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SUSTAINABLE APPROACH TO IMPROVE THE PRODUCTION PERFORMANCE OF MEDICINAL AND AROMATIC PLANTS BY APPLICATION OF VARIOUS TYPES OF BIOSTIMULANTS IN AN ORGANIC AGRICULTURAL SYSTEM

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1. INTRODUCTION

1.1. Medicinal and Aromatic Plants (MAPs)

The interest in the utilization of medicinal and aromatic plants (MAPs) has been globally increased over the past several decades (WHO, 2019). For millennia, MAPs have been a key category of plants for human health and culture, acting as a link between the health of humans and the natural world (Ninou et al., 2021).

MAPs show a long history dating back to ancient civilizations, when they were highly valued and carefully recorded in pharmacopoeias and medical writings. The basis for modern practices was laid by the ancient Greeks, Chinese, and Egyptians who used a variety of plants for their fragrant and medicinal virtues (Salmerón-Manzano et al., 2020).

These plants are distinguished by their therapeutic properties and aromatic compounds, making them precious in traditional and modern medicine, gastronomic sector, and pharmaceutical and cosmetics industry (Lubbe and Verpoorte, 2011; Michel et al., 2020; Pergola et al., 2024). Medicinal plants have long been utilized to cure a wide range of illnesses in humans. According to a survey provided by the World Health Organization (WHO), 80% of people worldwide prefer to use conventional medical practices. Due to claims that medicinal plants are safe to use and their shown efficacy in treating specific conditions, the usage of these plants is currently rising worldwide (Perez Gutierrez and Baez, 2009).

The FAO's Medicinal and Aromatic Plant Working Group defines MAPs as "botanicals that provide people with medicines to prevent disease, maintain health or cure ailments" (Marshall, 2012).

In the modern era, researchers, healthcare professionals, and consumers have a renewed interest in these plants because of the rise in natural and holistic health practices (Pergola et al., 2024). The need for natural alternatives has increased in response to growing knowledge of the drawbacks and restrictions of synthetic drugs, placing MAPs at the center of therapeutic uses and medical research (Sharma et al., 2021).

Various scientific sectors, including agricultural, botanical, chemical, food and pharmacological sciences have been impacting by the studies on MAPs (Andrade et al., 2018; Boukhatem and Setzer, 2020; Giannenas et al., 2020; Grigoriadou et al., 2020; Saha and Basak, 2020; Fierascu et al., 2021; Kisiriko et al., 2021; Kralova and Jampilek, 2021; Pinto et al., 2021; Maleš et al., 2022). Their responsibilities and potentials are understood holistically thanks to this multidisciplinary approach. Scientists are constantly finding new bioactive substances in these plants, which broadens our understanding and creates new opportunities for the creation of drugs and other uses (Fierascu et al., 2021; Kisiriko et al., 2021; Greff et al., 2023; Mitropoulou et al., 2023). Because overfishing, habitat loss, and climate change pose dangers to many species, it is also imperative that MAPs be conserved and cultivated sustainably (De Falco et al., 2013; Shafi et al., 2021).

According to the economic reports, the global herbal medicine market size was valued at USD 216.40 billion in 2023 and is projected to grow from USD 233.08 billion in 2024 to USD 437 billion by 2032, exhibiting a CAGR of 8.17% during the forecast period. Europe dominated the herbal medicine market with a market share of 44.82% in 2023 (https://www.fortunebusinessinsights.com/herbal-medicine-market-106320).

This growth is driven by increasing consumer awareness and preference for natural products, coupled with advancements in extraction and formulation technologies (Cadar er al., 2021; Capucho et al., 2023; Mendes et al., 2023; Spina et al., 2023)

Developing countries, in particular, benefit economically from the cultivation and export of MAPs. Countries such as India and China, are leader in the export of medicinal plants, herbs, and essential oils (Singh et al., 2022). These exports significantly contribute to their national economies, providing livelihoods for millions of people engaged in agriculture, harvesting, processing, and trade (Kala, 2015).

MAPs are widespread in the Mediterranean region and many species are part of the natural flora of this area. In the Mediterranean basin there are several agro-climatic areas that are ideal to produce and conserve MAPs (Taghouti et al., 2022).

Many MAPs are typical of some areas and have unique characteristics depending on the environment in which they have adapted. In fact, their presence in a given environment characterizes the biodiversity of a territory and in some cases, they represent the typical resource of a territory where ancient cultures and local products are linked by a strong bond of traditions and uses of these herbs (Giannenas et al., 2020).

The knowledge and use of aromatic and medicinal species present in a range become part of the tradition and cultural heritage of that territory in an essential way (Shafi et al., 2021; Taghouti et al., 2022).

The long history of using plants, the growing interest in natural ingredients, healthy diets, and the need for more traditional products with strong cultural heritage can all be used to explain why there is an increasing demand for such raw materials throughout the Mediterranean region (Cheminal et al., 2020; Stefanaki et al., 2021; Savvides et al., 2023).

The economic significance of MAPs also extends to the tourism sector. Ecotourism and wellness tourism often feature botanical gardens, herb farms, and traditional healing centers that attract visitors interested in natural health and wellness practices. This tourism generates revenue and supports local economies while promoting the conservation and sustainable use of MAPs (Capucho et al., 2023; Spina et al., 2023; Pergola et al., 2024).

1.1.2. Importance, area, production, diffusion, uses

MAPs are characterized by high adaptability and resistance even in the most disadvantaged environments, being able to withstand the most diverse climatic conditions. These characteristics have been exploited in order to use with benefits the marginal and often abandoned areas, such as those in the hills and mountains where it would be problematic to insert high-income species that need high inputs. The cultivation of MAPs is well integrated in marginal contexts because it positively responds to productive conditions characterized by low energy inputs (Argento et al., 2014).

Cultural practices and traditional knowledge systems worldwide have long recognized the value of MAPs (Shafi et al., 2021; Ivanova et al., 2022). Ethnobotany, the study of the relationship between people and plants, reveals the extensive use of these plants in traditional medicine, rituals, and daily life. Indigenous communities possess rich knowledge about the medicinal and aromatic properties of local flora, passed down through generations (El-Assri et al., 2021; Chaachouay et al., 2022; Savvides et al., 2023).

The ethnobotanical relevance of MAPs also extends to culinary traditions. Herbs and spices such as basil, oregano, thyme, rosemary, sage, are integral to various cuisines, enhancing flavor and preserving food. These plants often have health benefits, contributing to the cultural emphasis on food as medicine (Nieto, 2020; Maleš et al., 2022; Plati and Paraskevopoulou, 2022; Aziz et al., 2022; Speranza et al., 2023).

MAPs play vital roles in ecosystems, contributing to biodiversity, supporting wildlife, and maintaining ecological balance. Many MAPs are keystone species, meaning their presence and health significantly influence the structure and function of their ecosystems (Scherrer et al., 2023).

Additionally, MAPs can contribute to soil health and prevent erosion. Deep-rooted plants such as turmeric and ginger help maintain soil structure and fertility. The cultivation of these plants can enhance biodiversity and promote sustainable land use practices (Tuttolomondo et al., 2017; La Bella et al., 2021; Gupta et al., 2021).

MAPs have long been an essential part of culinary traditions all over the world because of their numerous health advantages as well as their capacity to improve the flavor and scent of food. Many cuisines use culinary herbs and spices like basil, oregano, thyme, rosemary, and cilantro as main ingredients because of their unique tastes and aromatic properties (Shahrajabian et al., 2020; Węglarz et al., 2020; Stefanaki et al., 2021; Aziz et al., 2022). Culinary MAPs also contribute to health beyond their flavor. Many herbs and spices possess bioactive compounds that can enhance digestion, reduce inflammation, and provide antioxidant benefits (Veenstra and Johnson, 2021; Marc et al., 2022; Kaur et al., 2023; Ansorena and Astiasaran, 2024).

Additionally, the idea that food may act as medicine is supported by the use of MAPs in cooking and is ingrained in a number of conventional medical systems. Culinary MAPs are a key component of this paradigm, which highlights the significance that nutrition plays in preserving health and avoiding disease (Stefanaki et al., 2021).

Thanks to aromatic qualities, MAPs are widely used in the fragrance industry, including perfumes, cosmetics, and aromatherapy. Essential oils extracted from these plants are used for their pleasant scents and therapeutic benefits, influencing mood, cognition, and overall well-being (Pirzad and Mohammadzadeh, 2018; Mahajan et al., 2020; Fierascu et al., 2021). Demand for EO is primarily driven by the following markets: household (16%), pharmaceutical (15%), food and beverage (35%), perfumes, cosmetics, and aromatherapy (29%) (Barbieri and Borsotto, 2018).

In order to replicate the aromatic and chemical components of natural, plant-based oils, which are more costly to produce, many enterprises utilize synthetic scents that are created in laboratories. Synthetic perfumes, however, could not have the health benefits of natural essential oils derived from plants and could possibly be dangerous for use in human applications (Sharmeen et al., 2021).

The fragrance industry extensively uses MAPs to create natural scents. Essential oils have potential uses in perfumes due to their volatile nature, but this does not rule out additional uses in cosmetics (Guzmán and Lucia, 2021). Their intended usage has been mostly industrial for a long time, since standardizing fragrance has been a regular technique that involves combining essential oils from several plants to create a precise aroma (Butnariu, 2021). Essential oils from several plants are key ingredients in high-end perfumes, providing unique and complex aromas that synthetic compounds often cannot replicate (Sharmeen et al., 2021).

Aromatherapy, a holistic healing practice, leverages the aromatic properties of MAPs to promote physical and psychological well-being. Essential oils are used in various methods, including inhalation, topical application, and diffusion, to address a range of health issues such as stress, anxiety, pain, and respiratory problems (Thangaleela et al., 2022). The therapeutic potential of essential oils is supported by increasing scientific evidence, highlighting their role in complementary and integrative medicine (Yuan et al., 2021).

1.1.3. Plant metabolism

Metabolism is the set of coordinated and integrated chemical transformations that incessantly occurs in all cells, and it is at the basis of cellular life (Fang et al., 2019; Nicola and Scarpa, 2022). Metabolism comprises a wide range of reactions for the construction of molecules (anabolism) necessary for the life of the organism, and for the demolition of others molecules (catabolism) to produce energy or secondary metabolites (Alamgir, 2018). In particular, it is possible to distinguish:

- Primary metabolism, which includes those metabolic processes that allow the biosynthesis and modification of molecules essential to the development and growth of plant organisms, such as carbohydrates, proteins, lipids, nucleic acids, belonging to the primary metabolites group.
- Secondary metabolism, which includes those biosynthetic pathways that originate from precursors of primary metabolism; these biosynthetic processes lead to the formation of secondary metabolites, which are not indispensable for the development and growth of plant organisms, but are essential in the processes of environmental adaptation, reproduction, and inter-species communication. These substances are responsible for the characteristic odor, color and taste of plant organs and have medicinal or toxic properties.

1.1.3. Secondary metabolism

The biosynthesis of active substances takes place through metabolic pathways using intermediate products of primary metabolism which, due to a biochemical imperfection or normal physiological process, accumulate in plant cells (Julsing et al., 2006; Luckner, 2013). Secondary metabolism products are multifunctional substances that, following events that cause an alteration of the balance of the plant organism, participate in the processes of restoration of cellular characteristics; for this reason, the induction of biotic and abiotic stress can cause an increase in the production of secondary metabolites and active compounds (Borges et al., 2017; Li et al., 2020).

Inside the organs of MAPs are present different classes of active compounds, biologically active substances that belong to different chemical groups: alkaloids, glycosides, gums, mucilage, bitter principles, tannins, organic acids, enzymes, vitamins, resins, balms, and essential oils (Daniel, 2006; Alamgir, 2018).

These substances can exhibit a wide range of pharmacological activities, such as antimicrobial, anti-inflammatory, antioxidant, and antitumor effects (Li et al., 2020; Huang et al., 2022; Skrypnik et al., 2022). The secondary metabolism in plants is an intricate biochemical process that produces these compounds, often as a response to environmental stressors such as pathogens, herbivores, or physical injuries (Borges et al., 2017; Pant et al., 2021). These substances are stored in different parts of the plants, such as leaves, roots, flowers, seeds, or bark, and their concentrations can vary depending on environmental conditions, genetic factors, and the plant's stage of development (Julsing et al., 2006; Yadav et al., 2014; Huang et al., 2022)

The biosynthesis of secondary metabolites is controlled by complex genetic and enzymatic pathways (Khare et al., 2020; Jan et al., 2021; Zhan et al., 2022). Secondary metabolites are classified according to the biosynthetic pathway from which they originate and are distinguished into (Nicola and Scarpa, 2022):

- terpenoids, formed by the mevalonate (MVA) and methyl erythritol phosphate (MEP) pathways;
- phenolic compounds, divided into:
    - Simple phenols, formed by the shikimic acid pathway;
    - Phenylpropanoids, formed by cinnamic acid pathway;
    - flavonoids formed by the union of the cinnamic acid and malonate pathways;
    - Quinone compounds, formed by the polychetite pathway;
- alkaloid compounds, which are derived biosynthetically from certain amino acids.

For example, alkaloids are nitrogen-containing compounds synthesized primarily from amino acids, while terpenoids are derived from isoprene units and are part of the plant's defense against herbivores and microorganisms. Phenolic compounds, on the other hand, are primarily synthesized via the shikimic acid pathway and include flavonoids, tannins, and lignins, which contribute to plant pigmentation, UV protection, and structural integrity (Khare et al., 2020; Pinto et al., 2021; Huang et al., 2022). The precise metabolic pathways involved can be influenced by both intrinsic factors, such as the plant's genetic makeup, and extrinsic factors like soil composition, climate, and exposure to biotic or abiotic stress (Li et al., 2020; Mahajan et al., 2020).

Medicinal plants have been extensively studied for their secondary metabolites (De Falco et al., 2013; Borges et al., 2017; Boukhatem and Setzer, 2020; Mahajan et al., 2020; Michel et al., 2020; Shahrajabian et al., 2020; Tuttolomondo et al., 2020; Kisiriko et al., 2021; Pant et al., 2021; Kant and Kumar, 2022; Shinyuy et al., 2023; Speranza et al., 2023). These compounds not only offer therapeutic benefits but also help the plants adapt to their natural habitats by deterring pests and protecting against diseases (Giannenas et al., 2020; Fierascu et al., 2021; Billowria et al., 2024).

Secondary metabolites found in MAPs represent a vital area of research in both botany and pharmacology. Their ability to modulate physiological processes in humans, coupled with their ecological roles in the plant kingdom, underscores the importance of further investigating these compounds. Advances in biotechnology, including genomics, metabolomics, and bioengineering, hold promise for enhancing the production of these valuable metabolites, offering new possibilities for the development of natural and sustainable therapeutic agents. The continued exploration of plant secondary metabolites is essential for harnessing the full potential of medicinal plants in modern healthcare, particularly in the development of novel treatments for a range of diseases (Alamgir, 2017; Michel et al., 2020; Cadar et al., 2021; Mitropoulou et al., 2023; Shinyuy et al., 2023).

Furthermore, the pharmacokinetics of these bioactive compounds in the human body, including their absorption, distribution, metabolism, and excretion, can depend on the chemical structure of the compounds (Billowria et al., 2024). For example, flavonoids, which are abundant in many medicinal plants, are often metabolized by the gut microbiota before they are absorbed into the bloodstream, while terpenes may be absorbed more rapidly but excreted through the urine or via exhalation (Sova and Saso, 2020). Understanding these mechanisms is crucial for optimizing the use of medicinal plants in therapeutic applications, as it helps determine effective dosages, potential interactions with other medications, and possible side effects (Shinyuy et al., 2023).

Secondary metabolism can vary in different plant species and is conditioned by ecological and genetic factors; the synthesis of different metabolites is often stimulated under non-optimal conditions and stress for plant species (Pedretti, 2003).

The amount of nitrogen in soil also influences the production and composition of secondary metabolites; the carbon/nitrogen equilibrium theory (Bryant et al., 1983; Coley et al., 1985) and later the growth/differentiation equilibrium theory (Lorio et al., 1986; Herms and

Mattson, 1992), explain how plants, when in poor soil conditions, are use available substances for processes related to the growth or differentiation and production of primary or secondary metabolism products. When nitrogen is readily usable, plants produce mainly high-nitrogen compounds, such as nitrogen-containing proteins and secondary metabolites. When nitrogen is limited, the plant synthesizes carbon-containing substances such as starch, cellulose and secondary non-nitrogen metabolites (Brandt and Mølgaard, 2001).

Within the plant organism, secondary products have a defensive function against herbivores and attacks of pathogens and other plants, but at the same time they also play an attractive role towards beneficial organisms such as pollinators or symbionts; they also have protective actions in relation to abiotic stresses such as those associated with changes in temperature, water and nutrient availability, light levels, UV exposure. They are responsible for the odours, colours and tastes typical of the plants that produce them, as well as having medicinal and toxic properties (Marzi and De Mastro, 2008).

In addition, several authors have identified among the potential roles of secondary metabolites, the function of plant growth regulators at the cellular level and the accumulation of reserve substances (Bakkali et al., 2008).

1.1.4. Essential oil (EO)

Essential oils (EOs) are described as "aromatic products with a mixture of compounds derived from plant raw material, either separated by steam, dry distillation, or by a suitable mechanical technique without heating" in the seventh edition of the European Pharmacopeia (El Asbahani et al., 2015). Through physical means, essential oil (EO) is extracted from liquid phase without undergoing any chemical compositional changes. Because the majority of people rely on the products of these plants, herbal plants are extremely significant. These plant' products are utilized in the food industry, cosmetics, medicine, and other fields (Kant and Kumar, 2021). EOs may be extracted from a variety of medicinal plant components, including barks, peels, leaves, buds, seeds, flowers, and more, using a variety of extraction processes (Tongnuanchan and Benjakul, 2014).

The EO composition, content and yield mainly depends on genetic factors, geographic origin, climate and soil conditions, and the specific part of the plant used for extraction (De Falco et al., 2013; Lukas et al., 2015; O. Elansary et al., 2020; Król et al., 2020; Emrahi et al., 2021; Ninou et al., 2021; Tarasevičienė et al., 2021; Jafari Khorsand et al., 2022; Farruggia et al., 2023; Stasińska-Jakubas et al., 2023; Medeiros et al., 2024).

One of the most important classes of compounds found in EOs are the terpenes, which are subdivided into monoterpenes, sesquiterpenes, and diterpenes, based on the number of isoprene units in them (Hüsnü et al., 2007; Verdeguer et al., 2020). Monoterpenes, such as limonene and pinene, are often responsible for the oil's fragrance and can possess antimicrobial, antifungal, and anti-inflammatory properties. Sesquiterpenes, such as β-caryophyllene, tend to have more complex structures and are known for their anti-inflammatory and analgesic effects (Yadav et al., 2014; Verdeguer et al., 2020; Wojtunik-Kulesza, 2022). In addition to terpenes, EOs contain phenolic compounds, such as thymol and eugenol, which contribute to their antioxidant and antimicrobial activities. These bioactive compounds are what make EOs valuable for use in aromatherapy, pharmaceuticals, cosmetics, and even food preservation (Kosakowska et al., 2019; Boukhatem and Setzer, 2020; Guzmán and Lucia, 2021; Kaur et al., 2023).

Among the most used medicinal plants for EO extraction are *Lavandula angustifolia* Miller (lavender), *Melissa officinalis* L. (lemon balm), *Mentha piperita* L. (peppermint), *Origanum vulgare* L. (oregano), *Salvia officinalis* L. (sage), *Thymus vulgaris* L. (thyme), and *Salvia rosmarinus* Spenn. (rosemary). Lavender oil, rich in linalool and linalyl acetate, is renowned for its calming, sedative, and anti-anxiety effects, making it popular in aromatherapy for stress relief and sleep disorders (Ciocarlan et al., 2021). The main bioactive compounds of *M. officinalis* are volatile compounds (geranial, neral, citronellal and geraniol), triterpenes (ursolic acid and oleanolic acid), phenolic compounds (rosmarinic acid, caffeic acid, and protocatechuic acid) and flavonoids (quercetin, rhamnocitrin, luteolin). Lemon balm EO is characterised by a fresh lemon odor and light yellow and is usually considered to be the responsible therapeutic principle for most biological activities. The extract from lemon balm possesses a variety of pharmacological actions with possible medicinal applications, such as antioxidant, antimicrobial, and cytotoxic activities, and many others (Petrisor et al., 2022).

Peppermint oil, dominated by menthol and menthone, is widely used for its analgesic, anti-inflammatory, and digestive properties (Zhao et al., 2022).

Terpenes such as thymol, carvacrol, p-cymene, γ-terpinene, and linalool are the main constituents of oregano EOs and based on their prevalence, different chemotypes can be commonly attributed to this species. Several researchers reported the antibacterial, antifungal, antiparasitic, antioxidant, anti-inflammatory, antitumor, skin disorders beneficial effects, next to antihyperglycemic and anti-Alzheimer activities of oregano extract (Lombrea et al., 2020).

EO of *S. officinalis* is known for its remarkable variability in the main monoterpene constituents β-pinene, 1,8-cineole, α-thujone, β-thujone and camphor. The extracts and EO obtained from sage have health-beneficial properties, such as antioxidant, antibacterial, hypoglycemic, anti-inflammatory, fungistatic, astringent, eupeptic, and anti-hydrolytic activity, hypotensive properties, central nervous system depressant actions, and anti-spasmodic activity; sage EO also possess bactericidal and bacteriostatic effects (Karalija et al., 2022).

Thyme oil contains high concentrations of thymol, which exhibits strong antimicrobial and antifungal properties, making it useful in both medicine and natural cleaning products (Posgay et al., 2022).

Rosemary oil, with its high levels of 1,8-cineole and camphor, is known for its stimulant and memory-enhancing effects, as well as its use in treating respiratory and circulatory issues. Rosemary extracts, thanks to the high phenolic contents, also present antioxidant, antibacterial, antifungal, hepatoprotective and insecticide properties. Several medicinal applications for *S. Rosmarinus* have been identified, such as treatment of disorders associated with the nervous, cardiovascular, gastrointestinal, genitourinary, menstrual, hepatic, and reproductive systems and with respiratory and skin conditions (de Macedo et al., 2020)

The pharmacological efficacy of EOs depends not only on their chemical composition but also on their method of application. Inhalation of EOs, for instance, allows volatile compounds to directly interact with the olfactory system and limbic system in the brain, influencing emotions and physiological responses. Topical application enables the active compounds to penetrate the skin and exert localized effects, such as reducing inflammation or pain. Some EOs are also used internally, although this practice requires careful dosage and consideration due to the high potency of these compounds and the risk of toxicity if misused (Cimino et al., 2021).

Scientific research into EOs has expanded significantly in recent years, with studies exploring their potential antimicrobial, antioxidant, anticancer, and neuroprotective effects.

The variability in chemical composition based on factors like plant genetics, environmental conditions and cultivation practices can affect the consistency of therapeutic outcomes. Moreover, while essential oils are generally considered safe when used properly, inappropriate usage, such as ingesting undiluted oils or applying them to sensitive skin, can lead to adverse effects, including allergic reactions, burns, and toxicity. Therefore, it is crucial to standardize the production and application of EOs to ensure their safe and effective use (Abdellaoui et al., 2020; Napoli et al., 2020; Cimino et al., 2021; Ni et al., 2021; Angane et al., 2022; Crișan et al., 2023).

1.1.5. Beneficial Properties of Medicinal and Aromatic Plants

MAPs are usually celebrated for their numerous beneficial properties, which significantly contribute to human health and well-being. These properties stem from a rich array of bioactive compounds that exhibit various pharmacological activities. MAPs plants have been utilized in traditional medicine for their therapeutic properties. These plants contain bioactive compounds that offer a range of health benefits (Julsing et al., 2006; Alamgir, 2017; Fierascu et al., 2021; Kisiriko et al., 2021; Thangaleela et al., 2022; Li et al., 2023; Billowria et al., 2024; Bouloumpasi et al., 2024). Some beneficial properties of oregano, sage, rosemary and lemon balm, which were investigated and tested, are described in the following paragraphs.

Oregano (*Origanum vulgare* L.) is well-known for its antimicrobial properties, which are primarily due to phenolic compounds such as carvacrol and thymol. Oregano oil is used to treat respiratory infections, gastrointestinal disorders, and skin conditions. It is also believed to boost immune function (De Falco et al., 2013; Kosakowska et al., 2019; Lombrea et al., 2020; Ninou et al., 2021; Jafari Khorsand et al., 2022).

Sage (*Salvia officinalis* L.) has a long history of medicinal use, known for its anti-inflammatory, antioxidant, and antimicrobial activities. Sage tea is traditionally consumed to soothe sore throats, improve digestion, and alleviate menopausal symptoms. Recent studies also suggest sage may enhance cognitive function and memory (Delamare et al., 2007; Abu-Darwish et al., 2013; Es-Sbihi et al., 2021; Aslani et al., 2023; Ben Akacha et al., 2023; Speranza et al., 2023)

Rosemary (*Salvia rosmarinus* Spenn.) is recognized for its cognitive-enhancing effects. Compounds like carnosic acid and rosmarinic acid in rosemary have antioxidant and anti-inflammatory properties, which can help protect the brain and improve memory and concentration. Rosemary is also used to alleviate muscle pain and improve digestion (Pirzad and Mohammadzadeh, 2018; de Macedo et al., 2020; La Bella et al., 2020; Veenstra et al., 2021; Aziz et al., 2022; Eid et al., 2022; Kaur et al., 2023; Ansorena et al., 2024).

Lemon balm (*Melissa officinalis* L.) is valued for its calming effects. It is commonly used to reduce stress and anxiety, promote sleep, and improve digestive health. Lemon balm contains compounds such as rosmarinic acid, which contribute to its soothing properties

(Ghazizadeh et al., 2021; Petrisor et al., 2022; Stasińska-Jakubas et al., 2023; Medeiros et al., 2024).

1.1.5.1. Antimicrobial Properties

Many MAPs possess potent antimicrobial properties, making them effective against a wide range of pathogens, including bacteria, fungi, and viruses. These antimicrobial effects are primarily due to the presence of essential oils and other bioactive compounds that can inhibit microbial growth and prevent infections (Ciocarlan et al., 2021; Angane et al., 2022; Huang et al., 2022; Posgay et al., 2022; Mitropoulou et al., 2023; Bouloumpasi et al., 2024).
Oregano contains high levels of carvacrol and thymol, which have been shown to disrupt the cell membranes of bacteria and fungi, inhibiting their growth. Oregano EO is used as a natural antibiotic and antifungal agent. It is also exploited in both culinary and medicinal contexts to prevent food spoilage and treat infections (Granata et al., 2021; Mutlu-Ingok et al., 2021; de Almeida et al., 2023; Walasek-Janusz et al., 2024).
The EO of sage contains thujone and camphor, which are effective against bacterial and fungal infections. Sage is used in mouthwashes and gargles to treat oral infections and sore throats (Delamare et al., 2007; Abu-Darwish et al., 2013; Ben Akacha et al., 2023; Speranza et al., 2023).
Rosemary shows antimicrobial activity against various bacteria and fungi. The compounds in the rosemary essential oil, such as cineole and camphor, contribute to its preservative qualities, making it useful in food preservation and natural disinfectants (Jafarı-sales and Pashazadeh, 2020; Pieracci et al., 2021; Günther et al., 2022; Kaur et al., 2023).
Lemon balm exhibits antiviral properties, particularly against herpes simplex virus. The rosmarinic acid in lemon balm helps to inhibit viral replication, making it a valuable natural remedy for cold sores and other viral infections (Schnitzler et al., 2008; Parham et al., 2020; Abdellatif et al., 2023).

1.1.5.2. Antioxidant Properties

The antioxidant properties of MAPs are crucial for neutralizing free radicals and reducing oxidative stress, which is implicated in the development of chronic diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders. Antioxidants in MAPs scavenge free radicals, protecting cells from damage and supporting overall health (Parham et al., 2020; Mutlu-Ingok et al., 2021; Skrypnik et al., 2022; Bouloumpasi et al., 2024).

Oregano is one of the herbs with the highest antioxidant capacity, primarily due to its high phenolic content. Regular consumption of oregano can help reduce oxidative stress and lower the risk of chronic diseases such as cancer and cardiovascular diseases (Mutlu-Ingok et al., 2021; Jafari Khorsand et al., 2022; Bouloumpasi et al., 2024; Walasek-Janusz et al., 2024).

Sage contains powerful antioxidants, including rosmarinic acid, carnosic acid, and flavonoids. These compounds help protect the body from oxidative damage and have been shown to improve brain function and reduce inflammation (Brindisi et al., 2021; Mot et al., 2022; Al-Mijalli et al., 2022; Mokhtari et al., 2023; Bouloumpasi et al., 2024).

