



**OPTIMIZATION OF SECONDARY METABOLITES PRODUCTION  
BY THE APPLICATION OF ELICITORS**

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**Doctoral (Ph.D.) dissertation**

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Budapest, Hungary

## **DECLARATION**

I declare that the thesis titled “Optimization of secondary metabolites production by the application of elicitors” is my work and that it has not been submitted before for any degree or examination in any other University.

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## List of abbreviations

AAE	Ascorbic acid equivalent
ANOVA	Analysis of variance
AOC	Antioxidant capacity
DAD	Diode-array detector
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
EO	Essential oil
FRAP	Ferric ion reducing antioxidant power
GC-FID	Gas chromatography- flame ionization detector
GC-MS	Gas chromatography- mass spectrometry
GAE	Gallic acid equivalent
HPLC	High-performance liquid chromatography
IC	Isochorismate
JA	Jasmonic acid
LOX	Lipoxygenases
MeJa	Methyl Jasmonate
OPDA	<i>Cis-(+)-12-oxophytodienoic acid</i>
PAL	Phenylalanine ammonia-lyase
PCA	Principal component analysis
Ph. Eur	European Pharmacopoeia
RI	Retention indices
ROS	Reactive oxygen species
SA	Salicylic acid
SMs	Secondary metabolites
TiO <sub>2</sub>	Titanium dioxide
TMAO	Trimethylamine <i>N</i> -oxide
TPC	Total phenolic content

## I. Introduction

### 1.1. Background

Since the beginning of time, humans have depended on natural resources to survive, prevent, and treat diseases. Among these resources, plants emerge as plentiful and diverse reservoirs of natural metabolites, obtained from different plant parts, including flowers, leaves, seeds, and more (Bachheti & Bachheti, 2023).

The metabolites encompass two groups; primary and secondary metabolites (SMs); the former one plays a pivotal role in the growth and physiological development of plants, such as sugars, amino acids, and lipids, which show a lot of uniformity in plenty of taxa. In contrast, the latter group consists of low-molecular-weight compounds that play significant roles in their interaction with the surrounding environment as well as establishing defense mechanisms. Despite the term 'secondary metabolites', these compounds are far from being secondary in the viability of plants. They can indirectly influence the growth, development, and reproduction of plants (Sati et al., 2022; Singh & Ram Avtar, 2020). The type and concentration of SMs may vary greatly among plant species and within the same plant species as well (Ahmad et al., 2018).

Unlike primary metabolites, certain SMs are synthesized only after a plant's exposure to various stresses; biotic or/and abiotic; or during specific developmental stages. Meanwhile, some SMs are constitutively produced and remain stored in inactive forms within specific cells or organelles until activated by a specific stimulus (Ahmad et al., 2018; Garagounis et al., 2021; Wu et al., 2023).

These compounds have long been employed for various purposes, including human therapeutic use, flavor and fragrance enhancement in foodstuffs and cosmetics.

Unfortunately, the burgeoning demand for these bioactive compounds exceeds their current bio production capacity. Hence, in the pursuit of alternatives to enhance their production, diverse biotechnological approaches have proven effective. One of the most promising methods is elicitation which involves the exogenous application of tolerable amounts of specific compounds referred to as elicitors. This process induces immune responses in plants, triggering defense reactions and ultimately resulting in an increased production of SMs (Kandoudi & Németh-Zámboriné, 2022; Largia et al., 2023). Hormonal elicitors play a crucial role in the elicitation process, they have the capacity to mimic or enhance the inherent hormonal signaling pathways of plants linked to their defense mechanisms and activate a cascade of biochemical reactions (Baenas et al., 2014; Davies, 2010; Rohwer & Erwin, 2008). Among the most extensively used elicitors are the plant growth regulators, methyl jasmonate (MeJa) and salicylic acid (SA).

Osmolytes, on the other hand, are organic compounds mostly involved in abiotic stress responses. Stressed plants produce these molecules to protect their cell contents against damage, scavenge radicals, adjust osmotic pressure, store carbon, and stabilize protein structures (Ghosh et al., 2021; Jogawat, 2019). Several studies have investigated the use of osmolytes, such as glycine betaine and proline, to mitigate abiotic stresses and enhance their physiological and biochemical traits (Armin & Miri, 2014; Ben Ahmed et al., 2010; Cirillo et al., 2016; Wang et al., 2019). Trimethylamine *N*-oxide (TMAO), an important osmolyte found in humans and especially marine animals, has lately been detected in plants and confirmed its involvement in abiotic stress tolerance (Catalá et al., 2021). However, no studies have been found to study the effects of this compound on the SMs accumulation.

Unfortunately, achieving the desired outcomes is not as straightforward as it appears. Numerous factors come into play during the process of elicitation. Plant species may exhibit varying responses to elicitors, and the effectiveness of elicitation can be influenced by the specific conditions and characteristics of each individual plant, such as genetic and environmental aspects. Other factors include the type and concentration of the elicitor used, the timing of elicitation, and potential interactions with other signaling pathways (Kandoudi & Németh-Zámoriné, 2022).

Existing literature provides insightful information about elicitation in different plant species. However, it seems that there is a gap in research for *in-vivo* elicitation in medicinal and aromatic plants (MAPs). Most studies focus on *in vitro* investigations such as tissue and hairy root cultures. While *in vitro* research offers a meticulously regulated environment where various parameters can be controlled, ensuring consistent and reproducible results, *in vivo* research can only be the basics of agrotechnological developments. That is why for practical innovations, only open field *in vivo* studies may serve as a background.

The above-mentioned reasons formed the foundation of our research. Therefore, it was crucial to develop a clear understanding of the effects of elicitation on selected model MAPs *in vivo* and collect data during different conditions on their reaction concerning biomass and SM production.

## 1.2. Aims of the research

The main goal of this research was to optimize the accumulation of SMs, notably the essential oil volatiles and phenolics, by the foliar application of two hormonal elicitors: MeJa and SA. The study focused on five valuable MAP species. Basil (*Ocimum basilicum* L.), hyssop (*Hyssopus officinalis* L.), marjoram (*Origanum majorana* L.), peppermint (*Mentha piperita* L.), and yarrow (*Achillea collina* Becker). To achieve this goal, the research was focusing on the main question:

- How do MeJa, SA, and TMAO influence the accumulation of volatile compounds in the essential oil (EO) and total phenolics (TPC) of the drugs?

In order to evaluate the results on a broader approach and to gain a deeper understanding of the plants' reactions, the following additional questions were defined:

- How do elicitor concentrations, treatment frequency, and exposure duration differences impact the observed plant characteristics?
- Could we maintain an acceptable dry matter production (height, biomass, drug ratio) in parallel with changing active compounds' accumulation?
- Are the effects of the studied elicitors similar to each other?
- Are the responses exhibited by the five model species uniform to each other?
- To what extent do environmental and weather conditions play a role in influencing the effects on the studied plant species?

Within the limits of our possibilities, we also carried out some additional investigations to detect the backgrounds of the plants' reactions to elicitation.

To answer these questions, we carried out open field plot experiments in 4 vegetation years together with 2 greenhouse and 2 phytotron trials. We intended to summarize specific findings on the five model species in order to establish scientific basics for optimization of their cultivation through elicitation.

## II. Literature review

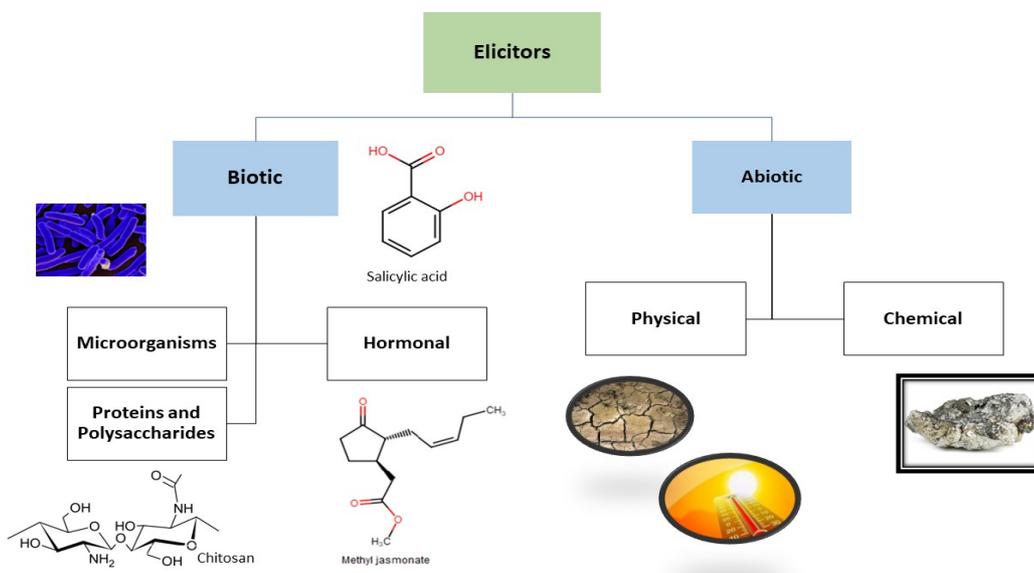
### 2.1 Elicitation

Elicitation is a method used to stimulate the accumulation of SMs within plants by introducing a controlled and appropriate amount of chemicals or biofactors. As plants frequently encounter various stresses like drought, extreme temperatures, diseases, and herbivore attacks, they have evolved intricate defense mechanisms. These mechanisms involve triggering morphological, physiological, biochemical, and molecular changes, including the synthesis of SMs to cope with challenges effectively. Elicitors seem to function by mimicking these stresses, prompting the initiation of a chain of reactions that ultimately lead to the accumulation of specific SMs in the plant (Potters et al., 2007). The series of events starts with the perception and recognition of the elicitor's signal by plant receptors in the plasma membrane, leading to the activation of effectors like ion channels and GTP-binding proteins. The activated effectors transmit signals from the elicitor to second messengers, subsequently amplifying the signal, triggering downstream reactions. These reactions include the activation of messengers in the hormonal signaling pathway, among others, SA and MeJa, as well as processes like ion fluxes and the production of reactive oxygen species (ROS). Consequently, this cascade of events results in the activation of transcription and translation processes involving defense-responsive genes and enzymes, thus resulting in the synthesis of SMs (Zhao et al., 2005).

Elicitors can be categorized as biotic and abiotic ones based on their nature, as shown in the figure below (Figure 1). Abiotic elicitors originate from non-biological sources, encompassing physical stresses like drought, heat, and salinity, as well as chemical stresses such as heavy metals and osmolytes when applied externally. Biotic elicitors, on the other hand, include various living organisms like microorganisms (rhizobacteria and fungi), plant originating proteins and polysaccharides (chitin, chitosan, and lectins), as well as plant growth regulators as hormonal elicitors (Largia et al., 2023).

Plant and tissue cultures have served as a model system to study and comprehend the intricate mechanisms behind elicitation, allowing for the isolation and manipulation of each factor involved in the process in a relatively simple and fast manner. Extensive research has demonstrated the effectiveness of elicitation *in vitro*. Recently, Gharari et al. (2020) tested chitosan and MeJa as elicitors on hairy root cultures of *Scutellaria bornmuelleri*. Their study demonstrated an induced over expression of the *MYB7* and *FNSII2* genes, key players in the biosynthesis of flavonoids. This led to a remarkable increase in the production of flavones, reaching levels up to 13.3-fold

higher than those detected in control cultures. Other studies, including cell cultures, shoot and callus cultures are discussed in detail in this review (Narayani & Srivastava, 2017).



**Figure 1 Classification of elicitors**

On the other hand, *in vitro* results cannot always be directly extrapolated to *in vivo* conditions, where a greater number of variables come into play within whole, intact living organisms. The intricate ecological system of a plant involves various environmental factors, such as soil composition, sunlight exposure, temperature fluctuations, and interactions with microorganisms. These elements significantly influence the composition of SMs, highlighting the importance of considering the broader context of a plant's natural habitat when studying the production of these bioactive compounds. An overview of the most recent studies on the effect of elicitors *in vivo* is demonstrated in Table 1.

**Table 1 Effects of elicitors on *in vivo* plants**

Species	Elicitor	Concentration	Application method	Effect	Reference
<i>Varronia curassavica</i>	SA	1 mM	Foliar spray	Increased anthocyanin, carotenoids, and EO content.	(Ramos Melo et al., 2023)
<i>Ocimum gratissimum</i>	SA	1 mM	Foliar spray	Increased EO content, eugenol, Superoxide Dismutase, catalase, hydrogen peroxide, and lipid peroxidation.	(Alvarenga et al., 2022)
<i>Salvia officinalis</i>	AgNO <sub>3</sub>	0.015 mM	Foliar spray	Increased phenolic acids: rosmarinic acid, salvianolic acid A, salvianolic acid B, and cinnamic acid.	(Pesaraklu et al., 2021)
<i>Medicago sativa</i>	Fe <sub>2</sub> O <sub>3</sub>	50–150 mg L <sup>-1</sup>	Hydroponic solution	Increased superoxide dismutase, peroxidase, catalase activity, and total chlorophyll content	(Y. Yang et al., 2023)
<i>Lavandula angustifolia</i>	Gibberellic acid	400 mg L <sup>-1</sup>	Foliar spray	Increased EO content, linalool, and terpinene-4-ol. Decrease in 1,8-cineole, camphor, and lavandulol.	(İzmirli & Yıldırım, 2023)
<i>Trigonella foenum-graecum</i>	<i>Pseudomonas fluorescens</i> + <i>Sinorhizobium meliloti</i>	10 <sup>4</sup> CFU L <sup>-1</sup>	Inoculation	Increased seed number per legume, seed weight per plant, seed mucilage, and nicotinic acid.	(Sharghi et al., 2018)
<i>Glycyrrhiza uralensis</i>	<i>Aspergillus niger</i>	10 g L <sup>-1</sup> of mycelium	Root culture	Increased glycyrrhetic acid, Total flavonoids	(Li et al., 2016)
<i>Datura stramonium</i>	Tomato mosaic virus	500 mg of virus-infected leaves	Inoculation	Increased hyoscyamine in capsules and roots.	(Grat et al., 2022)
<i>Dracocephalum kotschy</i>	Chitosan	100 mg L <sup>-1</sup>	Foliar spray	Increased phenylalanine ammonia lyase (PAL), hydrogen peroxide, catalase, rosmarinic acid, quercetin, apigenin, and nutrient absorption.	(Kahromi & Khara, 2021)
<i>Melissa officinalis</i>	Salt	100 mM	Irrigation solution	Decreased growth parameters, increased cyanidin-3-O-glycoside, rosmarinic acid, total phenolics, and flavonoids.	(Hawrylak-Nowak et al., 2021)
<i>Salvia officinalis</i>	Drought	40% (field capacity)	N. A*	Decreased growth parameters, EO content and yield, Chlorophyll, β-thujone, and 1,8-cineole. Increased camphor, α-thujone, and veridiflorol	(Aslani et al., 2023)
<i>Cuminum cyminum</i>	UV-B	290–320 nm	15 W lamp	Increased phenols, flavonoids, terpenoids, anthocyanins, β-carotene, and lycopene.	(Ghasemi et al., 2019)
<i>Mentha arvensis</i>	Heat	27–45 °C	N. A	Increased 1,8-cineole, menthone, pulegone, menthyl acetate and antimicrobial activity. Decreased menthol, and menthofurane,	(Heydari et al., 2018)

\* Not applicable

## 2.2 Phytohormones

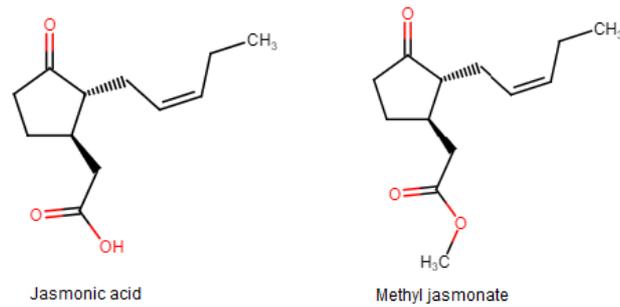
Phytohormones regulate plant growth and development, crucial factors in signal transduction that oversee numerous physiological, molecular, and biochemical processes within plants. These naturally occurring compounds act either in proximity to their sites of synthesis or at remote locations. Examples of phytohormones include auxin, gibberellins, cytokinins, salicylates, and jasmonates (Altaf et al., 2023).

### 2.2.1 Jasmonates

Jasmonic acid (JA) with its precursors and derivatives, including MeJa referred to as jasmonates (Figure 2), belongs to the oxylipin family. MeJa was first isolated in the 1960s from EO of jasmine (*Jasminium grandiflorum* L.) flowers (Demole et al., 1962). However, it was only after two decades that the first physiological effects of JA and MeJa were described. MeJa was identified as a senescence-promoting substance in wormwood (*Artemisia absinthium* L.) and a growth inhibitor in broad bean (*Vicia faba* L.) (Dathe et al., 1981; Ueda & Kato, 1980).

Jasmonates are synthesized through the octadecanoid pathway, originating from polyunsaturated fatty acids (Kombrink, 2012). In response to stress or developmental cues, the biosynthesis of jasmonates is initiated with the conversion of  $\alpha$ -linolenic acid, derived from chloroplast membrane galactolipids, into 13-hydroperoxylinolenic acid by lipoxygenase (LOX). Subsequently, this compound undergoes metabolism by allene oxide synthase and allene oxide cyclase, leading to the formation of *cis*-(+)-12-oxophytodienoic acid (OPDA). Facilitated by the JASSY protein in the chloroplast outer envelopes and the peroxisomal membrane transporter COMATOSE, OPDA is transported from the chloroplast to the peroxisomes. In the peroxisomes, OPDA undergoes a reductase reaction and experiences multiple  $\beta$ -oxidations, ultimately resulting in the production of JA (Footitt et al., 2002; Guan et al., 2019; Wasternack & Song, 2017). After the formation of JA, it undergoes conversion into its active, partially active, or inactive forms. The most active one is jasmonoyl-isoleucine, activating the transcription factors that control gene expression, which encode enzymes catalyzing the formation of various SMs (Wasternack & Strnad, 2018; Zhou & Memelink, 2016).

Jasmonates are essential for both defense mechanisms and the management of biotic and abiotic stress, as well as playing a significant role in the growth and development of plants (Huang et al., 2017). JA and its derivatives influence root growth, seed germination, tuber formation, tendril coiling, trichome initiation, flower development, and senescence (Kombrink & Wasternack, 2010; Santino et al., 2013).

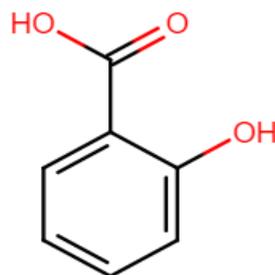


**Figure 2 Structures of JA and MeJa**

### 2.2.2 Salicylic acid

SA or 2-hydroxy benzoic acid is a phenolic compound synthesized by plants (Figure 3). Its name is derived from the name of the willow tree, *Salix*, from which salicin, a derivative of SA, was first extracted in 1828. It is essential in disease resistance as well as physiological and developmental processes in plants (Vlot et al., 2009). SA and derivatives can be synthesized via two distinct pathways: PAL or isochorismate (IC) pathways. However, both pathways require the same precursor chorismate (Peng et al., 2021). Upon stress, chorismic acid gets converted into chorismite, then to IC in the chloroplast. The IC is transported to the cytosol and converted to SA by the activity of isochorismate synthase and isochorismate pyruvate lyase. While in the PAL route, chorismate is converted to phenylalanine by the action of chorismate mutase. Following this, Phenylalanine undergoes conversion into *trans*-cinnamic acid, which can further transform into ortho-coumaric acid or benzaldehyde. SA can be directly synthesized from ortho-coumaric acid, whereas benzaldehyde requires additional conversions to benzoic acid and subsequently to 2-hydroxy benzoic acid. The IC route appears to be responsible for approximately 90% of SA production (Ding & Ding, 2020; Lefevere et al., 2020; Monte, 2023; Rieseberg et al., 2023).

SA has been recognized mostly as a regulatory signal molecule mediating plant response to biotic and abiotic stresses; however, this hormone has a crucial role also in seed germination, growth, photosynthesis and respiration, thermoregulation, flowering and senescence (Rivas-San Vicente & Plasencia, 2011).



**Figure 3. Structure of SA**

### 2.2.3 Role of jasmonates and SA in elicitation

The effects of exogenous application of JA/MeJa and SA on eliciting the production of SMs in MAPs have been well documented in *in vitro* studies (Hyeon et al., 2024; Sharma et al., 2015; Silva-Santos et al., 2023; Sukito & Tachibana, 2016). In contrast, their effects *in vivo* have been less extensively investigated, and more frequently, the results of elicitation are contradictory, which might be due to the complexity of influencing factors and physiological processes. Several factors may influence the effect of these elicitors including the plant species and cultivar used, developmental stage, growing conditions, homeostasis, or stress exposure. Also, the application method and timing, type and concentration of the elicitor may be of significance (Fatemi et al., 2020; Kandoudi & Németh-Zámboriné, 2022; Kianersi et al., 2021; Medeiros et al., 2024).

In parallel with the enhancement of SMs, plant tolerance to abiotic stress conditions could also be developed by the application of elicitors such as MeJa and SA. In the case of heavy metal toxicity, phytohormones have been demonstrated to mitigate their effects by regulating the mechanochemical components of cell walls and enhancing the production of antioxidant compounds (Fan et al., 2024; Giménez-Bañón et al., 2023; Per et al., 2016). Studies demonstrate the mitigating effects of JA and SA in various other abiotic stresses, including drought, salinity, light, heat, cold, and wounding (Estaji & Niknam, 2020; Hao et al., 2017; Otálora et al., 2022; L. J. Wang et al., 2010; Wongshaya et al., 2020). An overview of the published reviews in the last 10 years about the effects of MeJa and SA is presented in Table 2.

