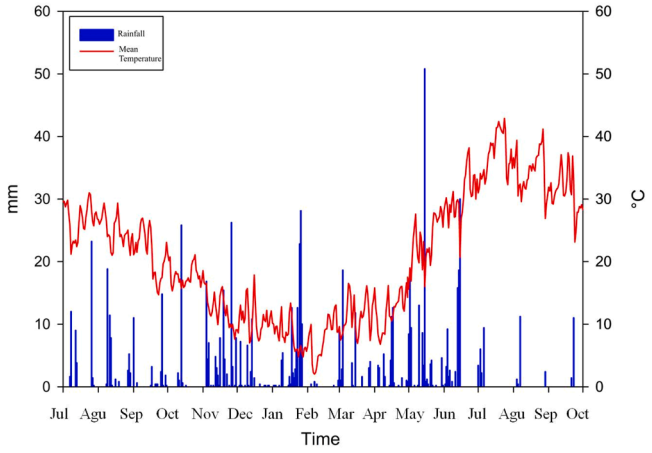


Fig. 1. Rainfall and temperature trends in the experimental site.

temperature), the highest in July (37.1 °C average monthly temperature), with an average annual temperature of 21.2 °C. These climatic factors significantly influenced the phenological stages of oregano. Elevated temperatures in July accelerated vegetative growth and flowering, leading to increased biomass and EO content. Conversely, cooler temperatures in February likely induced dormancy or slowed metabolic activity, allowing for energy conservation during less favorable conditions. The substantial rainfall in May ensured optimal soil moisture during critical vegetative stages, enhancing biomass production and EOconcentration. This interplay between temperature and rainfall highlights the significant impact of environmental conditions on both the quantity and quality of oregano.

2.3. Morphological and yield traits

During the full flowering phase for each treatment, the plant height was determined from the soil to the highest point of flowering stem. From the center rows of each plot and avoiding the border effect, ten randomly selected plants were harvested to measure the fresh and dry weights. One harvest was carried out, during July 2023. After the harvesting, plants were immediately weighted (fresh weight) using a precision balance (TE214S, Sartorius, Goettingen, Germany). To obtain the dry weight, the leaves and inflorescences were separated from the stems, were weighed and then were dried in a ventilated oven at 65 °C for 48 h until constant weight was reached (Gerami et al., 2016). Moreover, the following vegetative growth parameters were measured: plant height and diameter (cm), stem height and diameter (cm), inflorescence height and diameter (cm), biomass fresh yield (t ha^{-1}), fresh and dry yield of inflorescences and leaves (t ha^{-1}). Oregano plants before the harvesting phase are showed in Fig. 2.

2.4. Extraction andanalysis of EO

According to the procedure described in the European Pharmacopoeia (Council of Europe, 2020), 300 g dried aerial parts of each sample were subjected to hydrodistillation for 2 hours The EOs were solubilized in *n*-hexane and stored under N_2 at +4 °C and analysed by GC and GC-MS as reported by Amato et al. (2023). For the calculation of KI (relative retention indices), a series of n-alkanes (C10–C35) was used. Regarding the relative concentrations of the components, they were obtained through normalization of the peak area. The Fig. 3 reports a chromatogram of an EO.

2.5. Analysis of total phenolic content

The Folin-Ciocalteau assay was carried out to determine the total



Fig. 2. Oregano plants from experimental site.

phenol content (TPC), (Waterhouse, 2002). Ten μL of solution of EO or gallic acid, used to determine calibration, 790 μL of H_2O deionized and fifty μL of the Folin-Ciocalteau reagent were placed in the cuvettes. One hundred fifty μL of $CaCO_3$ were added after eight minutes. After 2 hours of incubation at room temperature, absorbance values at 765 nm were evaluated using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland). TPC was measured in mg s of gallic acid equivalent (per mL of EO GAE mg mL^{-1}). The analyses were performed in triplicate, and the values reported were the mean ± SD.

2.6. DPPH assay

The antioxidant activity was evaluated by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Xiang et al., 2019). In the radical form, DPPH has an absorption spectrum at 515 nm which varies if it is in the presence of substances that have anti-radical activity. Aliquots of EO were dissolved in MeOH to have final concentrations ranging from 25 to 500 μg mL^{-1}. The control used was a DPPH solution, while MeOH alone was used as a blank. After 45 minutes of incubation in the dark, the absorbance at 515 nm was measured using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland). The analyses were performed in triplicate and the results were expressed as IC_{50} value.

2.7. Statistical analysis

All assays were carried out in triplicate. The statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparisons test at the significance level of $p < 0.05$. Principal component analysis (PCA) and Cluster Heat Map were generated using *factoextra* and *gplots* packages of the R language for statistical computing, respectively (R Core Team, 2020).