Rosemary is rich in carnosic acid and rosmarinic acid, both of which have strong antioxidant properties. These chemicals support the health-promoting properties of rosemary by contributing to the elimination of free radicals and providing defense against oxidative stress. (Topal and Gulcin, 2022; Eid et al., 2022; Li Pomi et al., 2023; Bouloumpasi et al., 2024).

Lemon balm is a good source of antioxidants, particularly rosmarinic acid and flavonoids. These compounds contribute to reduce oxidative stress, support immune function, and promote skin health (Kamdem et al., 2013; Boneza and Niemeyer, 2018; Abdellatif et al., 2023; Bouloumpasi et al., 2024).

1.1.5.3. Anti-inflammatory Properties

Inflammation is a natural response to injury or infection, but chronic inflammation is a key factor in many diseases, including arthritis, cardiovascular diseases, and certain cancers. For the creation of anti-inflammatory and analgesic medications, secondary metabolites obtained from MAPs, such as polyphenols, flavonoids, terpenoids, and alkaloids, are crucial sources. According to recent findings, these molecules decrease pro- and neo-inflammatory mediators, chemokines, cytokines, and other inflammatory mediators involved in inflammatory processes, hence exhibiting an anti-inflammatory impact (Nunes et al., 2020; Brindisi et al., 2021; Gandhi et al., 2022; Sidiropoulou et al., 2022; Mitropoulou et al., 2023).

Oregano exhibits anti-inflammatory effects due to compounds such as carvacrol and rosmarinic acid. These compounds permit to reduce inflammation by inhibiting the production of pro-inflammatory cytokines (Avola et al., 2020; Sidiropoulou et al., 2022; Moghrovyan et al., 2023).

Various studies have documented the potential of sage to reduce inflammation in various studies. Its active compounds, including rosmarinic acid and carnosic acid, inhibit inflammatory pathways and help alleviate conditions such as arthritis and inflammatory bowel disease (Abu-Darwish et al., 2013; Han and Parker, 2017; Brindisi et al., 2021; Sidiropoulou et al., 2022).

Rosemary contains anti-inflammatory compounds like carnosic acid and rosmarinic acid, which contribute to reduce inflammation and pain. Rosemary is used to manage inflammatory conditions such as arthritis and muscle pain (Borges et al., 2019; Luo et al., 2020; Gonçalves et al., 2022).

It has been demonstrated that lemon balm has the potential to reduce inflammation due to its high content of rosmarinic acid. This compound inhibits the production of inflammatory molecules, making lemon balm effective in managing conditions like inflammatory skin diseases and gastrointestinal inflammation (Ramanauskienė et al., 2015; Kim et al., 2020; Ullah and Hassan, 2022).

1.1.6. Agronomic practices

MAPs are considered not only for their biomass production but also for their bioactive compounds, such as EOs, alkaloids, flavonoids, and phenolic compounds, which have important therapeutic and aromatic properties. Yield and quality parameters of MAPs are influenced by a variety of agronomic factors, including soil management, irrigation, fertilization, plant density, and harvest time (Chrysargyris et al., 2022; Nurzyńska-Wierdak et al., 2023). Understanding the impact of these agronomic practices can help to optimize quantity and quality of MAPs production, ensuring sustainability and maximizing economic value. The management of available resources and the choice of agronomic practices to be applied are fundamental aspects to obtain products with high and constant quality standards. MAPs accumulate bioactive substances throughout the biological cycle, but it is not excluded that in some cases temporary stress conditions may cause an increase in the content and yield of active molecules in plant tissues (Catizone et al., 2013). Crop manipulation or deliberate elicitation, a management strategy that consists in triggering temporary stress conditions for the plant organism during the growing cycle, can be used to increase the accumulation of secondary metabolites (Trivellini et al., 2016).

Soil management and tillage play a fundamental role in determining growth, yield, and quality of MAPs. Soil fertility, structure, and microbial activity directly affect water and nutrients availability for plants, influencing their overall health and productivity.

Water availability and irrigation management are critical factors that influence both the yield and quality of MAPs. In the Mediterranean region, MAPs are often unirrigated since this area is characterized by prolonged periods of drought (Virga et al., 2020). Water scarcity is one of the main factors leading to a decrease in crop production and inducing physiological and biochemical changes in plants. Stomatal closure is the first impact of water stress and causes the inhibition of carbon dioxide absorption and a decrease in photosynthesis; carbohydrate synthesis and distribution to belowground plant parts are negatively affected by water stress (Shaw et al., 2002). Similar to other MAPs, prolonged periods of water stress during the developmental stage may cause alterations in physiological and metabolic processes. Drought conditions have been found to enhance the EO content of some MAPS, because the plants produce more secondary metabolites as a defense mechanism against stress. However, prolonged or excessive water stress can severely reduce biomass production, compromising overall yield (Yadav et al., 2014; Morshedloo et al., 2017). According to many authors, limited water availability can have a negative effect on photosynthetic processes and transpiration, leading to a significant fall in growth and yield parameters. Low water availability tends to reduce nutrient absorption and translocation to the buds due to the slowing down of the transpiration process; limited water flow can occur while the availability of nutrients, such as potassium (K), nitrogen (N) and calcium (Ca), around the root area is scarce (Marschner and Rengel, 2023). A balanced irrigation strategy is essential for optimizing biomass production and the concentration of bioactive compounds. Deficit irrigation has been shown to increase the concentration of bioactive compounds such as essential oils, flavonoids, and alkaloids in many MAPs (Giannoulis et al., 2020; Gorgini Shabankareh et al., 2021). However, the intensity and duration of water stress has to be carefully controlled to avoid reducing plant productivity.

Nutrition plays a key role in the growth and development of all cultivated plants; in the case of MAPs, the availability of micro- and macro elements, fundamental for both primary and secondary metabolism, can effectively influence and increase plant growth and synthesis of secondary metabolites (Sotiropoulou and Karamanos, 2010; Elsayed et al., 2022; Farruggia et al., 2023; NurzyĔska-Wierdak, 2023). However, the increase in commercial production of MAPs due to fertilizers requires the application of an optimal dose of fertilizer (Giannoulis et al., 2020). Farmers should add nutrients to the soil, such as synthetic or natural fertilizers, to enrich it and thus obtain adequate yields (Urra et al., 2019; Pahalvi et al., 2021). The use of such fertilizers in agriculture boosts the output; however, their incorrect use has a negative influence on soil productivity and the quality of the environment (Chojnacka et al., 2020).

Different MAPs have specific nutrient requirements, and fertilization management can significantly enhance both biomass production and the concentration of bioactive compounds.

For instance, nitrogen is essential for vegetative growth and is known to increase biomass yield in as number of studies carried out both in pots and open fields have showed (Sotiropoulou and Karamanos, 2010; Karamanos and Sotiropoulou, 2013; Dordas, 2017)

However, an excessive amount of nitrogen can have negative effects on essential oils and other secondary metabolites, thereby reducing the medicinal value of plants. Phosphorus and potassium are often linked to improved root development and can enhance the synthesis of certain secondary metabolites. As reported by literature, phosphorus and potassium fertilization can affect production and qualitative characteristics of various MAPs. Potassium is implicated in enzymatic activation, photosynthesis and protein synthesis. Application of potassium increased biomass production and the EOs content in spearmint (*Mentha spicata* L.) and marigold (*Calendula officinalis* L.) (Chrysargyris et al., 2017). Phosphorus plays a crucial role in the biosynthesis of primary and secondary metabolites of MAPs. Kapoor et al. (2004) in fennel (*Foeniculum vulgare* Mill.), Trivino and Johnson (2000) in marjoram (*Origanum majorana* L.) and Ramezani et al. (2009) in basil (*Ocimum basilicum* L.) observed an increase in EO yield with P-fertilization.

Organic fertilizers, such as compost and manure, can also enhance soil fertility by improving microbial activity, leading to better nutrient uptake and higher quality plant materials. MAPs can coexist with organic farming methods, which are popular among both growers and buyers (Lubbe and Verpoorte, 2011; Kosakowska et al., 2019; Najar et al., 2021). Biological fertilizers are applied as an alternative to chemical fertilizers in organic and sustainable agricultural systems to boost soil fertility, soil organic matter and plant development (De Pascale et al., 2017; Ye et al., 2020). Using bio-based fertilizers in agriculture is a viable and effective way to increase production stability and nutrient usage efficiency, even in less-than-ideal circumstances (Chojnacka et al., 2020; Puglia et al., 2021).

1.2. Biostimulants

1.2.2. General context

For many years, farmers have extensively applied synthetic fertilizers and pesticides to guarantee a consistent crop yield throughout the growing season, even under favorable or unfavorable cultivation circumstances. But in recent decades, several environmentally friendly innovations have been put out that reduce the impact of agriculture on the environment, taking the place of synthetic agrochemicals (Balaban et al., 2016).

The replacement models for these systems of production include using technology that enable more effective management of water and mineral resources, as well as higher-quality and healthier output (Colla and Rouphael, 2015). In this regard, due to the increasing knowledge both of people and researchers, greater attention has been devoted to the preservation of human health and the environment, underlining the need to decrease the use of synthetic chemicals in agriculture. As a result of ongoing research, new technical methods with minimal environmental effect have been introduced.

As reported by several authors (Murillo-Amador et al., 2013; Kosakowska et al., 2019), MAPs can be successfully cultivated in organic farms located in temperate areas in accordance with European guidelines. The cultivation of medicinal plants using suitable and recommended agricultural practices, such as those concerning fertilization and irrigation, can achieve optimal agronomic output and supply the industry with standard bioactive compounds (Sotiropoulou and Karamanos, 2010; Murillo-Amador et al., 2015; Samani et al., 2019; Khammar et al., 2021; Farruggia et al., 2023). MAPs can coexist with organic farming methods, which are popular among both growers and buyers (Lubbe and Verpoorte, 2011; Kosakowska et al., 2019; Najar et al., 2021). Biological fertilizers are applied as an alternative to chemical fertilizers in organic and sustainable agricultural systems to boost soil fertility, soil organic matter and plant development (De Pascale et al., 2017; Ye et al., 2020). Using bio-based fertilizers in agriculture is a viable and effective way to increase production stability and nutrient usage efficiency, even in less-than-ideal circumstances (Chojnacka et al., 2020; Puglia et al., 2021). Many biostimulants are bio-based products (Calvo et al., 2014; Corsi et al., 2022). Biostimulants can influence both primary and secondary plant metabolism and improve the plants' ability to tolerate adverse soil pH, heat, salinity, drought, and nutritional stress (Ertani et al., 2013; Colla et al., 2014; Lucini et al., 2015; Di Miceli et al., 2023).

The application of biostimulants in agriculture, which has recently received global interest, represents a sustainable and successful practice to improve nutrient use efficiency and to obtain yield stability, also under sub-optimal conditions (Di Mola et al., 2021; Consentino et al., 2022). Plant biostimulants are widely used in various cropping systems, including organic, conventional, and integrated agricultural systems (Rojas-Rodríguez et al., 2023).
By encouraging greater macro- and micronutrient absorption and accumulation into the plants, biostimulants complement current fertilizers rather than replacing them and improve their overall effectiveness. Furthermore, foliar or radical application of biostimulant promote a rapid emergence of the seedlings and/or a rapid rooting process, anticipates yields, improves growth, fruit set, flowering, and the quality of the production (Colla and Rouphael, 2015). The effect of biostimulant application depends on several elements, including genetic, agronomic, environmental, and dose-related factors. Regarding these factors, a number of studies have been conducted recently to comprehend the actual working processes and the ideal settings of administration in order to give farmers who wish to apply these cutting-edge technologies effectively some guidance (Baltazar et al., 2021; Omoarelojie et al., 2021; Franzoni et al., 2022).

1.2.3. Definition and legislation

The first review regarding biostimulants was published in 1994 by Herve (Herve, 1994) and it reports some characteristics common to all products: low dosage of application and low environmental impact. In 1999, Zhang and Schmidt (Zhang and Schmidt, 1999) of the Virginia Polytechnic Institute and State University wrote an article in the online magazine "Ground Maintenance" describing biostimulants as "*materials that, in minimal quantities, promote plant growth*". In this article the authors emphasize the quantities of product used (“minimal quantities”), underlining the substantial difference between biostimulants (used in low dosages) and fertilizers/soil amendments (applied in high quantities). The plant biostimulants cited in this article were two remarkable group: humic acids and seaweed extracts. Their effect on plants was suggested to be mainly hormonal.
It is only in 2007 that Kauffmann and collaborators (Kauffman et al., 2007) officially introduce the definition of biostimulant products, describing them as "*materials other than fertilizers that promote plant growth when applied in low doses*", they also distinguish three groups of biostimulants: humic substances, algae extracts and amino acid-based products.
In 2011, a consortium of companies active in the fertilizer sector was established. In 2013 it evolved into the European Biostimulants Industry Council (EBIC). This consortium

represents a connecting platform between companies operating in the biostimulants sector and aims to promote the creation of the biostimulants category at European level to overcome the regulatory differences between Member States.

In 2012, du Jardin (du Jardin, 2012) reported that plant biostimulants were heterogeneous materials and he suggested to divide them into 8 categories: humic substances, organic materials, beneficial chemical elements, inorganic salts, seaweed extracts, chitin and chitosan derivated, antitraspirants, free amino acids and N-containing substances. Interestingly, in this first attempt of classification, du Jardin did not incorporate any microbial biostimulants. A few years later, to clarify and order the fertilizers and biostimulants sectors, the European Commission has entrusted Professor Patrick du Jardin with the task of carrying out an in-depth bibliographic study on biostimulants and drawing up a well-defined subdivision. In 2015, du Jardin (du Jardin, 2015) published his classification which included the following categories: humic and fulvic acids, protein hydrolysates, algae extracts, chitosan and other biopolymers, inorganic compounds, mycorrhizal fungi, and growth promoting bacteria. Moreover, he defined plant biostimulant as follows; "*A plant biostimulant is any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrient content*". In the same year, Colla and Rouphael (Colla and Rouphael, 2015) suggested to divide plant biostimulant in 6 non-microbial (chitosan, humic and fulvic acids, protein hydrolysates, phosphites, seaweed extract and silicon) and 3 microbial (arbuscular mycorrhizal fungi, plant growth-promoting bacteria and *Trichoderma* spp.) categories (Battacharyya et al., 2015; Canellas et al., 2015; Gómez-Merino and Trejo-Téllez, 2015; López-Bucio et al., 2015; Ruzzi and Aroca, 2015; Savvas and Ntatsi, 2015). On 27 March 2019, the European Parliament approved the new Regulation on fertilizers and inserted plant biostimulants in the Product Function Categorie (PFCs) 6. As reported in the Regulation (EU) 2019/1009 of the European Parliament, plant biostimulants are "product that stimulates the nutritional processes of plants regardless of its nutrient content with the only purpose of improving one or more of the following characteristics of the plant or of the plant rhizosphere: a) efficiency of the use of nutrients; b) tolerance to abiotic stress; c) qualitative characteristics; d) availability of nutrients confined in the soil or in the rhizosphere". Therefore, plant biostimulants are described based on agronomic effects and not based on their nature or mode of action. In addition, the regulation introduces two classes of biostimulants:

- microbial plant biostimulants (PFC A), a micro-organism or a consortium of micro-organisms referred to in CMC 7 in Part II of Annex II of the Regulation;
- non-microbial plant biostimulants (PFC B), a plant biostimulant other than a microbial plant biostimulant.

1.2.4. Non-microbial biostimulants

1.2.4.1. Seaweed extracts

Seaweed extracts (SWE) are a group of non-microbial biostimulants and their application in agriculture expanded in the 1950s as a result of extraction techniques that broke down algal tissue cells to produce molecules that stimulate plants (Battacharyya et al., 2015). SWE are products obtained from the processing of red, green, or brown algae and contain polysaccharides, alcohols, phenols, and compounds with hormone-like activity. Algae are harvested manually or mechanically along the ocean shores (Canada, Ireland, South Africa, China) and then cleaned, chopped, and extracted to generate the biostimulant. These days, there are several ways to extract: high-pressure treatment, cryoprocessing, water, acid, or alkaline. Furthermore, contemporary methods including microbial fermentation of the fundamental plant matrix were created. Currently, nevertheless, the most used technique is cold extraction in high-pressure water because it keeps the extremely biologically active molecules from undergoing chemical changes (Michalak and Chojnacka, 2015). Phytohormones, such as cytokinins, auxins, gibberellins, abscisic acid, and other compounds with hormone-like action are some of the bioactive compounds found in SWE (Nelson & Van Staden, 1985). According to Di Stasio et al. (2018), SWE also includes polysaccharides and derivatives as alginates, laminarin, mannitol, carrageenan, and fucoidans that stimulate the defense systems of plants in response to stress (Rouphael et al., 2017).

Seaweed extracts obtained from *Eklonia maxima* and *Ascophillum nodosum* can affect the productive response of crops by improving the absorption of nutrients and water, increasing the photosynthesis rate, and promoting compounds, such as auxins, cytokinins and gibberellins (Briceño-Domínguez et., 2014; Hernández-Herrera et al., 2014). A number of studies (Xu and Leskovar, 2015, O. Elansary et al., 2019; Sabatino et al., 2023; Consentino et al., 2023) report that the application of various seaweed extracts to MAPs and other crops can increase their resistance to various abiotic stresses, such as drought, high temperature, and lack of nutrients in the soil. The mechanisms responsible for the improved tolerance to abiotic stress are different and not yet fully known. The presence of abscisic acid, which controls stomata's opening and shutting to enhance a plant's tolerance to drought, is one of these factors, along with improved crop nutrition and increased root growth (Rouphael and Colla, 2020). Tursun (2022) has demonstrated that foliar application of seaweed extract as an organic fertilizer produces significant impact in terms of increasing the production of *Coriandrum sativum* L. The results obtained by Rasouli et al. (2022) show that the application of seaweed extract has a positive effect on the morphological, productive and biochemical traits of lettuce.

1.2.4.2. Protein hydrolysates

Protein hydrolysates (PH) represent another type of biostimulant, and their application can improve primary and secondary plant metabolism (Rouphael et al., 2022; Di Miceli et al., 2023). They are mixtures of polyprotein hydrolysate, oligo-protein hydrolysate and amino acids (Colla et al., 2014; Rouphael et al., 2017) and are obtained by chemical or enzymatic hydrolysis of vegetable or animal protein matrix. Collagen derived from tanning industry waste is the most utilized matrix for producing animal protein hydrolysates, whereas material from legumes is frequently used to generate plant protein hydrolysates (Colla and Rouphael, 2015; du Jardin, 2015). Betaines are another nitrogen complex and, in particular, glycine-betaine is known for its positive effect to plants stress (Chen and Murata, 2011).

Hydroxyproline and hydroxylysine, which are strongly linked to collagen, are present in biostimulants derived from animals. Vegetable protein hydrolysates have less glycine and proline, which are important in the processes of osmoregulation and stress tolerance, but more aspartic acid and glutamic acid, which are critical for the metabolism of inorganic nitrogen, than animal protein hydrolysates (Colla et al., 2015).

Protein hydrolysates can be applied to leaf or root and can promote various plant physiological responses, increase crop yield and quality, and enhance the tolerance of plants to drought, high salinity and temperatures levels, lack of nutrients, and unfavorable soil pH (Colla et al., 2014; Rouphael et al., 2017; Rouphael et al., 2022; Di Miceli et al., 2023). They may also increase the nutritional availability of substrates, nutrient absorption and improve nutrient usage efficiency in plants (Halpern et al., 2015; Nardi et al., 2016; Colla et al., 2017; Paul et al., 2019). Peptides and amino acids are primarily responsible for the biostimulating action of protein hydrolysates. After being absorbed by the plant, amino acids are utilized by it 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 (Colla and Rouphael, 2019).

Di Miceli et al. (2023) observed an increase in growth, yield parameters and quality parameters in eggplants treated with protein hydrolysate in open fields. Similar results have been obtained by Sabatino et al. (2021) in lettuce 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. (2019) claimed that protein hydrolysate application on tomato plants can be considered as a sustainable crop enhancement technology for agricultural productivity under water-limited conditions.

1.2.4.3. Humic substances

The organic material consists of humic compounds and organic residues. After the decomposition of organic materials operated by bacteria, complex macromolecules known as humic compounds are produced (Nardi et al., 2021). Humic substances include fulvic acids. Abiotic stress may be mitigated using fulvic acids as a biostimulant (Baltazar et al., 2021). These products also have a positive impact on several molecular processes, including protein synthesis, photosynthetic activity, and enzyme activity (Zanin et al., 2019; Nardi et al., 2021). Fulvic acids are organic chemicals formed when dead biota decomposes in the soil; they contain a high number of carboxylic groups (COOH), a high number of phenolic compounds, and a low number of aromatic structures (Canellas et al., 2015; do Rosário Rosa et al., 2021). The humic substances employed to generate biostimulants derived from fossil humus deposits, agri-food by-products, compost or vermicompost. Humic substances originating from peat and compost seem to have a greater bio-stimulating activity than those obtained from fossil humus deposits. Humic substances have the ability to alter the chemical and physical characteristics of soil, creating a structure that can support various processes such as air exchange, water drainage, root penetration, the growth of telluric microorganisms, cation exchange capacity, and decreased nutrient loss.

The application of fulvic acids has been tested to increase flavonoids, glutathione, and ascorbate by activating the genes involved in their metabolism, with the aim of reducing the harmful consequences of drought stress (Suh et al., 2014; Fang et al., 2020). Fulvic acids can increase the absorption of nutrients due to the influence that these substances have on the synthesis and functionality of membrane transport proteins (Rouphael and Colla, 2019). Their use can also produce positive effects on plant growth and increase the content of photosynthetic pigments, carotenoids, total phenols, as well as nitrogen, phosphorus and potassium (NPK) concentration (Halpern et al., 2015; Nardi et al 2016; Colla et al., 2017; Paul et al., 2019; Baltazar et al., 2021; Bayat et al., 2021).

In *Origanum vulgare* subsp. *hirtum*, Aytaç et al. (2022) obtained an increase in yields and EO content with the application of humic acid to soil. Abdel-Baky et al. (2019) observed significant increases in the morphological and productive attributes of four *Vicia faba* L. cultivars fertilized with foliar fulvic acid.

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2. AIMS OF THE RESEARCH

The use of biostimulant is an agronomic tool capable of increasing yield, quality, and nutraceutical properties of vegetables. Although many studies have been conducted about these subjects, according to the literature, there are few studies concerning MAPs cultivated in stressful environments, such as the Mediterranean basin. The aim of the present doctoral thesis was to assess the effects of the application of various types of foliar biostimulant and several strategies of application on the morphological, yield and chemical parameters of some MAPs cultivated in organic agricultural system in mediterranean environment. With this in mind, the following studies were performed:

1) Biostimulants improve plant performance of rosemary growth in agricultural organic system.
2) Foliar application of various biostimulants produces contrasting response on yield, essential oil and chemical properties of organically grown sage (*Salvia officinalis* L.).
3) Seaweed extract and fulvic acid application affect the biomass performance, the essential oil yield and composition of Sicilian oregano grown in an organic agricultural system.
4) Effect of two protein hydrolysate on yield and chemicals of organic rosemary.
5) Foliar biostimulants and frequency of application affect some yield and chemical properties of organically grown lemon balm

Studies n. 1, 2 and 3 have been already published in peer-reviewed journals. The studies n. 4 and 5 will be submitted in a few weeks. Other studies on this topic were conducted during the three years and, in some cases, published. However, it was preferred to show only the most relevant ones. The experimental part is presented below, and each study is composed by six different sections: (i) objectives (ii) materials and methods, (iii) results, (iv) discussions, (v) conclusions, and (vi) references.

3. EXPERIMENTAL PART

Experiment 1

Biostimulants Improve Plant Performance of Rosemary Growth in Agricultural Organic System

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Objective

The aim of this study was to evaluate the impact of four different biostimulants on the morphological and productive parameters of *Salvia rosmarinus* Spenn. organically cultivated in a Mediterranean environment without irrigation.

Materials and Methods

Experimental Site and Cultivation Practices

Tests were conducted on a local farm (Figure 1) located in Aragona (Sicily, Italy) (330 m a.s.l., 37°22′32.71″ N, 13°38′33.59″ E Google Earth), during the growing seasons of 2020–2021 and 2021–2022. The soil was categorized as Regosol (United States Department of Agriculture (USDA) classification: typic xerorthents) and sandy clay loam (48% sand, 28% clay and 24% silt) with a pH of 7.4, 16 g kg^{-1} organic matter, 1.22% total nitrogen, 20.4 ppm assimilable phosphate, and 364 ppm assimilable potassium. Agamic propagation was carried out by dividing the bushes. The plants were transplanted at the beginning of spring 2019. The plant density was 4000 plants ha^{-1}. The distance between rows and within rows was 2.50 m and 1.00 m, respectively. Rosemary plants were managed under rainfed conditions adopting a low-input growth method following the common agronomic practices of the cultivation area. Before transplanting, the experimental field received organic fertilization through the distribution of 2 t ha^{-1} of manure which was buried at a depth of 0.30 m. No pesticides were applied in either year. Weeds were controlled mechanically at the beginning of spring and before harvesting. In both years, plants were harvested during the third 10-day period of June.

Figure 1. Experimental site

Weather Data

Rainfall and temperature data were taken from a meteorological station that belonged to the Sicilian Agro-Meteorological Information Service (SIAS, 2023). The station has a datalogger and sensors for measuring air temperature (TAM platinum PT100 sensor, heat resistance with anti-radiation screen) and total rainfall (PPR sensor with tilting bucket rain gauge). Data about the average daily maximum and minimum temperatures (°C) and total decadal (10-day period) precipitation (mm) have been considered.

Treatments

Four commercial biostimulant formulations were used for the tests, based on *Eklonia maxima* (Kelpstar®, Mugavero fertilizers, Termini Imerese, Italy), *Ascophyllum nodosum* (Algastar®, Mugavero fertilizers, Termini Imerese, Italy), fulvic acids (Niger L®, Mugavero fertilizers, Termini Imerese, Italy), and protein hydrolysate (Tyson®, Mugavero fertilizers, Termini Imerese, Italy). Water (C) was used as the control. A total number of 6 foliar applications of biostimulants were made. The first application was performed during the first week of April in each year; the others were then carried out every two weeks. The biostimulant dosage was planned in order to provide the same total amount of N, considering the N content of each type of product. The doses are listed in Table 1.

Table 1. Doses of foliar biostimulants.

Biostimulant	Dose [1] [l hl^{-1}]	Total Amount [2] [l ha^{-1}]
EM = *Eklonia maxima*	0.25	6
AN = *Ascophyllum nodosum*	0.25	6
FA = Fulvic acids	0.05	12
PH = Protein hydrolysate	0.50	1.2

[1] For each application; [2] for the 6 total applications in 2400 L of water.

For each foliar application, 400 L of water ha^{-1} was used. A portable sprayer with an operating pressure of 250 kPa was adopted. Foliar treatments were applied at dawn, when temperature (10–12 °C), relative humidity (91–66%), and stomatal aperture were optimal for foliar absorption (Ruiz-Navarro et al., 2019). A randomized complete block design with three replicates was used for the tests. Year (Y) and biostimulants (B) were used as fixed effects in the linear model/ANOVA. Each block comprised 5 plots of 25 m^2. Foliar treatments were applied for each randomized plot in the block. The plots were well spaced in the block. During foliar applications, each plot was defined by plastic panels, which also prevented drift.

Plant Measurement

At harvest, plant height, number and diameter of primary stems, relative water content (RWC), chlorophyll content, total fresh yield, total dry yield, EO content, and EO yield were determined. In both years, plants were harvested during the third week of June. Young shoots formed in the year of harvest were collected to determine yield parameters. The plant material was dried in a shaded and ventilated environment for approximately 10 days at a temperature of 25–30 °C. Plant dry-matter weight was then calculated. The chlorophyll content was measured using Dualex 4 Scientific (Force A, Orsay, France) portable Chlorophyll meter. For each plot, thirty fully grown leaves were used. These readings were automatically averaged by the device.

The RWC of leaves was determined as follows. Thirty fresh leaves per plot have been taken, and the fresh weight (FW) was recorded. The leaves were floated in a falcon tube with distilled water for 24 h. The leaves were then removed from the water and placed on absorbent paper to remove the excess water, and the turgid weight (TW) was recorded. The leaves were then dried in an oven for twenty-four hours, and the dry weight was noted (Alyemeni et al., 2018). The following formula was used to determine the RWC:

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

Essential Oil Extraction

EO was obtained by hydro distillation in a Clavenger-type apparatus (Albrigi Luigi, Verona, Italy) of air-dried plant material (500 g) and 6 L of water (Figure 2). The extraction was carried out for 3 h in accordance with international guidelines (European Pharmacopoeia, 2008). After, the EO samples were stored at 4 °C.