**Table 2 Review papers and their main topics dealing with elicitation by MeJa and/ or SA**

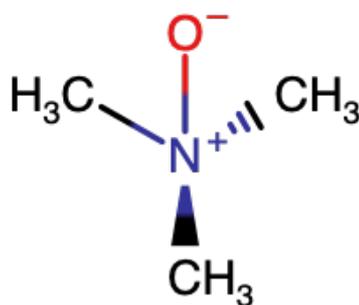
<b>Publication</b>	<b>Main topics</b>
Baenas et al., 2014	Elicitation in general, biotic and abiotic elicitors, effects on SMs *
Ramirez-Estrada et al., 2016	Elicitation <i>in vitro</i> and enhancing the production of SMs
Narayani & Srivastava, 2017	Elicitation <i>in vitro</i> and enhancing the production of SMs, biotic and abiotic elicitors, factors affecting elicitation
Wasternack & Song, 2017	Jasmonates: genes, enzymes and pathways of biosynthesis and signaling
Jamwal et al., 2018	The effects of growth regulators (including Ja, MeJa, SA) on SMs of MAPs *
Thakur et al., 2019	Chemical elicitation results by various molecules on SMs *
Wasternack & Hause, 2019	Jasmonates: biosynthesis, signaling network, involved genetic mechanisms, hormonal crosstalk
Ho et al., 2020	Role of exogenous MeJa in oxidative stress and accumulation of SMs in plant cell and organ cultures
Ali, 2021	The effect of SA on the elicitation of different SMs*
Gutiérrez-Gamboa et al., 2021	The effects of exogenous MeJa applications to grapevines on grape and wine quality*
Jan et al., 2021	SMs responses to biotic and abiotic elicitors*
Jogawat et al., 2021	Phytohormones crosstalk in crop plants under drought stress and the role of their exogenous application in SMs accumulation*
Nabi et al., 2021	Responses of <i>in vitro</i> cell cultures to elicitation by JA/MeJa
Assaf et al., 2022	The effects of growth regulators (including Ja, MeJa, SA) on SMs of Lamiaceae species*
Kandoudi & Németh-Zámboriné, 2022	<i>In vivo</i> elicitation of SMs of medicinal plants by MeJa and SA *
Jeyasri et al., 2023	<i>In vitro</i> elicitation of SMs of medicinal plants by MeJa and SA
Rehman et al., 2023	Role of JA in plant stress mitigation
Ali et al., 2024	Role of SA in plant abiotic stress tolerance and phytohormone crosstalk
Jalota et al., 2024	Stimulating effect of elicitors on cultured cells (including MeJa and SA)
Min et al., 2024	Role of exogenous application of MeJa in regulating postharvest fruit and vegetable disease resistance (Meta analysis) *

\**in vivo* plant results are *also* mentioned.

### 2.3 Trimethylamine N-oxide

TMAO is an organic solute belonging to the amine oxides class (Figure 4). It is a naturally occurring osmolyte in humans, animals, and plants. It acts by protecting the cellular components against osmotic stresses and protecting protein stability. In animals, TMAO plays a crucial role in maintaining the proper folding of proteins and neutralizing perturbation effects caused by denaturing agents such as pH changes, urea, high pressure, high salt concentrations, and low temperatures. While in humans, high levels of the compound in plasma serve as an indicative marker of an increased cardiovascular risk (Ufnal et al., 2015; Yancey, 2001).

Osmolytes like sucrose, trehalose, proline, and glycinebetaine, play crucial roles in safeguarding plants through various mechanisms. These include facilitating cellular osmotic adjustment and stabilization, inhibiting ROS production, and preserving membrane integrity (Slama et al., 2015; Tognetti et al., 2013). Under drought stress, the osmoprotectants are synthesized and transported into the cell, resulting in a strong negative osmotic potential, thus leading to an inward flow of water into the cell to sustain turgor pressure (Sharma et al., 2019). Osmolytes have been proven to enhance the accumulation of certain SMs with high antioxidant properties. (Abdelkader et al., 2019; Gholami Zali & Ehsanzadeh, 2018; Ozden et al., 2009). Surprisingly, evidence about the endogenous accumulation of TMAO in plants was lacking until Catalá et al. (2021) detected its presence in *Arabidopsis thaliana*, *Solanum lycopersicum*, *Hordeum vulgare*, and *Nicotiana benthamiana*. Moreover, abiotic stresses, such as drought, low temperature, and salt, were found to remarkably elevate its accumulation. The transcriptome analyses revealed TMAO's ability to induce the expression of genes involved in the abiotic stress through still unknown pathways.



**Figure 4 Structure of TMAO**

## 2.4 Basil

The *Ocimum* genus belongs to the Lamiaceae family and includes commercially important MAPs spread all over the temperate parts of the world; they are extensively used in the food, flavor, and perfume industries. *Ocimum* comprises a variety of species, among which sweet basil (*Ocimum basilicum* L.) seems to be the most popular (Gurav et al., 2021; Singh et al., 2015). Sweet basil is an annual herbaceous plant native to tropical and warm regions but has been naturalized in various regions worldwide. The plant can reach up to 60 cm in height, the leaves are generally ovate or oblong, long petioled, and decussate, spotted with numerous oil glands. The small flowers are typically white and the nutlets are dark in color (Hiltunen & Holm, 1999; Suddee et al., 2005).

There is considerable intraspecific variability in basil, observed both morphologically and biochemically (Carović-Stanko et al., 2011). Basil plants thrive in sunny and warm conditions and prefer well-drained soils. Propagation of basil can be achieved mostly through seed sowing, and planting typically occurs once the threat of frost has subsided. In temperate climates, two harvests are practiced (Pushpangadan & George, 2012).

In numerous countries, fresh or dried sweet basil leaves or their EO hold popularity as a culinary herb and are widely utilized as a food ingredient to enhance the flavor of baked goods, meat products, sauces, oils, and vinegars (Charles, 2013b). Infusions of *O. basilicum* are often used in traditional medicine to treat various ailments. In Morocco they are utilized against hyperlipidemia and to prevent atherosclerosis (Harnafi et al., 2009; Rachmawati et al., 2019). In Ayurveda, the whole plant is used to address respiratory diseases such as asthma and bronchitis, eye inflammation, fever, ear pain, cephalalgia, digestive problems, and malaria (Shahrajabian et al., 2020; Udayan & Balachandran, 2011). Basil extracts have antiviral, antibacterial, and antifungal activity against multiple strains, including multi-resistant ones (Chiang et al., 2005; Stanojevic et al., 2017). Besides, several scientific studies have ascertained the antioxidant, antitumor, anti-inflammatory, antidiabetic, and neuroprotective effects of basil, at least in *in vitro* studies (Ahmed et al., 2019; Eid et al., 2023; Rodrigues et al., 2016; Singh et al., 2022; Stanojevic et al., 2017).

*O. basilicum* is characterized by diverse chemical composition. Varga et al. (2017) proposed an intraspecific classification of *O. basilicum* into five chemotypes, and they concluded that 2 chemotypes can be considered European, one tropical, and another one specific to Reunion. Furthermore, they noticed that there is no correlation between the chemotypes and the morphotypes. Appendix 2 Table 1 shows examples of EO composition corresponding to different chemotypes of basil.

Considerable seasonal variations in EO composition have been observed in Brazil (Pinto et al., 2019). Factors such as fertilization type, temperature, and light intensity also significantly impact EO profiles (Milenković et al., 2019; Teliban et al., 2022). *O. basilicum* extracts reveal a rich polyphenol profile as well. A methanolic extract analysis confirms the presence of the phenolic acids rosmarinic acid, 3,4-dihydroxyphenylacetic acid, caffeic acid, caftaric acid, ferulic acid, chlorogenic acid, and rutoside (Bajomo et al., 2022; Elansary et al., 2020)

Several former studies about the elicitation of basil SMs exist. Significant differences were observed with SA treatments on the EO content (Gharib, 2006; Mirzajani et al., 2015), phenolics (Nazir et al., 2021), as well as in the ability to mitigate salt stress (Elhindi et al., 2017). The application of JA also changed the composition of *O. basilicum* oil with a notable increase in linalool and eugenol. Besides, new compounds, absent in the control, were detected (Złotek et al., 2016). The accumulation of phenolic compounds such as rosmarinic acid and caffeic acid was enhanced significantly after foliar treatments with MeJa in basil (Kim et al., 2006). Li et al. (2007) reported the same results along with elevated accumulation of enzymes involved in the biosynthesis of terpenoids and phenolics such as LOX and the P450 monooxygenase cinnamate-4-hydroxylase. Environmental stress, such as drought, can also enhance the accumulation of EO, glandular hairs density, and TPC. However, the water deficit results in significant yield losses, limiting its potential as an effective elicitor (Mulugeta et al., 2023).

## 2.5 Hyssop

Hyssop (*Hyssopus officinalis* L.) is a perennial sub-shrub belonging also to the Lamiaceae family. The name originates from the Hebrew word “ezob” which means sacred herb. Hyssop is commonly found in the wild flora of the Mediterranean region and cultivated in many countries (Fathiazad & Hamedeyazdan, 2011; Kokkini et al., 2003). Hyssop plants can reach up to 80 cm tall, the shoots are branched and woodening at the bottom. The leaves are small, lanceolate, with a dark green color. The spikes inflorescence consists of tubular flowers purple, pink, violet, or white. (Judžentienė, 2016; Ravindran et al., 2007). Hyssop can be propagated through seed sowing or vegetatively by cuttings. This plant can thrive in soils ranging from dry to moderately moist and flourishes in sunny and warm climates (Judžentienė, 2016; Sharifi-Rad et al., 2022).

The aromatic leaves and flowering shoots of *H. officinalis* serve as flavoring additives to salads, soups, meat and they are also utilized in the production of bitters and liquors. Its EO finds application in the perfumery industry for making soaps, creams, and fragrances. The aerial parts are traditionally used as part of spiritual cleansing rituals in churches and temples. Due to its colorful flowers, hyssop is also used as an ornamental plant (Fathiazad & Hamedeyazdan, 2011;

Fernández-López et al., 2003; Kazazi et al., 2007). Hyssop oil, infusions, or extracts are used in folk medicine to treat digestive problems, laryngitis, asthma, bronchitis, coughs, and colds due to their anti-inflammatory, antiviral, and antimicrobial effects (Ma et al., 2014; Mićović et al., 2022; Özer et al., 2006; Toby et al., 2010). Externally, hyssop can be used to accelerate wounds and contusion healing (Alexandru et al., 2015; Larki-Harchegani et al., 2021). Moreover, hyssop is believed to be expectorant, sedative, diaphoretic, carminative, and has antioxidant and myorelaxant activities (Dragland et al., 2003; Lu et al., 2002). Overall, hyssop oil is considered safe in small dosages. Nevertheless, when administered in high dosages, it may lead to convulsions and seizures due to the presence of high levels of monoterpene ketones in the oil (Burkhard et al., 1999).

Generally, the most abundant compounds of the hyssop EO are  $\alpha$ -pinene,  $\beta$ -pinene, camphene, isopinocamphe, and pinocamphe along with other constituents (Judžentienė, 2016). Examples of the EO composition from hyssop grown all over the world is presented in Appendix 2 Table 2.

The volatile composition of hyssop also depends on ontogenetic phase, proportion of flowers and leaves, and weather conditions (Németh-Zámbori, 2020). Like most *Lamiaceae* species, hyssop also accumulates polyphenols in significant concentrations. The analysis of alcoholic extracts of hyssop from Turkey identified chlorogenic acid (major phenolic acid), caffeic acid, p-coumaric acid, protocatechuic acid, and catechin as major phenolic components (Hatipoğlu et al., 2013). In Romanian accessions, chlorogenic acid was detected only at a concentration below 0.2%. Additionally, three flavonoid glycosides (isoquercitrin, rutin, and quercitrin) and two flavonoid aglycones (quercetin and luteolin) were found in the extracts (Vlase et al., 2014). The concentration of phenolics may also depend on the nutrient supply: foliar application of selenium significantly increased the TPC, total hydroxycinnamic acids, rosmarinic acid, and chlorogenic acid (Skrypnik, Feduraev, et al., 2022).

Elicitation of hyssop has been rarely discussed compared to the other species. Concerning jasmonates, only one study explored the effect of JA on the plant and found that 200  $\mu$ L JA significantly decreased the ratio of major monoterpenes, among others that of *cis*-pinocamphe and *trans*-pinocamphe (Ghasemi Pirbalouti et al., 2013). A study about the effect of 1 mM SA treatment increased the biomass, height and EO content (Sharifi, 2017). On the other hand, Fouad et al. (2023) showed that the effect of SA depended on the irrigation level and the elicitor's dosage, where the highest levels of TPC and antioxidant activity were obtained when the plants were watered once a week and treated with 200 ppm SA, while the highest EO content and yield were

found when irrigation was applied twice a week and treatments with 100 ppm SA. Therefore, choosing the best elicitation condition depends on the desired metabolites (Skrypnik, Feduraev, et al., 2022).

## 2.6 Marjoram

Sweet marjoram (*Origanum majorana* L.), previously known as *Majorana hortensis* Moench; is a perennial plant native to Cyprus and southern Turkey and distributed in many Mediterranean countries such as Morocco, Italy, Spain, and Portugal. However, it is cultivated as an annual plant in many countries all over the world (Ietswaart & Hague, 1980). Marjoram belongs to the *Lamiaceae* family and grows up to 30-60 cm high with descending, reddish-brown stems and hairy branches (Singla & Vasudeva, 2014). Leaves are petiolate, highly aromatic, oblong-ovate, and their surface is covered with hairs. The flowers are small, tubular, with white or light pink colors. The fruit is a single-seeded nutlet (Bouyahya et al., 2021; Charles, 2013a; Ietswaart & Hague, 1980; Wilson, 2016). Sweet marjoram is a frost-sensitive plant that typically thrives in fertile and well-drained loamy soil with a neutral to alkaline pH. Propagation can be achieved mostly by seed sowing. Harvesting is carried out once the plants start flowering and can be repeated two to three times, depending on the region. (El-Wahab, 2013; Nurzyńska-Wierdak & Dzida, 2009).

The leaves of sweet marjoram, dried or fresh, as well as its EO or even whole plant, have versatile uses. It is employed in cuisine as a condiment to season dishes, including meat, sausages and salamis, soups, pizza, and stews. Additionally, it is used as an ingredient in salads and serves as a food preservative. Beyond the kitchen, marjoram extracts and oil are commonly used to impart scent to fragrances, lotions, and soaps (Bhardwaj & Dubey, 2020; Charles, 2013a). In traditional folk medicine, marjoram is used to treat colds, rhinitis, toothaches, coughs, diabetes, and gastrointestinal problems. Externally, marjoram oil is applied to alleviate menstrual cramps, address insomnia, and provide relief from rheumatism (Cinbilgel & Kurt, 2019; El Hafian et al., 2014; El-Hilaly et al., 2003; Fleming, 2000; Loi et al., 2005).

It has been proven that marjoram has a strong antimicrobial activity (Amor et al., 2019; Marques et al., 2015; Pepa et al., 2019; Ramos et al., 2011) and high antioxidant capacity (Deuschle et al., 2018; Chaves et al., 2019; Khadhri et al., 2019; Leeja et al., 2007; Mossa et al., 2013; Vasudeva et al., 2014). Ethanolic extracts from marjoram have exhibited potential antidiabetic activity (Perez Gutierrez, 2012), antiparasitic and insecticidal (El-Akhal et al., 2014; N. Sharma et al., 2016), antispasmodic (Makrane et al., 2019), chemopreventive (Abdellatif & Alsharidah, 2023; Al Dhaheri et al., 2013), and hepatoprotective effects (El-Ashmawy et al., 2005; Mossa et al., 2013).

The most important biological constituents are volatiles. The EO of marjoram is rich in monoterpenes hydrocarbons and oxygenated monoterpenes, along with sesquiterpenes, the latter in small ratios. Several studies classified marjoram into two chemotypes: the terpinen-4-ol/*cis*-sabinene hydrate type and the carvacrol/thymol type, the latter type primarily found in wild-growing populations. The presence of this chemotype has been questionable and may be attributed to taxonomic ambiguities between two closely related species, *O. dubium* and *O. vulgare* (Baser et al., 1993; Lukas et al., 2013; Sarer et al., 1982; Sellami et al., 2009; Vera & Chane-Ming, 1999).

Similarly to other species, multiple factors may influence the composition of the EO, such as the cultivar, phenological stage, climate conditions, post-harvest techniques and method of extraction (Arnold et al., 1993; Böttcher et al., 1999; Farsi et al., 2019; Fischer et al. (1987, 1988), Komaitis et al., 1992; Ragab et al., 2019; Sellami et al., 2009). Some examples are collected in Appendix 2 Table 3. Besides the terpenoids, sweet marjoram is rich in nonvolatile phenolic compounds like phenolic acids, flavonoids, tannins, and proanthocyanidin, including hesperetin, 5,6,3'-Trihydroxy-7,8,4'-trimethoxyflavone, hydroquinone, rosmarinic acid, and arbutin (Erenler et al., 2016; Khadhri et al., 2019).

There are several references discussing the morphological, physiological, and biochemical responses of marjoram to elicitation. SA treatments revealed distinct effects depending on their concentration. 0.1 mM SA increased fresh and dry mass, number of branches, as well as concentration of photosynthetic pigments, macro- and micronutrients in the plant. In contrast, EO content, sabinene, p-cymene, and  $\gamma$ -terpinene proportions were predominantly affected by the concentration of 1 mM (Gharib, 2006). Jasmonates have also been proven to enhance the accumulation of phenolics, such as rosmarinic, caffeic, and chlorogenic acids, in callus cultures of marjoram (Korkor et al., 2017), chlorophylls, carotenoids, and the antioxidant activity in *in vivo* plants (Złotek, 2017). Abiotic elicitors have also been studied in marjoram. Salt treatments of 75 mM NaCl significantly increased the accumulation of TPC and the content of flavonoids and tannins (Baâtour et al., 2011). On the other hand, moderate drought conditions (50% field capacity) significantly decreased the biomass, the EO yield, in addition to sabinene,  $\gamma$ -terpinene, and  $\alpha$ -terpineol components. However, the EO percentage and the main volatile *trans*-sabinene hydrate increased under water deficit conditions. Moreover, the addition of 100  $\mu$ M MeJa treatments to stressed plants could not mitigate the negative effects of drought (Farsi et al., 2019).

## 2.7 Peppermint

The genus *Mentha* belongs to the Lamiaceae family and includes 42 species, 15 hybrids, and numerous subspecies, varieties, and cultivars. The taxonomy of this genus is intricate and complicated owing to its genetic diversity, polymorphism, and the ease with which hybridization occurs (Lawrence, 2006; Salehi et al., 2018; Tafrihi et al., 2021). Some of the species that hold significant economic benefits are *M. arvensis*, *M. canadensis*, and *M. spicata*, in addition to the hybrids *M. gracilis*, and *M. piperita*. They have long been used in food flavoring, pharmaceuticals, and perfumery (Tucker, 2006). Peppermint (*Mentha x piperita* L.) is a perennial aromatic herb, a triploid, sterile hybrid. The plant's characteristic flavor profile, peppery and spicy, is behind its nomenclature (Tyler et al., 1988). Due to its sterility, peppermint plants do not produce seeds; its propagation is achieved traditionally with its underground stems called stolons. The plant can reach up to 80 cm in height and has dark green leaves whose blade contains glandular trichomes on both sides. The stem is purplish, erect, and branches towards the top. The flowers are small and light purple, arranged in a dense whorl-like cylindrical terminal spike (Pushpangadan & Tewari, 2006).

Peppermint has a long history of use. Fresh or dried leaves are often utilized alone or with other herbs to prepare tea and different types of aromatic beverages, as a curing agent against throat infections, bronchitis, coughs, nausea, and digestive problems, to relieve menstrual cramps; and to reduce the symptoms of irritable bowel syndrome (Alammar et al., 2019; Boon et al., 2004). Moreover, peppermint oil is extensively added as a flavoring agent in cosmetics (soaps, shampoo, deodorant, and lotions), oral hygiene products such as mouthwash and toothpastes, and food products (ice creams, chocolates, and sweets) (Anwar et al., 2019; Mahendran & Rahman, 2020). Peppermint drugs and products have antimicrobial, antioxidant, antiseptic, antiparasitic, carminative, and antidiabetic properties (Table 3) (Camele et al., 2021; McKay & Blumberg, 2006; Nayak et al., 2020; Tucker, 2006). Peppermint leaves are beneficial to boost the quality of broiler chickens and eggs (Abdel-Wareth et al., 2019; Abdel-Wareth & Lohakare, 2014) or the growth of fishes (Adel, Abedian Amiri, et al., 2015; Adel, Safari, et al., 2015; Talpur, 2014). Peppermint EO has herbicidal, insecticidal, and antifungal properties, rendering it an excellent alternative to chemical pesticides (Kumar et al., 2012; Mahdavia & Saharkhiz, 2015; Morais et al., 2015). Table 3 summarizes some of the biological effects of peppermint extracts and EO.