3. Results

3.1. Morphological and quantitative traits of oregano

Tables 1 and 2 summarize the results of the analyses of variance for the traits examined in the present research. All morphological traits were significantly affected by the biostimulant foliar applications, except for the diameters of stem and inflorescence. The highest plant and inflorescence heights, and plant diameter were obtained by plant treated

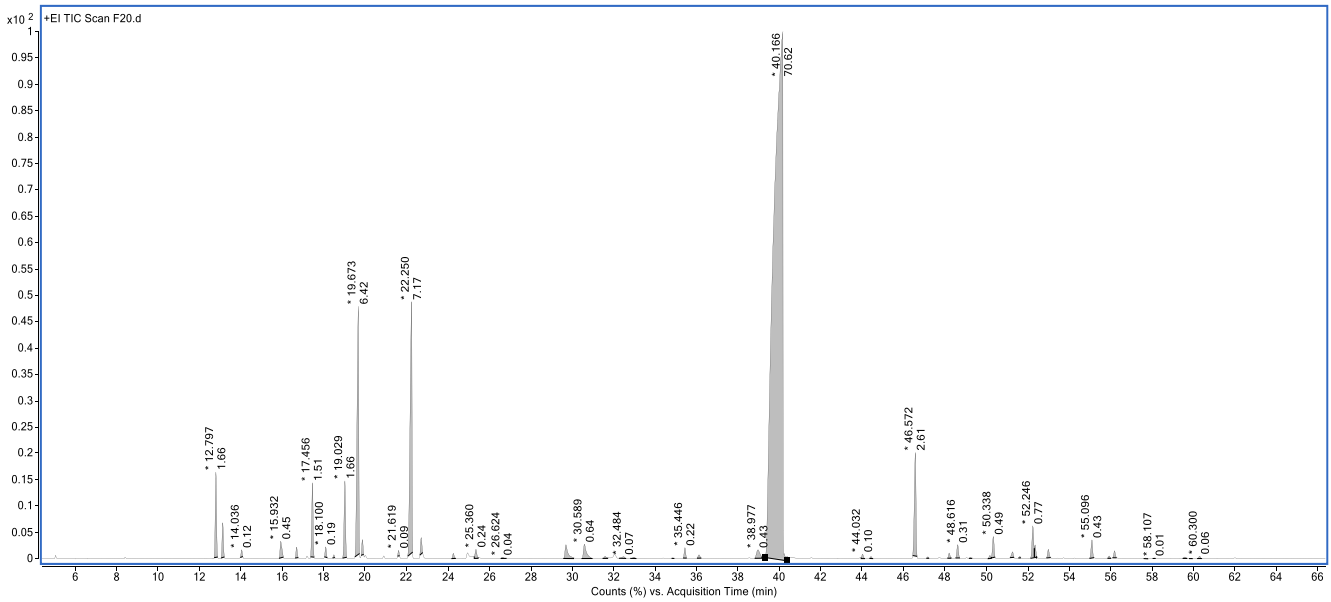


Fig. 3. A chromatogram of an EO.

Table 1
Effect of biostimulant foliar applications on the morphological traits of oregano.

Treatment	Plant height (cm)	Plant diameter (cm)	Stem height (cm)	Stem diameter (cm)	Inflorescence height (cm)	Inflorescence diameter (cm)
Control	68.3^{a}	101.7^{a}	42.8^{a}	2.8^{a}	13.3^{a}	7.1^{a}
Biostimol Plus + Peptamin-V Plus	72.0^{b}	106.0^{a}	52.6^{c}	3.1^{a}	17.6^{b}	7.1^{a}
Acadian®	75.7^{bc}	105.7^{a}	51.8^{bc}	3.1^{a}	20.2^{bc}	8.6^{a}
Megafol®	75.7^{bc}	108.0^{a}	53.1^{c}	3.1^{a}	20.5^{bc}	8.7^{a}
BlueN®	78.0^{c}	109.3^{a}	50.3^{b}	3.2^{a}	22.2^{c}	8.1^{a}

The same letters indicate that are not significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

Table 2
Effect of biostimulant foliar applications on the oregano yield parameters.

Treatments	Total fresh yield (t ha^{-1})	Inflorescences+Leaves fresh yield (t ha^{-1})	Inflorescences +Leaves dry yield (t ha^{-1})	Dry matter yield (%)
Control	2.4^{a}	1.03^{a}	0.37^{a}	35.9^{ab}
Biostimol Plus + Peptamin-V Plus	2.8^{ab}	1.36^{b}	0.52^{bc}	38.2^{b}
Acadian®	2.9^{ab}	1.54^{c}	0.53^{bc}	34.4^{a}
Megafol®	3.1^{b}	1.20^{ab}	0.43^{b}	35.8^{ab}
BlueN®	3.0^{ab}	1.46^{bc}	0.56^{c}	38.3^{b}

The same letters indicate that are not significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

with BlueN®, whilst the highest stem height was obtained by plant treated with Megafol® (Table 1).

Fresh and dry yield of inflorescences and leaves, and dry matter yield were considerably higher in plants treated with BlueN® (Table 2). Generally, the treatment with the biostimulant improved the growth and increased the yield. In particular, the treatment with BlueN, compared to the control, obtained the best performance increasing the plant height, the inflorescence height, the inflorescences and leaves dry yield of 14, 67, and 52 %, respectively (Tables 1 and 2).

3.2. Yield and chemical composition of the EOs

The yield in EO varied between the control (2.33 %) and the treatments. In fact, the sample treated with the Acadian® had a lower yield (1.46 %) than the control; otherwise, the sample treated with the BlueN® biostimulant gave a higher yield than the others (3.45 %). The samples treated with Biostimol Plus + Peptamin-V Plus and Megafol® gave a yield similar to the control (2.34 and 2.05 %, respectively) (Fig. 4).