Figure 2. Essential oil extraction.

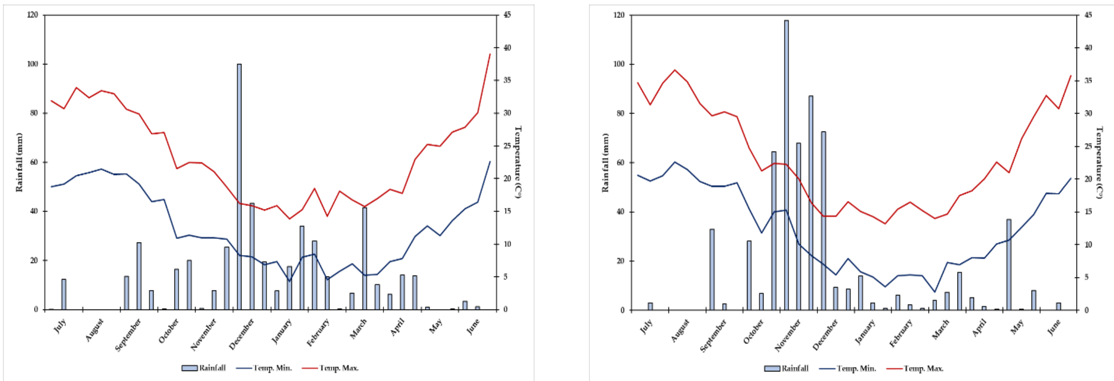
Statistical Analysis

Statistical analyses were performed using the package MINITAB 19 (State College, PA, USA) for Windows. Data were compared using analysis of variance (ANOVA). The difference between means was analyzed using Fisher's LSD test ($p \leq 0.05$). The percentage data for RWC were subjected to arcsine transformation. Pearson's correlation analysis was carried out to evaluate the relationships between the morphological and productive parameters of rosemary plants.

Results

Analysis of Rainfall and Air Temperature Trends at the Experimental Site

Air temperature and rainfall trends are shown in Figure 3. Over the two years, annual rainfall levels were 493 mm (2020–2021) and 611 mm (2021–2022). In the first growing season, rainfall was mainly distributed from December to March, reaching a peak during the first 10-day period of December (96 mm). In the second growing season, rainfall was mainly distributed from October to December. Significant rainfall occurred in the first 10-day period of May (37 mm). In both years, temperatures trends were similar and consistent with the average temperature of the experimental area (Figure 3).



(**a**) (**b**)

Figure 3. Temperature and rainfalls trends at the experimental site: **(a)** growing season 2020-2021; **(b)** growing season 2021-2022.

Effects of Year and Biostimulants on Morphological and Yield Parameters of Rosemary

The year (Y) factor significantly influenced plant height, stem diameter, chlorophyll content, and RWC (Table 2). The highest values of stem diameter and chlorophyll content were observed in the first year, while the highest values of plant height and RWC were in the second year (Table 2).

Table 2. Morphological and yield parameters in response to year (Y), biostimulants (B), and their interaction Y × B.

	Plant Height [cm]	**Primary Stem [n.]**	**Stem Diameter [mm]**	**Chlorophyll Content [μg cm^{-2}]**	**RWC [%]**	**Total Fresh Yield [t ha^{-1}]**	**Total Dry Yield [t ha^{-1}]**	**Essential Oil Content [% *v*/*w*]**	**Essential Oil Yield [kg ha^{-1}]**
Year (Y)									
2020–2021	52.0 ± 1.3 ^{b}	n.s.	3.1 ± 0.3 ^{a}	29.1 ± 0.6 ^{a}	67.0 ± 0.9 ^{b}	n.s.	n.s.	n.s.	n.s.
2021–2022	54.7 ± 1.5 ^{a}	n.s.	8.8 ± 0.2 ^{b}	28.3 ± 0.6 ^{b}	70.1 ± 1.8 ^{a}	n.s.	n.s.	n.s.	n.s.
Biostimulant (B)									
C	48.8 ± 0.6 ^{d}	8 ± 0.2 ^{d}	7.4 ± 0.2 ^{d}	25.7 ± 0.4 ^{c}	60.3 ± 0.7 ^{d}	8.3 ± 0.2 ^{c}	2.7 ± 0.1 ^{d}	1.72 ± 0.03 ^{a}	46.5 ± 1.96 ^{d}
EM	52.8 ± 2.1 ^{c}	19 ± 1.2 ^{a}	9.6 ± 0.2 ^{ab}	28.2 ± 0.3 ^{b}	68.6 ± 1.4 ^{c}	12.3 ± 0.8 ^{b}	4.0 ± 0.2 ^{b}	1.61 ± 0.05 ^{b}	65.0 ± 3.85 ^{a}
AN	53.7 ± 0.5 ^{bc}	12 ± 0.5 ^{c}	9.1 ± 0.3 ^{c}	29.2 ± 0.8 ^{b}	71.1 ± 1.1 ^{b}	11.8 ± 0.3 ^{b}	3.9 ± 0.2 ^{c}	1.44 ± 0.03 ^{d}	55.9 ± 1.93 ^{b}
FA	54.0 ± 2.0 ^{b}	17 ± 0.5 ^{b}	9.7 ± 0.3 ^{a}	29.4 ± 1.0 ^{b}	67.8 ± 0.8 ^{c}	13.1 ± 0.6 ^{a}	4.3 ± 0.2 ^{a}	1.14 ± 0.04 ^{e}	48.5 ± 3.66 ^{c}
PH	60.5 ± 0.3 ^{a}	13 ± 0.4 ^{c}	9.2 ± 0.2 ^{bc}	31.0 ± 0.5 ^{a}	74.2 ± 2.0 ^{a}	13.6 ± 0.3 ^{a}	4.4 ± 0.1 ^{a}	1.52 ± 0.13 ^{c}	66.5 ± 6.99 ^{a}
***p*-value**									
Y	0.000	0.493	0.028	0.022	0.000	0.730	0.751	0.378	0.131
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Y × B	0.000	0.198	0.116	0.002	0.000	0.001	0.000	0.010	0.010

Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$. n.s. = not significant. C = control; EM = *Eklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids; PH = protein hydrolysate.

Analysis of variance revealed that factor (B) had a highly significant effect ($p \leq 0.01$) on all parameters (Table 2). The highest value of plant height (60.5 cm) was recorded in PH-treated plants, while the highest number of primary stems (19) was in EM-treated plants. The stem diameter was on average higher in FA-treated plants, followed in descending order by EM, PH, and AN (Table 2). PH-treated plants showed the highest chlorophyll content (31.0 µg cm^{-2}) and RWC (74.2%), on average. The lowest chlorophyll content (25.7 µg cm^{-2}) and the lowest RWC (60.3%) were observed in C-plants (Table 2).

The highest values of yield parameters were obtained with the application of treatments FA and PH (Table 2). Particularly, FA-treated plants obtained average values of fresh biomass and dry biomass of 13.1 and 4.3 t ha^{-1}, respectively. Similar average values of fresh biomass (13.6 t ha^{-1}) and dry biomass (4.4 t ha^{-1}) were found in PH-treated plants. C showed the lowest average values at 8.3 t ha^{-1} for fresh biomass and 2.7 t ha^{-1} for dry biomass. The lowest values for all parameters in the study, except for the EO content, were consistently observed in the control plants. Regarding the EO content, the highest average value (1.72%) was found in C, followed by those obtained with EM (1.61%), while the lowest average value (1.14%) was recorded in the FA-treated plants. However, the lowest EO yield value per unit area was observed in C. Treatments EM (65.0 kg ha^{-1}) and PH (66.5 kg ha^{-1}) showed the highest EO yield values.

The year-by-biostimulant interaction significantly affected ($p \leq 0.01$) the plant height, chlorophyll content, RWC, total fresh yield, total dry yield, EO content, and EO yield (Table 3).

Table 3. Morphological and yield parameters in response to interaction year (Y) × biostimulants (B).

	Plant Height [cm]	Chlorophyll Content [μg cm^{-2}]	RWC [%]	Total Fresh Yield [t ha^{-1}]	Total Dry Yield [t ha^{-1}]	Essential Oil Content [% v/w]	Essential Oil Yield [kg ha^{-1}]
Y × B							
Y1 × C	47.0 ± 0.3 ^{f}	26.5 ± 0.3 ^{cd}	61.6 ± 0.5 ^{g}	8.2 ± 0.2 ^{e}	2.7 ± 0.1 ^{d}	1.73 ± 0.03 ^{a}	46.1 ± 0.42 ^{f}
Y1 × EM	48.2 ± 0.6 ^{ef}	28.8 ± 0.4 ^{b}	65.6 ± 0.7 ^{f}	12.0 ± 0.2 ^{cd}	3.9 ± 0.1 ^{c}	1.64 ± 0.02 ^{b}	63.2 ± 0.57 ^{c}
Y1 × AN	54.6 ± 0.2 ^{c}	30.6 ± 0.6 ^{a}	68.8 ± 0.8 ^{de}	12.0 ± 0.3 ^{cd}	4.1 ± 0.0 ^{b}	1.39 ± 0.03 ^{e}	56.6 ± 0.07 ^{d}
Y1 × FA	49.7 ± 0.8 ^{e}	28.2 ± 0.4 ^{b}	68.8 ± 0.8 ^{de}	13.6 ± 0.3 ^{a}	4.5 ± 0.1 ^{a}	1.11 ± 0.02 ^{f}	49.4 ± 0.46 ^{e}
Y1 × PH	60.3 ± 0.2 ^{a}	31.5 ± 0.6 ^{a}	69.8 ± 0.4 ^{cd}	13.4 ± 0.3 ^{ab}	4.3 ± 0.1 ^{b}	1.53 ± 0.01 ^{cd}	65.1 ± 0.86 ^{bc}
Y2 × C	44.6 ± 0.3 ^{g}	24.9 ± 0.3 ^{d}	59.1 ± 0.6 ^{g}	8.5 ± 0.1 ^{e}	2.7 ± 0.0 ^{d}	1.71 ± 0.02 ^{a}	46.9 ± 1.08 ^{f}
Y2 × EM	57.3 ± 0.2 ^{b}	27.7 ± 0.1 ^{bc}	71.6 ± 0.6 ^{bc}	12.6 ± 0.2 ^{c}	4.2 ± 0.1 ^{b}	1.58 ± 0.01 ^{bc}	66.8 ± 1.34 ^{ab}
Y2 × AN	52.7 ± 0.2 ^{d}	27.7 ± 0.4 ^{bc}	73.4 ± 0.3 ^{b}	11.6 ± 0.1 ^{d}	3.7 ± 0.0 ^{c}	1.50 ± 0.01 ^{d}	55.2 ± 0.99 ^{d}
Y2 × FA	58.3 ± 0.2 ^{b}	30.5 ± 0.5 ^{a}	66.9 ± 0.9 ^{ef}	12.7 ± 0.2 ^{bc}	4.1 ± 0.1 ^{b}	1.17 ± 0.01 ^{f}	47.6 ± 0.84 ^{ef}
Y2 × PH	60.6 ± 0.5 ^{a}	30.5 ± 0.5 ^{a}	78.3 ± 1.1 ^{a}	13.9 ± 0.1 ^{a}	4.5 ± 0.0 ^{a}	1.51 ± 0.03 ^{d}	68.0 ± 0.49 ^{a}
***p*-value**							
Y × B	0.000	0.002	0.000	0.001	0.000	0.010	0.010

Means and standard error are reported. Values with different letters are significantly different at $p \leq 0.05$. Y1 = 2021–2021; Y2 = 2021–2022; C = control; EM = *Eklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids; PH = protein hydrolysate.

In both years, the highest average values of plant height were observed in PH-treated plants. For treatments EM and FA, the tallest plants were obtained in the second year, while for AN, they were obtained during the first year. In both years, C-plants showed the lowest plant heights (Table 3). Regarding chlorophyll content, the highest values (ranged from 30.5 to 31.5 µg cm^{-2}) were obtained by the interactions Y1 × AN, Y1 × PH, Y2 × FA, and Y2 × PH (Table 3). The lowest average value of the chlorophyll content (24.9 µg cm^{-2}) was observed in C during both growing seasons (Table 3). In the second growing season, PH-treated plants showed the highest average value of RWC (78.3%), followed by AN-treated plants (73.4%). The lowest average values (ranged from 61.6% to 59.1%) were obtained by C in both years (Table 3). The highest values of fresh yield were obtained with the interactions Y1 × FA, Y1 × PH, and Y2 × PH (Table 3). The highest values ranged from 13.4 to 13.9 t ha^{-1}. In both years, the lowest values of fresh yield (8.2 and 8.5 t ha^{-1}) and the lowest values of dry yield (2.7 t ha^{-1}) were observed in C (Table 3). FA-treated plants (4.5 t ha^{-1}), in 2020–2021, and PH-treated plants (4.5 t ha^{-1}), in 2021-2022, revealed the highest average values of dry yield (Table 3). In both years, similar EO content values were observed. The highest values (1.73% and 1.71%) were recorded in C, while the lowest (1.11% and 1.17%) values were recorded in FA-treated plants (Table 3). Plants treated with PH and EM showed comparatively higher EO yields over both years, however with overall higher values in 2022. In 2021-2022, PH-treated plants showed the highest value of EO yield (68.0 kg ha^{-1}) followed by EM-treated plants (66.8 kg ha^{-1}) (Table 3). The lowest average values (46.1 and 46.9 kg ha^{-1}) were obtained in the control in both years and in FA-treated plants (Table 3).

Concerning the first growing season, a correlation analysis (Table 4) showed that plant height was significantly correlated with chlorophyll content (r = 0.844) and RWC (r = 0.728); an increase in the total fresh yield was related to morphological parameters, such as the number of primary stems (r = 0.668), diameter of primary stems (r = 0.837), and RWC (r = 0.826). The total dry yield was positively correlated with RWC (r = 0.879) and the total fresh yield (r = 0.969). Negatively significant relationships were found between the EO content and RWC (r = -0.641), total fresh yield (r = -0.681), and total dry yield (r = -0.761). The EO yield was positively correlated with the chlorophyll content (r = 0.733). In the second growing season, a correlation analysis revealed positive relationships between morphological and yield parameters. The EO content was negatively correlated with the chlorophyll content (r = -0.757). The increase in EO yield was due to an increase in RWC (r = 0.802), total fresh yield (r = 0.655), and total dry yield (r = -0.730). In both years, no significant correlation was found between the EO content and EO yield (Table 4).

Table 4. Pearson's correlation coefficients for morphological and productive parameters of rosemary.

		2021–2022								
		Plant Height [cm]	**Primary Stems [n.]**	**Primary Stems Diameter [mm]**	**Chlorophyll Content [μg L^{-1}]**	**RWC [%]**	**Total Fresh Yield [t ha^{-1}]**	**Total Dry Yield [t ha^{-1}]**	**EO Content [%]**	**EO Yield [kg ha^{-1}]**
2020–2021	**Plant height [cm]**		0.797 **	0.931 **	0.865 **	0.762 **	0.975 **	0.974 **	-0.586	0.630
	Primary Stems [n.]	-0.115		0.819 **	0.604	0.392	0.754 **	0.788 **	-0.627	0.400
	Primary Stems diameter [mm]	0.077	0.779 **		0.791 **	0.709 **	0.931 **	0.909 **	-0.624	0.549
	Chlorophyll content [μg L^{-1}]	0.844 **	0.127	0.385		0.617	0.839 **	0.814 **	-0.757 **	0.309
	RWC [%]	0.728 **	0.382	0.544	0.680 **		0.846 **	0.814 **	-0.146	0.802 **
	Total Fresh Yield [t ha^{-1}]	0.472	0.668 **	0.837 **	0.574	0.826 **		0.976 **	-0.562	0.655 **
	Total Dry Yield [t ha^{-1}]	0.525	0.584	0.797	0.620	0.879 **	0.969 **		-0.489	0.730 **
	EO Content [%]	-0.134	-0.299	-0.595	-0.161	-0.641 **	-0.681 **	-0.761 **		0.233
	EO Yield [kg ha^{-1}]	0.621	0.469	0.402	0.733**	0.479	0.541	0.488	0.191	

** Significant at 0.01 probability level.

Discussion

The cultivation of MAPs has become increasingly widespread throughout the world, mainly due to the very varied uses of the products (Rao et al., 2022). The increase in crop yields by reducing the use of common fertilizers represents one of the goals of modern agriculture (Colla et al., 2015). The results of the present study highlight the positive effects on yields and qualitative parameters of the foliar application of biostimulants on rosemary plants under rainfed conditions. In both growing seasons, the highest plant height values were found in plants treated with protein hydrolysate. The main reason for protein hydrolysate biostimulatory actions is their peptide and amino acid content (Di Miceli et al., 2023; Colla et al., 2015). Plants use amino acids for a variety of processes, including the synthesis of high-biological-activity compounds, energy production, and protein biosynthesis (Colla et al., 2014). Peptides also have a significant impact on plant responses to stress, information transfer between cells, and growth and development regulation (Rouphael et al., 2022). Waly et al. (2019) investigated the effects of seaweed extract foliar application on rosemary using doses varying between 200 and 600 mL hL^{-1} and obtained plant height values which were similar to those found in the present study. Al-Fraihat et al. (2023) obtained rosemary plant heights of between 35 and 45 cm when applying different foliar amino acids. The foliar application of biostimulants allowed us to obtain a significant increase in the number and diameter of primary stems. Biostimulants can operate on the primary metabolism by raising photosynthetic activity and derived chemicals, thereby increasing plants growth (Mao et al., 2022; Rao et al., 2022). According to other authors, applying biostimulants generates gibberellin- and auxin-like activities, which improve crop performances (Colla et al., 2015; Colla et al., 2014).

The rosemary plants treated with biostimulants obtained an increase in yield per unit area with respect to the control plants. In particular, the foliar application of fulvic acids and protein hydrolysates led to increases in fresh yield of approximately 5 t ha^{-1} higher than that of untreated plants and of 1.6–1.7 t ha^{-1} in terms of dried yield. This was due to the effects on the regulation of those enzymes involved in nitrogen metabolism, which improve the absorption and assimilation of this element (Di Miceli et al., 2023). Indeed, several physiological and biochemical processes, including the activation of enzymes involved in carbon and nitrogen metabolic pathways, the Krebs cycle and glycolysis, the elicitation of phytohormones, and increases in mineral absorption/accumulation in biostimulated plants through the modification of root morphology, contribute to improvements in crop production (Del Buono and Can, 2020; Bulgari et al., 2019). This is the case, in particular, when induced by biostimulant application under unfavorable growing conditions, such as drought stress (Colla et al., 2015; Battacharyya et al., 2015). The treated roots of rosemary plants can improve water absorption and the uptake of various nutrients and promote their distribution in plant tissues (Rao et al., 2022; Colla et al., 2015). Our yields are similar to those found by Singh and Wasnik (2013), who obtained yields ranging from 10 to 21 t ha^{-1} when applying organic and inorganic fertilizers and amounts of nitrogen varying between 50 and 300 kg N ha^{-1}. In contrast, Tawfeeq et al. (2016) observed a notable decrease in biomass production in rosemary plants which were treated with algae extracts.

Regarding the chlorophyll content and RWC, our findings highlight that the application of biostimulants obtained higher values with respect to untreated plants. Al-Fraihat et al. (2023) found similar results in rosemary plants managed with different types of amino acids through foliar treatments, obtaining lower contents in untreated plants. Many authors (Ciriello et al., 2022; Abdel-Rahman et al., 2020) reported increases in leaf chlorophyll content in plants treated with foliar biostimulants. A crucial aspect of controlling stomatal conductance and photosynthetic activity in plant tissues is related to an appropriate RWC (Bandurska et al., 2022). Modifications in the water balance can cause molecular change, growth retardation, and occasionally, plant tissue death (Harb et al., 2010; Chetouani et al., 2019). The values of RWC obtained in our work were similar to those found in previous studies (Bandurska et al., 2022; Munné-Bosch et al., 1999). Rahimi et al. (2022) and Elansary et al. (2020) observed an increase in RWC by applying different types of foliar biostimulants to *Thymus vulgaris* L. and *Mentha longifolia* L., respectively. Due to the fact that biostimulants promote root development and improve the ability of plants to absorb water and nutrients, they are predicted to have a positive effect on the relative water content (Shafie et al., 2021; Khorasaninejad et al., 2018). As reported by various authors (Aytaç et al., 2022; Colombo et al., 2012), the application of fulvic acids led to increases in the yield and quality of many crops since these substances promote the absorption, assimilation, and translocation of micro- and macro-nutrients.

In our study, the highest EO content was recorded by untreated plants. On the contrary, higher biomass and EO yields were found in plants treated with protein hydrolysate, in particular. As is well known, the production of secondary metabolites and EO is affected by stressful conditions for the plants (Figueiredo et al., 2008; Tawfeeq et al., 2016). In the present study, the lack of irrigation and water availability in the soil increased the EO content in the control plants with respect to the plants treated with biostimulants. Elansary et al. (2020) and Rahimi et al. (2022) investigated the effects of the application of biostimulants on mint and thyme, respectively; however, they found increases in the EO content of plants treated with biostimulants. It is important to emphasize that the EO content in the present study in all treatments was, on average, higher than that found in previous tests on rosemary (La Bella et al., 2021; Elsayed et al., 2022; Tawfeeq et al., 2016; Mwithiga et al., 2022). As reported in several studies, secondary metabolite synthesis is linked to abiotic and biotic factors, such as climate, soil, agronomic practices, post-harvest management, genetic makeup, plant age, development stage, and plant material (La Bella et al., 2021; Méndez-Tovar et al., 2016; Novák et al., 2010; Figueiredo et al., 2008; Virga et al., 2020; Di Mola et al., 2021; Barreca et al., 2021; Tuttolomondo et al., 2015). It is also worth noting that greater increases in the biomass of those plants treated with foliar application biostimulants could lead to increases in extract yields per unit of surface area. Giannoulis et al. (2020) and Truzzi et al. (2021) reported similar results in lavender (*Lavandula angustifolia* Mill.) and Lavandin (*Lavandula x intermedia* Emeric ex Loisel). The results obtained in this study, in particular those obtained in plants treated with protein hydrolysates, could be valuable for growing organic plants with a greater biomass and EO yield to be used in food, pharmaceutical, and chemical industries. In organic farming systems, plants are frequently at risk of nutrient deficiencies (De Pascale et al., 2017). The effectiveness of small amounts of protein hydrolysate is due to the direct and indirect effects on the plant's primary and secondary metabolism, also under stress conditions, such as limited water availability (Rouphael et al., 2022; Colla et al., 2014; Consentino et al., 2020).

Conclusions

The results obtained in this research confirm that biostimulants represent a valid tool when seeking to increase yields in the organic farming of rosemary. In particular, the application of fulvic acids and protein hydrolysates leads to increases in plant growth and biomass yields. The highest EO yield values were found in plants treated with *E. maxima* and protein hydrolysates. It is necessary to enhance sustainable agronomic methods in order to reduce the use of chemical inputs, improve environmental quality, and boost agricultural productivity. The use of biostimulants and other bio-based products are technological advancements also for the MAP sector. These results are of extreme interest to organic companies concerned with the cultivation of rosemary for essential oil and fresh and dry biomass production. Additional investigation is necessary to evaluate the impact of foliar biostimulant application on the morphological, productive, and chemical characteristics of other MAPs.

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Experiment 2

Foliar application of various biostimulants produces contrasting response on yield, essential oil and chemical properties of organically grown sage (*Salvia officinalis* L.)

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Objective

The aim of this study was to assess how four distinct biostimulants and two frequencies of application affected morphological, productive, and chemical characteristics of *S. officinalis* L. grown organically in a Mediterranean climate without irrigation. In particular, the following hypothesis were tested: 1) the foliar application of biostimulants improves productive parameters in sage plants cultivated in open field; 2) the foliar application of biostimulants affects the chemical parameters of the extract depending on the type of biostimulant and frequency of application.

Materials and methods

Experimental site and plant material

Tests were carried out at a local farm located in Aragona (Sicily, Italy) (330 m a.s.l., 37°22′32.71″ N, 13°38′33.59″ E), during the growing seasons of 2022 and 2023. The soil was classified as Regosol (United States Department of Agriculture (USDA) classification: typic xerorthents) and sandy clay loam (46% sand, 27% clay and 27% silt) with a pH of 7.3, 14 g kg^{-1} organic matter, 1.25% total nitrogen, 19.8 ppm assimilable phosphate, and 358 ppm assimilable potassium. Agamic propagation was carried out. The seedlings were produced by a commercial nursery and grown in plastic pots for 60 days. The plants were transplanted at the beginning of spring 2021. The distance between rows and within rows was 2.00 m and 0.50 m, respectively (Figure 1).

Figure 1. Experimental site

Sage plants were managed under rainfed conditions. Before transplanting, the experimental field received organic fertilization through the distribution of 2 t ha^{-1} of manure which was buried at a depth of 0.30 m. No pesticides or chemical fertilizer were used in either year. Weeds were removed mechanically at the end of winter and before harvesting.

Weather data

Data on rainfall and air temperature were recorded at a weather station belonging to the Sicilian Agro-Meteorological Information Service (SIAS, 2023). The station is equipped with a datalogger and various sensors for the measurement of air temperature (TAM platinum PT100 sensor, heat resistance with anti-radiation screen) and total rainfall (PPR sensor with tilting bucket rain gauge). Data regarding average daily maximum and minimum temperatures (°C) and total 10-day period precipitation (mm) were taken into consideration. Air temperature and rainfall trends are shown in Figures 2 and 3.

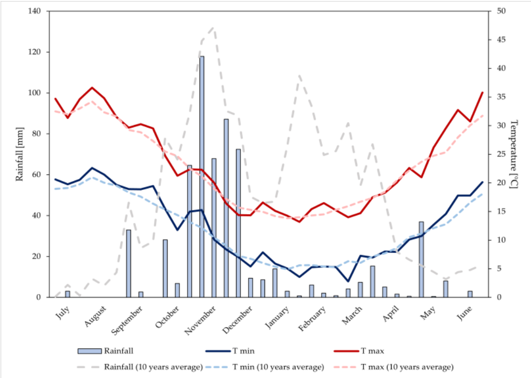

Figure 2. Temperature and rainfalls trends at the experimental site during growing season 2021-2022.

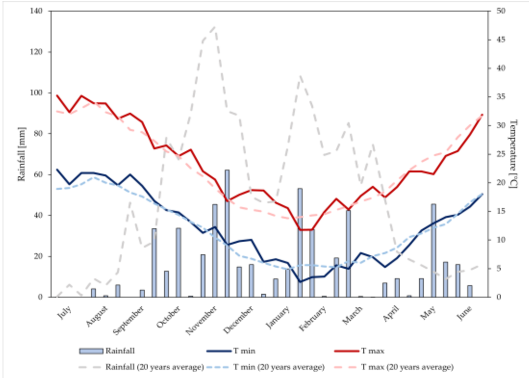

Figure 3. Temperature and rainfalls trends at the experimental site during growing season 2022-2023.

Over the two years, annual rainfall levels were 611 mm (2021-2022), and 538 mm (2022-2023). In the first growing season, rainfall was mainly distributed from October to December. During the period of biostimulant application and until the harvest, a total rainfall of 56 mm was observed. Significant rainfall occurred in the first 10-day period of May (37 mm). In the second growing season, rainfall was well-distributed from the end of September to March. During the period of biostimulant application and until the harvest, double rainfall levels (110 mm) compared to the first year were observed. Significant rainfall occurred in the second 10-day period of May (45 mm). In both years, temperatures trends were similar and consistent with the average temperature of the experimental area.

Treatments

Four commercial biostimulant formulations provided by the company "Mugavero fertilizers" were used for the tests.

- Protein Hydrolysate (PH), obtained from *Fabaceae* and containing amino acids and plant peptides (31%), organic nitrogen (5%) and organic carbon (25%).
- *Ecklonia maxima* (EM), containing organic nitrogen (1%), organic carbon (10%), auxin (11 ml l^{-1}) and cytokinin (0.03 mg l^{-1}), and organic substances with nominal molecular weights < 50 kDa (30%).
- *Ascophyllum nodosum* (AN), containing organic nitrogen (1%), organic carbon (10%), phytohormones and organic substances with nominal molecular weights < 50 kDa (30%).
- Fulvic Acids (FA), extracted from leonardite and containing organic nitrogen (0.5%) and organic carbon (30%).

The biostimulant dosage was planned to provide the same total amount of N, considering the N content of each type of product. The same total amount of biostimulant were applied following two frequencies of application, weekly frequency (F1) for a total of six applications and two-weeks frequency (F2) with three total applications. The doses are listed in Table 1.