**Table 3 Biological activities of peppermint extract and EO**

<b>Extract</b>	<b>Biological activity</b>	<b>Reference</b>
<b>Aqueous</b>	Antimicrobial	(Oh et al., 2013)
	Antioxidant	(Dorman et al., 2009)
	Antiviral	(Geuenich et al., 2008)
<b>Methanolic/ethanolic</b>	Antioxidant	(Farnad et al., 2014)
	Antiviral	(Yucharoen et al., 2012)
	Insecticidal	(Sharma & Vidyarthi, 2010)
	Anti-inflammatory	(Li et al., 2017)
<b>Essential oil</b>	Antimicrobial	(Camele et al., 2021)
	Antidiabetic	(Abdellatif et al., 2017)
	Antiviral	(Schuhmacher et al., 2003)
	Antifungal	(Desam et al., 2019)
	Antispasmodic	(de Sousa et al., 2010)
	Antiallergic	(Park et al., 2022)
	Anti-inflammatory	(Kehili et al., 2020)

Peppermint plants accumulate a diverse range of bioactive phytochemicals, contributing to their array of biological properties. Peppermint EO has been extensively studied and utilized due to its high economic importance (Orio et al., 2012; Radivojac et al., 2021). The main chemical components of peppermint oil include menthol, menthone, menthyl acetate, isomenthone, limonene, 1,8-cineole, menthofuran, and pulegone, the last one being limited to a maximum of 3% of EO according to the European Pharmacopeia (Council of Europe, 2019; HMPC, 2020). The main sesquiterpenes are germacrene D and  $\beta$ -caryophyllene (Mahendran & Rahman, 2020). The influencing factors of component ratios in peppermint oil are rather well studied and include cultivar, morpho-phenological factors, weather and stress conditions, among others (Abdi et al., 2019; Figueiredo et al., 2008; Kandoudi et al., 2023; Németh-Zámbori, 2020). Differences of some oil samples from diverse geographical locations are shown in Appendix 2 Table 4. It has long been known that ontogenesis strongly influences the composition of the EO, with ratios of menthol and menthyl-acetate increasing during the development while menthone decreases (Grulova et al., 2015). Other factors, including drying and distillation methods, can also contribute to the chemical variability of the oil (Beigi et al., 2018; Dai et al., 2010).

Peppermint plants contain several polyphenols, including the flavanone eriocitrin, the phenolic acids rosmarinic and caffeic acid, as well as flavonoids such as hesperidin, luteolin, and quercetin (Areias et al., 2001; Dorman et al., 2009; Fecka & Turek, 2007). Much like the chemical diversity

of the EO, variance in the ratio of polyphenolic compounds is also present in peppermint extracts (Farnad et al., 2014).

Numerous elicitation methods have been applied to peppermint plants to enhance their SMs. Titanium dioxide (TiO<sub>2</sub>) nanoparticles enhanced the biomass, the EO content, its menthol ratio (Ahmad et al., 2018). Similarly, the accumulation of rosmarinic acid, cinnamic acid, TPC, menthol, as well as the growth parameters were enhanced by the application of TiO<sub>2</sub> on peppermint (Shenavaie Zare et al., 2022). SA and MeJa treatments of *in vitro* propagated peppermint plants elevated the ratio of menthol, 1,8-cineole, β-pinene, and menthofuran. For menthone it was found that SA reduced, while MeJa increased its accumulation (Qaderi et al., 2023). In case of SA elicitation, the molecular background investigation has revealed the up-regulation of the gene encoding the enzyme responsible for the biosynthesis of p-coumaroyl-CoA, an important precursor of rosmarinic acid, hesperidin, and naringin, thereby explaining their enhanced accumulation in peppermint extracts (Figuroa Pérez et al., 2014; Figuroa-Pérez et al., 2019).

It appears that peppermint's SMs are highly susceptible to change when the plant encounters stress, either biotic or abiotic. Figuroa-Pérez et al. (2014) and Németh-Zámbori et al. (2017) emphasized the eliciting effects of drought on the phenolic accumulation and composition. Under water deficit, the protective effect of MeJa was demonstrated to enhance the accumulation of TPC, phenolic acids, and flavonoids, as well as the growth parameters (Abdi et al., 2019).

## 2.8 Yarrow

The Asteraceae family (or Compositae) ranks as one of the largest flowering plant families, boasting over 23,000 species and 1,600 genera, including the *Achillea* genus. The genus *Achillea* itself encompasses approximately 130 species, primarily native to Eurasia, with a few species found in northern Africa and North America. The taxonomy classification of *Achillea* species proves intricate due to intraspecific differentiation, polyploidy, and spontaneous hybridization, leading to morphological, genetic, and chemical diversity. One of the most significant species of this genus is *Achillea collina* Becker (Bessada et al., 2015; Németh & Bernath, 2008). *A. collina* is a spontaneous hybrid of *A. setacea* and *A. asplenifolia*, native to Europe and Asia, and belongs to the highly diverse group *Achillea millefolium* (Table 4). The plant is named after the ancient figure “Achilles” in Greek mythology who used it to heal his soldiers' wounds. While *millefolium* refers to a thousand featherlike leaves (Chandler et al., 1982; Kindlovits & Németh, 2012).

**Table 4 Achillea species belonging to the Millefolium group**

<b>Chromosome number</b>	<b>Group Millefolium</b>
2n	<i>A. asplenifolia</i> Vent.
2n	<i>A. setacea</i> W. et K.
2n	<i>A. roseo-alba</i> Ehrend.
4n	<i>A. collina</i> Becker
6n	<i>A. millefolium</i> L.
8n	<i>A. pannonica</i> Scheele
2n, (4n)	<i>A. ceretanica</i> Sennen
4n	<i>A. asiatica</i> Serg.
4n	<i>A. lanulosa</i> Nutt.
4n	<i>A. partensis</i> Saukel & Länger
4n	<i>A. styriaca</i> Saukel
6n	<i>A. borealis</i> Bong
<b>Group Distans</b>	
6n	<i>A. distans</i> W. et K.
6n	<i>A. stricta</i> (Koch) Scheich et Gremlí
6n	<i>A. tanacetifolia</i> All.

Yarrow, a perennial herbaceous plant, can achieve a height of up to 120 cm, producing one to several erect or ascending stems. The leaves are evenly distributed, with those near the bottom being the largest. These leaves are lanceolate, twice or thrice pinnately cut. The flowers are situated upon small disks and typically white or pink (Jan et al., 2021). Yarrow is drought- and salt tolerant, flourishes particularly well in warm and temperate regions. The species is propagated by seed sowing or by stock division. Due to its low ecological requirements, yarrow can be found in various habitats, including wetlands, steppes, grasslands, and along roadsides. It can be considered a weedy and invasive species in some habitats (Ijaz et al., 2020; Lakshmi et al., 2011).

The aerial parts of yarrow can be employed either fresh or dried to create infusions, decoctions, and tinctures. Additionally, they can be used externally in applications such as ointments and poultices (Chandler et al., 1982; Gourhan, 1975). Recent pharmacological findings have confirmed several traditional uses. The largest number of data have been accumulated about the antioxidant and anti-inflammatory effects, alongside, mostly *in vitro*, antimicrobial activities. Moreover, there are positive results on the analgesic, spasmolytic, anti-ulcer, choleric, hepatoprotective, and wound healing activities among others (Nemeth & Bernath, 2008).

The EO quality and quantity may vary greatly within the *Achillea millefolium* complex. However, according to the Ph. Eur, the EO of *Millefolii herba* should yield at least 2 ml/kg EO and should contain a minimum of 0.02% of proazulenes (Committee on Herbal Medicinal Products, 2020). The distilled EO is characterized by blue color due to the azulenogenic compounds (Todorova et al., 2007). The quality of the oil shows a large spectrum in the literature references, and the background is often not clear. Anomalies in identification, genetically fixed chemical diversity, differences in sampling or extraction methods, among others, might contribute to the mixed results (Németh-Zámbori, 2020). Besides, plant organ and differences in the phenological stage can also lead to both qualitative and quantitative changes in the oil (Kindlovits et al., 2016; Nemeth et al., 2007). Yarrow extracts are rich in phenolic compounds, including flavonoids and phenolic acids, too. The flavonoids are predominantly found as glycosides of apigenin, luteolin, and quercetin. In terms of phenolic acids, chlorogenic acid is present alongside caffeoylquinic acids (Giorgi et al., 2010, 2014). Flavonoid composition may have taxonomic correlations and therefore chemotaxonomic significance (Valant, 1978), however, mainly at the genus level. The intraspecific variability of phenolics in *A. collina* has been much less frequently studied than that of the volatile components.

According to our knowledge, limited research has been conducted on the effects of elicitation on *A. collina*. Most published data deal with *A. millefolium*. For instance, JA treated plants showed the presence of  $\alpha$ -fenchene, 4-methyl-acetate, *o*-cymene, and cubenol in the EO, compounds not present in control samples (Giorgi et al., 2015). Moreover, Giorgi et al. (2009) showed that yarrow plants grown in nitrogen deficient conditions increased their phenolic content. However, other parameters were negatively affected, such as growth, concentration of amino acids, proteins, chlorophylls, and carotenoids.

### III. Materials and methods

#### 3.1 Experimental site, design and plant material

##### 3.1.1 Open field experiments

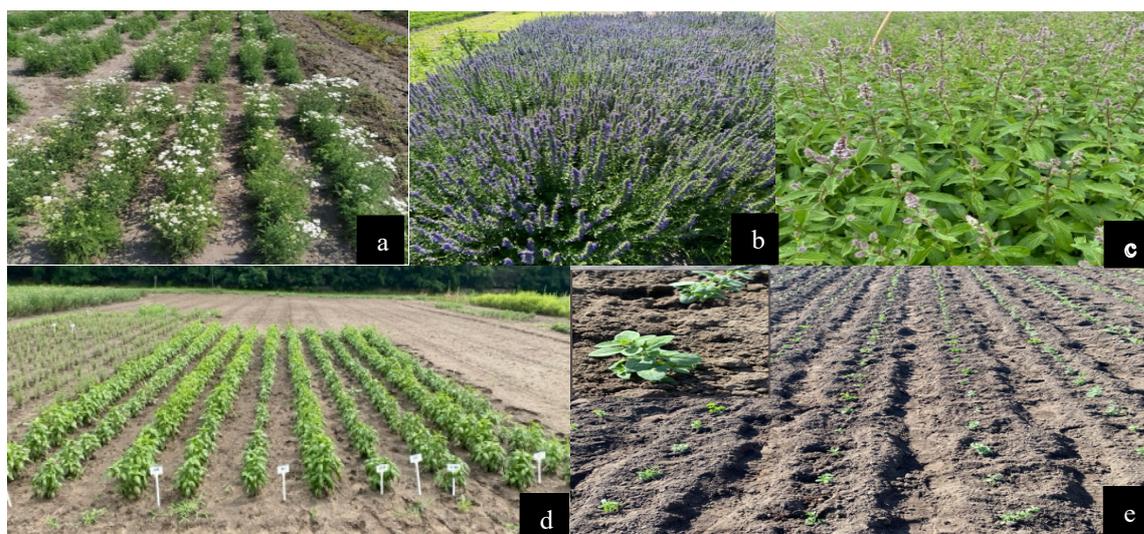
The open-field experiments were conducted at the Experimental Station of the University of Agricultural and Life Sciences (MATE) in Soroksár (Pest County, Hungary) (47.398820, 19.149270) over the summer of four consecutive years (2020–2023). The used genotypes are demonstrated in Table 5. All propagation material originated from the gene bank of the MAP Department, except for the 2020 *H. officinalis* plantation, where the seeds were collected from a city garden in Meran, Italy. However, in the subsequent years, we used the "Sophie" variety from the department's stock. Existing perennial plantations of *M. piperita* were used in the first year (2020), and new plots were prepared in the following year for the subsequent trials (2021 and 2022) propagated by healthy stolons from the mother plantation of MATE. As for *A. collina* Becker, the material was planted in 2020, and the same plantation was used for a second year in 2021. In the third year, new yarrow plants were propagated. *O. basilicum* and *O. majorana* were planted annually for four years from 2020 to 2023 and were the only species included in the 2023 experimental year. Seeds were sown in trays in the greenhouse, and after pricking out the seedlings, they were transplanted into open-field plots at the end of May/ beginning of June each year.

The plants were planted into plots or rows (varying between 10-20 m<sup>2</sup> according to species and year), well separated from each other by 50x50 cm spacing. In all experiments, it was assured that at least 10 healthy plants were available for each treatment (Figure 5).

During the vegetation period, regular irrigation was carried out every second day with a water dosage of 20–30 mm to maintain approximately 70% soil water content, along with manual weed control. The aerial parts of the plants were harvested by cutting them approximately 10 cm (in the case of hyssop 15 cm) above the soil surface, then air-dried in a sun-protected, dark but well-ventilated room for approximately two weeks. The phenological phase of the harvest was determined according to the usual agrotechnology of these crops, thus, at the beginning of flowering.

**Table 5 Genotypes and age of the experimental plants during the study years**

Species	2020	2021	2022	2023
<i>Achillea collina</i>	‘Azulenka’ (1 <sup>st</sup> year)	‘Azulenka’ (2 <sup>nd</sup> year)	‘Azulenka’ (1 <sup>st</sup> year)	-
<i>Hyssopus officinalis</i>	Meran gene bank accession (3 <sup>rd</sup> year)	‘Sophie’ (1 <sup>st</sup> year)	‘Sophie’ (2 <sup>nd</sup> year)	-
<i>Mentha x piperita</i>	‘Mexian’ (4 <sup>th</sup> year)	‘Mexian’ (1 <sup>st</sup> year)	‘Mexian’ (2 <sup>nd</sup> year)	-
<i>Ocimum basilicum</i>	‘Genovese’ (1 <sup>st</sup> year)	‘Genovese’ (1 <sup>st</sup> year)	‘Genovese’ (1 <sup>st</sup> year)	‘Genovese’ (1 <sup>st</sup> year)
<i>Origanum majorana</i>	‘Magyar’ (1 <sup>st</sup> year)	‘Magyar’ (1 <sup>st</sup> year)	‘Magyar’ (1 <sup>st</sup> year)	‘Magyar’ (1 <sup>st</sup> year)

**Figure 5 Open field set of our experimental species: yarrow (a), hyssop (b), peppermint (c), basil (d), marjoram (e) (Photo: Kandoudi, 2022)**

### 3.1.2 Greenhouse experiments

Semi-controlled environment experiments were carried out for two consecutive years (2021 and 2022) in the spring at the MATE Buda campus with two species, basil and marjoram. In 2021, the seedlings of both species were purchased from a commercial company, Zöldpont Kft. (Albertirsa, Hungary), whereas in 2022, only marjoram was planted from ‘Magyar’ seedlings, sown at Soroksár Experimental Station of MATE before transporting the seedlings to Buda campus. All plants were grown in 12 cm diameter pots filled with a commercial soil mixture (Florasca B) (Florasca Kft, Osl, Hungary) and watered regularly with optimum water supply until harvest. The aerial parts of the plants were harvested at phenological stages indicated in Table 9 by cutting them approximately 5 cm above the soil surface and then air-dried in a sun-protected, dark but well-ventilated room for approximately 10 days (Figure 6).

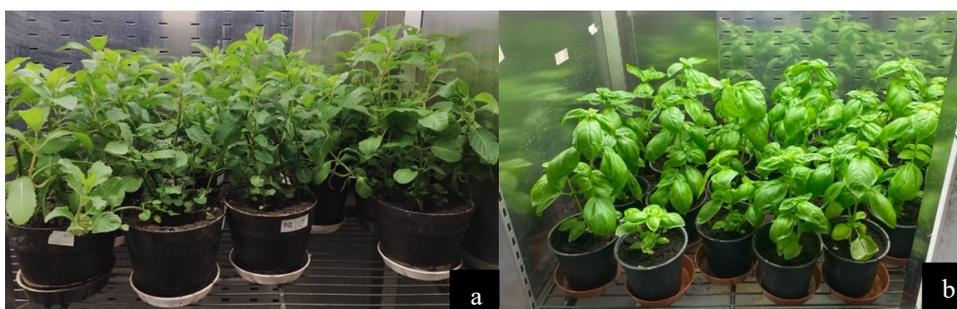


**Figure 6** Greenhouse experiments of marjoram (a) and basil (b), and airdried greenhouse basil (c) (Photo: Kandoudi, 2021)

### 3.1.3 Climatic chamber experiments

Controlled environment experiments were conducted during the autumn and winter seasons over two consecutive years (2021 and 2022) at the MATE Buda Campus climate chambers (Fitotron SGC120, Weiss Gallenkamp Ltd., Loughborough, Leicestershire, UK) with two species: peppermint (‘Mexian’) in 2021, propagated from a mother plantation at MATE, and basil (‘Genovese’) in 2022, propagated from seeds at the Soroksár Experimental Station of MATE.

The climate chambers were programmed according to each plant's specific needs and previous experiences of the department staff (Kandoudi et al., 2023). For basil, temperatures ranged between 24 and 27 °C during the day and 18–20°C at night, while peppermint conditions were set to 25–27°C during the day and 15–17°C at night. The light cycle for both species was regulated at 14 hours of light and 10 hours of darkness, with a light intensity of 14,500 lux provided by fluorescent (4200K) and incandescent (2700K) lamps. Air humidity was consistently maintained at 65%. All plants were grown in 16 cm diameter pots filled with a commercial soil mixture (Florasca B) (Florasca Kft, Osli, Hungary) (Table 6) and watered regularly every second day until harvest, assuring approximately a water content of 70% soil water capacity. The aerial parts of the plants were harvested by cutting them before budding (peppermint) or at the early flowering stage (basil) approximately 5 cm above the soil surface, then air-dried in a sun-protected, dark but well-ventilated room for approximately 10 days (Figure 7).



**Figure 7** Climatic chamber experiments, peppermint (a), basil (b) (Photo: Kandoudi, 2022)

Soil properties and weather conditions during the 2020–2023 experimental periods are summarized in Table 6 and Figure 8.

**Table 6 Soil properties of open-field, greenhouse, and phytotron experiments**

<b>Experiment and year</b>	pH H <sub>2</sub> O	Humus content %	Lime content %	NO <sup>2+</sup> NO <sup>3-</sup> N mg/kg	P <sub>2</sub> O <sub>5</sub> mg/kg	K <sub>2</sub> O mg/kg	Zn mg/kg	Mg mg/kg	Mn mg/kg
<b>Soil in the experimental station 2020</b>	7.82	2.84	0.34	6.90	412.89	245.54	4.09	131.78	25.64
<b>Soil in the experimental station 2021</b>	7.47	1.62	4.20	16.70	398.67	826.33	8.08	174.19	138.99
<b>Soil in the experimental station 2022</b>	7.48	1.71	3.85	12.30	400.10	535.10	8.11	155.20	130.76
<b>Soil in the experimental station 2023</b>	7.62	1.96	2.55	5.50	523.00	235.00	8.87	143.00	120.00
<b>Soil in the greenhouse and phytotron pots</b>	5.49	8.16	<0.20	1401.50	875.50	3357.4 0	12.4	829.00	54.70

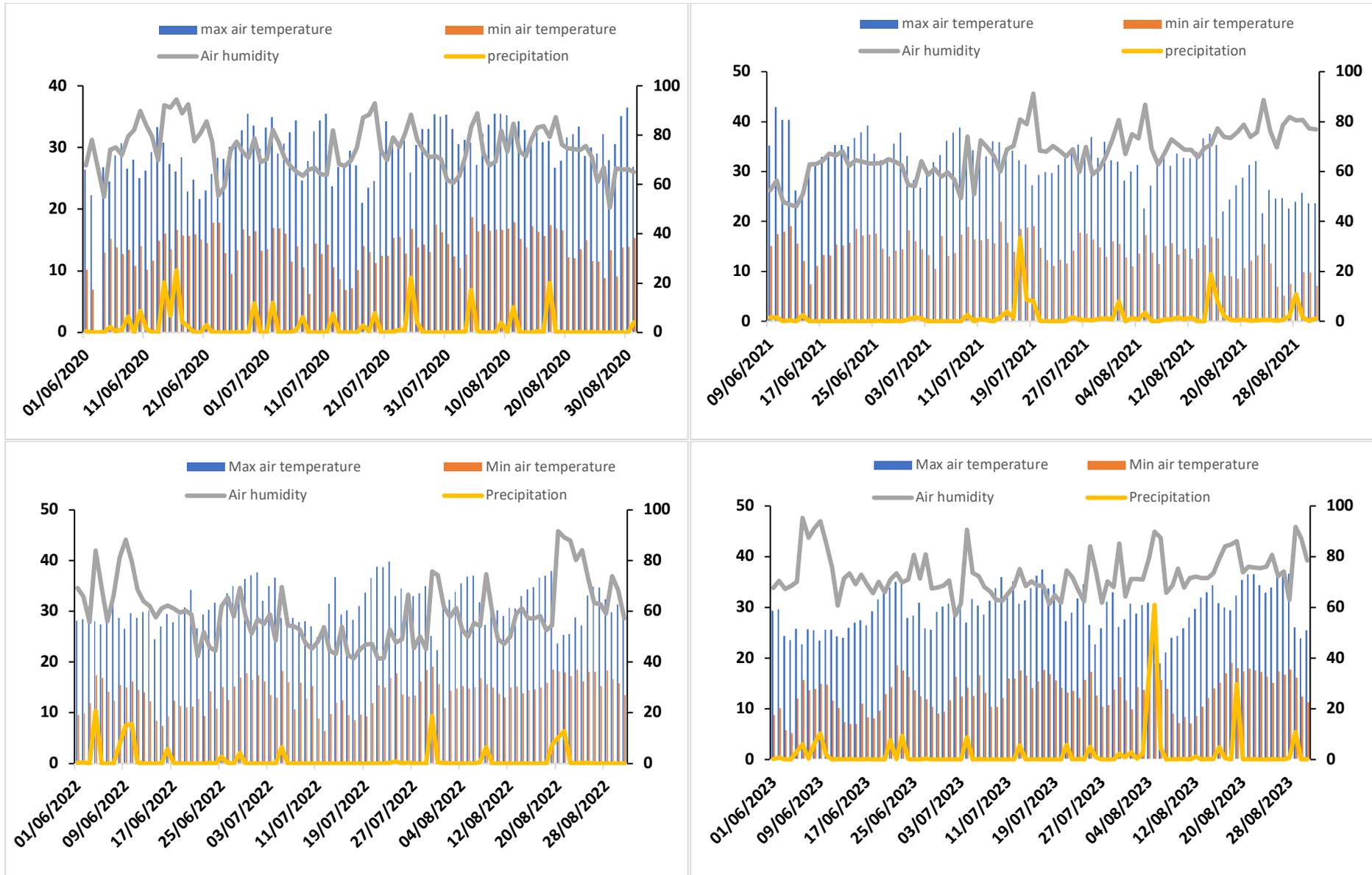


Figure 8 Main weather data of the experimental station, from June to August 2020-2023

## 3.2 Treatments

### 3.2.1 Open field elicitation

To evaluate the effects of elicitors on various plant species, we conducted open-field trials over three consecutive years (2020–2022) under consistent conditions. Treatments were started two weeks prior to each species' optimal harvest stage. Plants were sprayed with MeJa and SA, supplied by Sigma-Aldrich (Schnelldorf, Germany) and Kévés Béla Kft. (Soltvadkert, Hungary), respectively, in two concentrations (0.1 and 2.0 mM), dissolved in water with 0.3% ethanol. Control plots were sprayed with only water and ethanol. The experiment was repeated annually under similar conditions. In the second experimental year (2021), a third elicitor, TMAO (Merk Life Science Ltd., Hungary), was introduced at a single concentration (2.0 mM). Each solution was uniformly applied to the aboveground shoots using a hand sprayer (approx. 50 mL plant<sup>-1</sup>) (Figure 9). Treatments were applied twice, with a one-week interval between applications. Samples of each species were collected one week after the second treatment, determined after evaluating numerous literature data.