The number of identified components also varied between the control (44) and the biostimulant treatments, whose number varied in the range 48–56, with a parallel increase from Biostimol Plus + Peptamin-V Plus (44) to Megafol® and BlueN® (both 56 components). Oxygenated monoterpenes predominated in all the EOs: the control contained 82.2 % of this class: this value resulted significantly higher than in treatments (72.9–77.4 %). Carvacrol was found the main component of all EOs: also, in this case the control showed a greater amount of this compound (78.9 %) compared to the treatments (70.1–72.9 %, with an increase from Biostimol Plus + Peptamin-V Plus to BlueN®). The comparison between the control and the treated samples also highlighted other differences, both in the number of compounds identified and in their quantity (Table 3). In fact, bicyclogermacrene, calamenene, α-bisabolene were absent in the control but present in the other samples; sabinene, however, is only absent in the sample treated with Acadian®. Other differences regarded the percentages of thymol and γ-terpinene: thymol is present in lower percentages in the Biostimol Plus+ Peptamin-V Plus (0.4 %) and Megafol ® (0.6 %) samples, while it is present in major amounts in the Acadian® sample (3.3 %). γ-Terpinene, on the other hand, was less present in the control (3.3 %) while it is more

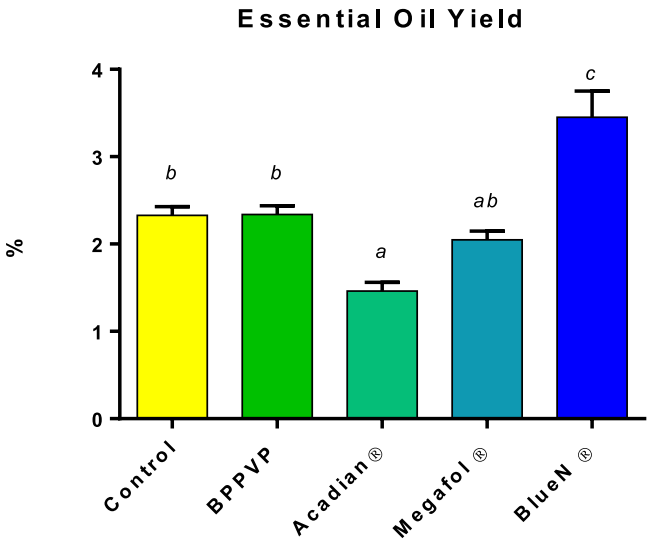


Fig. 4. Yield of the essential oils on a dried basis (w/w) of *O. vulgare* obtained by different biostimulant treatments. The same letters indicate that are not significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

abundant following treatment with BlueN® (7.9 %) (Fig. 5). On the contrary, the amount of monoterpene hydrocarbons (Fig. 6) resulted lower in the control (13.8 %) than in the treatments, which decreased from 20.9 % (Biostimol Plus + Peptamin-V Plus) to 17.5 % (BlueN®). Among these compounds α-thujene, β-myrcene, *p*-cymene, and γ-terpinene were the main components, whose amounts reflect those of their class, with a general increase in treatments. Sesquiterpene hydrocarbons also resulted lower in the control compared to the treatments, while there were no significant differences, among control and treatments, in the presence of oxygenated sesquiterpenes.

3.3. Antioxidant activity and total phenolic compounds of oregano

The antioxidant capacity of EOs was evaluated using the DPPH assay (Table 4). The results showed capacitance of all samples with IC_{50} values between 261 and 287.6 μg/mL. There is no significant difference between the samples regarding the antioxidant capacity. However, disginfican differences were found among the treatments regarding the TPC, evaluated through the Folin assay (Table 4). The control and the Acadian® sample were the richest in total phenolics (50.86 and 60.29 GAE mg mL^{-1} respectively).

3.4. Principal component analysis (PCA)

A principal component analysis (PCA) has been used in order to allow a combined study of 21 traits in a single multivariate analysis. The results are shown in Figs. 5–6, where the score plot and the loading plot of the morphological, quantitative and chemical variables are reported. The two principal components account for 75.83 % of the total variance, 51.11 % of which involves PC1 and 24.72 % involves PC2. The variables that most contributed to the PC1 were those related to the morphology and yield of the oregano as stem height, inflorescence height, total fresh yield, inflorescences+leaves dry yield, α-terpinene, γ-terpinene, as well as carvacrol, oxygenated monoterpenes and monoterpenes hydrocarbons which displayed high negative correlations with the PC1 (Fig. 7). The variables that most contributed to the PC2 were those related to plant height, dry matter yield, total phenolic content, α- thujene, α-pinene, *p*-cymene and thymol (Fig. 6).

The score plot (Fig. 8) shows a clear separation between the control and the treatments with biostimulants. In particular, the control tended to be differentiated on the negative side of the PC1 axis. Positive and significant correlations between stem height, inflorescence height, total fresh yield, inflorescences+leaves fresh and dry yields with α-terpinene, λ-terpinene, caryophyllene and sesquiterpene hydrocarbons were found (Fig. 7). TPC was positively correlated with heights of plant and inflorescence, and total fresh yield. Dry matter yield was positively correlated with EO yield, α- thujene, α-pinene and *p*-cymene (Fig. 7).

3.5. Heat map analysis

The data heat-map analysis (Fig. 9) identified two main clusters: the first corresponding to the control, and the second to all the treatments with biostimulants. The latter cluster associated the treatments with Megafol® and BlueN®, and Biostimol Plus + Peptamin-V Plus in a sub-group and Acadian® in another sub-group (Fig. 9). In particular, the control clustered separately because of its lower values of fresh and dry inflorescences and leaves yield, total fresh yield, plant height, inflorescence height, stem height, sesquiterpene hydrocarbons, monoterpene hydrocarbons, α-terpinene, γ-terpinene and caryophyllene contents. The Biostimol Plus + Peptamin-V Plus showed the highest *p*-cymene, α-thujene, α-terpinene, monoterpene hydrocarbons, β-myrcene. The Acadian® treatment sub-clustered separately from the BlueN® and Megafol® treatments because of its higher thymol and oxygenated monoterpenes content (Fig. 9).