Table 1. Doses of foliar biostimulants.

Biostimulant	**Dose[1] Frequency 1 week [l hl^{-1}]**	**Dose[1] Frequency 2 weeks [l hl^{-1}]**	**Total Amount[2] [l ha^{-1}]**
PH = Protein hydrolysate	0.025	0.050	1.2
EM = *Ecklonia maxima*	0.125	0.250	6.0
AN = *Ascophyllum nodosum*	0.125	0.250	6.0
FA = Fulvic acids	0.250	0.500	12.0

[1] = doses for each application; **[2] =** total quantity of biostimulant applied.

The first application was performed during the first week of April in each year. For each foliar application, 4 hl of water ha^{-1} were used. A portable sprayer with an operating pressure of 250 kPa was adopted. A randomized complete block design with three replicates was used for the tests. Biostimulant (B) and frequency (F) were used as fixed effects in the linear model/ANOVA. Each block comprised 10 plots of 30 m^2. Treatments were applied for each randomized plot in the block. The plots were well spaced in the block; plastic panels were used to delimit each plot and to avoid drift during foliar applications.

Plant Measurement

At harvest, plant height, chlorophyll content, relative water content (RWC), total fresh yield, total dry yield, essential oil (EO) content and essential oil (EO) yield were determined. In both years, plants were harvested during the third week of June at the stage of full flowering. Plants were cut at 5 cm above ground level and then dried in a shaded and ventilated environment for approx. 10 days at a temperature of 25-30°C. The chlorophyll content was measured using a Dualex Scientific (Force A, Orsay, France) portable Chlorophyll meter. Thirty fully developed leaves were used per plot. The instrument automatically averaged these readings. The RWC of leaves was estimated using fresh leaves, and the fresh weight (FW) was recorded. The leaves were floated in a falcon tube with distilled water for 24 h. The leaves were then removed from the water and placed on absorbent paper to remove the excess water, and the turgid weight (TW) was recorded. Subsequently, the leaves were dried in an oven for 24 h and the dry weight (DW) was recorded (Alyemeni et al., 2018). The RWC was calculated using the following equation:

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

Essential Oil Extraction

EO content was obtained by hydro distillation of air-dried plant material (500 g) for 3 h in accordance with international guidelines (European Pharmacopoeia, 2008) (Figure 4). The EO samples were stored at -18°C. Prior to GC/MS the essential oils obtained during the second growing season were diluted 1:100 with hexane and transferred to GC vials and stored at -18°C.

Figure 4. Essential oil extraction.

Essential Oil Profile, Gas Chromatography-Mass Spectrometry (GC-MS)

EO compounds were identified using a HP 6890 gas chromatograph coupled with the quadrupole mass spectrometer HP5972 MSD (Hewlett-Packard, Palo Alto, CA, USA) fitted with a DB5-MS capillary column (30 m × 0.25 mm inner diameter, film thickness: 0.25 µm; Agilent, Palo Alto, CA, USA). Helium was used as carrier gas (average velocity: 42 cm s^{-1}), the injector temperature was set to 250°C and the split ratio to 100:1. The temperature program started with 60°C for 4 min, rising to 100°C with 5°C min^{-1} increase, and from 100 to 280°C with 9°C min^{-1}. The retention indices of the essential oil compounds were determined in comparison to n-alkane hydrocarbons (retention index standard for GC, Sigma-Aldrich, Vienna, Austria) under the same conditions. The compounds were identified comparing their mass spectra and retention indices to published data. The composition was obtained by peak-area normalization, and the response factor for each compound was considered to equal 1.

Total Phenolics, Antioxidant Activity and Rosmarinic Acid

0.15 g of the finely powdered dry biomass obtained during the second year were extracted with 25 mL aqueous methanol (70%) for 30 minutes in an ultrasonic bath. The extracts were filtered and kept at -18°C until further analysis.

Total Phenolics

The total phenolics content was assayed with the Folin-Ciocalteu reagent, following the methodology described by Lamien-Meda et al. (2010). In the wells of the microplate, 5 µL extracts were added to 105 µL distilled water followed by 5 µL of Folin-Ciocalteu reagent, 10 µL Na_2CO_3 (35% in distilled water) and again 125 µL distilled water. Caffeic acid (Sigma-Aldrich, Austria; 10 mg in 100 mL milli-Q water) was used as standard. Increasing volumes (0 to 25 µL) of caffeic acid made up to 110 µL with distilled water instead of the samples were used to obtain a calibration curve. A blank was used to correct the readings. Calibration points and samples were pipetted and measured as quadruplicates. After 1 h resting in the dark, the absorbance of the reaction mixture was measured at 750 nm using a microplate reader (i-mark, Bio-Rad, Austria). The results were expressed as milligram caffeic acid equivalents per gram dry weight (mg c.a.e. g^{-1} dw).

Antioxidant Activity

Antioxidants react with the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) which is then decolorized. In accordance with the methodology reported by Chizzola et al. (2008), in the wells of the microplate, 5 µL extracts were added to 95 µL methanol and 100 µL of solution (2.2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, Germany; 0.0038 g in 25 mL methanol). Increasing volumes (0 to 8 µL) of Trolox (0.62 mg mL^{-1} in ethanol) made up to 100 µL with methanol instead of the samples were used to obtain a calibration curve. A preparation consisting of 50 µL Trolox, 50 µL distilled water and 100 µL DPPH reagent (where the DPPH was completely decolorized) was taken as blank and subtracted from all measurements. Calibration points and samples were pipetted and measured as quadruplicates. Discoloration was measured at 490 nm using a microplate reader (i-mark, Bio-Rad, Austria). The results were expressed in milligram trolox equivalents per gram dry weight (mg t.e. g^{-1} dw).

Rosmarinic Acid

The content of rosmarinic acid was measured according to Chizzola et al. (2008) using a Waters HPLC system consisting of a 626 pump, a 600S controller, a 717plus autosampler, a column oven operated at 25°C, and a 996-diode array detector (Waters S.A.S, Saint-Quentin, France). The separation was carried out on a Symmetry C18, 5.0 µm particle size, 4.6 × 150 mm column. The mobile phase used was 1% acetic acid/acetontrile 85:15 (solvent A) and methanol (solvent B). The analysis started with a solvent ratio of A/B of 9:1, and a linear gradient was performed to reach 100% B within 30 min. The flow rate was 1.0 mL min^{-1} and the injection volume, 20 µL. The quantification of rosmarinic acid was done using the external standard method by preparing seven calibration standards ranging from 3.9 to 500 µg ml^{-1} and recording the calibration curve at 330 nm.

Statistical Analysis

Statistical analyses were performed using the package MINITAB 19 for Windows. Data were compared using analysis of variance (ANOVA). The difference between means was carried out using Tukey's test ($p \leq 0.05$). Before the statistical analysis, all data were tested for normality with a Shapiro–Wilk test, and for homogeneity of variance with Levene's test.

Results

Morphological and yield parameters

ANOVA revealed that Frequency factor significantly affected ($p \leq 0.01$) the RWC during both years (Table 2). The highest RWC (81.6% and 83.7%) were always observed in plants treated every two weeks (Table 2).

Table 2. Influence of Frequency (F), Biostimulant (B) and their interaction on *S. officinalis* plant height, chlorophyll content and relative water content (RWC) over the 2-years study.

Source of variation	Degree of freedom	Plant height [cm]		Chlorophyll content [µg cm^{-2}]		RWC [%]	
		I Year	II Year	I Year	II Year	I Year	II Year
Frequency (F)							
1 week		37.9 ± 1.2 ^{a}	51.0 ± 1.8 ^{a}	35.1 ± 0.7 ^{a}	36.4 ± 0.6 ^{a}	74.7 ± 0.8 ^{b}	76.7 ± 0.8 ^{b}
2 weeks		37.8 ± 1.6 ^{a}	51.1 ± 1.9 ^{a}	35.6 ± 0.7 ^{a}	36.6 ± 0.4 ^{a}	81.6 ± 0.8 ^{a}	83.7 ± 1.1 ^{a}
Biostimulant (B)							
C		28.0 ± 1.0 ^{c}	38.6 ± 0.8 ^{c}	32.5 ± 0.3 ^{c}	35.0 ± 0.4 ^{c}	76.7 ± 0.4 ^{b}	77.7 ± 0.8 ^{c}
PH		40.9 ± 0.6 ^{a}	56.0 ± 1.5 ^{a}	35.1 ± 1.0 ^{b}	36.3 ± 0.4 ^{bc}	77.8 ± 1.3 ^{b}	81.0 ± 1.4 ^{ab}
EM		41.8 ± 0.5 ^{a}	55.7 ± 0.6 ^{a}	33.5 ± 0.5 ^{bc}	35.4 ± 0.7 ^{c}	77.9 ± 2.5 ^{b}	80.3 ± 2.5 ^{b}
AN		38.5 ± 0.7 ^{b}	52.6 ± 1.1 ^{b}	37.8 ± 0.6 ^{a}	38.3 ± 1.0 ^{a}	77.6 ± 3.3 ^{b}	79.9 ± 3.5 ^{b}
FA		40.0 ± 0.9 ^{ab}	52.3 ± 1.5 ^{b}	37.8 ± 0.7 ^{a}	37.4 ± 0.6 ^{ab}	80.7 ± 1.1 ^{a}	82.2 ± 1.4 ^{a}
p-value							
Frequency	1	0.828	0.893	0.290	0.781	0.000	0.000
Biostimulant	4	0.000	0.000	0.000	0.000	0.000	0.000
F × B	4	0.000	0.000	0.004	0.000	0.000	0.000

Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C = control; PH = protein hydrolysate; EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids.

Biostimulant factor and the interaction F × B had significant effect ($p \leq 0.01$) on plant height, chlorophyll content and RWC (Table 2). During the first year, the highest plant heights were recorded in PH- and EM-treated plants every week (42 cm) and in EM- and FA-treated plants every two weeks (41.5 and 41.7 cm, respectively) (Figure 5A). During the second year, the highest plant height (59 cm) was recorded in PH-treated plants every week, followed by the values observed by the interactions Frequency 1 × EM and Frequency 2 × FA. During both years, the lowest plant heights were recorded in C-plants (Figures 5A, B).

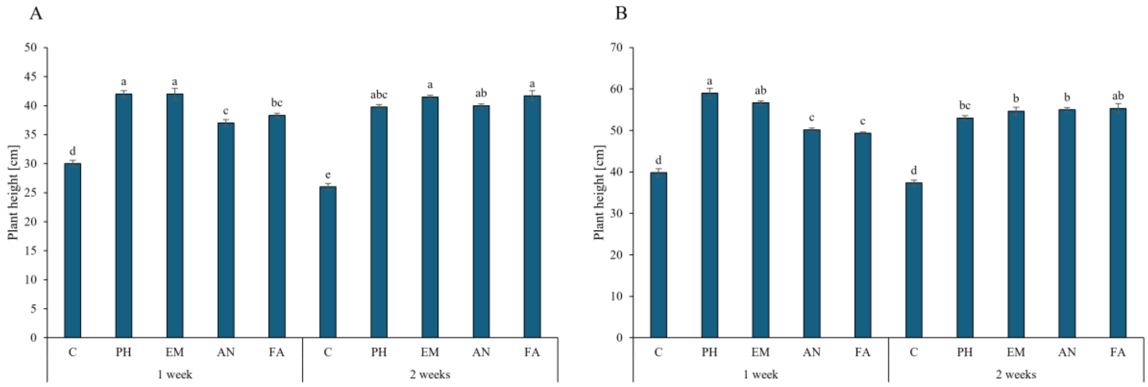

Figure 5. Influence of the interaction Frequency (F) × Biostimulant (B) on *S. officinalis* plant height, in the first year **(A)** and in the second year **(B)**. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C, control; PH, protein hydrolysate; EM, *Ecklonia maxima*; AN, *Ascophyllum nodosum*; FA, fulvic acids.

The application of AN and FA every week and the application of FA every two weeks produced the highest chlorophyll content (values between 37.4 and 39.1 µg cm^{-2}), during the first year. The lowest values were observed in C-plants following both frequencies (Figure 6A). The interaction Frequency 1 × AN produced the highest chlorophyll content during the second year. The lowest values were recorded by the interactions Frequency 1 × EM and Frequency 2 × C (Figure 6B).

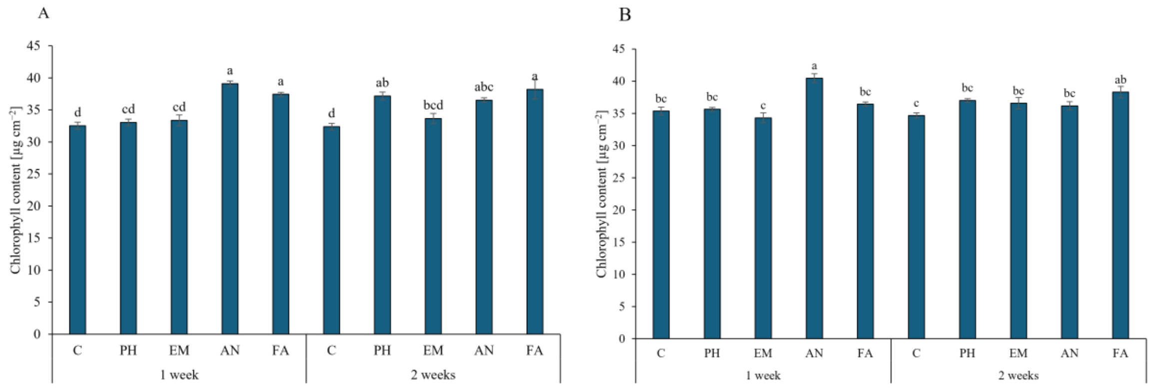

Figure 6. Influence of the interaction Frequency (F) × Biostimulant (B) on *S. officinalis* chlorophyll content, in the first year **(A)** and in the second year **(B)**. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C, control; PH, protein hydrolysate; EM, *Ecklonia maxima*; AN, *Ascophyllum nodosum*; FA, fulvic acids.

During both years, the highest RWC values were observed in EM-, AN- and FA-treated plants every two weeks (values between 83.2% and 87.6%). The lowest values were observed in AN-treated plants every week (Figures 7A, B).

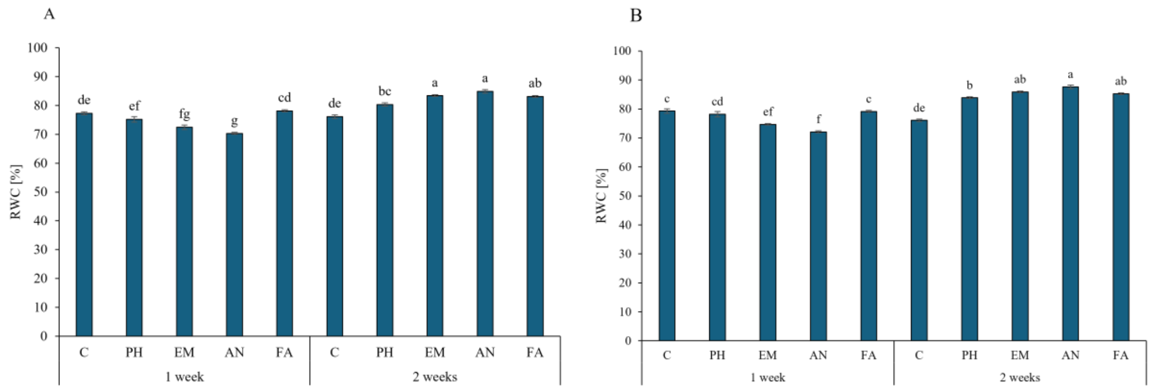

Figure 7. Influence of the interaction Frequency (F) × Biostimulant (B) on *S. officinalis* relative water content (RWC), in the first year **(A)** and in the second year **(B)**. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C, control; PH, protein hydrolysate; EM, *Ecklonia maxima*; AN, *Ascophyllum nodosum*; FA, fulvic acids.

Statistically analysis showed that frequency factor, biostimulant factor and the interaction F × B had significant effects ($p \leq 0.01$) on total fresh yield and total dry yield (Table 3).

Table 3. Influence of Frequency (F), Biostimulant (B) and their interaction on *S. officinalis* total fresh yield and total dry yield over the 2-years study.

	Degree of freedom	Total fresh yield [t ha^{-1}]		Total dry yield [t ha^{-1}]	
		I Year	II Year	I Year	II Year
Frequency (F)					
1 week		3.4 ± 0.2 [a]	6.5 ± 0.4 [a]	1.1 ± 0.1 [a]	2.1 ± 0.1 [a]
2 weeks		2.6 ± 0.1 [b]	4.8 ± 0.1 [b]	0.8 ± 0.0 [b]	1.4 ± 0.0 [b]
Biostimulant (B)					
C		2.4 ± 0.1 [d]	4.4 ± 0.1 [d]	0.7 ± 0.0 [d]	1.3 ± 0.1 [d]
PH		3.4 ± 0.2 [a]	6.5 ± 0.5 [a]	1.1 ± 0.1 [a]	2.0 ± 0.2 [a]
EM		2.7 ± 0.1 [c]	5.1 ± 0.1 [c]	0.9 ± 0.1 [c]	1.7 ± 0.1 [c]
AN		3.0 ± 0.2 [b]	5.7 ± 0.4 [b]	0.9 ± 0.1 [c]	1.7 ± 0.1 [c]
FA		3.4 ± 0.4 [a]	6.6 ± 0.9 [a]	1.0 ± 0.1 [b]	1.9 ± 0.3 [b]
p-value					
Frequency	1	0.000	0.000	0.000	0.000
Biostimulant	4	0.000	0.000	0.000	0.000
F × B	4	0.000	0.000	0.000	0.000

Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C = control; PH = protein hydrolysate; EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids.

Considering both years, the weekly application of FA produced the highest total fresh yield (4.3 and 8.7 t ha^{-1}), and the weekly application of PH and FA generated the highest total dry yields, 1.3 t ha^{-1} during the first year and 2.5 t ha^{-1} during the second year (Figures 8A, B). The lowest yield values (fresh and dry) were always observed in C-plants following the frequency 2 weeks (Figures 8A, B).

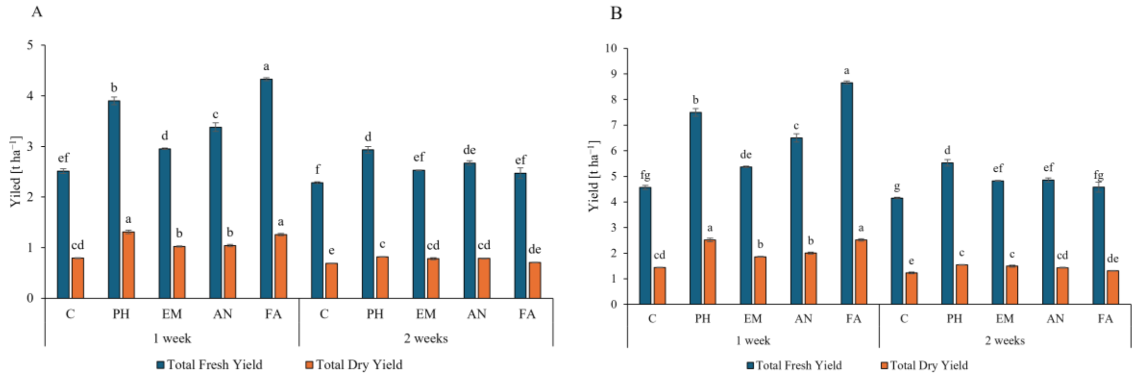

Figure 8. Influence of the interaction Frequency (F) × Biostimulant (B) on *S. officinalis* total fresh yield and total dry yield, in the first year **(A)** and in the second year **(B)**. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C, control; PH, protein hydrolysate; EM, *Ecklonia maxima*; AN, *Ascophyllum nodosum*; FA, fulvic acids.

Analysis of variance for EO content and EO yield displayed a significant effect ($p \leq 0.01$) of frequency, biostimulant and the interaction F × B (Table 4).

Table 4. Influence of Frequency (F), Biostimulant (B) and their interaction on *S. officinalis* essential oil (EO) content and essential oil (EO) yield over the 2-years study.

Source of variation	**Degree of freedom**	**EO content [%]**		**EO yield [kg ha^{-1}]**	
		I Year	**II Year**	**I Year**	**II Year**
Frequency (F)					
1 week		1.23 ± 0.05 ^{b}	1.11 ± 0.04 ^{b}	13.4 ± 0.9 ^{a}	22.9 ± 1.6 ^{a}
2 weeks		1.35 ± 0.03 ^{a}	1.24 ± 0.03 ^{a}	10.3 ± 0.3 ^{b}	17.5 ± 0.7 ^{b}
Biostimulant (B)					
C		1.30 ± 0.05 ^{b}	1.15 ± 0.03 ^{bc}	9.7 ± 0.6 ^{c}	15.4 ± 1.0 ^{d}
PH		1.46 ± 0.02 ^{a}	1.32 ± 0.02 ^{a}	15.7 ± 1.9 ^{a}	27.0 ± 3.1 ^{a}
EM		1.28 ± 0.08 ^{b}	1.18 ± 0.08 ^{b}	11.3 ± 0.2 ^{b}	19.5 ± 0.4 ^{bc}
AN		1.20 ± 0.05 ^{c}	1.10 ± 0.05 ^{c}	10.9 ± 0.3 ^{b}	18.6 ± 0.7 ^{c}
FA		1.22 ± 0.07 ^{c}	1.12 ± 0.06 ^{c}	11.6 ± 0.8 ^{b}	20.5 ± 1.8 ^{b}
p-value					
Frequency	1	0.000	0.000	0.000	0.000
Biostimulant	4	0.000	0.000	0.000	0.000
F × B	4	0.000	0.000	0.000	0.000

Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C = control; PH = protein hydrolysate; EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids.

Considering both years, PH-treated plants every week and EM-treated plants every two weeks produced the highest EO content (values between 1.34% and 1.51%). The lowest EO content were obtain in the other biostimulated plants every week (Figures 9A, B).

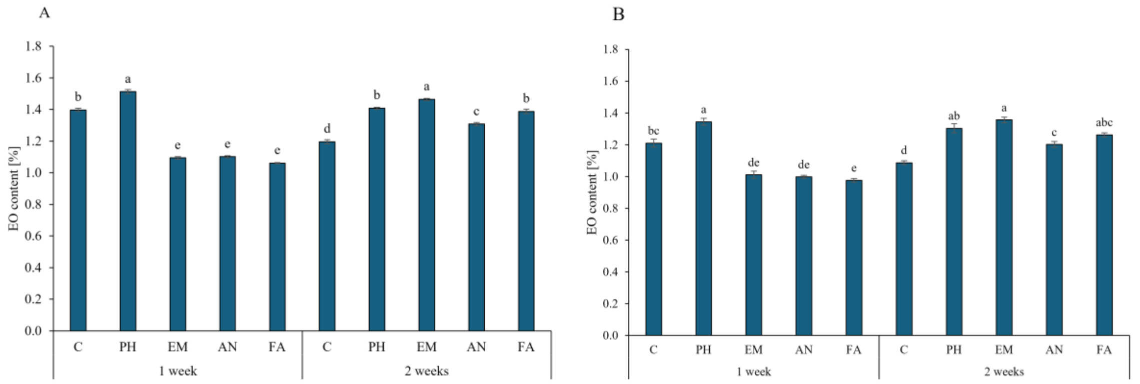

Figure 9. Influence of the interaction Frequency (F) × Biostimulant (B) on *S. officinalis* essential oil (EO) content, in the first year **(A)** and in the second year **(B)**. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C, control; PH, protein hydrolysate; EM, *Ecklonia maxima*; AN, *Ascophyllum nodosum*; FA, fulvic acids.

Regarding the EO yield, the application of PH every week generated the highest values in both years (19.8 kg ha^{-1} during the first year and 33.9 kg ha^{-1} during the second year). The lowest EO yield was obtained in C-plants following frequency 2 (Figures 10A, B).

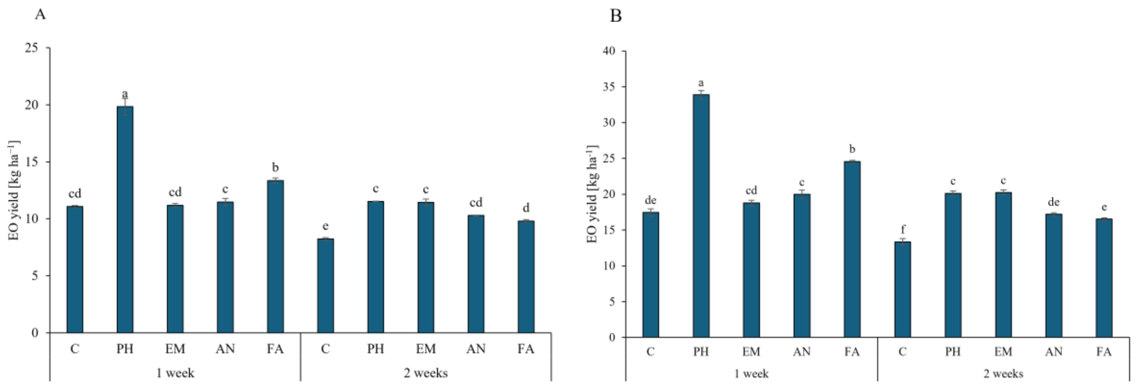

Figure 10. Influence of the interaction Frequency (F) × Biostimulant (B) on *S. officinalis* essential oil (EO) yield, in the first year **(A)** and in the second year **(B)**. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C, control; PH, protein hydrolysate; EM, *Ecklonia maxima*; AN, *Ascophyllum nodosum*; FA, fulvic acids.

Essential oil profile

The results of the analysis of variance of the EO profile are reported in Table 5. 44 compounds were identified, and the main components being α-pinene, camphene, β-pinene, 1,8-cineole, α-thujone, β-thujone, camphor, β-caryophyllene, aromadendrene, α-humulene, and viridiflorol.

Table 5. Chemical constituents of *S. officinalis* essential oil and *p*-value in response to Biostimulants (B), Frequency (F) and their interaction (B × F).

Peak	RI calc	RI lit	Compounds	Biostimulant (B)	Frequency (F)	Interaction B × F
1	918	919	tricyclene	0.002	0.918	0.290
2	922	923	α-thujene	0.081	0.039	0.618
3	929	933	α-pinene	0.006	0.896	0.458
4	942	952	camphene	0.003	0.989	0.289
5	968	973	sabinene	0.008	0.122	0.198
6	971	981	β-pinene	0.003	0.877	0.198
7	989	991	β-myrcene	0.011	0.912	0.173
8	1002	1005	α -phellandrene	0.051	0.108	0.306
9	1013	1018	α-terpinene	0.010	0.158	0.519
10	1021	1026	*p*-cymene	0.005	0.237	0.597
11	1027	1030	1,8-cineole	0.002	0.721	0.104
12	1036	1040	*cis*-β-ocimene	0.654	0.141	0.318
13	1054	1059	γ-terpinene	0.010	0.201	0.442
14	1062	1069	*trans*-sabinene hydrate	0.427	0.447	0.783
15	1084	1084	α-terpinolene	0.058	0.118	0.339
16	1103	1102	α-thujone	0.005	0.369	0.415
17	1113	1110	β-thujone	0.001	0.911	0.583
18	1133	1139	*trans*-sabinol	0.001	0.435	0.232
19	1140	1143	camphor	0.020	0.428	0.273
20	1160	1165	borneol	0.009	0.472	0.559
21	1172	1178	terpinen-4-ol	0.005	0.277	0.326
22	1185	1185	α-terpineol	0.071	0.204	0.992
23	1286	1285	bornyl acetate	0.087	0.463	0.938
24	1373	1376	α-copaene	0.008	0.419	0.259
25	1375	1375	β-elemene	0.010	0.468	0.089
26	1407	1409	α-gurjunene	0.007	0.375	0.319
27	1428	1428	β-caryophyllene	0.011	0.092	0.414
28	1434	1436	geranyl acetone	0.004	0.229	0.056
29	1439	1439	aromadendrene	0.010	0.293	0.284
30	1444	1444	α-caryophyllene	0.014	0.338	0.331
31	1456	1452	α-humulene	0.005	0.931	0.310
32	1462	1461	allo-aromadendrene	0.487	0.432	0.529
33	1478	1482	citronellyl isobutyrate	0.502	0.162	0.137
34	1490	1490	valencene	0.117	0.371	0.694
35	1492	1493	viridiflorene	0.012	0.305	0.362
36	1496	1502	cuparene	0.014	0.200	0.316
37	1516	1512	γ-cadinene	0.038	0.059	0.115
38	1526	1524	δ-cadinene	0.157	0.028	0.396
39	1573	1575	spathulenol	0.023	0.124	0.460
40	1583	1581	caryophyllene oxide	0.172	0.040	0.804
41	1598	1590	viridiflorol	0.004	0.997	0.192
42	1609	1607	humulene epoxide II	0.043	0.057	0.514
43	1616	1611	epicedrol	0.004	0.997	0.391
44	2070	2056	manool	0.231	0.018	0.677

RI calc = Retention Indices calculated based on C9-C27 n-alkenes from a HP-5MS-column; RI lit = Retention Indices according to literature.