The trials followed a completely randomized block design in three replications per treatment. The phenological stage at harvest, along with the timelines of treatments and harvests for each plant species, is summarized in Table 7.



**Figure 9 Elicitor spraying to basil (a), hyssop (b) (Photo: Kandoudi, 2022)**

**Table 7 Treatment and harvest timelines with corresponding phenological stages in open field experiments**

		<b>Basil</b>	<b>Hyssop</b>	<b>Marjoram</b>	<b>Peppermint</b>	<b>Yarrow</b>
<b>2020</b>	<b>1<sup>st</sup> treatment</b>	29 July	12 June	26 June	26 June	14 July
	<b>2<sup>nd</sup> treatment</b>	5 August	19 June	2 July	2 July	21 July
	<b>Harvest date</b>	13 August	26 June	10 July	10 July	29 July
	<b>Phenological stage</b>	Full flowering	Full flowering	Budding	Full flowering	Full flowering
<b>2021</b>	<b>1<sup>st</sup> treatment</b>	28 June	12 July	28 June	28 June	7 June
	<b>2<sup>nd</sup> treatment</b>	5 July	19 July	5 July	5 July	14 June
	<b>Harvest date</b>	12 July	27 July	12 July	12 July	21 June
	<b>Phenological stage</b>	Beginning of flowering	Full flowering	Budding	Full flowering	Beginning of flowering
<b>2022</b>	<b>1<sup>st</sup> treatment</b>	1 July	6 June	1 July	20 June	13 June
	<b>2<sup>nd</sup> treatment</b>	8 July	13 June	8 July	28 June	20 June
	<b>Harvest date</b>	15 July	20 June	15 July	6 July	28 June
	<b>Phenological stage</b>	Full flowering	Full flowering	Budding / beginning of flowering	Beginning/ Full flowering	Full flowering

### 3.2.2 Elicitation under well-watered and non-irrigated conditions

To study the influence of elicitors on plants under abiotic stress, we applied two levels of water supply: irrigated (control) and non-irrigated (drought stress). Basil plants were selected for this experiment, which was conducted over two years (2020 and 2022) in open field conditions. Cultivation and treatment procedures followed the previously described protocol, using MeJa and SA as elicitors at two concentrations (0.1 and 2.0 mM) in both years, with TMAO (2.0 mM) added in 2022.

Two plots were designated for each water treatment, each containing six rows. Different elicitors were applied to each row, with approximately eight plants per row. Irrigated plots received 20 mm of water twice weekly, while non-irrigated plots relied solely on natural precipitation. Six healthy plants from each treatment and plot were selected for harvest. The timeline for treatments and harvest was consistent with that used for basil plants in the elicitation experiment (Table 7).

### 3.2.3 Effect of increased elicitor concentration

To further investigate the effects of hormonal elicitation and optimize their impact, elevated concentrations of MeJa and SA (10.0 mM) were selected for this study. Peppermint was chosen for experimentation in both open-field and controlled-environment settings. An additional high-concentration treatment was included with the other treatments of peppermint plantation on open

field plot in 2022, while the phytotron experiment with the elevated concentrations was conducted in November 2021.

The solutions, prepared in water with 0.3% ethanol, were applied as a foliar spray using a hand-pump sprayer, distributing approximately 20 mL per plant in the phytotron experiments and 50 mL per plant in the open-field experiments, ensuring uniform coverage of the leaves. To prevent cross-contamination, 12 pots from each treatment group were temporarily removed from the phytotron, sprayed individually, and allowed to dry before being returned to the chamber. Two weeks after the first treatment, bulk samples were harvested from the open-field plots, and 10 pots from each treatment group were selected from the experiment. The phenological stage at sampling and the timelines of treatments and harvests are provided in Table 8.

**Table 8 Treatment and harvest timelines with corresponding phenological stages in high concentration elicitation experiments**

	1 <sup>st</sup> treatment	2 <sup>nd</sup> treatment	Harvest	Phenological stage
<b>Climatic chamber</b>	21 January 2022	28 January 2022	3 February 2022	Vegetative
<b>Open field</b>	20 June 2022	28 June 2022	6 July 2022	First half of flowering

### 3.2.4 Effect of elicitation duration

Our research also focused on identifying optimal treatment schedules to maximize SM accumulation. For study of this aspect, marjoram plants were chosen. The experiments were conducted over three years (2021–2023) and aimed to track time-dependent dynamics in response to elicitor treatments. In 2021 a semi-controlled environment experiment was set up in a greenhouse, while in 2022 and 2023 open field trials were carried out according to the methodologies described above (chapters 3.1.1. and 3.1.2.).

However, only one treatment was applied across all trials: 2.0 mM MeJa. The harvest was made at varying time intervals. The plants were divided into five groups: control and treatment groups harvested 48 hours, 120 hours, 1 week, and 2 weeks after the elicitor treatment. The phenological stage at sampling and the timelines of treatments and harvests are provided in Table 9.

**Table 9 Treatment and harvest timeline with corresponding phenological stages in time interval elicitation**

	Treatments	Harvest	Phenological stage
<b>Greenhouse 2021</b>	From 28th April to 10th May	12 May 2021	Budding
<b>Greenhouse 2022</b>	From 23rd June to 5th of July	7 July 2022	Budding
<b>Open field 2023</b>	From 28th June to 10th of July	12 July 2023	Budding

### 3.2.5 Effect of repeated elicitor treatment

The frequency of elicitor treatments may influence elicitation outcomes, varying with the elicitor type, plant species, and growing conditions. In this study, the effects of applying 2.0 mM MeJa either once or twice were compared on two species, marjoram and basil, cultivated in different environments. For basil, the first experiment was conducted in a climate chamber at the Buda campus and the second in an open field setting in 2023. For marjoram, the first experiment was carried out in a greenhouse in spring 2021, and the second one took place in an open field setting in 2023.

In each experiment, plants were divided into three distinct groups: a control group (similarly to the above experiments), a group treated with a single application of 2.0 mM MeJa and harvested after two weeks, and a third group receiving two MeJa applications at a one-week interval and harvested one week after the second spraying. For sampling, 10 individual plants were randomly selected from each group. The phenological stage at sampling and the timelines of treatments and harvests are provided in Table 10.

**Table 10 Treatment and harvest timelines with corresponding phenological stages in repeated treatment experiments**

	1 <sup>st</sup> Treatment	2 <sup>nd</sup> treatment*	Harvest	Phenological stage
<b>Basil (climatic chamber)</b>	11 November 2022	18 November 2022	25 November 2022	Budding
<b>Basil (open field)</b>	28 June 2023	5 July 2023	12 July 2023	Beginning of flowering
<b>Marjoram (greenhouse)</b>	28 April 2021	5 May 2021	12 May 2021	Budding
<b>Marjoram (open field)</b>	28 June 2023	5 July 2023	12 July 2023	Budding

\* The second treatment included only water and 0.3% ethanol for the group receiving only a single application.

## 3.3 Methods of the measurements and analyses

### 3.3.1 Morphological and yield measurements

In open field experiments, plant height was measured for each treatment group prior to sample collection, with 10 plants measured from ground level to shoot tip in 2021 and 2022. Sampling involved cutting three plants per plot per treatment for basil and yarrow and ten plants per treatment for marjoram. For peppermint and hyssop, bulk samples were harvested in three replications due to the high plant density, making individual sampling impractical. In controlled and semi-controlled environments, plant height was measured similarly for 10 plants, and biomass was assessed by collecting three replications with three individuals/replicate. After harvest, fresh

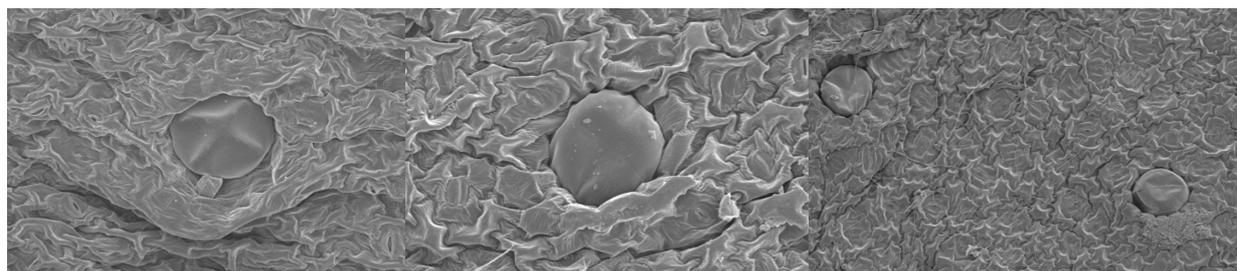
mass was determined by a laboratory scale. Plant height was expressed in cm, while fresh and dry shoot weights were determined as  $\text{g plant}^{-1}$  (Figure 10).



**Figure 10** Weight measurements of the plant material after drying, yarrow (a), basil (b), hyssop(c) (Photo: Kandoudi, 2020)

### **Glandular hair density**

The glandular hair density (Figure 11) was measured by cutting 5.5 mm diameter circles from the center of the leaf blade, excluding the main vein, from three species: peppermint (2<sup>nd</sup> cut), marjoram, and basil, grown in an open field setting in 2020, 2022, and 2023, respectively. Then the number of glandular peltate hairs on the abaxial surface of these blade samples was counted under a stereo microscope (type BMS 74959). Ten replicates per treatment were carried out.

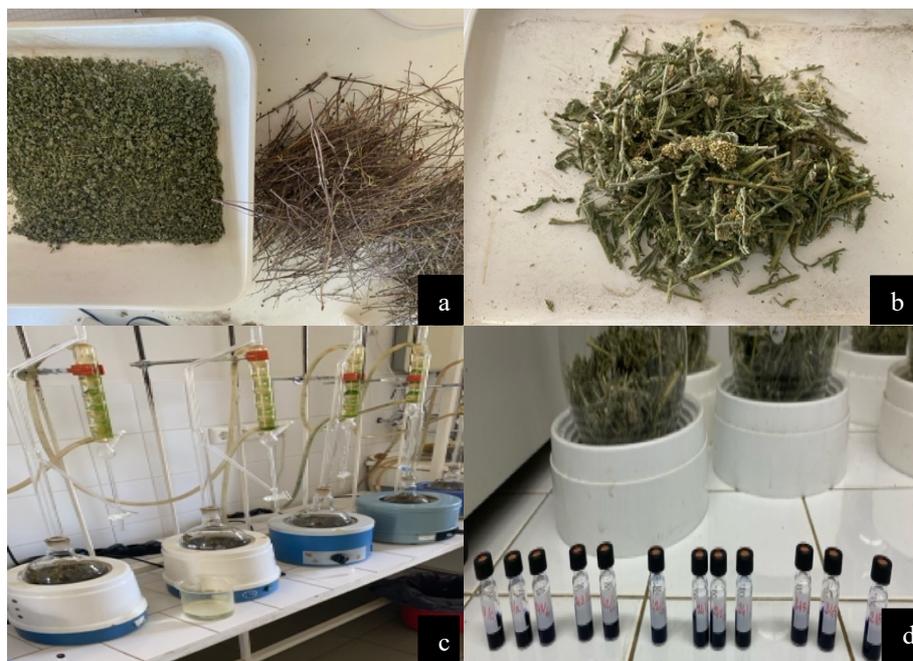


**Figure 11** Electron microscopic pictures of basil glandular hairs (Photo: Buczkó Krisztina, 2023)

## **3.3.2 Biochemical analysis**

### **Essential oil extraction**

After drying the plant material, the entire aerial parts of hyssop and yarrow were used for EO extraction (Figure 12). For the other species, the leaves and flowers were separated from the stems, and 20 g of dried, stemless plant material from each sample was hydro-distilled to extract the EO by using a Clevenger-type apparatus, along with 500 mL of water. The distillation process took 1.5 hours for peppermint and 2 hours for the rest of plant species, following the method recommended by the VII Hungarian Pharmacopoeia (Hungarica, 1986). The content was measured and expressed as  $\text{mL } 100 \text{ g}^{-1} \text{ DW}$  (dry weight). Once the oils were collected, any traces of water were eliminated. The sealed vials were stored in a refrigerator at  $4^{\circ}\text{C}$  until analysis.



**Figure 12 Hydro-distillation process steps: separation of leaves from stems (marjoram) (a), cutting the plant material into small pieces (yarrow) (b), distillation of the plant material (basil) (c), final EO product (yarrow) (d) (Photo: Kandoudi, 2020)**

### Essential oil composition

GC-FID and GC-MS analysis methods were used to determine the composition of the essential oils. An Agilent Technologies 6890N GC System with an HP-5 (5% phenyl methyl siloxane) capillary column (length: 30 m, i.d = 350  $\mu\text{m}$ , film thickness: 0.25  $\mu\text{m}$ ) was used for GC-FID analysis supplied by Agilent technologies international Sàrl (Rolle, Switzerland) (Figure 13). The GC was programmed as follows: an initial temperature of 50  $^{\circ}\text{C}$  for 10 min, followed by an increase from 50 to 150  $^{\circ}\text{C}$  at a rate of 4 $^{\circ}\text{C min}^{-1}$ , an increase from 150 to 220  $^{\circ}\text{C}$  at a rate of 12  $^{\circ}\text{C min}^{-1}$ , and a hold at 220  $^{\circ}\text{C}$  for 10 min. Helium was used as the carrier gas with a constant flow rate of 0.5  $\text{mL min}^{-1}$ . The injector and detector temperatures were set to 250  $^{\circ}\text{C}$ , and the split ratio was 22.6:1. An injected quantity of 0.2 mL was used. The percentage composition of the essential oil was determined from the GC peak areas.

Using the aforementioned instrument equipped with an Agilent Technologies MS 5975 inert mass selective detector, gas chromatography-mass spectrometry (GC-MS) analyses were conducted. Temperature program was as follows: initial temperature of 60  $^{\circ}\text{C}$  and increasing at a rate of 3  $^{\circ}\text{Cmin}^{-1}$  up to 240 $^{\circ}\text{C}$ . The final temperature was maintained for 5 min. Helium was used as carrier gas with a flow rate of 1  $\text{mL min}^{-1}$ , the injector and detector temperatures were set to 250  $^{\circ}\text{C}$ . The split ratio was 30:1, and an injected quantity of 0.2  $\mu\text{L}$  (solvent: n-hexane) was used. The ionization energy was set at 70 eV, and the mass spectra were recorded in full scan mode to produce total ion current (TIC) chromatograms in the mass range of m/z 50-550 uma. Identification of compounds was accomplished by calculating linear retention indices using the generalized equation of Van

Den Dool and Kratz (van Den Dool & Dec. Kratz, 1963) with literature data and matching their recorded mass spectra with those in a mass spectra library mass and spectral library references (NIST MS Search 2.0 library, Wiley 275) (Adams, 2007).

In case of yarrow, we have determined only the proazulene content of the drug, in harmony with the official method of the Ph.Eur. VIII. (*Millefolii herba*). The distilled oil was diluted with xylene to 50 ml, and then the absorption of the mixture was measured in a Thermo Evolution 201 spectrophotometer at 608 nm. Liquid xylene was used as compensation. Proazulene content was expressed as chamazulene percentages using the following formula:  $(2.1 \times A)/m$  where  $A$  is the absorbance and  $m$  is the mass of the sample in grams.



**Figure 13** Agilent Technologies GC instrument (a) for EO chemical analysis (b) (Photo: Kandoudi, 2022)

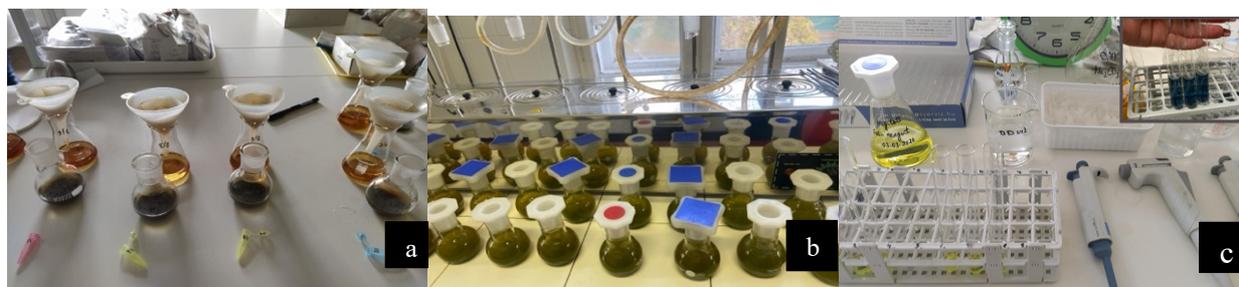
### **Total phenolic content (TPC)**

The extraction process involved adding 100 mL of boiling distilled water to 1 g of powdered plant material obtained by grinding the dry leaves and sifting them with a 500  $\mu\text{m}$  diameter sieve. The extracts were filtered and finally stored in a freezer after soaking for 24 h (Figure 14).

The modified method of Singleton and Rossi (Singleton & Rossi, 1965) was used to quantify the total phenolic content (TPC). In this method, 0.5 mL of the sample solution was placed in a test tube, followed by the addition of 2.5 mL of Folin-Ciocalteu's reagent (10 v/v%). After 1 min of incubation, 2 mL of sodium carbonate (700 mM) was added, and the resulting solution was incubated in hot water (50°C) for 5 min. The absorbance was then measured at 760 nm using a Thermo Evolution 201 spectrophotometer (Unicam Magyarország Kft., Budapest, Hungary). Gallic acid (300 mM) was used as the chemical standard for calibration, and the total phenolic content of the sample was expressed as mg of gallic acid equivalents per g of dry weight of extract (GAE  $\text{mg}\cdot\text{g}^{-1}$  DW). To prepare the blank, distilled water was used instead of the extract. The measurements were performed in six replications.

### Antioxidant capacity (AOC)

The antioxidant capacity was determined by the application of the ferric reducing antioxidant power (FRAP) assay developed by Benzie and Strain (Benzie & Strain, 1996), with a few modifications. FRAP reagent was prepared fresh in order to contain three things: sodium acetate buffer (pH 3.6), TPTZ (2,4,6-tripiridil-s-triazin) in HCl, and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  solution ( $20 \text{ mmol L}^{-1}$ ), in the proportion 10:1:1 (v/v/v); 10  $\mu\text{L}$  of the previously extracted test sample was added to 1.5 mL of acting FRAP reagent and 40  $\mu\text{L}$  distilled water. The absorbance of the solution was then measured at 593 nm after 5 min using the above-mentioned spectrophotometer. A blank was made to contain distilled water instead of the sample, and ascorbic acid was used as a positive control. FRAP values of samples were calculated from the standard curve equation and expressed as mg ascorbic acid equivalent (AAE)  $\text{g}^{-1}$  of dry extract. The measurements were performed in six replications.



**Figure 14** Determination of TPC from plant extraction (a & b) to analysis (c) (Photo: Kandoudi, 2021,2022)

### Composition of phenolic compounds

The analysis was carried out in the “Tudásközpont Laboratory” of MATE, Gödöllő. 0.5 g of freshly harvested and dried plant leaves was taken and crushed in a crucible mortar in the presence of 1-2 grams of quartz sand. The phenolic compounds were extracted by the addition of boiled 50 % ethanol in 2% ortho-phosphoric acid solution. The macerate was then transferred to an Erlenmeyer flask and shaken for 15 min at  $80^\circ\text{C}$  followed by ultrasonication for 5 min at  $80^\circ\text{C}$  in a water-bath ultrasonic device (model RK-165-BH Bendelin Sonorex, Germany). The extract was centrifuged for 5 min at 5000 rpm (M-Universal, MPW Med. Instrument, Poland). The supernatant was decanted and purified by passing through a  $22\mu$ , 13mm glass fibre syringe filter before injection onto HPLC apparatus.