4. Discussion

4.1. Effect of biostimulants on the morphological traits and yield of oregano

O. vulgare traits were influenced by the different origin and compostion of biostimulants and the highest ones were reached with the biostimulant BlueN®. The latter is composed of *Methylobacterium symbioticum* SB23, an exclusive Gram-negative endophytic bacterium, capable of spreading within the vegetal tissue of plants and fixing atmospheric nitrogen, regardless of its availability in the soil. Generally, *Methylobacterium* strains are rod-shaped and aerobic microbes which adapt to different conditions of temperature, pH and salinity and show a greater tolerance to chlorine and γ- irradiation. When applied to plants, *M. symbioticum* penetrates through the leaf stomata and colonizes the parenchyma, producing an enzymatic complex called nitrogenase, capable of converting atmospheric nitrogen (N_2, not available for plant's metabolism) into ammonium (NH_4^+), which is promptly transferred to plant cells to synthesize proteins and nucleic acids (Vera et al., 2023). *M. symbioticum* establishes a symbiotic relationship with the plant by using the one-carbon alcohol methanol (waste product of the pectin metabolism) as sole source of carbon and energy (Kutschera, 2007). Different studies evaluated the beneficial effects of *Methylobacterium* spp. on maize and strawberry (Vera et al., 2023), rice and wine grape (Pascual et al., 2020), peanut, citrus, pinus (Dourado et al., 2015) but no studies on oregano have been conducted. In fact, among the evaluated bacteria on oregano, there are some studies about beneficial effects of *Pseudomonas fluorescens* and *Bradyrhizobium* sp. on *Origanum majorana* L. (Banchio et al., 2008), and other bacteria strains isolated from rizhospheric soil on *O. onites* L. (Çakmakçı et al., 2023), and *O. syriacum* subsp. *sinaicum* (Alraey et al., 2019).

The positive results obtained in this study can be attributed to different factors a) the production of methylobamine, a natural pink pigment able to tolerate the ultraviolet (UV) radiation and to stimulate photosynthetic activity of plants (Vera et al., 2023); b) the synthesis of plant hormonal as cytokinins (*trans*-zeatin), abscisic acid and indole-3-acetic acid (Palberg et al., 2022). All these characteristics allow to classify this bacterium as Plant Growth Promoting Bacteria (PGPBs) (Dourado et al., 2015). Certainly, the most important advantage of this biostimulant is to reduce nitrogen fertilization of the soil and therefore economic waste and environmental impact. Concerning the nitrogen application, different studies reported the importance of this

Table 3
Chemical composition of five EOs of *O. vulgare* L.