The frequency factor significantly ($p \leq 0.05$) influenced the percentage of 38 compounds (Table 5). Considering only the main components, the highest percentages of α-pinene, camphene, β-pinene, 1,8-cineole, β-Thujone, β-caryophyllene, aromadendrene were observed in plants treated following frequency 1. In plants treated following frequency 2 the highest percentages of α-thujone, camphor α-humulene, viridiflorol were measured (Table 6).

Table 6. Influence of Frequency (F) of application on chemical constituents of *S. officinalis* essential oil.

Peak	Compounds	Frequency 1 week [%]	Frequency 2 weeks [%]	*p*-value
1	tricyclene	0.15 [a]	0.07 [b]	0.918
2	α-thujene	0.24	0.29	0.039
3	α-pinene	3.44 [a]	2.25 [b]	0.896
4	camphene	3.88 [a]	2.74 [b]	0.989
5	sabinene	0.06 [b]	0.12 [a]	0.122
6	β-pinene	2.71 [a]	2.16 [b]	0.877
7	β-myrcene	2.20 [a]	1.85 [b]	0.912
8	α -phellandrene	0.10	0.11	0.108
9	α-terpinene	0.40 [b]	0.54 [a]	0.158
10	*p*-cymene	0.50 [b]	0.72 [a]	0.237
11	1,8-cineole	18.81 [a]	16.45 [b]	0.721
12	*cis*-b-ocimene	0.12	0.11	0.141
13	γ-terpinene	0.51 [b]	0.70 [a]	0.201
14	*trans*-sabinene hydrate	0.14	0.14	0.447
15	α-terpinolene	0.37	0.42	0.118
16	α-thujone	10.58 [b]	16.01 [a]	0.369
17	β-thujone	4.74 [a]	4.38 [b]	0.911
18	*trans*-sabinol	0.10 [a]	0.08 [b]	0.435
19	camphor	16.06 [b]	18.51 [a]	0.428
20	borneol	1.43 [a]	1.07 [b]	0.472
21	terpinen-4-ol	0.24 [b]	0.30 [a]	0.277
22	α-terpineol	0.33	0.29	0.204
23	bornyl acetate	0.71	0.56	0.463
24	α-copaene	0.30 [a]	0.17 [b]	0.419
25	β-elemene	0.11 [a]	0.08 [b]	0.468
26	α-gurjunene	1.94 [a]	0.87 [b]	0.375
27	β-caryophyllene	8.82 [a]	7.20 [b]	0.092
28	geranyl acetone	0.49 [a]	0.28 [b]	0.229
29	aromadendrene	3.55 [a]	2.00 [b]	0.293
30	α-caryophyllene	0.38 [a]	0.22 [b]	0.338
31	α-humulene	5.74 [b]	8.16 [a]	0.931
32	allo-aromadendrene	0.47	0.64	0.432
33	citronellyl isobutyrate	0.17	0.16	0.162
34	valencene	0.26	0.2	0.371
35	viridiflorene	0.08 [a]	0.03 [b]	0.305
36	cuparene	1.42 [a]	0.91 [b]	0.200
37	γ-cadinene	0.09 [a]	0.08 [b]	0.059
38	δ-cadinene	0.31	0.28	0.028
39	spathulenol	1.15 [a]	0.53 [b]	0.124
40	caryophyllene oxide	0.77	0.62	0.04
41	viridiflorol	3.11 [b]	4.67 [a]	0.997
42	humulene epoxide II	0.75 [a]	0.48 [b]	0.057
43	epicedrol	0.25 [b]	0.40 [a]	0.997
44	manool	0.5	0.59	0.018

Means are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test.

The Biostimulant factor significantly ($p \leq 0.05$) influenced the percentage of α-thujene, δ-cadinene, caryophyllene oxide and manool (Table 7). The highest α-thujene (0.34%) content was observed in PH-treated plants, this value was statistically similar to those obtained in EM-, AN- and FA treated plants. The lowest content (0.20%) was found in C-plants (Table 7). In C-plants, the highest content of δ-cadinene (0.36%) and caryophyllene oxide (1.02%) was observed, while the lowest content was found in PH-treated plants (δ-cadinene 0.25% and caryophyllene oxide 0.48%). The values obtained with the other foliar biostimulants were statistically similar to each other (Table 7). The highest manool content (0.80%) was observed in C-plants, followed by those statistically similar obtained in AN-, PH- and EM-treated plants. In FA-treated plants, the lowest manool content was observed (0.38%) (Table 7).

Table 7. Influence of Biostimulant (F) on chemical constituents of *S. officinalis* EO.

Peak	Compounds	C [%]	PH [%]	EM [%]	AN [%]	FA [%]	*p*-value
1	tricyclene	0.12	0.09	0.10	0.11	0.12	0.918
2	α-thujene	0.20 [b]	0.34 [a]	0.28 [ab]	0.25 [ab]	0.25 [ab]	0.039
3	α-pinene	2.84	2.72	2.57	2.93	3.16	0.896
4	camphene	3.33	3.33	3.17	3.27	3.45	0.989
5	sabinene	0.05	0.14	0.11	0.09	0.07	0.122
6	β-pinene	2.35	2.59	2.35	2.43	2.47	0.877
7	β-myrcene	1.94	2.12	1.99	2.01	2.07	0.912
8	α -phellandrene	0.09	0.12	0.11	0.10	0.10	0.108
9	α-terpinene	0.37	0.57	0.51	0.45	0.45	0.158
10	*p*-cymene	0.47	0.72	0.69	0.59	0.58	0.237
11	1,8-cineole	16.87	18.14	17.39	17.61	18.14	0.721
12	*cis*-b-ocimene	0.10	0.13	0.12	0.11	0.12	0.141
13	γ-terpinene	0.51	0.73	0.68	0.60	0.60	0.201
14	*trans*-sabinene hydrate	0.12	0.15	0.15	0.15	0.14	0.447
15	α-terpinolene	0.33	0.45	0.43	0.38	0.38	0.118
16	α-thujone	10.38	15.69	14.83	12.75	12.81	0.369
17	β-thujone	4.59	4.49	4.56	4.55	4.62	0.911
18	*trans*-sabinol	0.10	0.08	0.09	0.10	0.08	0.435
19	camphor	15.69	18.09	18.47	16.92	17.27	0.428
20	borneol	1.36	1.04	1.18	1.31	1.34	0.472
21	terpinen-4-ol	0.23	0.28	0.29	0.28	0.28	0.277
22	α-terpineol	0.32	0.27	0.30	0.33	0.33	0.204
23	bornyl acetate	0.76	0.53	0.58	0.68	0.63	0.463
24	α-copaene	0.31	0.18	0.20	0.25	0.24	0.419
25	β-elemene	0.11	0.09	0.10	0.11	0.10	0.468
26	α-gurjunene	2.06	0.94	1.14	1.50	1.41	0.375
27	β-caryophyllene	9.53	6.94	7.51	8.35	7.73	0.092
28	geranyl acetone	0.51	0.28	0.33	0.39	0.42	0.229
29	aromadendrene	3.84	1.97	2.37	2.95	2.74	0.293
30	α-caryophyllene	0.41	0.21	0.26	0.32	0.30	0.338
31	α-humulene	6.40	7.36	7.26	6.98	6.74	0.931
32	allo-aromadendrene	0.53	0.37	0.41	0.45	1.04	0.432
33	citronellyl isobutyrate	0.19	0.15	0.16	0.17	0.16	0.162
34	valencene	0.29	0.17	0.20	0.24	0.25	0.371
35	viridiflorene	0.09	0.03	0.05	0.06	0.06	0.305
36	cuparene	1.58	0.85	1.03	1.26	1.13	0.200
37	γ-cadinene	0.11	0.07	0.08	0.09	0.08	0.059
38	δ-cadinene	0.36 [a]	0.25 [b]	0.28 [ab]	0.31 [ab]	0.28 [ab]	0.028
39	spathulenol	1.49	0.42	0.66	0.89	0.74	0.124
40	caryophyllene oxide	1.02 [a]	0.48 [b]	0.58 [ab]	0.70 [ab]	0.68 [ab]	0.040
41	viridiflorol	3.93	3.99	3.78	3.97	3.78	0.997
42	humulene epoxide II	1.00	0.38	0.52	0.61	0.55	0.057
43	epicedrol	0.32	0.34	0.32	0.32	0.32	0.997
44	manool	0.80 [a]	0.51 [ab]	0.51 [ab]	0.53 [ab]	0.38 [b]	0.018

Means are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. C = control; PH = protein hydrolysate; EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids.

The analysis of variance showed that the interaction factor did not statistically affect the percentage of EO compounds (Table 6).

Total phenolics, antioxidant activity and rosmarinic acid

Analysis of variance revealed that biostimulant (B) and frequency (F) factors had significant effects ($p \leq 0.05$) on total phenolic, antioxidant activity and rosmarinic acid (Table 8).

Table 8. Effect of Biostimulant (B), Frequency (F) and their interaction on total phenolic, antioxidant activity and rosmarinic acid.

	Total Phenolic [mg c.a.e. g^{-1} dw]	**Antioxidant Activity [mg t.e. g^{-1} dw]**	**Rosmarinic Acid [%]**
Frequency (F)			
1 week	57.0 ± 2.8^{b}	70.0 ± 6.5^{b}	1.1 ± 0.7^{b}
2 weeks	62.3 ± 3.5^{a}	94.2 ± 5.3^{a}	1.5 ± 0.6^{a}
Biostimulant (B)			
C	76.0 ± 2.1^{a}	108.2 ± 2.3^{a}	2.4 ± 0.1^{a}
PH	68.3 ± 1.9^{b}	65.3 ± 12.4^{b}	0.8 ± 0.4^{b}
EM	58.4 ± 1.9^{c}	78.3 ± 8.0^{ab}	1.1 ± 0.0^{b}
AN	43.9 ± 1.4^{e}	81.6 ± 9.2^{ab}	1.3 ± 0.6^{b}
FA	51.8 ± 1.2^{d}	77.4 ± 11.4^{ab}	1.0 ± 0.4^{b}
p-value			
F	0.000	0.003	0.016
B	0.000	0.017	0.000
F × B	0.001	0.612	0.666

Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$. c.a.e. = Caffeic acid equivalent; t.e. = Trolox equivalent. C = control; PH = protein hydrolysate; EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids.

The highest total phenolic (76.0 mg c.a.e. g^{-1}) and rosmarinic acid (2.4%) values were observed in Control plants. The lowest total phenolic value was observed in FA-treated plants, recorded as 24.2 mg c.a.e. g^{-1} lower than the highest content (Table 8). The lowest rosmarinic acid values were observed in all biostimulated plants (between 0.8% and 1.3%) (Table 8).

Considering antioxidant activity, the highest value (108.2 mg t.e. g^{-1}) was obtained in C-plants, statistically similar to those obtained in EM-, AN- and FA-treated plants (Table 8).

Regardless of the biostimulant factor, sage plants showed higher total phenolic, antioxidant activity and rosmarinic acid levels following Frequency 2.

Statistical analysis highlighted that the interaction B × F has a significant effect ($p \leq 0.01$) only on total phenolic content (Table 8). As reported in Figure 3, the highest total phenolic content (80.4 mg c.a.e. g^{-1}) was observed in C-plants following Frequency 2. The lowest total phenolic value (41.3 mg c.a.e. g^{-1}) was recorded in AN-treated plants following Frequency 1. This value was statistically similar to those obtained in AN-treated plants following Frequency 2 (Figure 11).

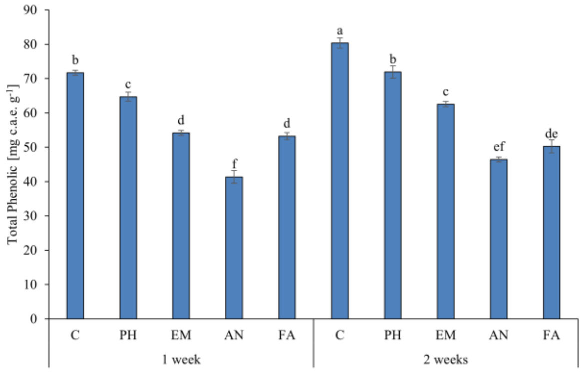

Figure 11. Influence of the interaction F × B on total phenolic. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to Tukey's test. c.a.e., Caffeic acid equivalent; C, control; PH, protein hydrolysate; EM, *Eklonia maxima*; AN, *Ascophyllum nodosum*; FA, fulvic acids.

Discussion

Nowadays, the cultivation of medicinal and aromatic plants has garnered increasing attention, necessitating the improvement of agronomic practices to maximize yields and harvest quality in a sustainable and logical manner (Sharifi-Rad et al., 2017; Rao et al., 2022). In addition to being used in the kitchen to add flavors to food, the food, cosmetic, and chemical sectors are increasingly using the MAPs to look for particular properties and/or compounds that can be found on various MAPs (Bernardini et al., 2018; Giannenas et al., 2020; Ovidi et al., 2021). Because of this, it's essential to develop and assess the impact that various agronomic techniques have on the chemical composition and qualitative characteristics of the extracts obtained from MAPs, in addition to refining the agronomic technique to boost yields (Elansary et al., 2019; Kosakowska et al., 2019; Giannoulis et al. 2020; Shahrajabian et al., 2021). Increased crop yields with the use of uncommon agricultural practices are one of the modern agriculture's goals (Krein et al., 2023; Di Miceli et al., 2022; Licata et al., 2022)

This study demonstrated how the foliar application of various biostimulants, a technique that is being used more and more and has produced excellent results on a variety of species, has improved the agronomic performance of *S. officinalis* biologically cultivated in a Mediterranean environment. In addition, both qualitative and chemical measures were impacted by the foliar treatment of biostimulants.

The quantity and frequency of applications, which may affect nutrient uptake and plant metabolic processes, are additional factors to consider (Bulgari et al., 2019; Li et al., 2022). At the meantime, the most efficient and financially beneficial course of action must be determined while keeping in mind that a higher number of applications translate into higher costs for farmers (Kocira et al., 2020; Li et al., 2022).

Over the course of the two years of the test, the largest heights were achieved with the foliar application of protein hydrolysates and *Ecklonia maxima*. Protein hydrolysates are combinations of amino acids and peptides that, when applied to plants, can cause a range of physiological reactions that support growth, improve the yield and quality of the product, and strengthen the plants' resistance to heat stress, salinity, drought, and nutritional stress (Schaafsma 2009; Colla et al., 2014). The positive effect of applying *E. maxima* may be related to the content of macro- and micronutrients, sterols, polysaccharides, betaines, and, additionally, enhancing-promoting compounds (auxins, cytokinins, gibberellins) (Tarakhovskaya et al. 2007; Briceño-Domínguez et al. 2014; Hernandez-Herrera et al. 2014; Arioli et al. 2015).

The height values are comparable to those found in the research conducted by Es-sbihi et al. (2020), which measured heights between 40 and 50 cm after applying salicylic acid foliar treatments to sage plants. Similar results were found by Soltanbeigi et al. (2021) on *S. officinalis* cultivated in greenhouses and fertilized with various methods.

During both years, treatment with *Ascophyllum nodosum* and fulvic acids increased the amount of chlorophyll in sage leaves. Numerous studies (Rasouli et al., 2022; Ciriello et al., 2022, Abdel-Rahman and Abdel-Kader 2020; Farruggia et al., 2024) have reported that plants treated with foliar biostimulants exhibited greater levels of chlorophyll in their leaves when compared to untreated plants. The values observed in this study agree with the chlorophyll content measured by Aslani et al. (2023) in *S. officinalis* under drought stress and inoculation treatments. The chlorophyll content of sage plants exposed to increasing nitrogen dosages showed a similar tendency into Khammar et al. work (2021).

Reduced cell turgor and relative water content (RWC) in water-stressed plants lead to decreased cell elongation and development, which in turn reduces leaf area. When antioxidant defenses and reactive oxygen species (ROS) are out of equilibrium in water-stressed plants, oxidative stress results (Mohammadi-Cheraghabadi et al., 2021).

A lower number of foliar seaweed applications produced an increase in the relative water content of the leaf. The application of the same biostimulants following the frequency 1 causes a significant decrease in RWC. Auxins, cytokinins, and gibberellins - enhancing-promoting compounds in algae extracts – in excessive quantity can be phytotoxic to plants and cause negative effects on the physiological processes and on the assimilation of water and nutrients (Illera-Vives et al., 2022). Due to the content of macro and micro-nutrients and plant hormones, the correct application of seaweed extracts can improve root growth and development and, at the same time, can maintain a balanced water content and a correct transpiration rate (Azizi et al., 2024).

During the second year of the research, the highest yield values were obtained thanks to the greater development of plants at the third year of cultivation. In general, a more frequent biostimulant application has allowed to obtain higher biomass and EO yield. During both years, the highest biomass yields (fresh and dry) were observed in plants treated with protein hydrolysate and fulvic acid. Peptide and amino acid content of protein hydrolysates is the primary cause of their biostimulatory activities (Di Miceli et al., 2023). Amino acids are used by plants for many different purposes, such as the synthesis of substances with high biological activity, the generation of energy, and the biosynthesis of proteins (Paul et al., 2019; Rouphael et al., 2022). Protein hydrolysates applied on leaves or on roots can alter the phyllosphere or rhizosphere's microbial community (Colla et al., 2017). Microorganisms in the rhizosphere and phyllosphere may release enzymes that break down peptides into smaller pieces that function as signaling molecules to promote plant development (Malécange et al., 2023). Fulvic acids are known to facilitate the absorption and translocation of micro- and macronutrients. For this reason, their application has been connected to increases in crop productivity and quality (Colombo et al., 2012; Aytaç et al., 2022). Additionally, these compounds positively influence several molecular functions, such as the production of proteins, photosynthetic activity, and enzyme activity (Nardi et al., 2021; Farruggia et al., 2024). Their application can boost the concentration of nitrogen, phosphorus, and potassium, as well as the content of photosynthetic pigments, carotenoids, and total phenols (Zanin et al., 2019; Aytaç et al., 2022). The yield obtained in this study are in line with those observed by Ostadi et al. (2022) in *S. officinalis* under drought stress and treated with TiO_2 nanoparticles and arbuscular mycorrhizal fungi. Also, Giannoulis et al. (2021) measured similar fresh yield in *S. officinalis* cultivated in Greece with different plant density and fertilization methods.

It is well known that the synthesis of secondary metabolites is affected by endogenous and exogenous factors (Rajabi et al., 2014; Ben Akacha et al., 2023). Several research report that the application of biostimulants can improve the beneficial qualities of plants by acting as elicitors for secondary metabolites, such as essential oils (Rahmani Samani et al., 2019; The et al., 2021; Ciriello et al., 2024). During the two-year study, the EO content ranged between 1.0% and 1.5%. These values are in line with those obtained by Ostadi et al. (2022) (0.6%-1.5%), Tarraf et al. (2017) (1.0%-1.2%), Govahi et al. (2015) (0.8%-2.1%), Bagdat et al. (2017) (1.2%-1.6%), Es-sbihi et al. (2020) (1.2%-2.8%) measured under different conditions. Otherwise, Geneva et al. (2010) observed a lower EO content (0.4%-0.5%) in *S. officinalis* fertilized with foliar NPK + microelements. Rahmani Samani et al. (2019) reported EO content from 0.6% to 0.8% after L-phenylalanine foliar application and inoculation of the roots of seedlings with plant grown promoters. Rioba et al. (2015) also obtained EO content lower than 1.0% in *S. officinalis* under different levels of nitrogen, phosphorus, and irrigation.

As reported by several authors (Delamare et al 2007; Abu-Darwish et al., 2013; Russo et al 2013; Rajabi et al., 2014; La Bella et al., 2015; Mot et al 2022), due to factors including geographic location, time of year, climate, genetic variations, phenological stages, sampling techniques, and extraction techniques, the percentage of EO constituents varies greatly. In this work, 44 constituents were identified, with the following being the most representative (above 2%): 1,8-cineole (average 17.6%), camphor (average 17.3%), α-thujone (average 13.3%), β-caryophyllene (average 8.0%), α-humulene (average 7.0%), β-thujone (average 4.6%), viridiflorol (average 3.9%), camphene (average 3.3%), aromadendrene (average 2.8%), α-pinene (average 2.8%), β-pinene (average 2.4%).

In accordance with other studies (Russo et al., 2013; Rajabi et al., 2014; Jažo et al., 2023; Schmiderer et al., 2023) the most represented class are monoterpenes, and 1.8-cineole, camphor and α-thujone. The presence of these compounds in sage essential oils has been linked to its antibacterial, antifungal, anti-inflammatory, antiseptic, antiscabies, and antisyphilitic qualities (Lahlou et al., 2002; Delamare et al., 2007; Kim et al., 2014; Abu-Darwish et al 2023; Jažo et al., 2023).

Results of interaction effects of frequency × biostimulant (not presented because no statistically significant difference observed) indicated that the highest concentrations of 1,8-cineole (21.0%) was observed in plants treated with fulvic acids following the frequency 1 week, an increase of 4.2% point compared to control plants. The other biostimulants foliar application following the same frequency allowed to obtain and increase between 1.8-2.1%

point compared to control plants. The camphor percentage increased by 5.1% point thanks to the application of fulvic acids following the frequency 2 compared to untreated plants. In general, the frequency 2 and biostimulant application allowed to obtain camphor percentage higher than 3.0-5.1% point compared to those observed in control plants. Similar trend has been observed in α-thujone content. In particular, the application of the four different biostimulant following the frequency 2 permitted to register an increase of camphor percentage between 1.8% and 3.9% point. In addition to the increase of biomass production, the use of biostimulants may promote the production of monoterpenes in sage leaves. The biostimulant may affect the activity of enzymes and the regulation of genes in the metabolic pathway linked to the creation of secondary metabolites (Vosoughi et al., 2018). Regarding other monoterpenes, such as α-pinene, camphene, β-pinene, the application of the four different biostimulant following the frequency 1 allowed to obtain a small increase in percentage content compared to control plant. β-Thujone values were similar in all treatment and ranged between 4.3-4.9%. β-Caryophyllene percentage was 9.5% in control plants following both frequency and in FA-treated plants following frequency 1, while in other treated plants ranged from 6.0% to 8.7%, with a particular decrease following the frequency 2. A similar trend was observed for the sesquiterpene aromadendrene. Regarding the sesquiterpene α-humulene, relevant increase of the content was measured in biostimulated plants following the frequency 2, with an increase of 1.5-2.9% point compared to control plants. Viridiflorol content decreased in biostimulated plant following frequency 1 but the percentage increased in biostimulated plant following frequency 2 compared to control plants.

Biostimulant application can change certain metabolic pathways and biochemical characteristics in plants (Amer et al., 2021). These substances have the potential to alter the pathway of secondary metabolites, impact plastid and chlorophyll, modify stress tolerance, and eventually manipulate the amount and quality of EO (Alkharpotly et al., 2024). According to Salehi et al. (2019), biostimulant helps plants to better access nutrients which promote the growth and division of glandular trichomes, and EO channels. In addition, these products can increase the photosynthetic activity of enzymes and precursors of EO (Rehman et al., 2016). In this study, biostimulant application resulted in an increase in monoterpenes and in a decrease in the synthesis of sesquiterpenes and diterpenes. In MAPs, phytohormones and phytohormone-like substances are involved in the stimulation and synthesis of volatile chemicals as well as other compounds (Pirbalouti et al., 2019). The increase in total content of monoterpenes could be linked to the presence within the biostimulants of growth promoting substance such as auxine, cytochines and giberellic acid, involved in the metabolic pathways of monoterpenes (Tarraf et al., 2017).

Contrary to what is observed with EO content, regarding total phenolic content, antioxidant activity and rosmarinic acid content, the highest values were always observed in control plants. In biostimulated plants a decrease of these parameters was observed compared to control plants. It is commonly known that stressful conditions affect MAPs capacity to create secondary metabolites (Figueiredo et al., 2008; Tawfeeq et al., 2016; Kulak et al., 2020; Chaski et al., 2023; Farruggia et al., 2023). Presumably, the foliar biostimulant treatments and the rains that fell during the last stage of the cycle, before the sample collection, helped the sage plants evade stress situations and consequently lower the production of some secondary metabolites, like phenols and rosmarinic acid. However, a variety of writers (Elansary et al., 2019; Bonini et al., 2020, Saia et al., 2021; Rahimi et al., 2022) have noted that when various species are exposed to microbial and non-microbial biostimulants the quantity of secondary metabolites increases. Our data suggested to avoid generalization of effects on secondary metabolites and take stress or stress-relief into consideration as well.

Conclusion

The results of the present study highlight some positive effects of biostimulants foliar application on morphological, productive and yield parameters of sage plants under rainfed conditions. All biostimulants produced an improvement in plant growth and yields compared to control plants. In particular, the more frequent application of fulvic acid and protein hydrolysate allowed to obtain the highest biomass and EO yields. The highest EO content was observed in plant treated every week with protein hydrolysate. In the EO obtained from biostimulated plants every week was registered the highest 1,8-cineole percentage. The highest percentage increases in the content of α-thujone and camphor were observed in biostimulated plants following frequency 2. Otherwise, the biostimulant application has caused a decrease in total phenolic, antioxidant activity and rosmarinic acid values, compared to untreated plants. Sage yields can be increased by the foliar application of biostimulants, and this practice is advised particularly for organic cultivation. Further research is needed to better understand the mechanisms of action of biostimulants on medicinal and aromatic plants primary and secondary metabolism.

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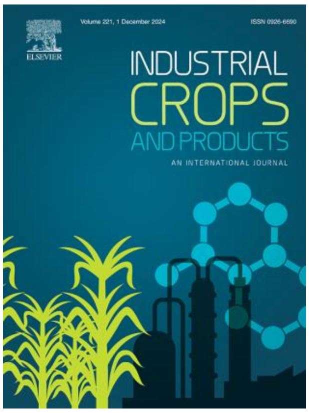
Experiment 3



Seaweed extract and fulvic acid application affect the biomass performance, the essential oil yield and composition of Sicilian oregano grown in an organic agricultural system

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Objective

The aims of this study were: (i) to investigate the effects of AN and FA at different dosages on morphological and yield traits of oregano grown under organic farming conditions, (ii) to assess the impact of AN and FA at different dosages on EO content, yield and composition, (iii) to evaluate the influence of AN and FA at different dosages on total phenolic content (TPC), antioxidant activity (AA) and rosmarinic acid content (RAC). Specifically, the next hypothesis was examined: the application of different doses of foliar biostimulants improves the agronomic characteristics of oregano grown in rainfed conditions and modulates the chemical characteristics of the extract obtained from this species.

Materials and methods

Test site

The study was conducted in an organic farm located in Aragona, a rural community in Western Sicily (Italy) during two growing seasons (2021–2022 and 2022–2023). This farm is specialised in the production of oregano plants. The soil is sandy clay loam (45% sand, 25% silt and 30% clay) with 1.3% organic matter, 1.2% total nitrogen, 19.1 ppm assimilable phosphate, 341.0 ppm assimilable potassium, pH of 7.2. The soil is classified as Regosols (typic xerorthents) in accordance with the United States Department of Agriculture (USDA). In accordance with the climate classification by Köppen–Geiger (Kottek et al., 2006), the climate of the site is warm temperate with dry and hot summers.

Weather data

During the trials, 10-day total rainfall and average maximum and minimum air temperatures were detected by a weather measurement station owned by the Sicilian Agro-Meteorological Information Service (SIAS, 2023) and located close to the farm. Temperature and rainfall trends during the two years are shown in Figure 1 (A and B).