A Chromaster Hitachi HPLC instrument containing a Model 5160 gradient pump, a Model 5260 autosampler, a Model 5310 column oven, and a Model 5430 diode-array detector (DAD) was used with a parad\_KB0\_2dat software for operation and data processing.

The separation of phenolic compounds was performed on Ascentis phosphor-conditioned C18 phase (C18-PCP, from Supelco, USA) with gradient elution of 1% ortho-phosphoric acid (A) and acetonitrile (B) according to a recently developed protocol (Under publication). The gradient elution started with 1% B in A, changed to 20% B in 20 min, stayed isocratic for 10 min, changed to 30% B in 5 min, stayed isocratic for 10 min and finally turned to 1% B in 5 min. The DAD detection was between 190nm and 700nm. The quantification was based on recording the area at the maximum absorbance wavelength of each compound and relating it to that of the standard solution.

Stock solutions for different phenolics (Sigma-Aldrich via Merck, Budapest Hungary) were prepared by dissolving 2-3 mg in 10 ml absolute ethanol or methanol and diluted 10 times with 50% ethanol in 1% ortho-phosphoric acid. The working solutions were used for calibration curves, identification, and quantification of phenolic compounds. In case of no standard available, the compounds were tentatively identified on the basis of comparison of their spectral characteristics and chromatographic behaviour with literature data.

### **Phenylalanine ammonia-lyase activity**

The PAL enzyme activity was measured at the Hun-REN Research Institute, Martonvásár. Samples were taken from basil and marjoram in three experimental trials under MeJa 2 treatments, harvested two weeks after elicitation with two treatments (Table 11.). In each case, leaf samples were collected from at least 5 plants and immersed immediately in liquid nitrogen, then kept frozen until the analyses (Figure 15). 1 g of frozen leaves was homogenized at 4°C in 4 mL of 50 mM Tris-HCl buffer (pH 8.8) containing 5 mM  $\beta$ -mercaptoethanol and 4% (w/v) polyvinylpyrrolidone. The homogenate was then centrifuged at 10,000 g for 10 minutes, and the resulting supernatant was used for the enzymatic assay. 2.75 mL of 50 mM L-phenylalanine in 50 mM Tris-HCl buffer (pH 8.8) combined with 250  $\mu$ L of the enzyme extract was used to initiate the reaction, which was incubated at 37°C for 1 hour. The reaction was stopped by adding 10% trichloroacetic acid and centrifuged at 10,000 g for 5 minutes. PAL activity was quantified spectrophotometrically by measuring the absorbance at 290 nm, reflecting *trans*-cinnamic acid formation according to Gao et al. (2008). One unit of PAL activity was defined as an increase in absorbance of 0.01  $\text{min}^{-1}$  and expressed as enzyme units per gram of fresh weight (U  $\text{g}^{-1}$  FW).

### **Lipoxygenases activity**

The LOX enzyme activity was measured at the Hun-REN Research Institute, Martonvásár. Samples were taken and processed similarly, as mentioned in the case of PAL measurements. Then, to prepare the enzymatic extract, 250 mg of frozen leaves were ground with a pestle and mortar at

4°C in the presence of 1% w/w polyvinylpyrrolidone and 1 mL of sodium phosphate buffer (50 mM, pH 6.5) containing 0.25% v/v Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The homogenate was then centrifuged at 20,000g for 30 minutes at 4°C, and the resulting supernatant was kept on ice for subsequent enzyme assays. LOX activity was measured following the method of Axelrod et al. (1981) using linoleic acid as a substrate. Enzyme assays were prepared by adding 25 µL of enzyme extract to a mixture containing 100 µL of 10 mM linoleic acid and 0.875 mL of phosphate buffer (50 mM, pH 6.0), with the reaction conducted at 25°C. LOX activity was assessed by monitoring the increase in absorbance at 234 nm over 1, 2.5, and 5 minutes. A blank reaction was performed using phosphate buffer (pH 6.5) in place of the leaf extract. The hydroperoxides formed during the enzymatic reaction were quantified using a molar extinction coefficient of 25,000 M<sup>-1</sup> cm<sup>-1</sup>.



**Figure 15 Whole basil leaf (a), basil leaf cut in small pieces and preserved in liquid nitrogen for enzymatic activity (b) (Photo: Kandoudi, 2022)**

### **Statistical analysis**

IBM SPSS version 29 software (International Business Machines Corporation, North Castle, USA) was used to analyze the data. A one-way analysis of variance (ANOVA), followed by either Tukey's test or the Games-Howell test, was performed at a 5% significance level. Shapiro-Wilk's test and Levene's test were used to assess the normality of distribution and homogeneity of variances, respectively. The relationship between glandular hair density and EO content was analyzed using Pearson's correlation coefficient (r). Principal component analysis (PCA) of the EO composition was performed using OriginPro 2023b software (OriginLab Corporation, Northampton, USA).

All the measurements and treatments carried out are summarized in Table 11.

**Table 11 Summary of the experiments conducted between 2020-2023**

Year	Environment	Species	Treatment	Morphological traits	EO content and composition	TPC and AOC	Phenolic composition	PAL and LOX	Glandular hair density	
2020	Open field	Basil	MeJa (0.1 & 2.0 mM)		X	X				
		Hyssop	SA (0.1 & 2.0 mM)		X	X				
		marjoram			X	X				
		Peppermint (1 <sup>st</sup> cut)			X	X				
		Peppermint (2 <sup>nd</sup> cut)			X				X	
		Yarrow			X	X				
2021	Open field	Basil	MeJa (0.1 & 2.0 mM)	X	X	X				
		Hyssop	SA (0.1 & 2.0 mM)	X	X	X				
		Marjoram	TMAO (2.0 mM)	X	X	X				
		Peppermint		X	X	X				
		Yarrow	MeJa (0.1 & 2.0 mM) SA (0.1 & 2.0 mM)	X	X	X				
	Greenhouse	Marjoram (commercial variety)	MeJa (2.0 mM)	X	X	X				
2022	Open field	Basil	MeJa (0.1 & 2.0 mM)	X	X	X				
		Hyssop	SA (0.1 & 2.0 mM)	X	X	X				
		Marjoram	TMAO (2.0 mM)	X	X	X			X	
		Peppermint	MeJa (0.1, 2.0 & 10.0 mM) SA (0.1, 2.0, & 10 mM) TMAO (2.0 mM)	X	X	X	X			
		Yarrow	MeJa (0.1 & 2.0 mM) SA (0.1 & 2.0 mM)	X	X	X				
	Phytotron	Basil	MeJa (2.0 mM) SA (2.0 mM)	X		X		X		
		Peppermint	MeJa (10.0 mM) SA (10.0 mM)			X	X			
	Greenhouse	Marjoram	MeJa (2.0 mM)			X				
	2023	Open field	Basil	MeJa (0.1 & 2.0 mM)	X	X			X	X
			Marjoram	SA (0.1 & 2.0 mM)	X	X			X	

## IV. Results

### 4.1 Open field elicitation

#### 4.1.1 Morphological traits

The effect of elicitors on the height of our model species was assessed over two consecutive years (2021 and 2022) in an open-field setting. Overall, the treatments resulted in negligible height differences, except for marjoram in 2022, which exhibited a significant response. Detailed results are presented in Table 12 and Tables 1 to 5 in Appendix 3.

In 2021, basil height experienced a slight increase with SA treatments, with mean heights varying from 45.5 cm in control plants to 54.3 and 51.8 cm with SA 1 and SA 2, respectively. However, these treatments had an opposite effect in the following year, resulting in a decrease in height by approximately 6% with both concentrations.

In a similar manner, the highest heights of hyssop plants in 2021 were attained through SA treatments, resulting in increases of 8 and 5% with SA 1 and SA 2, respectively.

As for marjoram, slight decreases were observed with the treatments in the first year, except for the SA 2 treated plants. The height of marjoram ranged from 29.88 cm after MeJa 2 elicitation to 34.2 cm with SA 2. However, in a contrasting pattern, the concentration of MeJa 2 that caused a reduction in height in 2021, the same parameter significantly increased by 17% in 2022. While the other treatments had positive effects on height as well, none of them were significant.

The higher concentration of SA treatment influenced the height of yarrow plants in a comparable manner in both experimental years. The highest values of the studied trait were observed with plants treated with SA 2, reaching 69.4 and 61.2 cm in 2021 and 2022, respectively.

**Table 12 The effect of elicitors on the height (cm) of the studied species in 2021 and 2022**

Year	Treatment	Basil	Hyssop	Marjoram	Yarrow
2021	C	45.5± 5.13 <sup>a</sup>	50.3±5.05 <sup>a</sup>	33.7±5.23 <sup>a</sup>	67.8±3.03 <sup>a</sup>
	MeJa 1	51.0±6.42 <sup>a</sup>	51.3±2.88 <sup>a</sup>	33.2±2.82 <sup>a</sup>	67.0±4.53 <sup>a</sup>
	MeJa 2	49.7±8.21 <sup>a</sup>	51.5±3.45 <sup>a</sup>	29.9±2.10 <sup>a</sup>	68.4±1.95 <sup>a</sup>
	SA 1	54.3±6.71 <sup>a</sup>	54.3±7.03 <sup>a</sup>	31.3±3.24 <sup>a</sup>	65.0±3.32 <sup>a</sup>
	SA 2	51.8±6.88 <sup>a</sup>	52.7±3.78 <sup>a</sup>	34.2±4.89 <sup>a</sup>	69.4±3.51 <sup>a</sup>
	TMAO	45.5±5.44 <sup>a</sup>	52.60±3.33 <sup>a</sup>	30.7±3.62 <sup>a</sup>	-
2022	C	47.4±3.11 <sup>a</sup>	-	25.1±1.55 <sup>b</sup>	55.7±2.50 <sup>a</sup>
	MeJa 1	44.5±4.90 <sup>a</sup>	-	27.2±1.58 <sup>ab</sup>	58.8±1.94 <sup>a</sup>
	MeJa 2	44.9±3.00 <sup>a</sup>	-	29.4±3.62 <sup>a</sup>	56.8±2.79 <sup>a</sup>
	SA 1	44.8±2.71 <sup>a</sup>	-	27.7±1.58 <sup>ab</sup>	57.0±4.38 <sup>a</sup>
	SA 2	44.4±2.44 <sup>a</sup>	-	27.1±2.53 <sup>ab</sup>	61.2±5.74 <sup>a</sup>
	TMAO	46.2±5.39 <sup>a</sup>	-	27.6±1.85 <sup>ab</sup>	59.5±4.14 <sup>a</sup>

Values are presented as Mean ± SD. Different letters are for significantly different groups. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO.

Similarly to the height parameter, elicitors had minimal significant effects on plant biomass. Instead, most variations were observed between plantation years rather than among treatments. The results of fresh and dry weight measurements from the 2021 and 2022 open-field experiments, presented in Table 13 and Appendix 3 (Tables 1 to 5), indicate that basil plants had the highest biomass in both years, with the second year yielding the maximum fresh and dry weight. Similar effects resulted in both years from the foliar application of the phytohormones, where MeJa 1, SA 2, and TMAO reduced the fresh weight by 13, 11, and 31% in 2021, and 12, 17, and 8% in 2022, respectively. On the other hand, the highest fresh biomass was obtained after the treatment by SA 1, which reached an increase of 25 and 12% in the first and second years, respectively. However, none of these changes were statistically significant. As for the dry weight, TMAO was the only treatment reducing significantly the parameter in basil by 28% in 2021.

There was significant variation in the yield of fresh and dry biomass among hyssop plants in the first and second year. However, differences between the control group and treated plants were not significant. Notably, there was a remarkable contrast between MeJa 1 and MeJa 2 treatments. The fresh weight varied from 88.56 g plant<sup>-1</sup> to 118.78 g plant<sup>-1</sup>, respectively. Similarly with the dry biomass, there is an increase of 35% between these two concentrations. Conversely, in the second year, there were no notable changes observed in either trait.

The elicitation of marjoram plants did not have any significant differences in the fresh biomass, except for a reduction in 2021 achieved by TMAO. The highest value was determined due to the

foliar treatment with SA 2 in 2021, however, in 2022 the highest fresh biomass was observed in MeJa 2 treated marjoram. Moreover, in both years, the TMAO had a suppressing effect by 37 and 17% in 2021 and 2022, respectively with the effect from the former year being significant compared to the control. As for the dry weight, a significant reduction was spotted with MeJa 1, MeJa 2, and TMAO by 35, 23, and 40%, respectively, in the first year. No significant decreases were reported in the second year.

In contrast to the previously mentioned species, peppermint plants exhibited significant enhancement in fresh biomass across all treatments in 2021. The most substantial increase was observed with the lower concentration of MeJa (MeJa 1), followed by SA 2, with increases of 38 and 28%, respectively. Dry mass also showed elevation following foliar treatments in 2021, with only MeJa 1, SA 1, and SA 2 treatments resulting in significant increases of 47, 43, and 41%, respectively. Nonetheless, these treatments could not replicate the same effects in the subsequent year, with both parameters showing insignificant differences.

No significant variations were observed in the case of yarrow following elicitor treatments, either in fresh or in dry mass, showing consistency across both years. Yet, MeJa treatments could enhance the fresh mass by 40% with both concentrations of the elicitor in 2021. Similarly to that, in the dry mass, the phytohormone MeJa caused the highest increase by 42 and 55%, respectively, with increasing concentrations. In 2022, no significant changes were noticed; however, the highest values were obtained by non-treated yarrow samples concerning both the fresh and dry biomass.

**Table 13 The effect of elicitors on the production (g plant<sup>-1</sup>) of the studied species in 2021 and 2022**

		2021					2022						
		C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO
<b>Fresh weight</b>	<b>basil</b>	132.33±7.00 <sup>ab</sup>	115.22±1.59 <sup>9ab</sup>	141.33±1.08 <sup>4ab</sup>	165.33±1.90 <sup>4a</sup>	117.44±1.01 <sup>ab</sup>	90.89±2.04 <sup>b</sup>	163.67±2.31 <sup>7a</sup>	144.00±1.24 <sup>9a</sup>	150.11±27.95 <sup>a</sup>	183.00±1.63 <sup>7a</sup>	135.67±8.5 <sup>a</sup>	150.67±16.86 <sup>a</sup>
	<b>hyssop</b>	104.44±1.01 <sup>2ab</sup>	88.56±4.53 <sup>b</sup>	118.78±8.08 <sup>a</sup>	99.33±7.55 <sup>ab</sup>	81.22±8.51 <sup>b</sup>	103.44±5.71 <sup>ab</sup>	93.11±3.42 <sup>a</sup>	87.56±3.98 <sup>a</sup>	86.22±6.00 <sup>a</sup>	81.56±3.29 <sup>a</sup>	80.22±4.02 <sup>a</sup>	87.11±6.62 <sup>a</sup>
	<b>marjoram</b>	30.80±4.01 <sup>a</sup>	22.27±1.36 <sup>ab</sup>	27.20±3.02 <sup>a</sup>	31.20±7.28 <sup>a</sup>	31.67±3.70 <sup>a</sup>	19.53±5.67 <sup>b</sup>	33.33±7.21 <sup>a</sup>	35.53±3.70 <sup>a</sup>	36.00±8.31 <sup>a</sup>	33.07±3.50 <sup>a</sup>	34.80±4.41 <sup>a</sup>	27.80±4.76 <sup>a</sup>
	<b>peppermint</b>	103.44±7.21 <sup>c</sup>	143.44±1.43 <sup>a</sup>	120.11±8.53 <sup>b</sup>	121.67±9.02 <sup>b</sup>	132.33±1.07 <sup>9ab</sup>	126.22±10.12 <sup>b</sup>	63.67±8.58 <sup>a</sup>	68.44±7.45 <sup>a</sup>	69.78±7.14 <sup>a</sup>	59.33±6.75 <sup>a</sup>	65.56±6.78 <sup>a</sup>	59.56±6.30 <sup>a</sup>
	<b>yarrow</b>	69.20±11.71 <sup>a</sup>	97.20±16.78 <sup>a</sup>	97.33±15.59 <sup>a</sup>	94.00±17.74 <sup>a</sup>	83.60±13.74 <sup>a</sup>	-	79.00±13.26 <sup>a</sup>	69.33±6.11 <sup>a</sup>	66.67±12.10 <sup>a</sup>	68.67±16.80 <sup>a</sup>	57.67±1.09 <sup>a</sup>	-
<b>Dry weight</b>	<b>basil</b>	27.69±3.09 <sup>ab</sup>	23.35±1.90 <sup>bc</sup>	30.93±2.02 <sup>ab</sup>	33.96±1.11 <sup>a</sup>	24.03±1.37 <sup>bc</sup>	19.84±2.78 <sup>c</sup>	29.67±4.15 <sup>a</sup>	24.73±1.88 <sup>a</sup>	25.88±4.60 <sup>a</sup>	32.51±5.75 <sup>a</sup>	25.07±1.49 <sup>a</sup>	27.33±3.34 <sup>a</sup>
	<b>hyssop</b>	24.77±0.59 <sup>ab</sup>	20.91±1.29 <sup>b</sup>	28.24±2.63 <sup>a</sup>	23.56±2.60 <sup>ab</sup>	20.44±1.53 <sup>b</sup>	22.82±3.28 <sup>ab</sup>	29.68±1.67 <sup>a</sup>	28.30±1.84 <sup>a</sup>	29.48±2.47 <sup>a</sup>	25.86±1.62 <sup>a</sup>	25.64±1.20 <sup>a</sup>	26.34±0.95 <sup>a</sup>
	<b>marjoram</b>	8.99±1.29 <sup>a</sup>	5.81±0.33 <sup>bc</sup>	6.92±0.74 <sup>bc</sup>	8.51±1.60 <sup>ab</sup>	8.36±0.93 <sup>ab</sup>	5.37±1.16 <sup>c</sup>	8.27±2.00 <sup>a</sup>	9.07±1.09 <sup>a</sup>	9.14±2.52 <sup>a</sup>	8.08±0.72 <sup>a</sup>	8.44±1.64 <sup>a</sup>	7.17±1.38 <sup>a</sup>
	<b>peppermint</b>	20.46±1.73 <sup>b</sup>	30.04±2.98 <sup>a</sup>	22.64±1.88 <sup>ab</sup>	29.22±3.24 <sup>a</sup>	28.92±3.04 <sup>a</sup>	23.31±1.56 <sup>ab</sup>	21.62±1.58 <sup>a</sup>	22.46±1.47 <sup>a</sup>	22.79±1.26 <sup>a</sup>	19.99±1.02 <sup>a</sup>	22.33±1.96 <sup>a</sup>	20.60±1.14 <sup>a</sup>
	<b>yarrow</b>	17.99±2.51 <sup>a</sup>	25.55±8.20 <sup>a</sup>	27.88±6.26 <sup>a</sup>	23.43±8.37 <sup>a</sup>	19.63±3.61 <sup>a</sup>	-	25.57±9.09 <sup>a</sup>	19.56±5.17 <sup>a</sup>	21.17±6.41 <sup>a</sup>	19.38±7.74 <sup>a</sup>	17.37±5.79 <sup>a</sup>	-

Values are presented as Mean ± SD. Different letters are for significantly different groups. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO

### 4.1.2 Essential oil content

The effect of elicitors on the EO content was evaluated over the course of three years. The analysis showed significant differences between the years ( $p < 0.01$ ), the treatments ( $p < 0.01$ ), as well as the interaction between the year and the treatment factors in all our species ( $p < 0.01$ ) (The appendix 3 Tables 1 to 5) and Figure 16.

Basil plants grown in 2021 accumulated the highest EO contents ( $1.08 \text{ mL } 100 \text{ g}^{-1} \text{ DW}$ ) with MeJa 2 resulting in the highest EO content compared to all treatments in all experimental years. In the plantations of 2020 and 2021, the majority of the treatments did not have significant effects, except for the MeJa 2 treatment in the latter year, which resulted in a 24% higher EO content compared to the control. However, in the year 2022, the EO proportion of basil significantly changed after all treatments. Notably, besides MeJa 2, SA 1 also induced an almost 12% increase. In contrast, MeJa 1 and SA 2 caused a decrease of 15 and 27%, respectively. These negative tendencies, however, were not observed in previous years.