RT		Control	Biostimol Plus + Peptamin-V Plus	Acadian®	Megafol®	BlueN®	KIa	KIb	Identification[c]
		%							
12.8	α- Thujene	1.3 ± 0.1	1.7 ± 0.3	1.1 ± 0.1	1.4 ± 0.2	1.4 ± 0.2	928	930	1,2,3
13.1	α-Pinene	0.5 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	0.5 ± 0.2	932	932	1,2,3
14.0	Camphene	0.1 ± 0.05	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.04	0.1 ± 0.02	945	945	1,2,3
15.9	Sabinene	0.3 ± 0.03	0.5 ± 0.09	-	0.1 ± 0.04	T	971	956	1,2,3
16.0	β-Pinene	-	-	0.1 ± 0.03	0.2 ± 0.05	0.1 ± 0.04	971	971	1,2,3
16.7	1-Octen−3-ol	0.3 ± 0.04	0.2 ± 0.05	0.2 ± 0.04	0.2 ± 0.03	0.4 ± 0.08	981	982	1,2
17.2	3-Octanone	T	-	T	0.1 ± 0.04	0.1 ± 0.03	988	988	1,2
17.5	β-Myrcene	1.2 ± 0.2	1.5 ± 0.3	1.3 ± 0.3	1.4 ± 0.2	T	991	991	1,2,3
17.9	3-Octanol	-	-	-	-	T	997	990	1,2
18.1	α-Phellandrene	0.2 ± 0.04	0.2 ± 0.02	0.2 ± 0.06	0.2 ± 0.03	0.2 ± 0.03	1000	1000	1,2,3
18.5	δ−3-Carene	T	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.06	1006	1005	1,2,3
19.0	α-Terpinene	0.9 ± 0.1	1.7 ± 0.3	1.4 ± 0.2	1.4 ± 0.4	1.5 ± 0.3	1013	1022	1,2,3
19.6	*p*-Cymene	4.9 ± 0.5	6.5 ± 0.6	5.4 ± 0.4	5.2 ± 0.5	4.6 ± 0.3	1021	1023	1,2,3
19.9	D-Limonene	0.3 ± 0.05	0.3 ± 0.09	0.3 ± 0.04	0.3 ± 0.07	0.3 ± 0.06	1024	1025	1,2,3
20.0	1,8-Cineole	-	-	-	-	T	1026	1026	1,2,3
20.9	*trans*-β-Ocimene	-	-	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.06	1038	1036	1,2,3
21.6	*p*-Mentha−1(7),8-diene	-	0.1 ± 0.04	-	-	-	1048	1085	1,2
21.6	*cis*-β-Ocimene	-	-	-	0.1 ± 0.04	0.1 ± 0.02	1048	1043	1,2,3
22.3	γ-Terpinene	3.3 ± 0.4	7.1 ± 0.5	7.0 ± 0.6	6.6 ± 0.6	7.9 ± 0.4	1057	1055	1,2,3
22.7	Sabinene hydrate	0.7 ± 0.1	0.3 ± 0.04	0.5 ± 0.05	0.5 ± 0.04	0.5 ± 0.05	1063	1058	1,2,3
24.3	Terpinolene	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.06	0.1 ± 0.04	0.1 ± 0.02	1084	1084	1,2
25.3	Linalool	0.1 ± 0.03	0.2 ± 0.04	0.1 ± 0.03	0.2 ± 0.04	0.1 ± 0.03	1099	1099	1,2,3
26.6	*p*−2-Menthen−1-ol	-	T	T	T	T	1117	1116	1,2,3
27.4	*allo*-Ocimene	-	-	T	T	T	1128	1118	1,2,3
29.7	*endo*-Borneol	0.3 ± 0.04	0.5 ± 0.06	0.7 ± 0.1	0.6 ± 0.08	0.3 ± 0.04	1160	1160	1,2,3
30.6	Terpinen−4-ol	0.6 ± 0.07	0.7 ± 0.09	0.5 ± 0.04	0.6 ± 0.07	0.4 ± 0.04	1172	1172	1,2,3
31.6	α-Terpineol	0.1 ± 0.04	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.04	T	1186	1191	1,2,3
32.5	*trans*-Dihydrocarvone	0.1 ± 0.06	0.1 ± 0.03	0.1 ± 0.04	0.1 ± 0.06	0.1 ± 0.04	1200	1198	1,2,3
33.1	*cis*-Dihydrocarvone	0.1 ± 0.04	0.1 ± 0.05	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.02	1208	1207	1,2,3
34.9	*p*-Mentha−1(7),8(10)-dien−9-ol	-	T	-	T	T	1235	1287	1,2
35.4	Methyl carvacrol	0.8 ± 0.2	0.3 ± 0.1	0.3 ± 0.08	0.7 ± 0.2	0.4 ± 0.1	1242	1244	1,2,3
36.2	Carvone	0.1 ± 0.02	0.1 ± 0.03	0.2 ± 0.04	0.1 ± 0.02	0.1 ± 0.04	1254	1243	1,2,3
39.0	Thymol	1.1 ± 0.3	0.4 ± 0.2	3.3 ± 0.5	0.6 ± 0.2	1.1 ± 0.1	1294	1294	1,2,3
40.2	Carvacrol	78.9 ± 1.8	70.1 ± 1.6	71.9 ± 1.3	72.8 ± 1.5	72.9 ± 1.2	1313	1311	1,2,3
44.0	Carvacrol acetate	T	0.1 ± 0.03	0.1 ± 0.04	0.1 ± 0.02	0.1 ± 0.02	1373	1367	1,2,3
44.4	(-)-β-Bourbonene	T	T	T	T	T	1379	1380	1,2
46.6	Caryophyllene	1.0 ± 0.1	2.7 ± 0.5	1.5 ± 0.3	2.5 ± 0.3	2.4 ± 0.2	1413	1412	1,2,3
47.2	β-Copaene	T	T	T	T	T	1423	1416	1,2,3
47.7	Aromadendrene	-	-	T	T	T	1432	1439	1,2,3
48.6	Humulene	0.1 ± 0.02	0.3 ± 0.03	0.3 ± 0.02	0.3 ± 0.02	0.3 ± 0.01	1446	1446	1,2,3
49.2	*cis*-Muurola−4(15),5-diene	T	T	0.1 ±	0.1 ±	T	1457	1461	1,2
50.2	γ-Muurolene	0.1 ± 0.02	0.1 ± 0.03	T	T	T	1472	1472	1,2,3
50.3	Germacrene D	0.2 ± 0.05	0.5 ± 0.09	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.03	1474	1474	1,2,3
51.3	Bicyclogermacrene	-	0.2 ± 0.04	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.04	1490	1491	1,2
51.6	α-Muurolene	T	T	T	T	T	1496	1508	1,2,3
52.3	β-Bisabolene	1.2 ± 0.2	0.8 ± 0.1	0.9 ± 0.2	1.3 ± 0.3	1.2 ± 0.1	1506	1506	1,2,3
52.4	α- Farnesene	-	0.3 ± 0.06	-	-	-	1508	1509	1,2,3
53.0	Calamenene	-	0.2 ± 0.04	T	T	0.1 ± 0.03	1519	1516	1,2,3
53.1	Cadina−1(10),4-diene	0.1 ± 0.01	-	0.1 ± 0.03	0.1 ± 0.02	T	1520	1530	1,2
53.7	α-Cadinene	-	-	0.1 ± 0.02	0.1 ± 0.02	-	1532	1495	1,2
54.3	*cis*-α-Bisabolene	-	0.4 ± 0.08	T	T	T	1541	1522	1,2,3
54.7	*epi*-Cubebol	-	-	T	T	-	1549	1531	1,2,3
55.1	Thymoquinol	0.6 ± 0.1	0.1 ± 0.04	0.6 ± 0.09	0.3 ± 0.07	0.2 ± 0.04	1555	1522	1,2
55.9	Spathulenol	T	0.2 ± 0.04	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.02	1570	1570	1,2,3
56.2	Caryophyllene oxide	0.1 ± 0.02	T	0.2 ± 0.03	0.2 ± 0.04	0.1 ± 0.01	1574	1574	1,2,3
57.7	Humulene epoxide II	T	T	T	T	T	1600	1614	1,2
58.1	Epicubenol	T	0.1 ± 0.02	T	T	T	1608	1606	1,2,3
59.6	τ -Cadinol	T	T	T	T	T	1635	1479	1,2
60.3	α-Cadinol	T	0.1 ± 0.01	T	T	T	1648	1647	1,2
62.0	α-Bisabolol	0.1 ± 0.02	-	T	T	T	1680	1680	1,2,3
Monoterpene hydrocarbons		13.8	20.9	18.1	18.3	17.5			
Oxygenated monoterpenes		82.2	72.9	77.4	76.0	75.6			
Sesquiterpene hydrocarbons		2.7	5.5	3.2	4.6	4.2			
Oxygenated sesquiterpenes		0.8	0.5	0.9	0.4	0.4			
Others		0.3	0.2	0.2	0.5	0.5			
Total		99.8	99.9	99.9	99.8	98.2			