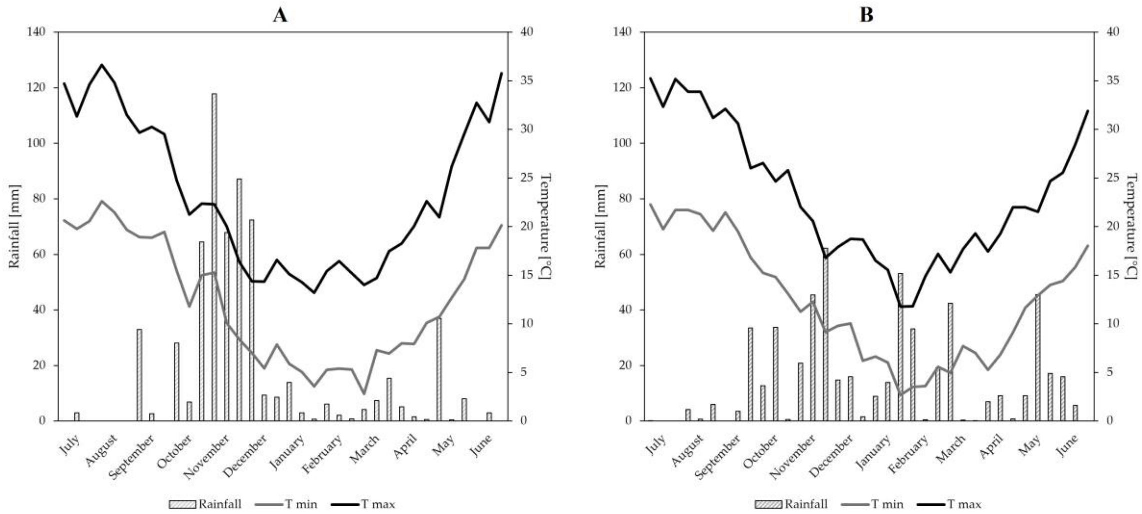

Figure 1. Temperature and rainfall trends during the first (A) and the second (B) year.

Total rainfall ranged from 611 mm (2021-2022) to 538 mm (2022-2023). Average rainfall over the 2-year period was 574 mm. In the 1st-growing season, approximately 80% of rainfall (480 mm) was recorded from October to January. From the beginning of the vegetative growth phase till the harvest date, 92 mm of rainfall was observed. The highest rainfall event (37 mm) happened during first 10-day period of May. In the 2nd-growing season, rainfall levels were well spread from September to March. Throughout the biostimulant application phase and up to harvest, 110 mm of rainfall were measured. The highest rainfall event occurred in the second 10-day period of May (45 mm).

The temperature trends in both years were comparable to the study area's average temperature. An increase in minimum and maximum temperatures was observed from pre-flowering stage until harvest. In general, higher maximum temperatures were observed in the second growing season, from May until harvest, compared with the first growing season.

Experimental field and cultivation practices

The experimental field of oregano was established in March 2019. Agamic propagation consisted in dividing the bushes. A plant density of 10000 plants ha^{-1} was obtained, adopting 2.00 m between rows and 0.50 m within rows (Figure 2).

Figure 2. Experimental site.

The field was fertilised using 2.0 t ha^{-1} of cattle manure (0.5% of N, 0.2% of P2O5, 0.7% K2O, approximately) prior to transplantation. Oregano plants were organically cultivated under rainfed conditions. Every year, weeds were mechanically controlled at the start of spring and before harvest. Oregano plants were manually harvested once a year, making a cut at 5 cm above ground level. The harvest occurred at full blooming phase, during the second 10-day period of June every year.

Treatments

Two biostimulant formulations were foliar applied:

- *Ascophyllum nodosum* (AN), having organic carbon (10.0%), organic nitrogen (1.0%), phytohormones (30%), and organic substances naturally contained in seaweed extracts.
- Fulvic Acid (FA), that was extracted from leonardite and having organic carbon (30%) and organic nitrogen (0.5%).

For each biostimulant, two doses were taken into consideration in order to supply the same total amount of nitrogen avoiding any differences in nitrogen content (Table 1). Control (C) treatment was only water. Four applications, using 400.0 L of water ha^{-1} for each event, were performed from vegetative growth stage until pre-flowering stage. Yearly, the first application was carried out during the first week of April. The other foliar treatments were performed every 10 days. Foliar applications were made through a portable hand-sprayer with an operating pressure of 250.0 kPa and equipped with a flat fan nozzle. Each plot was delimited during application with a plastic panel to prevent drift and contamination of adjacent plots. To ensure uniformity in the amount of biostimulant applied to the oregano, foliar treatments were carried out by a single operator.

Table 1. Biostimulants doses.

Foliar biostimulant	**Abbreviation**	**Doses for each application [L hL^{-1}]**	**Total amount[a] [L ha^{-1}]**
Ascophyllum nodosum	AN4	0.250	4
Ascophyllum nodosum	AN2	0.125	2
Fulvic acids	FA8	0.500	8
Fulvic acids	FA4	0.250	4

[a] = total quantity of biostimulant applied

Foliar applications were performed early in the morning, when temperature, relative humidity and stomatal aperture were ideal for foliar absorption (Ruiz-Navarro et al., 2019). The plot size was 30 m^2 (2.0 m × 15.0 m) with 15 plants per plot, that were taken into consideration for the subsequent measurements. For preventing drift during foliar sprays, plastic panels were utilized to separate each plot. The experimental scheme was a randomized complete block design using three replications.

Morphological and yield traits

At harvest (Figure 3), some traits such as plant height, relative water content (RWC), chlorophyll content, total fresh yield, were determined. After harvest, plants were dried in a shaded and ventilated environment for approx. 10 days at a temperature of 25–30 °C and total dry yield were determined. Stems, leaves, and flowers were manually separated, and each fraction has been weighed. The stems were not used for EO extraction because of the marginal amounts of EO.

Figure 3. Experimental field before harvest.

Chlorophyll content was measured using a Dualex Scientific (Force A, Orsay, France) portable Chlorophyll meter. For each plot, 30 leaves have been considered. The device calculated the mean of the values.

The relative water content of leaves was assessed by taking 30 fresh leaves from each plot and the fresh weight (FW) was measured. For 24 hours, the leaves floated in distilled water within a falcon tube. After that, leaves were removed from the water and put on absorbent paper to eliminate the excess water, and the turgid weight (TW) was recorded. The leaves were then dried in an oven for 24 hours, and the dry weight (DW) was noted (Alyemeni et al., 2018). The following equation was used to determine the RWC:

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

EO extraction and analysis

Essential oils were extracted by hydro-distillation of air-dried plant material (500.0 g) per each plot for 3 h, according to Ph. Eur. 7.0, 20812 (01/2008). In total 3 essential oil extractions were carried out per each treatment. The EO content was calculated dividing the volume of EO obtained by the weight of the biomass samples. The EO yield was estimated multiplying the EO content by the total dry yield. The EO samples were stored at -18°C. Prior to gas chromatography–mass spectrometry (GC/MS), the EOs were diluted 1:100 with hexane and moved to GC vials. The EO components were assessed with a HP 6890 gas chromatograph connected with the quadrupole mass spectrometer HP5972 MSD (Hewlett-Packard, Palo Alto, CA, USA). The operating parameters reported by Farruggia et al., (2024) were utilized. The EO compounds retention indices (RI) were calculated and compared with those of n-alkane hydrocarbons (RI standard for GC, Sigma-Aldrich, Vienna, Austria). Mass spectra and retention indices were compared with data reported in the literature in order to identify each compound. Peak-area normalization was used to obtain the composition, and the response factor of each component was taken to be equal to 1.

Determination of some chemical parameters

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

- Total phenolic content, expressed as milligram caffeic acid equivalents per gram dry weight (mg c.a.e. g^{-1} dw), following the method described by Lamien-Meda et al. (2010).
- Antioxidant activity, expressed as milligram trolox equivalents per gram dry weight (mg t.e. g^{-1} dw), following the method described by Chizzola et al. (2008).
- Rosmarinic acid content, as reported Farruggia et al., (2024).

Statistical analyses

One-way analysis of variance (ANOVA) was conducted to compare the data per year. The difference between means was carried out using Fisher's LSD test ($p \leq 0.05$). Foliar biostimulants were used as fixed effects in the linear model/ANOVA. Before ANOVA, the RWC data were subjected to arcsine transformation. Prior to statistical analysis, Levene's test and the Shapiro-Wilk test were used, respectively, to check the homogeneity of variance and normality of all the data.

Principal components analysis (PCA) was also carried out on the productive and chemicals parameters and on the most represented EO compounds (over 2.0% on average). In order to determine the ideal amount of principal components (PC), factors having eigenvalues over 1.0 were taken into account. The software MINITAB 19 (State College, PA, USA) for Windows was used for statistical analyses.

Results

Morphological and yield traits

ANOVA showed that foliar biostimulant significantly influenced ($p \leq 0.01$) plant height, chlorophyll content, and RWC during both years (Table 2).

Table 2. Influence of foliar application on oregano plant height, chlorophyll content and relative water content (RWC) during the two-years research.

Foliar application	Plant height [cm]		Chlorophyll content [μg cm^{-2}]		RWC [%]	
	I year	II year	I year	II year	I year	II year
C	37.0 ^{c}	44.5 ^{c}	25.4 ^{e}	29.5 ^{d}	58.5 ^{c}	88.1 ^{a}
AN4	51.0 ^{a}	65.2 ^{a}	31.3 ^{a}	33.3 ^{c}	59.6 ^{c}	89.0 ^{a}
AN2	42.5 ^{b}	61.2 ^{b}	30.7 ^{b}	34.8 ^{a}	68.6 ^{a}	84.0 ^{b}
FA8	41.7 ^{b}	60.0 ^{b}	27.1 ^{c}	32.9 ^{c}	55.3 ^{d}	87.5 ^{a}
FA4	42.5 ^{b}	64.0 ^{a}	26.4 ^{d}	34.1 ^{b}	62.3 ^{b}	87.4 ^{a}
p-value	0.000 **	0.000 **	0.000 **	0.000 **	0.000 **	0.004 **

Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. ** = significant at 0.01 probability level. C = control (only water); AN4 = 4 L ha^{-1} of *A. nodosum*; AN2 = 2 L ha^{-1} of *A. nodosum*; FA8 = 8 L ha^{-1} of fulvic acids; FA4 = 4 L ha^{-1} of fulvic acids.

The highest plant height values (51.0 and 65.2 cm) were observed in AN4-treated plants in both years. During the second year, the application of FA4 produced the highest plant height (64.0 cm). Regarding chlorophyll content, the highest value (31.3 μg cm^{-2}) was observed in AN4-treated plants in the first year and in AN2-treated plants (34.8 μg cm^{-2}) in the second year. The lowest plant height and chlorophyll content values were recorded in control plants (Table 2). Concerning the RWC, in the first year the highest value (68.6%) was observed in AN2-treated plants while the lowest in control plant and AN4-treated plants (Table 2). In the second year, comparable RWC values have been recorded in control plants, AN4-, FA8-, and FA4-treated plants with values ranging from 87.4% to 89.0%.

In both years, statistical analysis of inflorescence and leaf percentages, and stem percentages revealed a significant influence ($p \leq 0.01$) of the foliar biostimulant factor (Figure 4). The application of two doses of *A. nodosum* produced the highest percentage value for inflorescences (74.7%) and leaves (74.1%) and, consequently, the lowest percentage value for stems during the first year (Figure 4. A). However, control plants generated the highest percentage values for inflorescences and leaves (66.8%) during the second year (Figure 4. B).

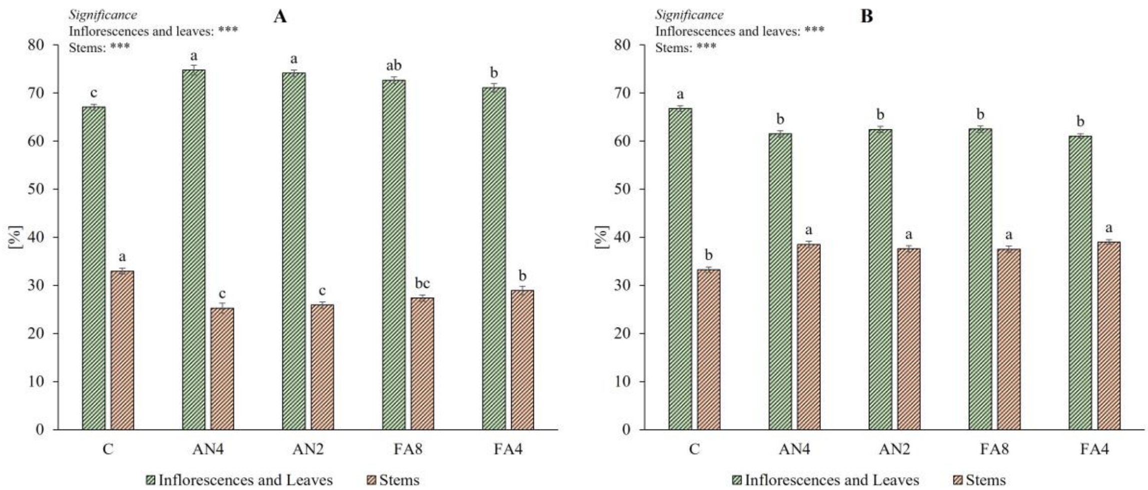

Figure 4. Influence of foliar application on oregano inflorescence and leaf percentages and stem percentages during first (A) and the second (B) year. Means and standard errors are shown. The values followed by the equal letter are not significantly different for $p \leq 0.05$ according to LSD test. *** = $p \leq 0.001$. C: control (only water); AN4 = 4 L ha^{-1} of *A. nodosum*; AN2 = 2 L ha^{-1} of *A. nodosum*; FA8 = 8 L ha^{-1} of fulvic acid; FA4 = 4 L ha^{-1} of fulvic acid.

Based on ANOVA outcomes, the foliar biostimulant had a significant effect ($p \leq 0.01$) on total yields, fresh and dry, during both years (Figure 5). The foliar application of AN2 and two doses of fulvic acid created the highest total fresh yield (with values ranged from 4.6 to 4.8 t ha^{-1}) and total dry yield (with values ranged from 2.6 to 2.7 t ha^{-1}) in the first year (Figure 5. A). During the second year, the highest total dry yield was found in oregano plants treated with both FA doses (Figure 5. B). The application of all biostimulants led to obtain similar total dry yields during the second year (values ranged from 4.6 to 4.9 t ha^{-1}). The lowest yields were observed in control plants in both years (Figure 5).

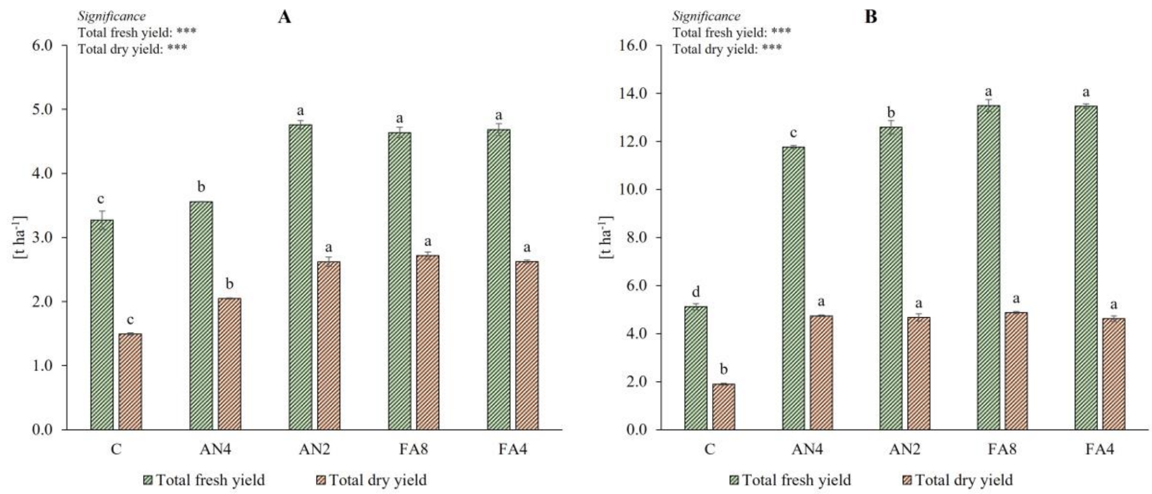

Figure 5. Influence of foliar application on oregano total fresh yield and total dry yield during first (A) and the second (B) year. Means and standard errors are shown. The values followed by the equal letter are not significantly different for $p \leq 0.05$ according to LSD test. *** = $p \leq 0.001$. C: control (only water); AN4 = 4 L ha^{-1} of *A. nodosum*; AN2 = 2 L ha^{-1} of *A. nodosum*; FA8 = 8 L ha^{-1} of fulvic acid; FA4 = 4 L ha^{-1} of fulvic acid.

Regarding EO content and EO yield, ANOVA revealed that foliar biostimulant produced significant differences ($p \leq 0.01$) in both years (Table 3). The highest EO content (3.93% and 3.46%) and EO yields (73.3 and 97.4 kg ha^{-1}) were obtained in FA4-treated plants (Table 3). The lowest EO content was observed in control plants and in AN2-treated plants during the first year (Table 3). The application of AN2 produced the lowest EO content during the second year (Table 3). Regarding EO yields, control plants had the lowest values (Table 3).

Table 3. Influence of foliar application on oregano essential oil (EO) content and yield during the two-year research.

Foliar application	**EO content [%$_{v/w}$]**		**EO yield [kg ha^{-1}]**	
	I year	II year	I year	II year
C	2.70 ^{c}	2.85 ^{c}	27.1 ^{e}	36.1 ^{d}
AN4	3.02 ^{b}	3.08 ^{b}	46.1 ^{d}	89.6 ^{b}
AN2	2.83 ^{c}	2.16 ^{d}	55.0 ^{c}	63.0 ^{c}
FA8	3.14 ^{b}	2.96 ^{bc}	61.9 ^{b}	90.2 ^{b}
FA4	3.93 ^{a}	3.46 ^{a}	73.3 ^{a}	97.4 ^{a}
p-value	0.000 **	0.000 **	0.000 **	0.000 **

Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. ** = significant at 0.01 probability level. C = control (only water); AN4 = 4 L ha^{-1} of *A. nodosum*; AN2 = 2 L ha^{-1} of *A. nodosum*; FA8 = 8 L ha^{-1} of fulvic acids; FA4 = 4 L ha^{-1} of fulvic acids.

Essential oil profile

The EO compounds, and the ANOVA results are reported in Table 4. Thirty-nine different compounds were found. Statistical analysis revealed that foliar biostimulant significantly influenced ($p \leq 0.05$) the percentage of γ-terpinene, *trans*-sabinene hydrate, carvacrol methyl ether, carvacrol, aromadendrene, α-humulene, alloaromadendrene in the first year and the percentage of carvacrol in the second year (Table 4).

Table 4. Components of oregano EO and *p*-value in response to foliar application during the two-years research.

Peak	Compounds	RI_{calc}	RI_{lit}	I year, Foliar application: C	AN4	AN2	FA8	FA4	Signif.	II year, Foliar application: C	AN4	AN2	FA8	FA4	Signif.
1	α-thujene	923	923	1.96	2.00	1.85	1.81	1.80	n.s.	0.89	1.10	1.28	1.15	1.13	n.s.
2	α-pinene	929	933	0.77	0.82	0.74	0.71	0.71	n.s.	0.54	0.49	0.58	0.54	0.49	n.s.
3	camphene	943	952	0.09	0.09	0.09	0.08	0.08	n.s.	0.12	0.07	0.08	0.08	0.04	n.s.
4	sabinene	971	973	0.17	0.17	0.15	0.15	0.14	n.s.	0.13	0.10	0.12	0.12	0.08	n.s.
5	myrcene	989	991	2.16	2.12	1.99	2.00	1.90	n.s.	1.12	1.13	1.28	1.19	1.14	n.s.
6	α-phellandrene	1002	1005	0.41	0.41	0.38	0.38	0.37	n.s.	0.24	0.25	0.28	0.26	0.25	n.s.
7	α-terpinene	1013	1018	3.82	3.80	3.59	3.58	3.38	n.s.	2.77	2.84	3.22	2.95	2.96	n.s.
8	p-cymene	1021	1026	6.44	6.49	6.52	6.53	6.02	n.s.	4.96	6.33	6.61	6.03	6.20	n.s.
9	limonene	1025	1031	0.53	0.52	0.49	0.49	0.46	n.s.	0.33	0.33	0.37	0.35	0.33	n.s.
10	cis-beta-ocimene	1036	1040	1.98	1.85	1.79	1.81	1.74	n.s.	1.76	1.47	1.62	1.56	1.31	n.s.
11	trans-beta-ocimene	1046	1050	0.31	0.27	0.26	0.27	0.25	n.s.	0.16	0.15	0.17	0.15	0.14	n.s.
12	γ-terpinene	1056	1059	16.10 a	16.09 a	15.21 ab	14.92 ab	13.95 b	*	13.17	13.34	15.02	14.01	14.00	n.s.
13	cis-sabinene hydrate	1062	1069	0.83	0.90	0.85	0.87	0.86	n.s.	0.65	0.63	0.67	0.62	0.60	n.s.
14	α-terpinolene	1084	1084	0.12	0.11	0.10	0.10	0.09	n.s.	0.11	0.11	0.12	0.11	0.10	n.s.
15	trans-sabinene hydrate	1094	1089	0.20 c	0.21 a	0.20 bc	0.21 a	0.21 ab	**	0.22	0.24	0.26	0.22	0.19	n.s.
16	linalool	1098	1098	0.31	0.30	0.30	0.27	0.26	n.s.	0.42	0.09	0.21	0.53	0.00	n.s.
17	borneol	1160	1165	0.15	0.14	0.14	0.14	0.14	n.s.	0.56	0.11	0.24	0.56	0.00	n.s.
18	terpinen-4-ol	1172	1177	0.34	0.31	0.32	0.30	0.30	n.s.	0.14	0.09	0.10	0.12	0.08	n.s.
19	α-terpineol	1186	1185	0.13	0.13	0.12	0.12	0.12	n.s.	0.21	0.22	0.24	0.22	0.18	n.s.
20	neral	1239	1235	2.48	2.66	2.65	2.63	2.60	n.s.	3.15	3.67	4.13	3.38	3.32	n.s.
21	carvacrol methyl ether	1249	1244	5.33 a	5.47 a	5.31 a	5.30 ab	5.08 b	*	5.04	5.27	5.52	5.27	5.02	n.s.
22	thymol	1298	1290	46.29	45.63	46.98	47.32	49.96	n.s.	52.79	51.44	47.64	50.25	53.39	n.s.
23	carvacrol	1302	1298	0.64 a	0.47 c	0.48 bc	0.55 ab	0.57 a	**	0.59 a	0.54 ab	0.59 a	0.43 c	0.47 bc	**

24	α-copaene	1375	1376	0.12	0.12	0.12	0.08	0.11	n.s.	0.11	0.12	0.13	0.13	0.10	n.s.
25	β-bourbonene	1384	1380	0.11	0.09	0.10	0.09	0.08	n.s.	0.09	0.08	0.08	0.09	0.02	n.s.
26	β-caryophyllene	1419	1428	1.89	1.97	2.02	1.96	1.85	n.s.	2.05	1.99	2.00	2.06	1.73	n.s.
27	aromadendrene	1439	1439	0.11 c	0.13 ab	0.14 a	0.13 ab	0.12 b	**	0.14	0.14	0.14	0.14	0.11	n.s.
28	α-humulene	1455	1452	0.21 b	0.24 a	0.26 a	0.25 a	0.25 a	*	0.39	0.26	0.29	0.48	0.20	n.s.
29	alloaromadendrene	1462	1461	0.11 b	0.12 a	0.14 a	0.13 a	0.13 a	**	0.19	0.20	0.20	0.19	0.16	n.s.
30	γ-muurolene	1478	1477	0.40	0.43	0.45	0.44	0.43	n.s.	0.44	0.49	0.48	0.49	0.43	n.s.
31	germacrene D	1483	1480	0.65	0.65	0.71	0.63	0.57	n.s.	1.03	1.28	1.10	1.11	1.15	n.s.
32	bicyclogermacrene	1496	1494	0.31	0.31	0.34	0.34	0.33	n.s.	0.46	0.42	0.41	0.40	0.36	n.s.
33	α-muurolene	1501	1499	0.15	0.16	0.17	0.16	0.16	n.s.	0.17	0.19	0.18	0.18	0.17	n.s.
34	β-bisabolene	1510	1509	2.02	2.07	2.21	2.09	2.02	n.s.	1.59	1.81	1.74	1.71	1.71	n.s.
35	γ-cadinene	1516	1512	0.49	0.52	0.54	0.55	0.56	n.s.	0.56	0.64	0.59	0.59	0.56	n.s.
36	δ-cadinene	1526	1524	0.84	0.87	0.92	0.93	0.92	n.s.	1.07	1.21	1.14	1.15	1.08	n.s.
37	(E)-α-bisabolene	1535	1549	0.09	0.10	0.10	0.10	0.11	n.s.	0.09	0.11	0.10	0.09	0.09	n.s.
38	caryophyllene oxide	1580	1581	0.19	0.26	0.26	0.28	0.26	n.s.	0.39	0.36	0.34	0.30	0.30	n.s.
39	viridiflorol	1589	1590	0.14 b	0.14 b	0.15 ab	0.16 a	0.17 a	*	0.11	0.13	0.12	0.10	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 literature. ** = significant at 0.01 probability level; * = significant at 0.05 probability level; n.s. = not significant. Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. C = control (only water); AN4 = 4 L ha^{-1} of *A. nodosum*; AN2 = 2 L ha^{-1} of *A. nodosum*; FA8 = 8 L ha^{-1} of fulvic acids; FA4 = 4 L ha^{-1} of fulvic acids.

The highest γ-terpinene values were noted in control plants (16.10%) and in AN4-treated plants (16.09%), while the lowest value in FA4-treated plants (13.95%) (Table 4). The highest *trans*-sabinene hydrate contents were recorded in AN4- and FA8-treated plants (0.21%) while the lowest in control plants (0.20%). Control plants, AN4-, and AN2-treated plants generated the highest carvacrol methyl ether (values ranged from 5.31% to 5.47%), and FA4-treated plants the lowest (5.08%). The highest carvacrol content was found in control plants (0.64%) and FA4-treated plants (0.57%) in the first year (Table 4). The same performance was obtained by control plants and by AN2-treated plants (0.59%) in the second year (Table 4). Considering the aromadendrene content, the highest value (0.14%) was recorded in AN2-treated plants while the lowest (0.11%) in control plants. The foliar application of all biostimulants produced higher values of α-humulene (ranged from 0.24% to 0.26%) and alloaromadendrene (ranging from 0.12% to 0.14%) compared with those observed in control plants (0.21% α-humulene and 0.11% alloaromadendrene (Table 4). The highest viridiflorol contents were recorded in FA8- (0.16%) and FA4-treated plants (0.17%) while the lowest in control and AN4-treated plants (0.14%).

Chemical parameters

ANOVA showed that foliar biostimulant significantly affected TPC and AA in both years (Table 5). The RAC was significantly influenced only in the second year (Table 5). In both years, the highest TPC was observed in control plants (128.3 mg c.a.e. g^{-1} and 112.7 mg c.a.e. g^{-1}). As reported in table 5, the lowest total phenolic was obtained in FA4-treated plants in the first year (109.9 mg c.a.e. g^{-1}) and in AN4-treated plants in the second year (91.3 mg c.a.e. g^{-1}). During the first year, the greatest antioxidant activity was recorded in the control, AN4- and FA8-treated plants (Table 5). In the second year, the control plant produced the highest AA (133.5 mg t.e. g^{-1}) and the highest RAC (2.4%) (Table 5).

Table 5. Influence of foliar application on oregano total phenolic content (TPC), antioxidant activity (AA), and rosmarinic acid content (RAC) during the two-years research.

Foliar application	**TPC [mg c.a.e. g^{-1}]**		**AA [mg t.e. g^{-1}]**		**RAC [%]**	
	I year	II year	I year	II year	I year	II year
C	128.3 ^{a}	112.7 ^{a}	157.2 ^{a}	133.5 ^{a}	3.0 ^{a}	2.4 ^{a}
AN4	119.2 ^{c}	91.3 ^{d}	154.6 ^{a}	128.2 ^{b}	3.0 ^{a}	1.6 ^{b}
AN2	124.7 ^{b}	100.5 ^{b}	150.9 ^{b}	117.6 ^{c}	2.8 ^{a}	1.9 ^{ab}
FA8	127.6 ^{ab}	96.6 ^{c}	154.5 ^{a}	120.9 ^{c}	2.9 ^{a}	1.6 ^{b}
FA4	109.9 ^{d}	95.6 ^{c}	146.9 ^{c}	106.0 ^{d}	2.7 ^{a}	1.3 ^{b}
p-value	0.000 **	0.000 **	0.000 **	0.000 **	0.103 n.s.	0.034 *

Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. ** = significant at 0.01 probability level; * = significant at 0.05 probability level; n.s. = not significant. c.a.e. = caffeic acid equivalent; t.e.= trolox equivalent. C = control (only water); AN4 = 4 L ha^{-1} of *A. nodosum*; AN2 = 2 L ha^{-1} of *A. nodosum*; FA8 = 8 L ha^{-1} of fulvic acids; FA4 = 4 L ha^{-1} of fulvic acids.