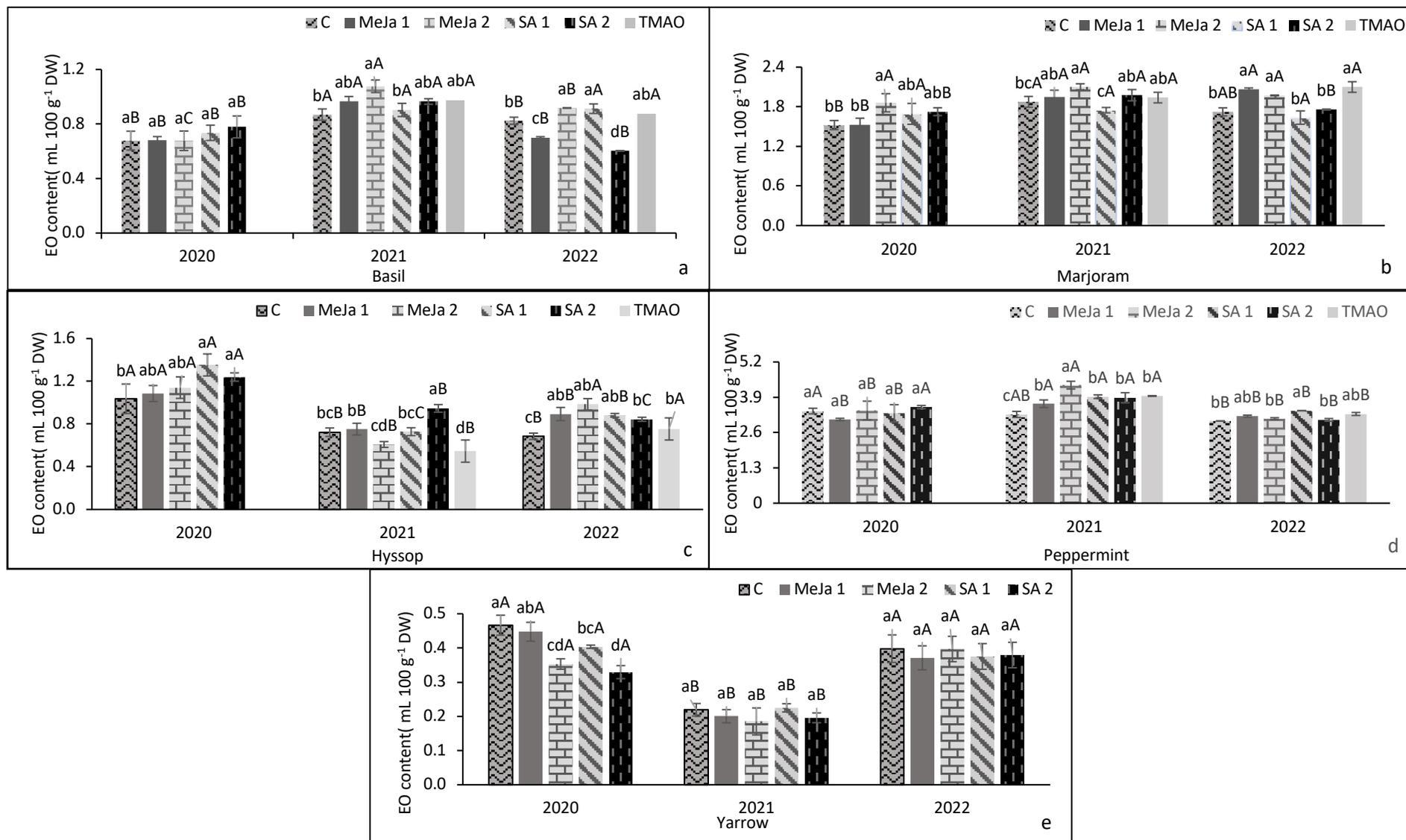
The EO content of hyssop was significantly higher in the first year compared to the subsequent experiments due to the genotype difference. The lowest EO content registered in the Meran accession was  $1.04 \text{ mL } 100 \text{ g}^{-1} \text{ DW}$ , while the highest contents in the Sophie variety could only reach  $0.95$  and  $0.98 \text{ mL } 100 \text{ g}^{-1} \text{ DW}$  with SA 2 in 2021 and with MeJa 2 in 2022, respectively. Concerning the elicitor effects, the lowest EO accumulations in hyssop were registered with the TMAO and MeJa 2 treatments in 2021, with contents of  $0.55$  and  $0.61 \text{ mL } 100 \text{ g}^{-1} \text{ DW}$ , respectively. Remarkably, in this species, SA appears to be more effective in changing the accumulation of EO. SA 2 treatments elevated their production by 20, 31, and 23% in 2020, 2021, and 2022, respectively. In 2021, SA 2 was the sole treatment that significantly enhanced the EO content, while in the other years, SA 1 exhibited a stimulating effect, which was even higher (by 31 and 29% in 2020 and 2022, respectively). MeJa effects were only detected in last year's hyssop plantation. MeJa 1 increased the hyssop oil accumulation by 30%, and MeJa 2 by almost 44%. However, TMAO treatments had opposite effects. In the year 2021, treatments with this compound lowered the oil content significantly by 25%, whereas, in 2022, TMAO could increase the oil by around 9%.

In the case of marjoram, EO production varied throughout the three years, with content ranging from  $1.52$  to  $2.10 \text{ mL } 100 \text{ g}^{-1} \text{ DW}$  obtained from the control plants of 2020 and the MeJa 2 treated plants of 2021, respectively. In the first experimental year, the plants exhibited significantly lower

EO contents compared to the other years, except for the MeJa 2 treated plants. This concentration was able to enhance the EO content significantly by 22%. Moreover, similar tendencies were observed throughout the subsequent years, where MeJa 2 elevated the accumulation of EO by 12 and 15% in 2021 and 2022, respectively. In the first two years, no other treatments resulted in significant changes. However, in 2022, both concentrations of MeJa and TMAO could elevate the content, with elevations reaching 20 and 22% increase with MeJa 1 and TMAO, respectively.

The highest EO contents of peppermint were obtained mostly in the year 2021, while the 2020 and 2022 plantations produced lower content. The content ranges from 3.02 to 4.35 mL 100 g<sup>-1</sup> DW obtained from control plants in 2022 and MeJa 2 treated plants in 2021, respectively. In the first year, the treatments did not affect the peppermint oil production significantly; plants treated with MeJa 1 had lower values than the control plants by 9%; however, this decrease was not statistically significant. Conversely, all treatments elevated the EO accumulation in the following year, with MeJa 2 having the most increase of 33%. However, this treatment could not enhance the accumulation of peppermint oil in 2022, when significant improvement in the content was observed with the lower concentration of SA (13% increase). SA 1, SA 2, and TMAO treatments were also capable of enhancing the accumulation of peppermint oil in 2021 by around 20%.

Evaluating the EO content of yarrow, in 2021 we experienced a significant decrease in the EO production compared to the other years, which was most remarkable in the case of the control plants (more than a 2-fold decrease compared to the first year). Regarding the treatments, no eliciting effects were observed with either MeJa or SA in all years. In fact, the higher concentrations of MeJa and SA treatments significantly reduced the accumulation in 2020 (by 24, 14, and 30% with MeJa 2, SA 1, and SA 2, respectively). In the following years, we noted no significant changes with any of the treatments.



**Figure 16** EO content of the five plant species between 2020-2022. Data are expressed as means  $\pm$  SD; Different letters are for significantly different groups. Capital letters to differentiate between the experimental years under fixed elicitation treatments and small letters are used to differentiate elicitors effects under fixed years. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO.

### 4.1.3 Essential oil composition

Based on the results of GC-MS analysis of basil EO over three experimental years (Table 14), significant variations could be observed with the treatments in the ratios of several constituents. In all our samples, linalool was detected as the major component, but its content fluctuated depending on the experimental year and treatment. Its ratio varied from 35.72% in SA 2 treated samples of 2022 to 60.77% in MeJa 2 samples in 2021. Each year, one single treatment changed significantly the compound's ratio; however, this effect was not consistent. In the first year, SA 2 elevated the ratio of linalool by 16%, whereas the same compound caused a decrease in the last year by 13%. The change of linalool also affected the ratio of total monoterpenes, which changed in these years by 10%. Interestingly, treatments with SA 2 had a similar pattern in the EO accumulation in those years, as discussed above (chapter 4.1.2.). In 2021, TMAO was responsible for the significant drop of linalool percentage by 13%, while its ratio changed only non-significantly in the other treatments. MeJa 2 affected the ratios of iso-bornyl acetate and eugenol, but the two compounds exhibited opposite responses. Specifically, this treatment consistently decreased the former compound by 35, 27, and 10% and stimulated the accumulation of the latter compound by 48, 89, and 19%, in 2020, 2021, and 2022, respectively. However, the changes observed in the last year were not considered significant. Interestingly, methyl cinnamate was detected solely in the 2021 experiment; the control plants exhibited only traces of this compound. Whereas TMAO treatment appeared to favor its biosynthesis and substantially enhanced its accumulation up to 3.90%. In the first year, the sesquiterpene compounds germacrene D,  $\alpha$ -bulnesene, and *cis*- $\gamma$ -cadinene reached their peak levels under the influence of the same elicitor, MeJa 2, increasing by 34, 37, and 27%, respectively. Consequently, the total sesquiterpenes in these basil samples were also enhanced by 14% compared to the control. In the following years, no remarkable changes were observed with the MeJa 2 treatment. Among these compounds, only  $\alpha$ -bulnesene showed a significant elevation of 27% with TMAO treatment in 2021 and an increase of 35% with SA 2 in 2022.

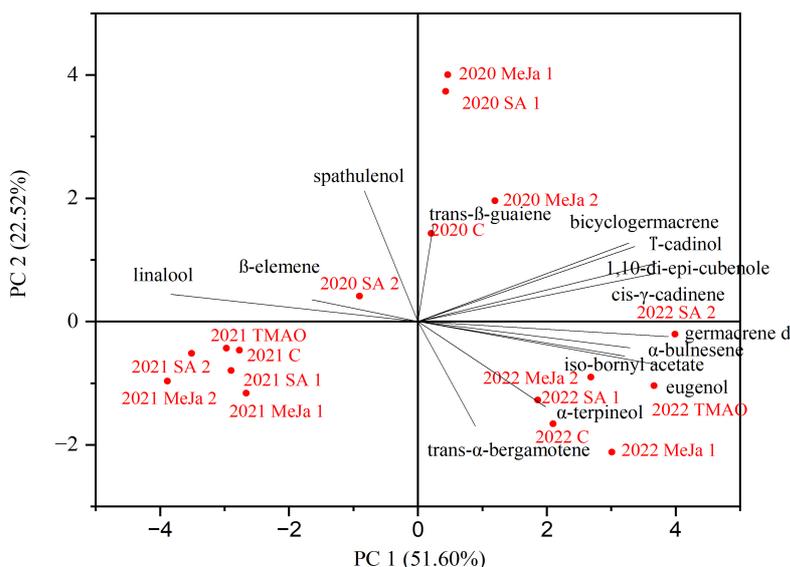
The relationship between the volatile composition of basil oil and the elicitors was examined using principal component analysis (PCA), as depicted in Figure 17. The two principal axes of the PCA plot accounted for a total of 74.12% of the variance. The arrangement of clusters revealed three distinct groups. The first and most distant cluster comprised treatments from 2022 and was characterized by higher ratios of germacrene D,  $\alpha$ -bulnesene, iso-bornyl acetate, and eugenol, which contributed to its clustering. The second cluster consisted of treatments from 2020, excluding SA 2. This group exhibited predominantly rich sesquiterpene ratios compared to the other clusters. The last cluster contained treatments from 2021, distinguished by high linalool

levels. Interestingly, this cluster also included the treatment SA 2 from 2020. Furthermore, the distances between treatments in the last two years were smaller than those in the first year.

**Table 14 EO composition (GC area %) of basil samples in open field between 2020-2022**

	RI <sup>1</sup>	2020					2021					2022						
		C	MeJa 1	MeJa 2	SA 1	SA 2	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO
<b>1,8-cineole</b>	<b>1034</b>	6.76 <sup>a</sup>	7.27 <sup>a</sup>	5.21 <sup>a</sup>	6.51 <sup>a</sup>	7.22 <sup>a</sup>	13.72 <sup>ab</sup>	12.51 <sup>ab</sup>	10.97 <sup>b</sup>	13.56 <sup>ab</sup>	14.03 <sup>a</sup>	14.45 <sup>a</sup>	9.04 <sup>a</sup>	9.14 <sup>a</sup>	7.27 <sup>a</sup>	7.73 <sup>a</sup>	7.85 <sup>a</sup>	8.54 <sup>a</sup>
<b>linalool</b>	<b>1097</b>	51.60 <sup>b</sup>	48.13 <sup>b</sup>	49.55 <sup>b</sup>	51.21 <sup>b</sup>	60.09 <sup>a</sup>	57.03 <sup>ab</sup>	54.84 <sup>b</sup>	60.77 <sup>a</sup>	55.54 <sup>b</sup>	58.09 <sup>ab</sup>	49.75 <sup>c</sup>	41.29 <sup>ab</sup>	38.73 <sup>bc</sup>	41.20 <sup>ab</sup>	43.23 <sup>a</sup>	35.72 <sup>c</sup>	38.27 <sup>bc</sup>
<b><math>\alpha</math>-terpineol</b>	<b>1189</b>	1.07 <sup>a</sup>	1.09 <sup>a</sup>	0.94 <sup>a</sup>	0.87 <sup>a</sup>	0.81 <sup>a</sup>	1.23 <sup>a</sup>	1.04 <sup>a</sup>	0.98 <sup>a</sup>	1.12 <sup>a</sup>	1.12 <sup>a</sup>	1.11 <sup>a</sup>	1.34 <sup>a</sup>	1.33 <sup>a</sup>	1.16 <sup>a</sup>	1.30 <sup>a</sup>	1.23 <sup>a</sup>	1.46 <sup>a</sup>
<b>iso-bornyl acetate</b>	<b>1281</b>	1.83 <sup>ab</sup>	1.89 <sup>a</sup>	1.19 <sup>c</sup>	1.48 <sup>bc</sup>	1.17 <sup>c</sup>	1.36 <sup>ab</sup>	1.61 <sup>a</sup>	0.99 <sup>b</sup>	1.71 <sup>a</sup>	1.03 <sup>b</sup>	1.40 <sup>a</sup>	2.02 <sup>bc</sup>	2.53 <sup>a</sup>	1.81 <sup>c</sup>	1.92 <sup>bc</sup>	2.25 <sup>ab</sup>	2.23 <sup>ab</sup>
<b>eugenol</b>	<b>1361</b>	2.74 <sup>b</sup>	2.85 <sup>b</sup>	4.05 <sup>a</sup>	2.39 <sup>b</sup>	2.24 <sup>b</sup>	1.40 <sup>b</sup>	1.90 <sup>b</sup>	2.65 <sup>a</sup>	2.01 <sup>ab</sup>	1.55 <sup>b</sup>	1.26 <sup>b</sup>	4.77 <sup>b</sup>	5.77 <sup>b</sup>	5.69 <sup>b</sup>	4.79 <sup>b</sup>	5.05 <sup>b</sup>	6.77 <sup>a</sup>
<b>(E)-methyl cinnamate</b>	<b>1376</b>	-	-	-	-	-	t <sup>2</sup>	t	0.14 <sup>b</sup>	0.15 <sup>b</sup>	0.35 <sup>b</sup>	3.90 <sup>a</sup>	-	-	-	-	-	-
<b><math>\beta</math>-elemene</b>	<b>1391</b>	0.75 <sup>b</sup>	0.85 <sup>ab</sup>	1.02 <sup>a</sup>	1.04 <sup>a</sup>	0.50 <sup>c</sup>	0.93 <sup>a</sup>	1.09 <sup>a</sup>	0.92 <sup>a</sup>	1.01 <sup>a</sup>	0.88 <sup>a</sup>	1.24 <sup>a</sup>	0.71 <sup>a</sup>	0.63 <sup>a</sup>	0.51 <sup>a</sup>	1.06 <sup>a</sup>	1.01 <sup>a</sup>	0.71 <sup>a</sup>
<b>trans-<math>\alpha</math>-bergamotene</b>	<b>1437</b>	5.13 <sup>a</sup>	4.31 <sup>a</sup>	5.39 <sup>a</sup>	2.87 <sup>b</sup>	5.14 <sup>a</sup>	4.72 <sup>b</sup>	6.48 <sup>a</sup>	5.41 <sup>ab</sup>	4.76 <sup>b</sup>	4.57 <sup>b</sup>	4.92 <sup>b</sup>	6.17 <sup>ab</sup>	6.89 <sup>a</sup>	6.00 <sup>ab</sup>	6.37 <sup>a</sup>	4.90 <sup>b</sup>	4.79 <sup>b</sup>
<b>germacrene D</b>	<b>1482</b>	2.30 <sup>b</sup>	2.59 <sup>ab</sup>	3.08 <sup>a</sup>	2.96 <sup>ab</sup>	2.71 <sup>ab</sup>	1.55 <sup>a</sup>	2.06 <sup>a</sup>	1.68 <sup>a</sup>	1.73 <sup>a</sup>	1.64 <sup>a</sup>	2.09 <sup>a</sup>	3.67 <sup>b</sup>	3.56 <sup>b</sup>	3.72 <sup>b</sup>	3.74 <sup>b</sup>	5.07 <sup>a</sup>	3.99 <sup>b</sup>
<b>bicyclgermacrene</b>	<b>1497</b>	0.76 <sup>b</sup>	1.05 <sup>a</sup>	1.10 <sup>a</sup>	1.23 <sup>a</sup>	0.79 <sup>b</sup>	0.30 <sup>a</sup>	0.44 <sup>a</sup>	0.32 <sup>a</sup>	0.35 <sup>a</sup>	0.29 <sup>a</sup>	0.43 <sup>a</sup>	0.75 <sup>b</sup>	0.68 <sup>b</sup>	0.89 <sup>b</sup>	0.70 <sup>b</sup>	1.28 <sup>a</sup>	1.03 <sup>a</sup>
<b>trans-<math>\beta</math>-guaiene</b>	<b>1499</b>	0.16 <sup>b</sup>	2.46 <sup>a</sup>	0.21 <sup>b</sup>	0.21 <sup>b</sup>	-	-	-	-	-	-	-	-	-	-	-	-	-
<b><math>\alpha</math>-bulnesene</b>	<b>1506</b>	2.21 <sup>b</sup>	-	3.03 <sup>a</sup>	2.92 <sup>a</sup>	2.49 <sup>ab</sup>	1.09 <sup>b</sup>	1.15 <sup>b</sup>	1.06 <sup>b</sup>	1.21 <sup>ab</sup>	1.10 <sup>b</sup>	1.38 <sup>a</sup>	3.02 <sup>b</sup>	3.04 <sup>b</sup>	2.89 <sup>b</sup>	2.65 <sup>b</sup>	4.07 <sup>a</sup>	3.23 <sup>b</sup>
<b>cis-<math>\gamma</math>-cadinene</b>	<b>1515</b>	2.61 <sup>b</sup>	2.92 <sup>ab</sup>	3.32 <sup>a</sup>	2.85 <sup>ab</sup>	2.67 <sup>b</sup>	2.11 <sup>a</sup>	1.87 <sup>a</sup>	1.58 <sup>a</sup>	1.83 <sup>a</sup>	1.97 <sup>a</sup>	1.87 <sup>a</sup>	2.68 <sup>a</sup>	2.86 <sup>a</sup>	3.14 <sup>a</sup>	2.81 <sup>a</sup>	3.08 <sup>a</sup>	2.98 <sup>a</sup>
<b>spathulenol</b>	<b>1584</b>	1.06 <sup>a</sup>	1.21 <sup>a</sup>	0.83 <sup>b</sup>	1.23 <sup>a</sup>	-	0.35 <sup>a</sup>	0.32 <sup>a</sup>	0.25 <sup>a</sup>	0.35 <sup>a</sup>	0.33 <sup>a</sup>	0.40 <sup>a</sup>	-	-	-	-	-	-
<b>1,10-di-epi-cubonole</b>	<b>1621</b>	1.29 <sup>a</sup>	1.35 <sup>a</sup>	1.32 <sup>a</sup>	1.35 <sup>a</sup>	0.84 <sup>b</sup>	0.67 <sup>a</sup>	0.61 <sup>a</sup>	0.50 <sup>a</sup>	0.58 <sup>a</sup>	0.54 <sup>a</sup>	0.61 <sup>a</sup>	1.14 <sup>a</sup>	1.23 <sup>a</sup>	1.25 <sup>a</sup>	1.23 <sup>a</sup>	1.35 <sup>a</sup>	1.30 <sup>a</sup>
<b><math>\tau</math>-cadinol</b>	<b>1644</b>	9.23 <sup>ab</sup>	9.74 <sup>a</sup>	9.53 <sup>a</sup>	9.65 <sup>a</sup>	8.36 <sup>b</sup>	6.85 <sup>a</sup>	5.77 <sup>ab</sup>	4.89 <sup>b</sup>	5.44 <sup>ab</sup>	5.51 <sup>ab</sup>	5.77 <sup>ab</sup>	8.06 <sup>a</sup>	8.49 <sup>a</sup>	8.59 <sup>a</sup>	8.76 <sup>a</sup>	9.14 <sup>a</sup>	8.87 <sup>a</sup>
<b>compounds &lt;1%</b>		7.64	9.62	8.28	8.57	3.96	4.09	4.88	4.05	5.07	4.58	5.21	4.58	4.53	5.44	4.42	6.00	4.99
<b>monoterpenes</b>		66.72	65.18	63.36	65.65	73.67	78.69	76.68	80.38	79.23	80.60	77.23	61.51	60.63	59.87	61.70	55.28	60.69
<b>sesquiterpenes</b>		30.65	32.55	34.88	32.09	25.67	19.28	20.72	17.28	18.02	17.43	19.64	28.52	29.63	30.26	29.55	33.40	29.27
<b>total</b>		97.38	97.73	98.24	97.75	99.34	97.97	97.39	97.67	97.25	98.03	96.87	90.03	90.26	90.12	91.25	88.68	89.96

Values are presented as Mean. <sup>1</sup> Retention indices. <sup>2</sup> trace (below 0.1%). C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%). Compounds that reached at least 1% GC area percentage are shown.



**Figure 17 Principal component plot analysis on the essential oil composition of basil samples originating from three years**

The analysis of the essential oil composition of hyssop over three years is presented in Table 15, demonstrating variance in the ratio of the dominant constituent isopinocampone as well as of other compounds. The ratio of the main compound varied from 29.07% in the control plants of the first year to 49.52% in the TMAO treated plants in the last year. In 2020, all treatments increased its ratio, with the highest elevations observed with MeJa 2 (28%) and SA 1 (60%) elicitors. In the next year, however, only SA 2 and TMAO influenced it, dropping the ratio by 29 and 17%, respectively. In 2022, only TMAO showed an effect on this compound, though in the opposite direction: an increase of 38% was registered. The isomer *trans*-pinocampone could not be detected in the first year but changed significantly afterwards (when we used another accession) due to MeJa and SA treatments (up to a six-fold increase in 2021 by SA 2) but dropped after TMAO spraying. The other characteristic monoterpene,  $\beta$ -pinene, was less affected by the treatments, except for significant elevations by MeJa and SA 1 in 2020, decreased in 2021 due to TMAO, and increased again in 2022 by the same treatment. The effect of the elicitors MeJa and SA on  $\beta$ -phellandrene manifested in decreasing its ratio on several occasions (the largest one of -54% in 2020), but TMAO increased it both in 2021 and 2022, by 67 and 168%, respectively.