[a] The Kovats retention indices determined relative to a series of *n*-alkanes (C_{10}–C_{35}) on the apolar HP-5 MS [b] Theoretical Kovats index for each compound [c] Identification method: 1 = comparison of the Kovats retention indices with published data, 2 = comparison of mass spectra with those listed in the NIST 02 and Wiley 275 libraries and with published data, and 3 = co-injection with authentic compounds; t = trace (<0.1 %). - = absent, T = traces, less than 0.05 %.

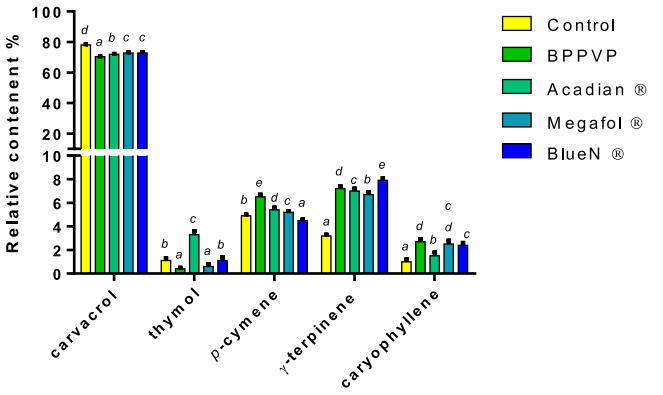


Fig. 5. Percentage of major components of five EOs of *O. vulgare* obtained by different biostimulant treatments. The same letters indicate that are not significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

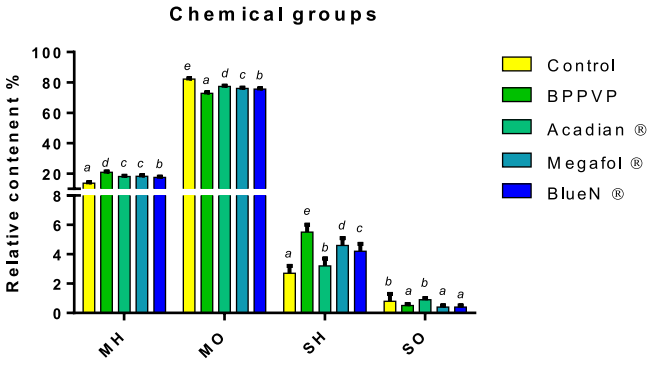


Fig. 6. Percent composition of main chemical groups in the essential oils subjected to different biostimulant treatments. MH: Monoterpenes hydrocarbons; MO: Monoterpenes oxygenated; SH: Sesquiterpenes hydrocarbons; SO: Sesquiterpenes oxygentated. The same letters indicate that are not significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

Table 4
Antioxidant activity (DPPH) and Total Phenolic Content (TPC).

	Control	Biostimol Plus + Peptamin-V Plus	Acadian®	Megafol®	BlueN®
DPPH IC_{50} (μg/mL)	287.60 ± 0.56[a]	263.80 ± 0.63[a]	263.00 ± 0.92[a]	261.00 ± 0.88[a]	272.10 ± 0.74[a]
TPC (GAE mg mL^{-1})	50.86 ± 0.81[b]	40.98 ± 0.98[a]	60.29 ± 0.79[c]	45.51 ± 0.77[ab]	41.55 ± 0.63[a]

The results are expressed as mean ± SD. The same letters indicate that are not significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

macroelement for the growth of oregano and EO yield (Farruggia et al., 2024a; Giannoulis et al., 2020). In general, the nitrogen increases number of stems, branches and inflorescences per plant, leaf area index, dry matter and oil yield (Farruggia et al., 2024a; Giannoulis et al., 2020). To contrast, Ninou et al. (2021), reported that the increased nitrogen supply level had a negative effect on EO content of five field grown greek oregano (*O. vulgare* subsp. *hirtum*) populations.