Principal components analysis (PCA)

The PCA for productive and chemical parameters and for the most represented EO compounds (over 2.0% on average) showed two principal components (PC) with eigenvalues higher than 1. The two PC described 93.9% of the total variance (Table 6).

Table 6. Eigenvalues, total variance explained (TVE) and cumulative total variance explained (cumulative TVE) of the two principal components (PCs) for production and chemical variables and the most relevant EO compounds.

Variables	**PC1**	**PC2**
Total fresh yield	-0.26	0.302
Total dry yield	-0.231	0.334
EO content	-0.35	-0.147
EO yield	-0.34	0.177
Total phenolic	0.315	-0.155
Antioxidant activity	0.315	-0.117
Rosmarinic acid	0.35	-0.169
α-terpinene	0.305	0.253
p-cymene	-0.047	0.429
γ-terpinene	0.314	0.24
neral	0.052	0.414
carvacrol methyl ether	0.222	0.337
thymol	-0.272	-0.296
Total eigenvalue	6.90	5.31
TVE [%]	53.10	40.90
Cumulative TVE [%]	53.10	93.90

The first principal component (PC1) provided 53.1% of the total variation and it mostly showed a favourable correlation with TPC, AA, RAC, α-terpinene, γ-terpinene, neral, carvacrol methyl ether. The second principal component (PC2) gave 40.9% of the total variation and was positively correlated with total fresh yield, total dry yield, EO yield, α-terpinene, p-cymene, γ-terpinene, neral, carvacrol methyl ether (Table 6). Foliar biostimulant application generated a distinct separation with the control in the bottom right quadrant of the plot and the biostimulant in the bottom left and in the two upper quadrants of the plot. In particular, the control treatment was placed in the bottom right quadrant because of the variables TPC, AA, and RAC acid. The lowest dose of fulvic acid was in the bottom left quadrant with the variables EO content and thymol contributing, while FA8 was in the upper left quadrant with the variables EO yield, total fresh yield, total dry yield, and *p*-cymene contributing. Both AN treatments were positioned in the upper right quadrant of the plot based on other EO compounds, such as α-terpinene, *p*-cymene, γ-terpinene neral, carvacrol methyl ether (Figure 6).

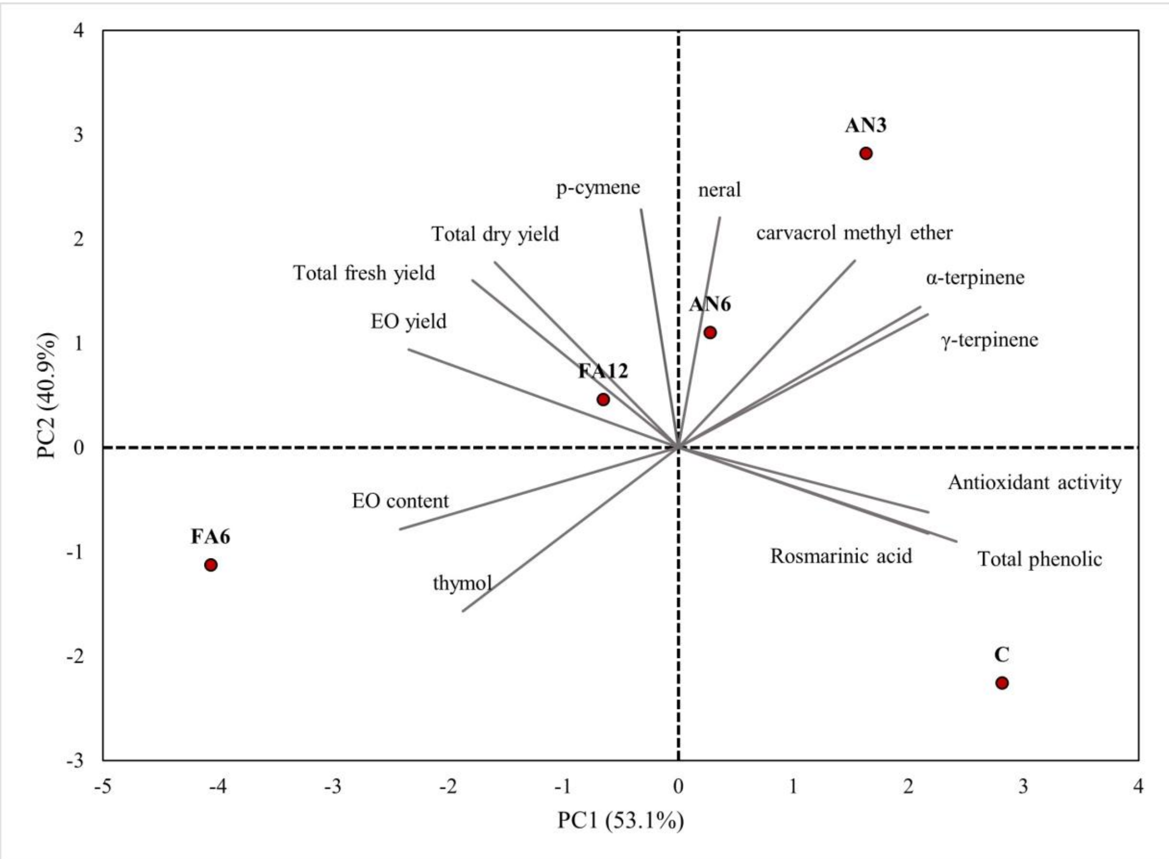

Figure 6. Graph of principal component analysis (PCA) for productive and chemical parameters and the most represented EO compounds of oregano affected by foliar biostimulant application. AN4 = 4 L ha^{-1} of *A. nodosum*; AN2 = 2 L ha^{-1} of *A. nodosum*; FA8 = 8 L ha^{-1} of fulvic acid; FA4 = 4 L ha^{-1} of fulvic acid.

Discussion

General aspects

Oregano is currently one of the most interesting and exploited MAPs throughout the world (Lukas et al., 2015). It is a valuable source of bioactive compounds characterized by a range of pharmacologic properties, including antibacterial, antioxidant, anti-cholinesterase, and cardioprotective activities (Morshedloo et al., 2018). The increase in yields and optimization of energy inputs for production are the main objectives of modern agriculture (Al-Karaki and Othman, 2023; Caruso et al., 2019). However, maintaining quality standards and production levels whilst reducing chemical fertiliser use are critical aspects for MAP farmers (Ninou et al., 2021; Kimera et al., 2021). To enhance nutrient absorption and utilisation effectiveness, stress tolerance, and quality features, foliar biostimulant application promotes the regulation and improvement of some physiological responses in plants (Rouphael and Colla, 2018). Using naturally produced plant biostimulants is a viable and sustainable method in both conventional and organic farming systems (De Pascale et al., 2017). This study highlights how the agronomic performance of organically grown oregano can be improved by the foliar spray of biostimulants. Foliar biostimulants have influenced the morphological, yield and qualitative characteristics of this species which was managed without the use of irrigation water. Future studies should consider the effects of biostimulants on oregano cultivated under different climatic conditions to validate their applicability in various global agricultural environments.

Morphological and yield traits

In this study, similar trends in morphological and yield characteristics were observed; however, the highest values were recorded during the second year. This can be explained by the greatest vegetative growth of plants, in accordance with previous studies (Ninou et al., 2021; Goliaris, 1997). In general, the application of biostimulants allowed the plant height to increase with respect to control plants. The highest doses of *A. nodosum* produced the greatest boost in plant height compared with control. As reported by Shukla et al. (2019), *A. nodosum* improves plant performance through the control of plant hormone signaling and boost of flavonoid, carotene, betaine, or carbohydrate biosynthesis. A wide range of complex bio-stimulatory substances, such as vitamins, minerals, and complex chemical compounds, are responsible for these effects, as described by Santaniello et al. (2017). In Greece, in oregano plants, Dordas (2009) obtained values of plant height which varied between 57.0

cm and 70.0 cm through the foliar treatments with calcium and magnesium. When adding various nitrogen rates to the soil, Król et al. (2020) reported values of plant height ranging from 24.0 cm to 35.0 cm and found the highest performance in plants which were fertilised using the highest nitrogen doses. Plant heights of 40–50 cm were recorded by Sotiropoulou and Karamanos (2010) by varying nitrogen fertilisation treatments. In a similar environment, some authors (Farruggia et al., 2023; Virga et al., 2020) observed similar plant heights to this study. In the first year, chlorophyll content and relative water content were found to be lower than those of the second year. This fact was related to greater rainfall in the second year and, consequently, higher soil moisture than the first year; increases in water availability promoted photosynthetic activity in oregano plants in accordance with Hancioglu et al. (2021). Biostimulant application led to higher chlorophyll content. Several authors (Ciriello et al., 2022; Abdel-Rahman et al., 2020) observed improvements in chlorophyll content in some species treated with foliar or radical biostimulants. Seaweed extracts contain cytokinins or cytokinin-like compounds, which may provide benefits on chlorophyll content and photosynthetic capacity of plants (Rouphael et al., 2017). The chlorophyll content in the present study was similar to that reported in studies on oregano carried out with different aims (Farruggia et al., 2023; Emrahi et al., 2021; Murillo-Amador et al., 2013). Regarding RWC, foliar application of different biostimulants allowed to achieve an increase in leaf water content during the first year. This result could be linked to the positive effect of biostimulant used on the root growth and nutrients and water uptake (Shafie et al., 2021; Khorasaninejad et al., 2018). Biostimulants improve the absorption, digestion, and translocation of micro- and macronutrients, which is why their use has been linked to improvements the yield and quality of crops (Farruggia et al., 2024; Aytaç et al., 2022). Abdali et al. (2023) evaluated the influence of several foliar biostimulants on biomass characteristics traits and the adaptive physiological reactions of oregano under water stress conditions. The authors found that RWC values varied between 46% and 79%. In a study carried out in Iran evaluating different varieties of oregano irrigated with varying water levels, Emrahi et al. (2021) obtained RWC values similar to those recorded during the first year of this study. Murillo-Amador et al. (2015) measured percentage values of RWC which were similar to those observed during the second year of the present study. These authors tested oregano plants in different environmental conditions and with different levels of organic fertilisers, obtaining RWC values ranging from 81% to 86%. During the first year, an increase of 4–7% in leaf and flower percentages was observed in biostimulated plant compared with control plants. During the second year, increased vegetative development caused a change in the percentage distribution of the different portions of the plant in favor of stems. Oregano is a perennial plant that over the years develops its root system and, consequently, increases the vegetative development of the plant (Goliaris, 1997). Greater growth with age of vegetation and the woody portion of the stems determined a total biomass yield of more than double compared with the first year (Ninou et al., 2021). The application of AN and FA produced positive impact on biomass production in both years. During the second year, the yield of plants treated with both products more than doubled compared with control plants. The second year was more effective thanks to the higher age of the plants and to increased rainfall during the biostimulant application period; adequate water accessibility enhances the biomass production and its constituent parts in MAPs (Virga et al., 2020). During the second year, the application of FA generated the highest fresh and dry biomass yields. It is worth noting that the two doses of fulvic acid led to similar results regarding yields in fresh and dry biomass. Plant growth can be stimulated by several substances contained in these products that act on metabolism processes by enhancing photosynthetic activity and related compounds (Bulgari et al., 2015). Several authors (Malécange et al., 2023; Bonini et al., 2020) state that biostimulants application has hormone-like effects, such those of auxin and gibberellin, that have a positive effect on biomass yield. Biostimulants contain plant growth regulators that encourage the formation of new structural biomass, and they consent to optimize the uptake, transfer, and absorption of available nutrients from the soil (Gupta et al., 2024; Basile et al., 2021). Yildiztekin et al. (2018) and Abdali et al. (2023) affirm that the evaporation/transpiration ratio, the production of growth-promoting agents, leaf water potential, root growth, and the oregano plant's ability to absorb water are all impacted by foliar treatment. It has also been shown that the use of biostimulants increases the permeability of cell membranes, allowing potassium to enter and promoting intracellular pressure, cell division, and production (Abdali et al., 2023; Yang et al., 2022). Nıkou et al. (2019), in a two-year study, found the highest values of fresh biomass yield in the second year. These authors obtained values of biomass yield varying from 7.1 t ha^{-1} to 17.2 t ha^{-1} using different types of organic and mineral fertilisers. In Greece, Dordas (2009) reported values of dry biomass yield which ranged between 2.9 t ha^{-1} to 4.1 t ha^{-1} by applying foliar calcium and magnesium on two oregano varieties. In Greece, Giannoulis et al. (2020) found flower and leaf dry yields of 1.8 t ha^{-1} and 4.2 t ha^{-1} in oregano plants grown in rainfed conditions and fertilised with different nitrogen levels. Abdali et al. (2023) recorded average dry yields of approximately 1.1 t ha^{-1} in plants treated with amino acids and foliar seaweeds. The EO content of MAPs is commonly altered by environmental and genetic factors and agronomic practices need to be modified to reduce fluctuations in secondary metabolite production (Mot et al., 2022; Bistgani et al., 2019; Ninou et al., 2017; La Bella et al., 2015). This aspect is important for companies operating in the herbal extract market which need to provide exact quantities. As reported by Amer et al. (2021), biostimulants can affect metabolic pathways and biochemical activity in plants. Some compounds contained in biostimulants modify the route of secondary metabolites, affect plastids and chlorophyll, influence ability to tolerate stress, and change the quantity and the composition of EOs (Alkharpotly et al., 2024). Considering the results obtained over the two-year tests, the lowest doses of fulvic acid produced the highest EO content and EO yields. Dordas (2009) obtained an EO content of more than 4% in oregano treated with foliar calcium and magnesium. Similar EO contents were observed by Król et al. (2020) in oregano fertilised with different nitrogen doses. In addition, these findings are related to those observed by Aytaç et al. (2022) who increased oregano yields by applying different doses of humic substances to the soil. Humic substances improve conditions in the rhizosphere and can promote plant development by improving the content of natural plant growth-promoting hormones, which are related to the EO synthesis (Rahimi et al., 2022). The results are consistent with those observed by Elansary et al. (2019) and Rahimi et al. (2022) which in wild mint (*Mentha longifolia* L.) and in thyme (*Thymus vulgaris* L.), respectively, found an increase in EO content in plants treated with foliar biostimulants compared with control plants. Tawfeew et al. (2016) also observed the highest values of EO content and yield in seaweed extract-treated rosemary plants.

Essential oil profile

In the EO samples analysed in this study, 39 compounds were discovered, of which 23 were monoterpenes and 16 sesquiterpenes. The most abundant compounds (over 2.0% on average) were α-terpinene, *p*-cymene, γ-terpinene, neral, carvacrol methyl ether and thymol. Considering the average values of the two years, biostimulant application did not influence the ratio between monoterpenes and sesquiterpenes compared with control plants. Many studies have been conducted on the chemical composition and content of oregano EOs, as well as their biological qualities and effects when added to food matrices (Tsitlakidou et al., 2022; Shafiee-Hajiabad et al., 2014). The EO composition is responsable for oregano aroma when used in food preparation (Asensio et al., 2015). In the present study no relevant changes were detected in the composition of EOs obtained from oregano plants treated with biostimulants and, consequently, the aromatic profile of the end-product is not altered.

According to Shahrajabian and Sun (2022), biostimulants enable plants to improve their nutrient uptake, promoting the development and proliferation of glandular trichomes and EO synthesis. Furthermore, these compounds can enhance the photosynthetic activity of EO enzymes and precursors, as reported by Rehman et al. (2016).

Chemical parameters

In general, TPC, AA, and RAC were found to be higher during the first year of study, and the highest values were often recorded in the control; when the highest doses of fulvic acid (FA8) were applied, a total phenolic content similar to that of control plants was generated. The application of the highest dose of both biostimulants produced similar results for antioxidant activity during the first year. Several authors (Rahimi et al., 2022; Saia et al., 2021; Bonini et al., 2020) have shown that the quantity of secondary metabolites rises in many species with exposure to microbial and non-microbial biostimulants. A number of factors produce effects on the secondary metabolite synthesis in MAPs and in oregano plants as well (Wenneck et al., 2023; Farruggia et al., 2023; Tawfeeq et al., 2016; Sharafzadeh, 2012; Figueiredo et al., 2008). Biostimulant application may have an impact on gene regulation and enzyme activity in secondary metabolic pathways (Vosoughi et al., 2018).

Principal components analysis (PCA)

Principal components analysis revealed a complete framework for assessing the impacts of foliar biostimulant treatment on oregano yield and quality properties. AN and FA treatments were clearly separated from control. Foliar biostimulant application negatively affected chemical parameters (total phenols, antioxidant activity, and rosmarinic acid). However, the application of foliar biostimulant produced positive effects in yields and the most represented EO compounds.

Conclusions

The creation and application of environmentally friendly practices that may enhance the quality and quantitative elements of crops are essential in the modern day. This study is the first to demonstrate that the application of two different biostimulants, such as seaweed extract and fulvic acid, under non-irrigated conditions can improve the production and chemical properties of Sicilian oregano, offering new solutions for organic farming in arid regions. Morphological traits and yield performance were positively influenced by foliar biostimulant applications. The highest plant growth and biomass yields were measured in plants treated with both doses of fulvic acid and with the lowest dose of *A. nodosum*. During both years, the lowest dose of fulvic acid produced the highest EO contents and, consequently, the highest EO yields. On the other hand, biostimulant treatment resulted in a drop in TPC, AA, and RAC. These results have implications for the agricultural, pharmaceutical, and food sectors. These results suggest that biostimulants should be used as a good practice to maximize crop output in unfavorable growing environments. Therefore, further research and field trials are necessary to investigate deeper how the biostimulants act on MAP primary and secondary metabolism in order to increase yields and quality but limit agronomic inputs.

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Experiment 4

Effect of two protein hydrolysate on yield and chemicals of rosemary

Objective

The aim of this study was to assess the effect of foliar application of two different type of protein hydrolysates, one plant-derived and another one animal-derived, on yield and chemical parameters of *Salvia rosmarinus* Spenn. grown in the Mediterranean environment.

Materials and methods

Experimental Site and Cultivation Practices

Tests were conducted on a local farm located in Aragona (Sicily, Italy) (330 m a.s.l., 37°22′32.71″ N, 13°38′33.59″ E Google Earth), during the growing seasons of 2021-2022 and 2022-2023. The soil was categorized as Regosol (United States Department of Agriculture (USDA) classification: typic xerorthents) and sandy clay loam (48% sand, 28% clay and 24% silt) with a pH of 7.4, 16 g kg^{-1} organic matter, 1.22% total nitrogen, 20.4 ppm assimilable phosphate, and 364 ppm assimilable potassium. Agamic propagation was carried out by dividing the bushes. The plants were transplanted at the beginning of spring 2019. The plant density was 4000 plants ha^{-1}. The distance between rows and within rows was 2.50 m and 1.00 m, respectively (Figure 1).

Figure 1. Experimental site.

Rosemary plants were managed under rainfed conditions adopting a low-input growth method following the common agronomic practices of the cultivation area. Before transplanting, the experimental field received organic fertilization through the distribution of 2 t ha^{-1} of manure which was buried at a depth of 0.30 m. No pesticides were applied in either year. Weeds were mechanically controlled at the beginning of spring and before harvesting. In both years, plants were harvested during the third 10-day period of June.

Weather data

During the trials, 10-day total rainfall and average maximum and minimum air temperatures were detected by a weather measurement station owned by the Sicilian Agro-Meteorological Information Service (SIAS, 2023) and located close to the farm. Temperature and rainfall trends during the two years are shown in Figure 2 (A and B).

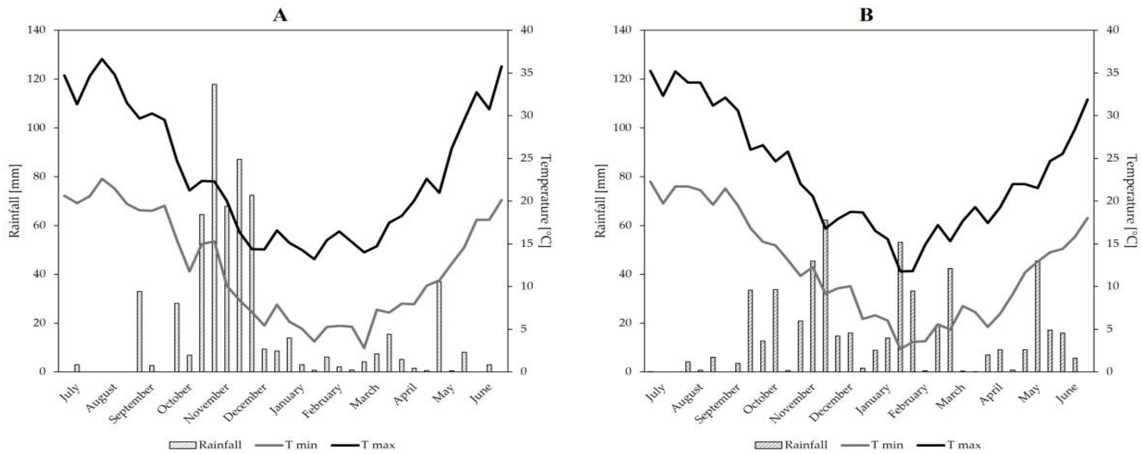

Figure 2. Temperature and rainfall trends during the first (A) and the second (B) year.

Total rainfall ranged from 611 mm (2021-2022) to 538 mm (2022-2023). Average rainfall over the 2-year period was 574 mm. In the 1st-growing season, approximately 80% of rainfall (480 mm) was recorded from October to January. From the beginning of the vegetative growth phase until the harvest date, 92 mm of rainfall was detected. The highest rainfall event (37 mm) happened during first 10-day period of May.

In the 2nd-growing season, rainfall levels were well-spread from September to March. Throughout the biostimulant application phase and up to harvest, 110 mm of rainfall were measured. The highest rainfall event occurred in the second 10-day period of May (45 mm). The temperature trends in both years were comparable to the study area's average temperature. An increase in minimum and maximum temperatures was observed from pre-flowering stage until harvest. In general, higher maximum temperatures were observed in the second growing season, from May until harvest, compared to the first growing season.

Treatments

Two biostimulant formulations were foliar applied:

- Animal protein hydrolysate (APH), (3 mL L^{-1}), derived from hydrolyzed animal epithelium and containing organic nitrogen 8.0%, organic carbon 27.0%, amino acids 50.0% and free amino acids 15%.
- Vegetal protein hydrolysate (VPH), (5 mL L^{-1}), obtained from *Fabaceae* and containing amino acids and plant peptides (31%), organic nitrogen (5%) and organic carbon (25%).

The biostimulant dosage was planned in order to provide the same total amount of N, considering the N content of each type of product. Control (C) treatment was only water. Four applications, using 400 L of water ha^{-1} for each event, were performed every 10 days from the first week of April. Foliar applications were made through a portable hand-sprayer with an operating pressure of 250 kPa and equipped with a flat fan nozzle. Each plot was delimited during application with a plastic panel to prevent drift and contamination of adjacent plots. The plot size was 30 m^2 (2 m × 15 m). To ensure uniformity in application of biostimulant, foliar treatments were carried out by a single operator. Foliar applications were performed early in the morning, when temperature, relative humidity and stomatal aperture were ideal for foliar absorption (Ruiz-Navarro et al., 2019).
The experimental scheme was a randomized complete block design using three replications.

Morphological and yield traits

At harvest total fresh yield was determined. After harvest, plants were dried in a shaded and ventilated environment for approx. 10 days at a temperature of 25-30 °C and total dry yield was determined.

EO extraction and analysis

EOs were extracted by hydro-distillation of air-dried plant material (500 g) for 3 h, according to Ph. Eur. 7.0, 20812 (01/2008). The EO content was calculated dividing the volume of EO obtained by the weight of the biomass samples. The EO yield was estimated multiplying the EO content by the total dry yield. The EO samples were stored at -18°C. Prior to gas chromatography–mass spectrometry (GC/MS), the EOs were diluted 1:100 with hexane and moved to GC vials. EO components were assessed with a HP 6890 gas chromatograph connected with the quadrupole mass spectrometer HP5972 MSD (Hewlett-Packard, Palo Alto, CA, USA) (Figure 3). The operating parameters reported by Farruggia et al., (2024b) were utilized. The EO compounds retention indices (RI) were calculated and compared to those of n-alkane hydrocarbons (RI standard for GC, Sigma-Aldrich, Vienna, Austria). Mass spectra and retention indices were compared to data reported in the literature in order to identify each compound. Peak-area normalization was used to obtain the composition, and the response factor of each component was taken to be equal to 1.

Figure 3. Gas chromatograph/mass spectrometer.

Determination of chemical parameters

Using 25 mL of 70% aqueous methanol, 0.15 g of the 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 minutes at room temperature. After being filtered, the extracts were stored at -20°C for further examination. The extracts were used to determine:

- Total phenolic content (TPC), expressed as milligram caffeic acid equivalents per gram dry weight (mg c.a.e. g^{-1} dw), as described by Lamien-Meda et al. (2010).
- Antioxidant activity (AA), expressed as milligram trolox equivalents per gram dry weight (mg t.e. g^{-1} dw), as described by Chizzola et al. (2008).
- Rosmarinic acid content (RAC), as reported Farruggia et al., (2024b).

Statistical analyses

One-way analysis of variance (ANOVA) was conducted to compare the data per year. The difference between means was carried out using Fisher's LSD test ($p \leq 0.05$). Foliar biostimulants were used as fixed effects in the linear model/ANOVA. Prior to statistical analysis, Levene's test and the Shapiro-Wilk test were used, respectively, to check the homogeneity of variance and normality of all the data. The software MINITAB 19 (State College, PA, USA) for Windows was used for statistical analyses.

Results and discussion

Statistical analysis revealed that foliar biostimulant significantly influenced ($p \leq 0.01$) total fresh yield, total dry yield, EO content and EO yield in two-yearstudy (Table 1).

Table 1. Effect of foliar biostimulant application on total fresh yield, total dry yield, EO content and EO yield of rosemary plants in two-year study.

Foliar Biostimolant	Total Fresh Yield [t ha^{-1}]		Total Dry Yield [t ha^{-1}]		EO Content [%$_{v/w}$]		EO Yield [kg ha^{-1}]	
	I year	II year	I year	II year	I year	II year	I year	II year
C	9.0 ^{c}	8.9 ^{b}	3.2 ^{c}	3.1 ^{c}	2.07 ^{b}	2.21 ^{b}	65.9 ^{c}	69.2 ^{c}
APH	14.3 ^{b}	15.2 ^{a}	4.5 ^{b}	4.6 ^{b}	2.28 ^{b}	2.50 ^{a}	102.6 ^{b}	112.3 ^{b}
VPH	15.0 ^{a}	15.3 ^{a}	4.8 ^{a}	5.1 ^{a}	2.66 ^{a}	2.52 ^{a}	128.9 ^{a}	127.4 ^{a}
***p*-value**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. C: control (only water); APH: animal protein hydrolysate (3 mL L^{-1}), vegetal protein hydrolysate (5 mL L^{-1}).

During the first year of trials, the highest total fresh yield (15.0 t ha^{-1}) was observed in VPH-treated plants, while similar values (15.2 and 15.3 t ha^{-1}) were recorded in plants treated with both protein hydrolysates during the second year. In the case of total dry yield, the same trends were detected during both years; the highest values (4.8 and 5.1 t ha^{-1}) were obtained in VPH-treated plants while the lowest ones (3.2 and 3.1 t ha^{-1}) in control plants. It is worth noting that the biostimulant effect of the two types of protein hydrolysates is mainly related to their peptide and ammino acid content. These substances significantly affect how the plants react to stress. Literature highlights that protein biosynthesis, energy generation, and the creation of molecules with high biological activity depend on peptide and ammino acid content (Colla et al., 2014; Rouphael et al., 2022; Di Miceli et al., 2023). The results of this study are in line with those observed by Singh and Wasnik (2013) and Farruggia et al. (2024a). The authors recorded values between 8 to 21 t ha^{-1}. The application of VPH produced the highest EO content (2.66%) in the first year of study and the highest EO yields

(128.9 and 127.4 kg ha^{-1}) in both years (Table 1). For all parameters the lowest values were obtained in control plants. Similar results were observed by Elansary et al. (2020) and Rahimi et al. (2022) on mint and thyme plants which were treated with different biostimulants. Regarding the two most represented compounds of rosemary EOs, α-pinene and 1.8 cineol, statistical analysis showed that the application of foliar biostimulants only affected the percentage of 1.8 cineol in the second year (Table 3). The highest percentage values, 25.7% and 25.1%, were recorded in control plants and VPH-treated plants, respectively. In the metabolic pathway connected to the production of secondary metabolites, the biostimulant may have an impact on the regulation of genes and the activity of enzymes (Vosoughi et al., 2018). The aromatic profile of the final product has been found to be unaffected since no significant alterations in the content of essential oils extracted from biostimulant-treated plants were found in the current investigation.