Concerning the sesquiterpenes, their overall ratio was decreased by all the treatments in most trials, except for the TMAO treatment in 2022 (+38% rise). This treatment also induced the most changes in the individual sesquiterpene compounds, especially in the last year. Elemol, the main compound among these sesquiterpenes, did not change significantly in the first year but showed an elevation

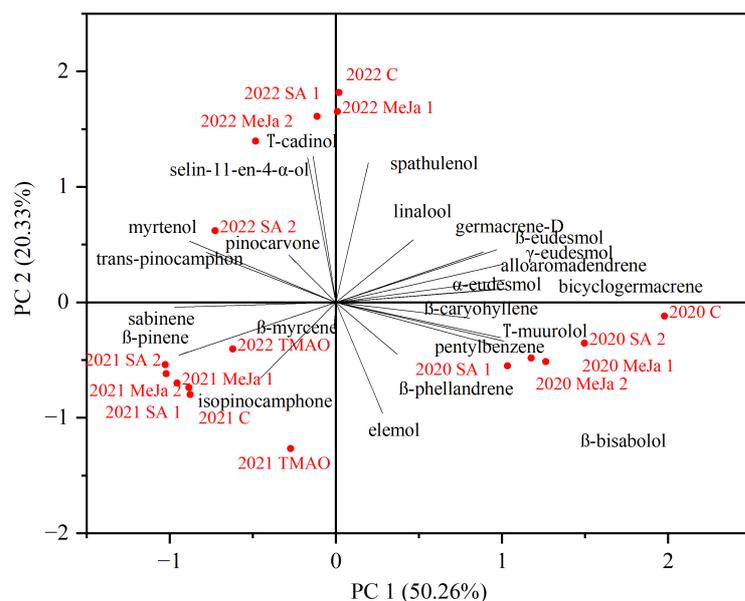
in the next years due to the TMAO treatment. At the same time, the same elicitor significantly decreased several other sesquiterpene compounds, such as  $\beta$ -caryophyllene, germacrene D,  $\beta$ -eudesmol, and selin-11-en-4- $\alpha$ -ol leading to a substantial reduction in total sesquiterpenes by 56%.

The principal component analysis for the EO of hyssop across the three experimental years is depicted in Figure 18. The two principal components (PC1 and PC2) explain 50.26% and 20.33% of the variance, respectively, accounting for a total of 70.59% of the data variability. The clustering indicates clear distinctions based on the year, which reflect considerable changes in the chemical composition of hyssop oil depending on the experimental year. The 2020 group aligns mostly with compounds like  $\beta$ -bisabolol, pentylbenzene, and  $\tau$ -muurolol. The 2021 cluster shows a dominance in sabinene,  $\beta$ -pinene, and isopinocampone compounds. While the 2022 samples are strongly associated with selin-11-en-4- $\alpha$ -ol, spathulenol, and  $\tau$ -cadinol. However, treatment-specific subtle variations were still evident within every year. For instance, the SA 1 treated samples are positioned relatively far from the rest of the treatments in 2020, which aligns with the results from Table 15, where this treatment had the highest differences in monoterpenes and sesquiterpenes. Similarly, TMAO-treated samples from the subsequent years also point toward a significant shift in their chemical profile, with TMAO samples from 2022 clustering more closely to the 2021 group.

**Table 15 EO composition (GC area %) of hyssop sample in open field between 2020-2022**

Compound	RI <sup>1</sup>	2020					2021					2022						
		C	MeJa 1	MeJa 2	SA 1	SA 2	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO
sabinene	976	0.74 <sup>a</sup>	1.04 <sup>a</sup>	1.06 <sup>a</sup>	1.05 <sup>a</sup>	0.89 <sup>a</sup>	2.37 <sup>a</sup>	2.49 <sup>a</sup>	1.93 <sup>a</sup>	1.96 <sup>a</sup>	2.44 <sup>a</sup>	1.57 <sup>a</sup>	1.74 <sup>a</sup>	1.48 <sup>a</sup>	1.95 <sup>a</sup>	1.76 <sup>a</sup>	1.63 <sup>a</sup>	1.45 <sup>a</sup>
β-pinene	981	3.44 <sup>b</sup>	5.20 <sup>a</sup>	5.74 <sup>a</sup>	5.93 <sup>a</sup>	4.47 <sup>ab</sup>	15.05 <sup>a</sup>	13.08 <sup>ab</sup>	11.22 <sup>ab</sup>	12.12 <sup>ab</sup>	12.39 <sup>ab</sup>	8.76 <sup>b</sup>	7.04 <sup>b</sup>	6.00 <sup>b</sup>	8.08 <sup>b</sup>	7.96 <sup>b</sup>	8.28 <sup>ab</sup>	9.66 <sup>a</sup>
β-myrcene	995	2.14 <sup>a</sup>	2.56 <sup>a</sup>	2.03 <sup>a</sup>	1.49 <sup>a</sup>	1.65 <sup>a</sup>	2.14 <sup>bc</sup>	1.76 <sup>cd</sup>	1.34 <sup>d</sup>	1.70 <sup>cd</sup>	2.40 <sup>b</sup>	3.35 <sup>a</sup>	2.08 <sup>ab</sup>	2.03 <sup>ab</sup>	1.61 <sup>ab</sup>	2.34 <sup>a</sup>	1.59 <sup>b</sup>	2.42 <sup>a</sup>
β-phellandrene	1029	12.85 <sup>ab</sup>	14.46 <sup>a</sup>	10.61 <sup>b</sup>	5.89 <sup>c</sup>	8.12 <sup>bc</sup>	8.16 <sup>b</sup>	4.14 <sup>c</sup>	3.35 <sup>c</sup>	6.25 <sup>bc</sup>	8.95 <sup>b</sup>	21.92 <sup>a</sup>	7.23 <sup>b</sup>	7.62 <sup>b</sup>	3.42 <sup>c</sup>	9.02 <sup>b</sup>	4.29 <sup>c</sup>	12.09 <sup>a</sup>
linalool	1097	1.10 <sup>a</sup>	1.12 <sup>a</sup>	1.05 <sup>a</sup>	1.00 <sup>a</sup>	0.99 <sup>a</sup>	0.92 <sup>b</sup>	0.73 <sup>b</sup>	0.90 <sup>b</sup>	0.92 <sup>b</sup>	0.91 <sup>b</sup>	1.17 <sup>a</sup>	1.44 <sup>a</sup>	1.15 <sup>a</sup>	0.95 <sup>a</sup>	1.04 <sup>a</sup>	0.91 <sup>a</sup>	0.99 <sup>a</sup>
pentylbenzene	1152	2.29 <sup>b</sup>	2.93 <sup>a</sup>	3.18 <sup>a</sup>	3.78 <sup>a</sup>	3.19 <sup>a</sup>	-	-	-	-	-	-	-	-	-	-	-	-
trans-pinocamphone	1163	-	-	-	-	-	3.13 <sup>dc</sup>	11.70 <sup>bc</sup>	15.04 <sup>ab</sup>	7.99 <sup>cd</sup>	20.92 <sup>a</sup>	1.58 <sup>c</sup>	6.17 <sup>c</sup>	10.60 <sup>b</sup>	15.48 <sup>a</sup>	10.26 <sup>b</sup>	12.66 <sup>ab</sup>	2.78 <sup>d</sup>
pinocarvone	1166	0.14 <sup>c</sup>	0.18 <sup>c</sup>	0.32 <sup>b</sup>	0.37 <sup>b</sup>	0.63 <sup>a</sup>	1.28 <sup>a</sup>	0.51 <sup>b</sup>	-	-	-	-	-	-	0.87 <sup>b</sup>	-	3.97 <sup>a</sup>	1.21 <sup>b</sup>
isopinocamphone	1170	29.07 <sup>c</sup>	36.33 <sup>b</sup>	37.30 <sup>b</sup>	46.36 <sup>a</sup>	37.16 <sup>b</sup>	45.56 <sup>a</sup>	42.64 <sup>ab</sup>	46.28 <sup>a</sup>	47.85 <sup>a</sup>	32.56 <sup>c</sup>	37.90 <sup>bc</sup>	35.76 <sup>b</sup>	33.90 <sup>b</sup>	35.26 <sup>b</sup>	34.64 <sup>b</sup>	34.94 <sup>b</sup>	49.52 <sup>a</sup>
myrtenol	1194	-	-	-	-	-	1.28 <sup>bc</sup>	1.34 <sup>abc</sup>	1.50 <sup>abc</sup>	1.73 <sup>ab</sup>	2.05 <sup>a</sup>	0.93 <sup>c</sup>	1.92 <sup>a</sup>	1.65 <sup>a</sup>	1.68 <sup>a</sup>	1.28 <sup>a</sup>	1.58 <sup>a</sup>	0.50 <sup>b</sup>
β-caryophyllene	1420	1.99 <sup>a</sup>	1.84 <sup>a</sup>	1.55 <sup>a</sup>	1.90 <sup>a</sup>	2.05 <sup>a</sup>	1.34 <sup>a</sup>	1.66 <sup>a</sup>	1.08 <sup>a</sup>	1.15 <sup>a</sup>	1.20 <sup>a</sup>	1.40 <sup>a</sup>	1.47 <sup>ab</sup>	1.33 <sup>abc</sup>	1.52 <sup>a</sup>	1.04 <sup>bc</sup>	0.98 <sup>c</sup>	0.45 <sup>d</sup>
alloaromadendrene	1462	2.10 <sup>a</sup>	1.89 <sup>a</sup>	1.51 <sup>a</sup>	1.97 <sup>a</sup>	2.29 <sup>a</sup>	0.83 <sup>a</sup>	0.82 <sup>a</sup>	0.50 <sup>a</sup>	0.83 <sup>a</sup>	0.70 <sup>a</sup>	0.72 <sup>a</sup>	1.34 <sup>a</sup>	1.38 <sup>a</sup>	1.15 <sup>a</sup>	1.28 <sup>a</sup>	1.21 <sup>a</sup>	0.56 <sup>b</sup>
germacrene D	1482	4.61 <sup>a</sup>	4.01 <sup>a</sup>	3.12 <sup>a</sup>	4.38 <sup>a</sup>	4.72 <sup>a</sup>	2.77 <sup>a</sup>	2.42 <sup>a</sup>	1.89 <sup>a</sup>	2.39 <sup>a</sup>	1.87 <sup>a</sup>	2.39 <sup>a</sup>	4.10 <sup>a</sup>	3.63 <sup>a</sup>	3.39 <sup>a</sup>	3.81 <sup>a</sup>	2.49 <sup>ab</sup>	1.18 <sup>b</sup>
bicyclogermacrene	1497	5.46 <sup>a</sup>	4.62 <sup>a</sup>	4.02 <sup>a</sup>	4.91 <sup>a</sup>	5.81 <sup>a</sup>	2.17 <sup>a</sup>	2.03 <sup>a</sup>	1.98 <sup>a</sup>	2.21 <sup>a</sup>	2.31 <sup>a</sup>	3.28 <sup>a</sup>	3.67 <sup>a</sup>	3.32 <sup>a</sup>	3.09 <sup>ab</sup>	3.56 <sup>a</sup>	2.77 <sup>ab</sup>	1.74 <sup>b</sup>
elemol	1553	6.03 <sup>a</sup>	5.75 <sup>a</sup>	5.42 <sup>a</sup>	5.10 <sup>a</sup>	5.68 <sup>a</sup>	4.36 <sup>b</sup>	4.34 <sup>b</sup>	4.69 <sup>b</sup>	4.81 <sup>b</sup>	5.54 <sup>ab</sup>	8.69 <sup>a</sup>	3.36 <sup>b</sup>	3.61 <sup>b</sup>	2.86 <sup>b</sup>	3.17 <sup>b</sup>	6.79 <sup>a</sup>	6.21 <sup>a</sup>
spathulenol	1584	0.79 <sup>a</sup>	0.51 <sup>a</sup>	0.55 <sup>a</sup>	0.41 <sup>a</sup>	0.54 <sup>a</sup>	-	-	-	-	-	-	1.25 <sup>ab</sup>	1.42 <sup>a</sup>	1.43 <sup>a</sup>	1.27 <sup>ab</sup>	1.30 <sup>ab</sup>	0.86 <sup>b</sup>
γ-eudesmol	1635	2.73 <sup>a</sup>	1.69 <sup>ab</sup>	1.77 <sup>ab</sup>	1.43 <sup>b</sup>	1.64 <sup>ab</sup>	-	-	-	-	-	-	1.01 <sup>a</sup>	1.50 <sup>a</sup>	0.52 <sup>b</sup>	0.74 <sup>b</sup>	-	-
τ-cadinol	1644	-	-	-	-	-	-	-	-	-	-	-	1.89 <sup>a</sup>	1.44 <sup>a</sup>	0.99 <sup>ab</sup>	1.10 <sup>ab</sup>	0.90 <sup>ab</sup>	-
τ-muurolol	1647	2.18 <sup>a</sup>	0.89 <sup>b</sup>	1.32 <sup>ab</sup>	0.70 <sup>b</sup>	1.25 <sup>b</sup>	-	-	-	-	-	-	-	-	-	-	-	-
β-eudesmol	1653	2.21 <sup>a</sup>	1.33 <sup>b</sup>	1.52 <sup>ab</sup>	0.99 <sup>b</sup>	1.31 <sup>b</sup>	-	-	-	-	-	-	1.03 <sup>a</sup>	1.01 <sup>a</sup>	0.56 <sup>ab</sup>	0.79 <sup>a</sup>	0.67 <sup>a</sup>	0.31 <sup>b</sup>
α-eudesmol	1656	2.40 <sup>a</sup>	1.60 <sup>ab</sup>	2.37 <sup>a</sup>	1.11 <sup>b</sup>	1.57 <sup>ab</sup>	-	-	-	-	-	-	0.75 <sup>a</sup>	0.74 <sup>a</sup>	0.47 <sup>a</sup>	0.60 <sup>a</sup>	0.83 <sup>a</sup>	0.53 <sup>a</sup>
selin-11-en-4-α-ol	1661	-	-	-	-	-	-	-	-	-	-	-	2.99 <sup>a</sup>	3.09 <sup>a</sup>	2.13 <sup>a</sup>	2.69 <sup>a</sup>	0.91 <sup>b</sup>	0.49 <sup>b</sup>
β-bisabolol	1671	6.18 <sup>a</sup>	4.53 <sup>ab</sup>	4.22 <sup>b</sup>	4.11 <sup>b</sup>	5.05 <sup>ab</sup>	-	-	-	-	-	-	-	-	-	-	-	-
unidentified		-	-	-	-	-	3.67	3.90	4.64	3.64	1.62	3.17	2.77	3.45	4.17	3.32	3.59	1.73
compounds <1%		1.45	2.19	2.43	2.00	2.63	4.54	5.93	3.40	3.56	3.77	2.58	7.46	6.30	6.93	7.10	4.84	2.43
monoterpenes		50.59	63.81	61.27	65.85	57.09	87.09	87.10	88.79	86.89	87.20	82.18	69.85	70.99	77.77	76.43	76.27	84.48
sesquiterpenes		39.31	30.86	29.82	29.03	34.56	12.46	12.38	10.95	12.22	12.43	17.23	26.61	25.65	20.76	22.32	20.81	12.60
total		89.90	94.67	91.09	94.88	91.65	99.55	99.48	99.74	99.11	99.64	99.41	96.46	96.64	98.53	98.75	97.08	97.08

Values are presented as Mean. <sup>1</sup> Retention indices. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%). Compounds that reached at least 1% GC area percentage are shown.



**Figure 18 Principal component plot analysis on the essential oil composition of hyssop samples originating from three years**

Table 16 shows the EO constituents identified in marjoram. The ratio of the major components, *cis*-sabinene hydrate, linalool, and terpinene-4-ol, reacted similarly towards the higher concentration of SA elicitor in the first and last year. *Cis*-sabinene hydrate and linalool were decreased significantly (by 15%) in 2020 and by 16 and 10%, respectively, in 2022, with the linalool decrease not being significant. Conversely, terpinene-4-ol was increased remarkably with this treatment by 29 and 17% in the first and last year, respectively. Similar, but not significant, increasing tendencies in this component were also registered due to other treatments in both years. In 2021, none of these major components changed significantly after foliar spraying of the elicitors, except *cis*-sabinene hydrate accumulation that was enhanced significantly with TMAO by 17%. Moreover, this elicitor seems to have a higher impact on the EO composition of marjoram in 2021, especially on the ratio of terpinene compounds (-10% of total terpenes). However, the ratios of terpinene-type compounds were elevated in several other cases. Each  $\alpha$ -terpineol,  $\alpha$ - and  $\gamma$ -terpinene were elevated due to the MeJa 2 treatment in the first and second year. Whereas the same treatment suppressed the accumulation of these volatiles in 2022. However, none of these changes in the terpinene components were statistically significant. When analyzing the combined ratio of terpinene-type compounds in the oil, it becomes evident that, in the majority of cases, the treatments were effective in increasing these ratios. On the other hand, it seems that the lowest accumulation of monoterpenes was obtained in each year by SA 1 treatment and was associated

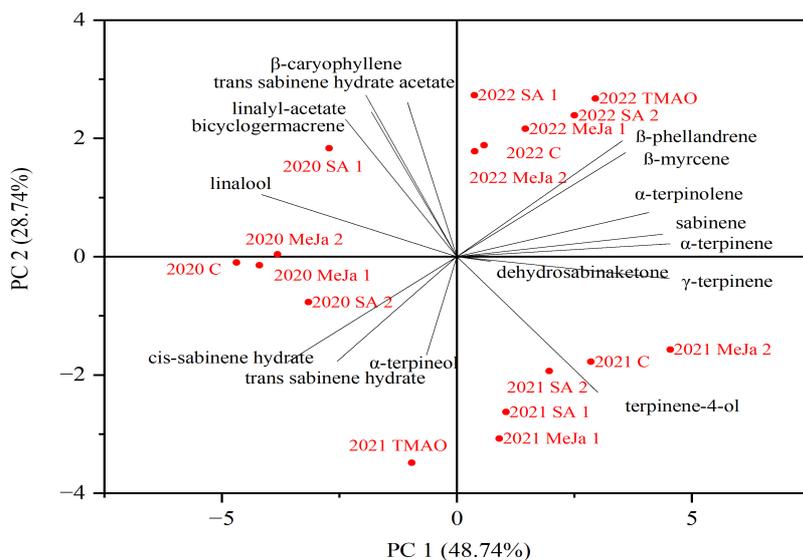
with a concomitant rise in sesquiterpenes reaching +33, 31, and 3% in 2020, 2021, and 2022, respectively.

The differences in EO of marjoram were further evaluated by a PCA analysis in Figure 19. The two principal axes of the PCA plot represent a total of 77.48% of the variance. The left side of the plot contains scattered treatments from 2020 separated from the other years, with SA 1 being distant from the other treatments and characterized by higher bicyclogermacrene,  $\beta$ -caryophyllene, *trans* sabinene hydrate acetate, and linalyl-acetate. The 2022 samples are closely located in the upper right side, being rich in monoterpene hydrocarbons such as sabinene,  $\beta$ -phellandrene, and  $\beta$ -myrcene. Whereas the samples from 2021 are clustered together in the lower right area of the plot, except for the TMAO that is more distant and shows greater dissimilarities with the other treatments from the same year.