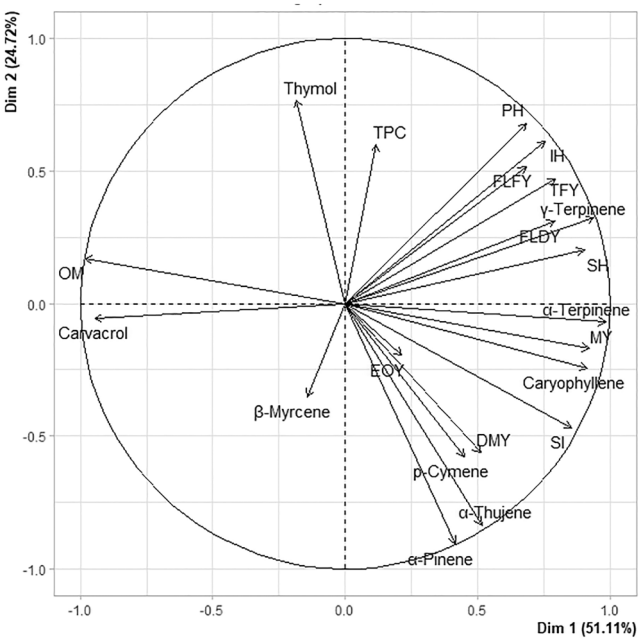


Fig. 7. Correlation biplot of the two first principal components variables in the two-dimensional space. PH: Plant Height; IH: Inflorescence Height; TFY: Total Fresh Yield; FLFY: Inflorescences+Leaves Fresh Yield; FLDY: Inflorescences+Leaves Dry Yield; SH: Stem Height; MY: Monoterpene Hydrocarbons; SI: Sesquiterpene Hydrocarbons; DMY: Dry Matter Yield; EOY: Essential Oil Yield; OM: Oxygenated Monoterpenes; TPC: Total Phenolic Content.

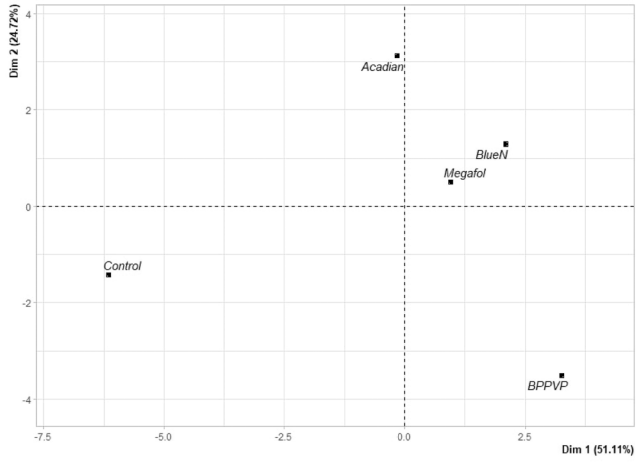


Fig. 8. Biplot showing the projection from the analysis of morphological, quantitative and qualitative traits. BPPVP: Biostimol Plus + Peptamin-V Plus.

4.2. Effects of biostimulants on EO yield and composition, antioxidant activity and TPC

In the literature, the chemical composition of EO *O. vulgare* is particularly varied. In fact, the composition of an EO is influenced by several factors, such as vegetal metabolism, climatic factors and the secretory activity of glandular hairs (Jerkoví et al., 2001). The analysis we obtained highlighted how our samples present a carvacrol chemotype: this is in agreement with the works carried out by Baj et al. (2020), Baycheva and Dobreva (2021), and Qiao et al. (2021). The percentage of carvacrol here reported (70–78.9 %) is similar to that obtained by Qiao et al. (2021) (75.72 %) However, as regards Baycheva and Dobreva (2021) and Baj et al. (2020), obtained a lower percentage of carvacrol was 45.09 % and 57.3 % respectively. Differences in terms of

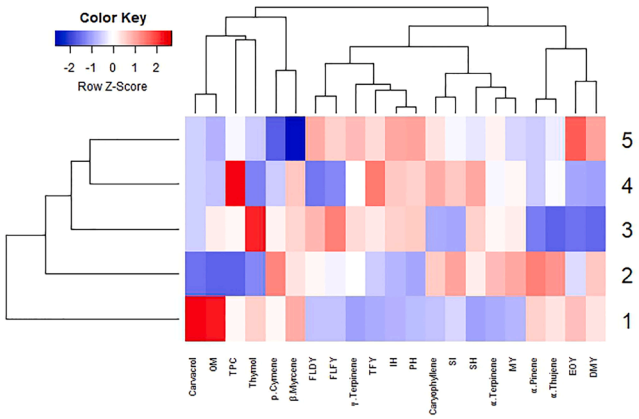


Fig. 9. Cluster heat map analysis summarizing open field *Origanum vulgare* L. responses to control (1), BPPV (2), Acadian® (3), Megafol® (4) and BlueN® (5) treatments.

percentages were also noted for the other compounds. In fact, the percentage of terpinene obtained in this study (7.9 %) appears to be higher compared to the afore mentioned works, while the percentage of thymol appears to be lower. Other works, however, report a chemical composition different from the one we obtained. Zhao et al. (2021) obtained an oregano EO in which the most abundant component was methyleugenol, while carvacrol accounted for 15 %. An EO with thymol chemotype was instead obtained by Simirgiotis et al. (2020). In fact, in their sample, thymol was found to be the most abundant component, while carvacrol represented only 3 %. Furthermore, hydrocarbon monoterpenes appear to be more present than those obtained by us (38.7 % vs 20.9 %), while the data regarding hydrocarbon and oxygenated sesquiterpenes appear to be similar. Kosakowska et al. (2021) reported sabinene as the main constituent (16.41 %), with *p*-cymene as the second most present component (10.45 %). The percentages of *p*-cymene were higher than those present in our samples (4.5–6.5 %), while the percentages of terpinene were similar to those obtained by us.

In our study less variations of carvacrol content were found among the treatments with biostimulants. The highest content of carvacrol was obtained in control samples. This agrees with a study of Giannoulis et al. (2020), who found that the unfertilized treatment had the lower thymol and the higher carvacrol content in inflorescences and leaves of oregano compared to plants fertilized with nitrogen. In addition, being the carvacrol content negatively correlated with morphological traits and yield (Fig. 6), this compound could be produced mainly when the plant grows under stress conditions (Emrahi et al., 2021). Similar result was obtained also by Farruggia et al. (2024b), who found that biostimulants application produced contrasting response on *Salvia officinalis* L., causing an increase in biomass yield and a decrease in total phenolic, antioxidant activity and rosmarinic acid content. Probably the oregano plants with biostimulant treatments and precipitation received before the harvesting period (May) manged to avoid stress producing less carvacrol and others bioactive compounds.