Table 3. Effect of foliar biostimulant on the main EOs compounds of rosemary, α-pinene and 1.8 cineol, in the two-year study.

Foliar Biostimolant	**α-pinene [%]**		**1.8 cineol [%]**	
	I year	**II year**	**I year**	**II year**
C	36.9 [a]	35.5 [a]	24.4 [a]	25.7 [a]
APH	33.6 [a]	32.9 [a]	24.9 [a]	24.2 [b]
VPH	33.3 [a]	35.3 [a]	24.4 [a]	25.1 [a]
***p*-value**	0.057	0.402	0.452	0.010

Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. C = control (only water); APH: animal protein hydrolysate (3 mL L^{-1}), vegetal protein hydrolysate (5 mL L^{-1}).

Considering both years, ANOVA showed that foliar biostimulant had significant impact ($p \leq 0.05$) on total phenolic content (TPC), antioxidant activity (AA) while it did not influence the rosmarinic acid content (RAC) (Table 2).

Table 2. Effect of foliar biostimulant on total phenolic content (TPC), antioxidant activity (AA), and rosmarinic acid content (RAC) of rosemary plants in the two-year study.

Foliar Biostimolant	TPC [mg c.a.e. g^{-1}]		AA [mg t.e. g^{-1}]		RAC [%]	
	I year	II year	I year	II year	I year	II year
C	107.9 [a]	110.7 [a]	145.6 [a]	142.0 [a]	1.65 [a]	1.58 [a]
APH	97.6 [b]	97.3 [b]	137.8 [b]	117.7 [b]	1.59 [a]	1.28 [a]
VPH	95.3 [b]	97.3 [b]	138.8 [ab]	111.1 [b]	1.59 [a]	1.28 [a]
***p*-value**	0.004	0.000	0.029	0.001	0.590	0.322

Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. c.a.e. = caffeic acid equivalent; t.e.= trolox equivalent. C = control (only water); APH: animal protein hydrolysate (3 mL L^{-1}), vegetal protein hydrolysate (5 mL L^{-1}).

As reported in Table 2, the highest TPC (107.9 and 110.7 mg c.a.e. g^{-1}) and AA (145.6 and 142.0 mg t.e. g^{-1}) were observed in control plants. The application of both protein hydrolysates produced similar values in both years. Stressful environment is well-recognized to affect the ability of MAPs to synthesize secondary metabolites (Tawfeeq et al., 2016; Chaski et al., 2023). Many authors (Bonini et al., 2020; Saia et al., 2021; Rahimi et al., 2022) have reported that the quantity of secondary metabolites tend to increase in different species with the exposure to microbial and non-microbial biostimulants. Our findings indicate that it is important to take stress or stress-relief into account to avoid generalizing impacts on secondary metabolites.

Conclusions

The findings of this study validate the use of protein hydrolysates as a useful tool for improve yields in organic rosemary. In general, the highest crop and EO yields were found in plants which were treated with plant-based protein hydrolysates. These results are of great interest to organic farms which are inclined to use sustainable and eco-friendly products to increase agricultural production. Technological advances for MAPs production also include the use of biostimulants and other bio-based products.

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Experiment 5

Foliar biostimulants and frequency of application affect yield and chemical properties of organically grown lemon balm

Objective

The goal of this study was to assess how four different biostimulants and two frequencies of application affected morphological, productive, and chemical characteristics of *Melissa officinalis* L. grown organically in a Mediterranean climate under rainfed conditions. In particular, the following hypothesis were tested: 1) the foliar application of biostimulants improves productive parameters in lemon balm plants cultivated in open field; 2) the foliar application of biostimulants affects the chemical parameters of the extracts depending on the type of biostimulant and frequency of application.

Materials and methods

Experimental site and plant material

Tests were carried out at a local farm located in Aragona (Sicily, Italy) (330 m a.s.l., 37°22′32.71″ N, 13°38′33.59″ E), during the 2022. The soil was classified as Regosol (United States Department of Agriculture (USDA) classification: typic xerorthents) and sandy clay loam (46% sand, 27% clay and 27% silt) with a pH of 7.3, 14 g kg^{-1} organic matter, 1.25% total nitrogen, 19.8 ppm assimilable phosphate, and 358 ppm assimilable potassium. Agamic propagation was carried out. The seedlings were produced by a commercial nursery and grown in plastic pots for 60 days. The plants were transplanted at the end of January 2022. The distance between rows and within rows was 2.00 m and 0.50 m, respectively (Figure 1). Before transplanting, the experimental field received organic fertilization through the distribution of 2 t ha^{-1} of manure which was buried at a depth of 0.30 m. Irrigation was managed using a drip system, returning 100% of the evapotranspiration. No pesticides or chemical fertilizer were used in either year. Weeds were removed mechanically before harvesting.

Figure 1. Experimental site.

Weather data

Data on rainfall and air temperature were recorded at a weather station belonging to the Sicilian Agro-Meteorological Information Service (SIAS, 2023). The station is equipped with a datalogger and various sensors for the measurement of air temperature (TAM platinum PT100 sensor, heat resistance with anti-radiation screen) and total rainfall (PPR sensor with tilting bucket rain gauge). Data regarding average daily maximum and minimum temperatures (°C) and total 10-day period precipitation (mm) were taken into consideration. Air temperature and rainfall trends are shown in Figure 2.

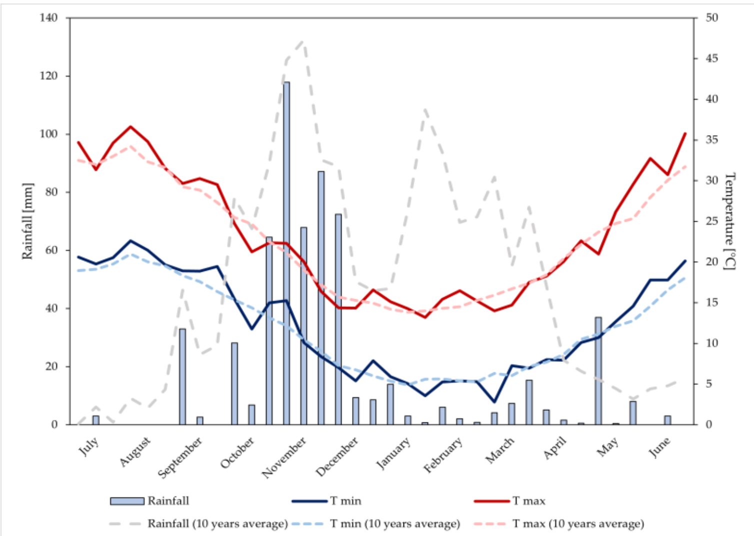

Figure 2. Temperature and rainfalls trends at the experimental site from June 2021 to July 2022.

From July 2021 to June 2022, a total rainfall levels were 611 mm (2021-2022). Rainfall was mainly distributed from October to December. During the period of biostimulant application and until the harvest, a total rainfall of 56 mm was observed. Significant rainfall occurred in the first 10-day period of May (37 mm). Temperatures trends were similar and consistent with the average temperature of the experimental area.

Treatments

Four commercial biostimulant formulations were used for the tests:

- *Ecklonia maxima* (EM), containing organic nitrogen (1%), organic carbon (10%), auxin (11 ml l^{-1}) and cytokinin (0.03 mg l^{-1}), and organic substances with nominal molecular weights < 50 kDa (30%).
- *Ascophyllum nodosum* (AN), containing organic nitrogen (1%), organic carbon (10%), phytohormones and organic substances with nominal molecular weights < 50 kDa (30%).
- Fulvic Acids (FA), extracted from leonardite and containing organic nitrogen (0.5%) and organic carbon (30%).
- Protein Hydrolysate (PH), obtained from *Fabaceae* and containing amino acids and plant peptides (31%), organic nitrogen (5%) and organic carbon (25%).

The biostimulant dosage was planned to provide the same total amount of N, considering the N content of each type of product. The same total amount of biostimulant was applied following two frequencies of application, weekly frequency (F1) for a total of six applications and two-weeks frequency (F2) with three total applications. The main doses are listed in Table 1.

Table 1. Doses of foliar biostimulants.

Biostimulant	**Dose[1] Frequency 1 week [l hl^{-1}]**	**Dose[1] Frequency 2 weeks [l hl^{-1}]**	**Total Amount[2] [l ha^{-1}]**
EM = *Ecklonia maxima*	0.125	0.250	6.0
AN = *Ascophyllum nodosum*	0.125	0.250	6.0
FA = Fulvic acids	0.250	0.500	12.0
PH = Protein hydrolysate	0.025	0.050	1.2

1 = doses for each application; **2** = total quantity of biostimulant applied.

The first application was performed during the first week of April in each year. For each foliar application, 4 hL of water ha^{-1} was used. A portable sprayer with an operating pressure of 250 kPa was adopted. A randomized complete block design with three replicates was used for the tests. Biostimulant (B) and frequency (F) were used as fixed effects in the linear model/ANOVA. Each block comprised 10 plots of 30 m^2. Treatments were applied for each randomized plot in the block. The plots were well spaced in the block; plastic panels were used to delimit each plot and to avoid drift during foliar applications.

Plant measurement

At harvest total fresh yield and total dry yield were determined. In both years, plants were harvested during the third week of June. Plants were cut at 5 cm above ground level and then dried in a shaded and ventilated environment for 10 days at a temperature of 25-30°C.

Determination of some chemical parameters

0.15 g of the finely powdered dry biomass were extracted with 25 mL aqueous methanol (70%) for 30 minutes in an ultrasonic bath. The extracts were filtered and kept at -18°C until further analysis.

Total phenolics

The total phenolics content was assayed with the Folin-Ciocalteu reagent, following the methodology described by Lamien-Meda et al. (2010). In the wells of the microplate, 5 µL extracts were added to 105 µL distilled water followed by 5 µL of Folin-Ciocalteu reagent, 10 µL Na_2CO_3 (35% in distilled water) and again 125 µL distilled water. Caffeic acid (Sigma-Aldrich, Austria; 10 mg in 100 mL milli-Q water) was used as standard. Increasing volumes (0 to 25 µL) of caffeic acid made up to 110 µL with distilled water instead of the samples were used to obtain a calibration curve. A blank was used to correct the readings. Calibration points and samples were pipetted and measured as quadruplicates (Figure 3). After 1 h resting in the dark, the absorbance of the reaction mixture was measured at 750 nm using a microplate reader (i-mark, Bio-Rad, Austria). The results were expressed as milligram caffeic acid equivalents per gram dry weight (mg c.a.e. g^{-1} dw).

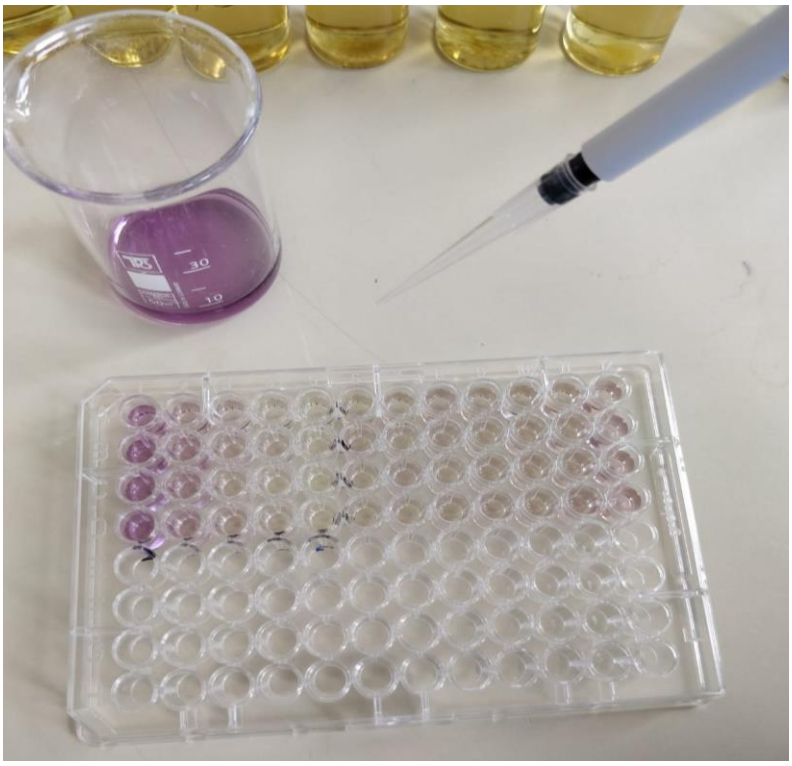

Figure 3. Preparation of plates for determination of phenolic content.

Antioxidant activity

Antioxidants reacted with the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) which wasthen decolorized. In accordance with the methodology reported by Chizzola et al. (2008), in the wells of the microplate, 5 µL extracts were added to 95 µL methanol and 100 µL of solution (2.2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, Germany; 0.0038 g in 25 mL methanol). Increasing volumes (0 to 8 µL) of Trolox (0.62 mg mL^{-1} in ethanol) made up to 100 µL with methanol instead of the samples were used to obtain a calibration curve. A preparation consisting of 50 µL Trolox, 50 µL distilled water and 100 µL DPPH reagent (where the DPPH was completely decolorized) was taken as blank and subtracted from all measurements. Calibration points and samples were pipetted and measured as quadruplicates. Discoloration was measured at 490 nm using a microplate reader (i-mark, Bio-Rad, Austria). The results were expressed in milligram trolox equivalents per gram dry weight (mg t.e. g^{-1} dw).

Rosmarinic acid

The content of rosmarinic acid was measured according to Chizzola et al. (2008) using a Waters HPLC system consisting of a 626 pump, a 600S controller, a 717plus autosampler, a column oven operated at 25°C, and a 996-diode array detector (Waters S.A.S, Saint-Quentin, France). The separation was carried out on a Symmetry C18, 5.0 µm particle size, 4.6 × 150 mm column. The mobile phase used was 1% acetic acid/acetontrile 85:15 (solvent A) and methanol (solvent B). The analysis started with a solvent ratio of A/B of 9:1, and a linear gradient was performed to reach 100% B within 30 min. The flow rate was 1.0 mL min^{-1} and the injection volume, 20 µL. The quantification of rosmarinic acid was done using the external standard method by preparing seven calibration standards ranging from 3.9 to 500 µg ml^{-1} and recording the calibration curve at 330 nm.

Statistical analyses

Statistical analyses were performed using the package MINITAB 19 for Windows. Data were compared using analysis of variance (ANOVA). The difference between means was carried out using LSD test ($p \leq 0.05$). Before the statistical analysis, all data were tested for normality with a Shapiro–Wilk test, and for homogeneity of variance with Levene's test.

Results and discussion

Statistical analysis revealed that biostimulant (B), frequency (F) and their interaction significantly influenced ($p \leq 0.01$) total fresh yield and total dry yield (Table 2).

Table 2. Effect of biostimulant (B), frequency (F) and their interaction on total fresh yield and total dry yield of lemon balm plants.

Source of variation	**Total Fresh Yield [t ha^{-1}]**	**Total Dry Yield [t ha^{-1}]**
Biostimulant (B)		
C	2.6 ^{d}	0.8 ^{d}
EM	4.1 ^{c}	1.2 ^{c}
AN	5.8 ^{b}	1.6 ^{b}
FA	6.6 ^{a}	1.8 ^{a}
PH	6.7 ^{a}	1.6 ^{b}
Frequency (F)		
1 week	4.3 ^{b}	1.2 ^{b}
2 weeks	6.0 ^{a}	1.6 ^{a}
***p*-value**		
B	0.000	0.000
F	0.000	0.000
B × F	0.000	0.000

Means are reported. Values with different letters are significantly different at $p \leq 0.05$ according to LSD test. C = control (only water); EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids; PH = protein hydrolysate.

The application of FA and AN produced the highest total fresh yields, 6.6 and 6.7 t ha^{-1} respectively, while the application of FA generated the highest total dry yields, 1.8 t ha^{-1}. The lowest fresh and dry yields were observed in control plants (Table 2).

Considering the interaction B × F, the highest total fresh yield (8.6 t ha^{-1}) and total dry yield (2.2 t ha^{-1}) were recorded in FA-treated plants every two weeks. The lowest values were obtained in control plants following both frequencies and in EM-treated plants every week (Figure 4).

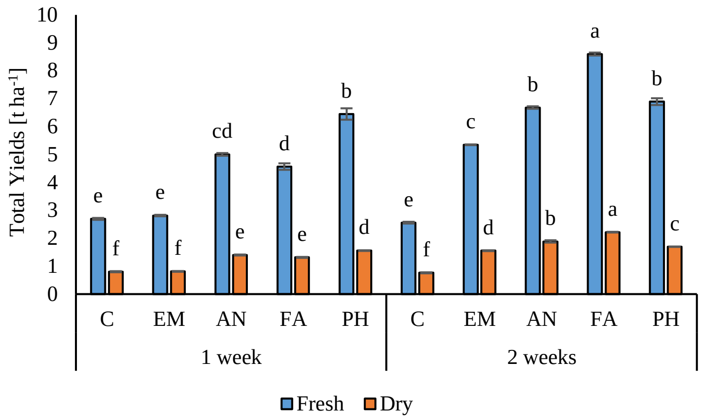

Figure 4. Influence of the interaction B × F on total fresh yield and total dry yield of lemon balm. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to LSD test. c.a.e. = caffeic acid equivalent; t.e.= trolox equivalent. C = control (only water); EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids; PH = protein hydrolysate.

The usage of PH produced similar values following both frequencies of application while the use of FA every week generated a significant decrease in biomass yield compared with values recorded in plants treated every two weeks. However, the recorded values were always higher than those measured in control plants. Excessive amounts of plant biostimulant can be phytotoxic to plants and have a severe impact on their ability to absorb water and nutrients as well as other physiological functions (Illera-Vives et al., 2022; Farruggia et al., 2024). Protein hydrolysates are mixture of amino acids and peptides and can induce a variety of physiological responses in plants that enhance growth, increase product yield and quality, and fortify the resistance to heat stress, salinity, drought, and nutritional stress. Micro- and macronutrient translocation and absorption are known to be aided by fulvic acids. Because of this, the use of them has been linked to improvements in crop quality and production (Colombo et al., 2012; Aytaç et al., 2022). Furthermore, the synthesis of proteins, photosynthetic activity, and enzyme activity are only a few of the molecular processes that these substances favorably impact (Nardi et al., 2021; Farruggia et al., 2024). Analysis of variance showed a significant effect ($p \leq 0.01$) of biostimulant (B) and frequency of application (F) on total phenolic content (TPC) (Table 2). The highest TPC (118.7 mg c.a.e. g^{-1}) was recorded in PH-treated plants, while the lowest one in AN-treated plants. In addition, plants treated every week produced higher TPC (Table 2). Antioxidant activity (AA) was significantly affected ($p \leq 0.05$) by the biostimulant factor. As reported in table 2, the highest AA was recorded in EM-, AN- and FA-treated plants, with values from 160.5 to 162.0 mg t.e. g^{-1}, while the lowest value (156.8 mg t.e. g^{-1}) was obtained in control plants.

Biostimulant (B) factor, frequency (F) factor and their interaction did not have significant effect on rosmarinic acid content (RAC).

Table 2. Effect of biostimulant (B), frequency (F) and their interaction total phenolic content (TPC), antioxidant activity (AA), and rosmarinic acid content (RAC) of lemon balm plants.

Source of variation	**TPC [mg c.a.e. g^{-1}]**	**AA [mg t.e. g^{-1}]**	**RAC [%]**
Biostimulant (B)			
C	114.6 ^{ab}	156.8 ^{b}	2.2 ^{a}
EM	115.8 ^{ab}	162.0 ^{a}	2.3 ^{a}
AN	105.3 ^{c}	160.6 ^{a}	2.1 ^{a}
FA	112.3 ^{b}	160.5 ^{a}	2.3 ^{a}
PH	118.7 ^{a}	160.3 ^{ab}	2.3 ^{a}
Frequency (F)			
1 week	115.9 ^{a}	159.3 ^{a}	2.3 ^{a}
2 weeks	110.8 ^{b}	160.8 ^{a}	2.2 ^{a}
***p*-value**			
B	0.000	0.006	0.301
F	0.001	0.071	0.379
B × F	0.000	0.001	0.072

Means are shown. The values followed by different letter are significantly different for $p \leq 0.05$ according to LSD test. c.a.e. = caffeic acid equivalent; t.e.= trolox equivalent. C = control (only water); EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids; PH = protein hydrolysate.

The interaction between biostimulant (B) and frequency had a significant effect on total phenolic content (TPC) of lemon balm (Table 2). The highest values were observed in FA- and PH-treated plants following frequency 1, 122.6 and 122.1 mg c.a.e. g^{-1} respectively, while the lowest in plants treated every week with AN and every two weeks with FA (Figure 5).

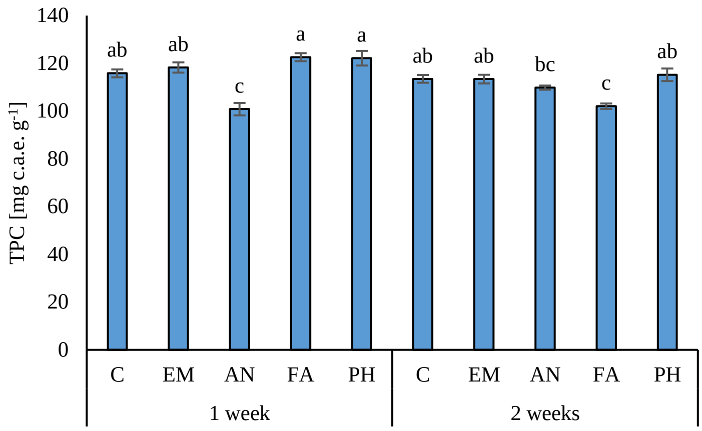

Figure 5. Influence of the interaction B × F on total phenolic content (TPC) of lemon balm. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to LSD test. c.a.e. = caffeic acid equivalent; t.e.= trolox equivalent. C = control (only water); EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids; PH = protein hydrolysate.

The interaction B × F significantly influenced the antioxidant activity (AA) of lemon balm (Table 2). As reported in Figure 6, values were obtained in the range from 155.2 mg c.a.e. g^{-1} to 164.0 mg c.a.e. g^{-1}. The highest value was recorded in EM-treated while the lowest in control plant, in both cases following a frequency of application every two weeks.

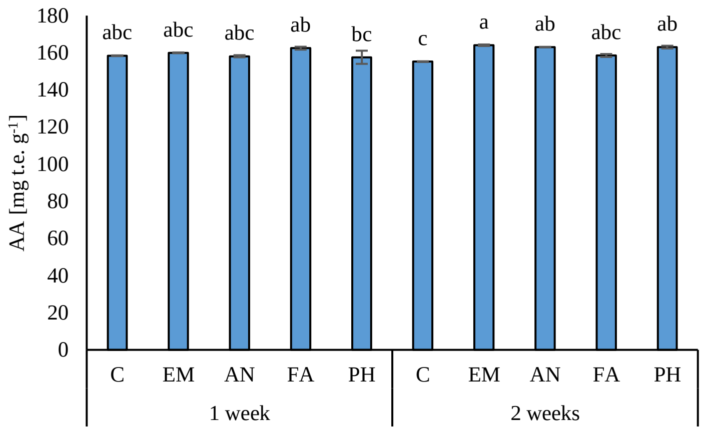

Figure 6. Influence of the interaction B × F on Antioxidant activity (AA) of lemon balm. Means and standard errors are reported. Values with different letters are significantly different at $p \leq 0.05$ according to LSD test. c.a.e. = caffeic acid equivalent; c.a.e.= caffeic acid equivalent. C = control (only water); EM = *Ecklonia maxima*; AN = *Ascophyllum nodosum*; FA = fulvic acids; PH = protein hydrolysate.

The results obtained in this study disagree with those observed by Chrysargyris et al. (2022) who conducted a study on lemon balm cultivated in organic and conventional systems. Particularly, the authors observed higher phenolic content and lower antioxidant activity than those observed in this research.

Conclusions

The findings of this study confirm the use of biostimulants as a useful tool for improve yields in organic lemon balm. It is interesting to note that a less frequent biostimulants application produced higher yields than more frequent treatments. In particular, the application of fulvic acids every two weeks produced the highest fresh and dry yield. Considering the chemical parameters, the interaction between biostimulant and frequency had a significant effect on total phenolic content (TPC) of lemon balm. The highest values were observed in FA- and PH-treated plants following frequency 1. The interaction B × F significantly affected also the antioxidant activity (AA). The highest value was recorded in EM-treated while the lowest in control plant, in both cases following frequency two weeks. Further research is needed to validate the obtained data and to verify the effect on other chemical parameters.

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4. CONCLUDING REMARKS

MAPs are extensively distributed over Europe, Asia, and Africa, with the Mediterranean basin serving as one of their favorite growing location. The demand for organic herbs is rising globally, and MAPS are frequently cultivated in this region through organic farming. Because they are recognized as a significant source of bioactive compounds with possible health benefits, these plants have drawn the interest of scientists, consumers, medical professionals, and businesses in recent years. These chemicals are extensively acknowledged to be pharmacologically active and are used in other significant areas including cosmetic, food and pesticide industries.

The Mediterranean region is progressively being impacted by climate change in which high temperatures and low water availability impact the quality and output of agricultural yields. For farmers and, in general for agriculture, climate change is a worrying scenario. Water shortage resulting from decreased precipitation in sensitive zones poses a severe danger to agricultural profitability, even beyond natural disasters and epidemics brought on by a quickly changing environment. Limited capacity of plants to absorb water results in a limited capacity to absorb nutrients, which is already a limitation in low-input cultivation methods like organic farming. In the Mediterranean area, MAPs are frequently grown under rainfed conditions in low input or agricultural organic systems.

Recently, biostimulants have been used as tool to accomplish the goal of creating a more sustainable agricultural output, lowering the demand for agrochemicals and irrigation water. In addition, the foliar application of these products has been strongly advised for quick nutrient absorption, which permits to maximize the plant nutrition without endangering the sustainability of the environment. It is well-known thar biostimulants can improve metabolic processes like photosynthesis, phytohormone regulation, nutrient and water uptake, and the activation of genes that confer resistance to abiotic stresses and produce changes in plant architecture and phenology.

Focusing on the main findings of this study, it is possible to affirm that the application of foliar biostimulants represents an innovative practice to improve productive and qualitative parameters for MAPs organically cultivated considering the climate characteristics of the Mediterranean region.

In general, the foliar application of fulvic acid produced an increase in biomass and EO yields for oregano, rosemary, sage and lemon balm plants. In addition, the application of vegetal protein hydrolysate positively influenced the yields of rosemary and sage plants.

Specifically, the foliar application of various biostimulants generated an increase in rosemary fresh and dry yields in the range 42-71% compared to control. Concerning sage, the main treatments produced an increase of fresh yield between 13% and 50% and of dry yield between 29% and 57%. In the case of oregano plants, the application of fulvic acids and seaweed extract allowed to obtain an increase ranged from 0.3 to 8.4 t ha^{-1} (fresh biomass) and from 0.5 t ha^{-1} to 3.0 t ha^{-1} (dry biomass) with respect to control. Finally, in the lemon balm plants treated with various types of biostimulants, increases of fresh biomass (1.5 - 4.1 t ha^{-1}) and dry biomass (0.4 - 1.0 t ha^{-1}) were detected. When comparing the four species in this study, the best results in terms of EO yields were recorded for oregano plants using fulvic acids. Increases of approximately 170% were observed compared with control plants. Appreciated findings in terms of EO yield were found when applying vegetal protein hydrolysate on rosemary plants.

A novelty of this study has been the impact of biostimulant application on EO composition. Nowadays, there is a lack of consensus between researchers regarding the effect of biostimulant application on EO composition of various MAPs. In this study, it is worth noting that biostimulant application significantly affected the percentage of some minor compounds of the sage EO, only. For oregano and rosemary plants, non-appreciated results were observed for both major and minor compounds.

Regarding the main chemical parameters, a decrease of total phenolic content, antioxidant activity and rosmarinic acid content were observed for oregano, sage, and rosemary plants. Otherwise, a weekly application of fulvic acid and protein hydrolysates produced an increase of total phenolic content for lemon balm, while the same frequency application of a biostimulant based on *E. maxima* generated the highest antioxidant activity.

The results of this study highlight that the application of low amounts of biostimulants can directly and indirectly affect the primary and secondary metabolism of MAPs mitigating the effects of stressful conditions. However, further research is needed to gain a deeper understanding the action mechanisms of these products on physiological and metabolic processes of medicinal and aromatic plants.