Negative correlations have been found between carvacrol and γ-terpinene, *p*-cymene and α-terpinene (Fig. 7). In fact, *p*-cymene and γ-terpinene are the final precursors in the pathway of carvacrol (Emrahi et al., 2021).

Phenolic and polyphenolic compounds can have an antioxidant action, preventing damage due to oxidative stress which is potentially dangerous for human health. Polyphenols are the antioxidants present especially in fruit and vegetable-based diets, providing nutritional advantages and contributing to the prevention of chronic diseases (Minatel et al., 2017). Furthermore, phenolic compounds can provide health benefits by counteracting the possible onset of metabolic disorders, such as type 2 diabetes mellitus (De Sales et al., 2012). Several studies have shown that the antioxidant activity of *O. vulgare* EO is linked to the presence of phenolic compounds, such as carvacrol and thymol. These phenolic compounds, thanks to their natural origin, could be used instead of synthetic antioxidants currently used in the food industry (Goyal et al., 2021; Rahman et al., 2021). The data obtained are in agreement with those reported by Laothaweerungsawat et al. (2020) where two EOs of *O. vulgare* were analyzed, one coming from tropical areas and one coming from the Mediterranean area: these samples are less active in comparison with the samples studied in our paper. Ilić et al. (2002) analyzed the antioxidant activity and TPC of *O. vulgare* EOs, obtaining an IC_{50} of approximately 4.5 mg/mL, thus highlighting a lower antioxidant capacity compared to the EOs we studied. Plants treated with Acadian® showed a higher content of total phenols than both the control and the other treated samples. This data is in agreement with the data present on the use of this biostimulant (Shafie et al., 2021). *Ascophyllum nodosum* is mostly present throughout the northwest coast of Europe and the northeastern coast of North America and, as the other seaweeds, produces many stress-related compounds that are important for its survival in these environments. Probably the application of Acadian® could have a positive influence on the expression of genes involved in the biosynthesis of phenols (Shukla et al., 2019). In fact, other studies reported that this biostimulant enhanced the phenolic content of *Spinacia oleracea* L. and improved fruit quality of *Vitis vinifera* L., by increasing the accumulation of anthocyanins and phenolics of grapevines (Shukla et al., 2019).

The responses of oregano plants to foliar biostimulant Biostimol Plus + Peptamin-V Plus were different compared to the other three biostimulants. The combination of these two biostimulants, could be allowed a different behavior of oregano plant. In particular, the extract of *Ascophyllum nodosum* could promote the activity of specific enzymes involved in the production of secondary metabolites (Abdali et al., 2023), in this case monoterpene and sesquiterpene hydrocarbons. On the other hand, the addition of micronutrients, as zinc and manganese could influence the composition of EO (Bhat et al., 2020).

In *Origanum* spp. the yield performance, EO content and composition are influenced also by the harvest time (Baranauskienė et al., 2013; Sonmez, 2019). Further studies are necessary to evaluate how the use of these biostimulants can influence these traits during different harvest times.

Although in recent years research on the effects of biostimulants has focused mainly on nitrogen-fixing microorganisms, further studies on microbial biostimulants are necessary. The quality of commercial inoculants depends on several factors, such as the storage method, the viability of the microorganisms and the effectiveness of the selected strains. The use of microbial consortia made up of several genetically different strains could increase their versatility and broad-spectrum action.

5. Conclusion

This study examined how four biostimulants affected the growth, yield and chemical composition of EO of oregano. The results of this study attest to the positive effect of the application of biostimulants both for yields and for volatile chemical profile of oregano. Although it is necessary to better investigate the influence of dose and modality of application (foliar and/or radical), the results already appear promising for the definition of sustainable cultivation protocols, which appear particularly useful in consideration of the peculiar methods of use of oregano. In this study, plants treated with BlueN® showed the best quantitative response compared to essential oil composition. Besides, the application of Acadian® biostimulant gave the highest total phenolic content.

In this work the biostimulant treatments influenced the chemical composition of EO by increasing the number of components, monoterpene hydrocarbons and sesquiterpene hydrocarbons contents. The production of EOs from organic cultivation, and in this case according to the application of biostimulants, could meet the needs of many

consumers, people mainly careful to the environment and information reported on the label. Moreover, with the increase in environmental awareness and the need to reduce the use of chemical fertilizers, biostimulants offer a promising solution for more sustainable and productive agriculture. Despite the oregano plant adapts to different climatic conditions, air temperature above 33 °C may limit plant growth. Therefore, the biostimulants could allow oregano plants to overcome critical periods, especially abiotic stress from high temperatures during the summer period in Mediterranean areas.

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CRediT authorship contribution statement

Vincenzo Candido: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization. **Michele Perniola:** Supervision, Resources, Project administration, Investigation, Funding acquisition. **Donato Casiello:** Methodology, Investigation. **Laura De Martino:** Writing – review & editing, Validation, Methodology, Investigation, Data curation, Conceptualization. **Vincenzo De Feo:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Giuseppe Amato:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Loriana Cardone:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nunzia Cicco:** Supervision, Investigation, Conceptualization. **Michele Denora:** Writing – review & editing, Software, Methodology, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Michele Perniola reports financial support was provided by Basilicata Region. None If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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