**Table 16 EO composition (GC area %) of marjoram samples in open field between 2020-2022**

	RI <sup>1</sup>	2020					2021					2022						
		C	MeJa 1	MeJa 2	SA 1	SA 2	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO
<b>sabinene</b>	<b>976</b>	3.52 <sup>a</sup>	3.42 <sup>a</sup>	3.19 <sup>a</sup>	4.52 <sup>a</sup>	3.77 <sup>a</sup>	6.92 <sup>a</sup>	5.06 <sup>b</sup>	7.32 <sup>a</sup>	5.12 <sup>b</sup>	6.33 <sup>ab</sup>	4.58 <sup>b</sup>	5.90 <sup>a</sup>	6.33 <sup>a</sup>	5.80 <sup>a</sup>	5.43 <sup>a</sup>	6.19 <sup>a</sup>	6.25 <sup>a</sup>
<b>β-myrcene</b>	<b>995</b>	0.8 <sup>a</sup>	0.76 <sup>a</sup>	0.70 <sup>a</sup>	1.03 <sup>a</sup>	0.86 <sup>a</sup>	1.28 <sup>ab</sup>	1.05 <sup>b</sup>	1.40 <sup>a</sup>	1.07 <sup>b</sup>	1.26 <sup>ab</sup>	0.95 <sup>b</sup>	1.60 <sup>a</sup>	1.68 <sup>a</sup>	1.58 <sup>a</sup>	1.42 <sup>a</sup>	1.64 <sup>a</sup>	1.61 <sup>a</sup>
<b>α-terpinene</b>	<b>1018</b>	2.12 <sup>a</sup>	2.24 <sup>a</sup>	2.29 <sup>a</sup>	2.87 <sup>a</sup>	2.75 <sup>a</sup>	5.20 <sup>ab</sup>	4.37 <sup>b</sup>	6.18 <sup>a</sup>	4.25 <sup>b</sup>	4.91 <sup>ab</sup>	3.47 <sup>c</sup>	4.44 <sup>ab</sup>	4.94 <sup>ab</sup>	4.16 <sup>b</sup>	4.32 <sup>b</sup>	4.80 <sup>ab</sup>	5.55 <sup>a</sup>
<b>β-phellandrene</b>	<b>1029</b>	2.08 <sup>a</sup>	2.18 <sup>a</sup>	2.22 <sup>a</sup>	2.48 <sup>a</sup>	2.19 <sup>a</sup>	2.66 <sup>a</sup>	2.32 <sup>a</sup>	2.84 <sup>a</sup>	2.42 <sup>a</sup>	2.66 <sup>a</sup>	2.15 <sup>a</sup>	2.98 <sup>a</sup>	2.96 <sup>a</sup>	2.91 <sup>a</sup>	2.74 <sup>a</sup>	3.07 <sup>a</sup>	3.04 <sup>a</sup>
<b>γ-terpinene</b>	<b>1056</b>	4.30 <sup>a</sup>	4.96 <sup>a</sup>	4.83 <sup>a</sup>	5.47 <sup>a</sup>	5.48 <sup>a</sup>	8.94 <sup>ab</sup>	8.20 <sup>b</sup>	10.17 <sup>a</sup>	8.34 <sup>b</sup>	8.55 <sup>b</sup>	6.89 <sup>c</sup>	7.27 <sup>bc</sup>	7.89 <sup>ab</sup>	7.06 <sup>c</sup>	7.58 <sup>bc</sup>	8.86 <sup>a</sup>	8.86 <sup>a</sup>
<b>trans-sabinene hydrate</b>	<b>1070</b>	6.57 <sup>a</sup>	7.10 <sup>a</sup>	6.99 <sup>a</sup>	6.65 <sup>a</sup>	7.18 <sup>a</sup>	6.40 <sup>a</sup>	6.95 <sup>a</sup>	6.49 <sup>a</sup>	7.03 <sup>a</sup>	6.66 <sup>a</sup>	7.18 <sup>a</sup>	6.46 <sup>a</sup>	6.57 <sup>a</sup>	6.66 <sup>a</sup>	6.56 <sup>a</sup>	6.50 <sup>a</sup>	6.28 <sup>a</sup>
<b>α-terpinolene</b>	<b>1085</b>	0.98 <sup>a</sup>	1.12 <sup>a</sup>	1.63 <sup>a</sup>	1.29 <sup>a</sup>	1.25 <sup>a</sup>	1.75 <sup>a</sup>	1.69 <sup>a</sup>	2.05 <sup>a</sup>	1.76 <sup>a</sup>	1.74 <sup>a</sup>	1.39 <sup>a</sup>	1.69 <sup>b</sup>	1.85 <sup>b</sup>	1.66 <sup>b</sup>	1.82 <sup>b</sup>	1.92 <sup>ab</sup>	2.15 <sup>a</sup>
<b>cis-sabinene hydrate</b>	<b>1096</b>	27.98 <sup>a</sup>	25.48 <sup>ab</sup>	24.80 <sup>ab</sup>	21.91 <sup>b</sup>	23.77 <sup>b</sup>	21.46 <sup>b</sup>	22.21 <sup>ab</sup>	19.38 <sup>b</sup>	20.34 <sup>b</sup>	21.59 <sup>b</sup>	25.20 <sup>a</sup>	19.15 <sup>a</sup>	18.05 <sup>a</sup>	19.22 <sup>a</sup>	17.15 <sup>ab</sup>	16.03 <sup>b</sup>	15.27 <sup>b</sup>
<b>linalool</b>	<b>1097</b>	20.26 <sup>a</sup>	18.28 <sup>ab</sup>	17.95 <sup>ab</sup>	15.86 <sup>b</sup>	17.22 <sup>b</sup>	9.20 <sup>ab</sup>	9.53 <sup>ab</sup>	7.82 <sup>b</sup>	8.72 <sup>ab</sup>	9.26 <sup>ab</sup>	10.80 <sup>a</sup>	12.93 <sup>a</sup>	13.07 <sup>a</sup>	13.87 <sup>a</sup>	12.47 <sup>a</sup>	11.59 <sup>a</sup>	11.07 <sup>a</sup>
<b>dehydrosabinaketone</b>	<b>1120</b>	t <sup>2</sup>	t	t	t	t	1.38 <sup>a</sup>	1.54 <sup>a</sup>	1.46 <sup>a</sup>	1.52 <sup>a</sup>	1.34 <sup>a</sup>	1.31 <sup>a</sup>	1.37 <sup>a</sup>	1.42 <sup>a</sup>	1.40 <sup>a</sup>	1.40 <sup>a</sup>	1.47 <sup>a</sup>	1.55 <sup>a</sup>
<b>terpinene-4-ol</b>	<b>1175</b>	11.62 <sup>b</sup>	14.02 <sup>ab</sup>	13.28 <sup>ab</sup>	12.67 <sup>ab</sup>	14.99 <sup>a</sup>	19.19 <sup>a</sup>	20.63 <sup>a</sup>	20.67 <sup>a</sup>	20.90 <sup>a</sup>	18.37 <sup>a</sup>	18.70 <sup>a</sup>	13.87 <sup>b</sup>	14.29 <sup>b</sup>	14.00 <sup>b</sup>	14.71 <sup>b</sup>	16.23 <sup>a</sup>	15.60 <sup>ab</sup>
<b>α-terpineol</b>	<b>1189</b>	4.70 <sup>a</sup>	4.14 <sup>a</sup>	4.56 <sup>a</sup>	4.52 <sup>a</sup>	4.57 <sup>a</sup>	4.35 <sup>b</sup>	4.82 <sup>ab</sup>	4.23 <sup>b</sup>	4.93 <sup>a</sup>	4.98 <sup>a</sup>	5.20 <sup>a</sup>	5.06 <sup>a</sup>	4.28 <sup>b</sup>	4.48 <sup>ab</sup>	4.43 <sup>ab</sup>	4.09 <sup>b</sup>	4.37 <sup>ab</sup>
<b>trans-sabinene hydrate acetate</b>	<b>1247</b>	2.47 <sup>a</sup>	2.48 <sup>a</sup>	2.76 <sup>a</sup>	3.96 <sup>a</sup>	3.95 <sup>a</sup>	0.87 <sup>a</sup>	1.17 <sup>a</sup>	1.24 <sup>a</sup>	1.09 <sup>a</sup>	1.18 <sup>a</sup>	1.31 <sup>a</sup>	2.42 <sup>b</sup>	2.41 <sup>b</sup>	2.87 <sup>b</sup>	4.14 <sup>a</sup>	5.17 <sup>a</sup>	3.79 <sup>ab</sup>
<b>linalyl-acetate</b>	<b>1250</b>	4.27 <sup>a</sup>	3.00 <sup>b</sup>	3.36 <sup>ab</sup>	3.44 <sup>ab</sup>	3.51 <sup>ab</sup>	2.33 <sup>b</sup>	2.08 <sup>ab</sup>	1.79 <sup>c</sup>	2.41 <sup>b</sup>	2.95 <sup>a</sup>	2.53 <sup>b</sup>	4.20 <sup>a</sup>	3.32 <sup>a</sup>	3.86 <sup>a</sup>	4.49 <sup>a</sup>	3.40 <sup>a</sup>	3.82 <sup>a</sup>
<b>β-caryophyllene</b>	<b>1420</b>	3.22 <sup>b</sup>	3.88 <sup>a</sup>	3.69 <sup>ab</sup>	4.14 <sup>a</sup>	3.11 <sup>b</sup>	2.13 <sup>b</sup>	2.50 <sup>ab</sup>	2.18 <sup>b</sup>	2.79 <sup>a</sup>	2.30 <sup>ab</sup>	2.59 <sup>a</sup>	3.56 <sup>b</sup>	3.78 <sup>b</sup>	3.67 <sup>b</sup>	4.68 <sup>a</sup>	3.92 <sup>b</sup>	3.49 <sup>b</sup>
<b>bicyclogermacrene</b>	<b>1497</b>	2.89 <sup>b</sup>	3.63 <sup>a</sup>	3.63 <sup>a</sup>	4.20 <sup>a</sup>	2.26 <sup>b</sup>	1.72 <sup>b</sup>	1.90 <sup>ab</sup>	1.64 <sup>b</sup>	2.24 <sup>a</sup>	1.77 <sup>b</sup>	2.05 <sup>a</sup>	3.61 <sup>a</sup>	3.47 <sup>a</sup>	2.88 <sup>a</sup>	3.02 <sup>a</sup>	2.91 <sup>a</sup>	3.06 <sup>a</sup>
<b>compounds &lt;1%</b>		0.85	0.96	1.08	1.07	1.11	4.77	4.64	4.94	5.47	4.62	2.87	4.73	5.00	5.09	5.26	5.86	4.15
<b>monoterpenes</b>		92.52	90.12	89.61	87.75	92.60	95.34	94.71	95.39	93.86	95.06	94.54	92.52	92.45	93.01	92.23	93.10	93.20
<b>sesquiterpenes</b>		6.11	7.51	7.32	8.33	5.38	3.85	4.41	3.81	5.03	4.07	4.64	7.34	7.43	6.76	7.56	6.76	6.72
<b>total</b>		98.63	97.63	96.92	96.08	97.98	99.19	99.12	99.21	98.89	99.13	99.19	99.86	99.88	99.77	99.78	99.86	99.93

Values are presented as Mean. <sup>1</sup> Retention indices. <sup>2</sup> trace (percentage <0.1%). C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%). Compounds that reached at least 1% GC area percentage are shown.



**Figure 19 Principal component plot analysis on the essential oil composition of marjoram samples originating from three years**

The GC-MS analysis of peppermint EO in three experimental years is presented in Table 17. There is a high variability in several components among the treatments in 2021, whereas the first year showed the least significant compositional differences. Limonene and 1,8-cineole were significantly enhanced by all treatments in 2021. The largest increases were observed with the higher concentrations of MeJa and SA on limonene (38 and 47% increase, respectively), and for 1,8-cineole, the ratios elevated by 31 and 34%, respectively. In the other years the effects were statistically insignificant. only SA 2 had an effect, reducing menthone by 9% while increasing menthol by 12%. Interestingly, the following year showed the opposite trend, with SA 2 stimulating menthone accumulation over menthol, leading to a 23% increase in the former and a 17% decrease in the latter. In 2022, both compounds followed a similar pattern, with menthone increasing by 30% and 18%, and menthol by 20% and 22%, under SA 1 and SA 2 treatments, respectively. Notably, menthol's isomers and acetate ester reacted similarly to the treatment, with their ratios being significantly decreased: neomenthol by 9%, isomenthol by 45%, and iso-menthyl acetate by 51%. The other treatments were able to change their ratios significantly as well, in most cases. Besides these changes, pulegone ratio increased by 140% in 2021 due to SA 2 and by 63 and 72% with SA 1 and SA 2, respectively in 2022. The most important compounds of peppermint EO, menthol and menthone were less frequently changed in the MeJa treatments, except for increasing the ratio of menthone in 2021 and increasing the menthol ratios in 2020 (only MeJa 1) and 2022. Neomenthol was also elevated by approximately 12% after MeJa treatments in this year, while decreased in 2021 with MeJa 1. The only sesquiterpenes detected above 1% ratio were germacrene D and viridiflorol; only slight variations were observed throughout the experiments,

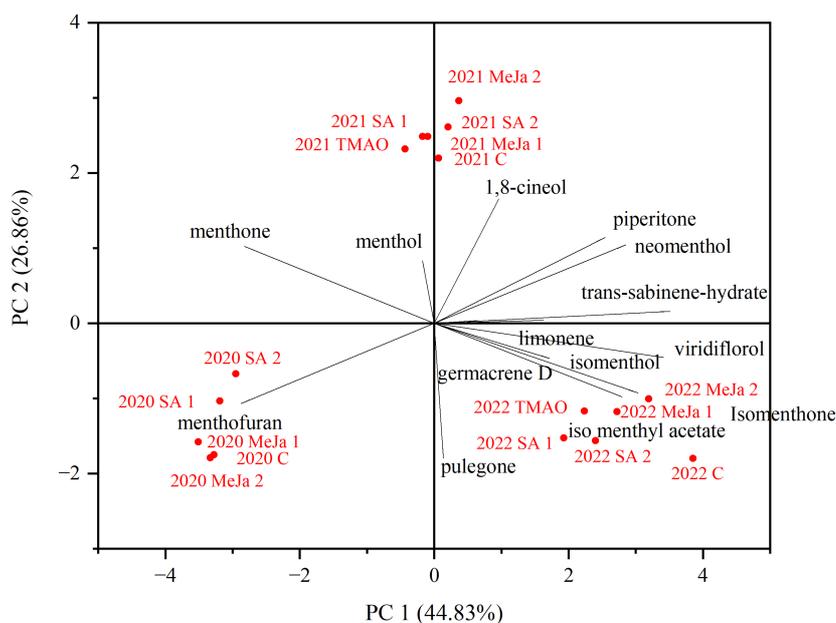
except for germacrene D, which decreased after foliar spraying of TMAO in 2022 by 34%. Similarly, the total monoterpenes and sesquiterpenes did not experience major changes with our treatments.

Further evaluation of the changes in the EO of peppermint was carried out by two-dimensional PCA in Figure 20. The principal components (PC1 and PC2) represented 71.69% of the total information. Three distinctive groups were identified as belonging to each experiment. The cluster from the first experimental year is situated in the lower left of the plot and characterized by high ratios of menthofuran; however, it is worth mentioning that in this year isomenthone and menthofuran could not be separated, so the ratios presented in Table 17 represent the mixture of these two compounds. Moreover, the distances indicate more pronounced dissimilarities between SA and the other treatments. Treatments from 2021 are clustered in the mid-upper side of the plot with relative homogeneity of the samples. Whereas the third cluster from 2022 is situated on the lower right side, featuring higher isomenthone, isomenthol, and *iso*-menthyl acetate content along with the sesquiterpenes. Moreover, the EO from the control seems to have higher dissimilarities with the other treatments from this year.

**Table 17 EO composition (GC area%) of peppermint samples in open field between 2020-2022**

	RI <sup>1</sup>	2020					2021					2022						
		C	MeJa 1	MeJa 2	SA 1	SA 2	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO
<b>limonene</b>	<b>1029</b>	5.51 <sup>a</sup>	5.33 <sup>a</sup>	5.23 <sup>a</sup>	6.02 <sup>a</sup>	6.15 <sup>a</sup>	4.61 <sup>d</sup>	6.28 <sup>ab</sup>	6.38 <sup>ab</sup>	5.76 <sup>bc</sup>	6.78 <sup>a</sup>	5.41 <sup>c</sup>	6.53 <sup>a</sup>	6.39 <sup>a</sup>	6.09 <sup>a</sup>	6.04 <sup>a</sup>	6.15 <sup>a</sup>	5.88 <sup>a</sup>
<b>1,8-cineole</b>	<b>1034</b>	4.29 <sup>a</sup>	4.28 <sup>a</sup>	3.94 <sup>a</sup>	4.70 <sup>a</sup>	4.99 <sup>a</sup>	5.50 <sup>d</sup>	6.80 <sup>bc</sup>	7.22 <sup>ab</sup>	6.60 <sup>bc</sup>	7.37 <sup>a</sup>	6.07 <sup>c</sup>	5.84 <sup>a</sup>	4.97 <sup>b</sup>	4.90 <sup>b</sup>	4.84 <sup>b</sup>	4.96 <sup>b</sup>	4.65 <sup>b</sup>
<b>trans-sabinene-hydrate</b>	<b>1070</b>	-	-	-	-	-	0.79 <sup>b</sup>	0.85 <sup>ab</sup>	1.08 <sup>a</sup>	0.88 <sup>ab</sup>	0.92 <sup>ab</sup>	0.78 <sup>b</sup>	1.56 <sup>a</sup>	1.44 <sup>a</sup>	1.53 <sup>a</sup>	1.32 <sup>a</sup>	1.39 <sup>a</sup>	1.42 <sup>a</sup>
<b>menthone</b>	<b>1158</b>	35.05 <sup>a</sup>	32.92 <sup>ab</sup>	35.16 <sup>a</sup>	34.12 <sup>a</sup>	31.81 <sup>b</sup>	30.06 <sup>c</sup>	34.29 <sup>b</sup>	34.44 <sup>b</sup>	37.10 <sup>a</sup>	34.08 <sup>b</sup>	37.53 <sup>a</sup>	18.87 <sup>c</sup>	20.55 <sup>bc</sup>	18.55 <sup>c</sup>	24.49 <sup>a</sup>	22.32 <sup>b</sup>	21.35 <sup>b</sup>
<b>menthofuran</b>	<b>1158</b>	7.70 <sup>a</sup>	7.57 <sup>a</sup>	7.92 <sup>a</sup>	7.52 <sup>a</sup>	7.41 <sup>a</sup>	0.29 <sup>ab</sup>	0.20 <sup>b</sup>	0.26 <sup>bc</sup>	0.29 <sup>ab</sup>	0.34 <sup>a</sup>	0.32 <sup>ab</sup>	0.86 <sup>b</sup>	1.11 <sup>ab</sup>	0.98 <sup>ab</sup>	1.26 <sup>a</sup>	1.39 <sup>a</sup>	1.06 <sup>ab</sup>
<b>neomenthol</b>	<b>1159</b>	-	-	-	-	-	6.86 <sup>a</sup>	5.87 <sup>b</sup>	6.11 <sup>bc</sup>	6.22 <sup>bc</sup>	6.26 <sup>bc</sup>	6.47 <sup>ab</sup>	5.26 <sup>c</sup>	5.82 <sup>b</sup>	5.90 <sup>b</sup>	5.10 <sup>c</sup>	5.26 <sup>c</sup>	6.59 <sup>a</sup>
<b>isomenthone</b>	<b>1168</b>	-	-	-	-	-	0.36 <sup>a</sup>	0.17 <sup>c</sup>	0.22 <sup>b</sup>	0.21 <sup>b</sup>	0.18 <sup>bc</sup>	0.15 <sup>c</sup>	3.04 <sup>a</sup>	3.06 <sup>a</sup>	2.89 <sup>a</sup>	3.12 <sup>a</sup>	3.15 <sup>a</sup>	2.96 <sup>a</sup>
<b>menthol</b>	<b>1171</b>	27.79 <sup>b</sup>	30.68 <sup>a</sup>	27.74 <sup>b</sup>	29.47 <sup>ab</sup>	31.02 <sup>a</sup>	35.10 <sup>a</sup>	31.69 <sup>b</sup>	30.34 <sup>bc</sup>	29.15 <sup>c</sup>	30.21 <sup>bc</sup>	29.57 <sup>c</sup>	24.74 <sup>b</sup>	30.48 <sup>a</sup>	31.17 <sup>a</sup>	29.73 <sup>a</sup>	30.12 <sup>a</sup>	29.79 <sup>a</sup>
<b>isomenthol</b>	<b>1182</b>	-	-	-	-	-	0.29 <sup>a</sup>	0.19 <sup>b</sup>	0.17 <sup>b</sup>	0.16 <sup>b</sup>	0.17 <sup>b</sup>	0.16 <sup>b</sup>	7.76 <sup>a</sup>	0.51 <sup>b</sup>	0.50 <sup>b</sup>	0.35 <sup>b</sup>	0.42 <sup>b</sup>	0.51 <sup>b</sup>
<b>pulegone</b>	<b>1236</b>	1.97 <sup>ab</sup>	1.81 <sup>ab</sup>	2.07 <sup>a</sup>	1.45 <sup>ab</sup>	1.13 <sup>b</sup>	0.10 <sup>b</sup>	0.13 <sup>b</sup>	0.13 <sup>b</sup>	0.14 <sup>b</sup>	0.24 <sup>a</sup>	0.13 <sup>b</sup>	1.41 <sup>b</sup>	1.85 <sup>ab</sup>	1.57 <sup>b</sup>	2.30 <sup>a</sup>	2.43 <sup>a</sup>	1.41 <sup>b</sup>
<b>piperitone</b>	<b>1249</b>	1.58 <sup>a</sup>	1.50 <sup>a</sup>	1.67 <sup>a</sup>	1.60 <sup>a</sup>	1.58 <sup>a</sup>	2.05 <sup>a</sup>	1.86 <sup>b</sup>	2.09 <sup>a</sup>	1.99 <sup>ab</sup>	1.88 <sup>b</sup>	1.97 <sup>ab</sup>	1.87 <sup>a</sup>	1.91 <sup>a</sup>	1.98 <sup>a</sup>	1.85 <sup>a</sup>	1.90 <sup>a</sup>	1.87 <sup>a</sup>
<b>iso-menthyl acetate</b>	<b>1291</b>	4.57 <sup>a</sup>	4.56 <sup>a</sup>	4.12 <sup>a</sup>	3.91 <sup>a</sup>	3.98 <sup>a</sup>	7.45 <sup>a</sup>	3.61 <sup>b</sup>	3.75 <sup>b</sup>	3.68 <sup>b</sup>	3.15 <sup>b</sup>	3.80 <sup>b</sup>	10.21 <sup>ab</sup>	10.70 <sup>ab</sup>	12.08 <sup>a</sup>	10.13 <sup>b</sup>	10.31 <sup>ab</sup>	12.06 <sup>a</sup>
<b>germacrene D</b>	<b>1482</b>	1.78 <sup>a</sup>	1.50 <sup>a</sup>	1.77 <sup>a</sup>	1.48 <sup>a</sup>	1.68 <sup>a</sup>	1.63 <sup>a</sup>	1.75 <sup>a</sup>	1.55 <sup>a</sup>	1.74 <sup>a</sup>	1.68 <sup>a</sup>	1.76 <sup>a</sup>	1.91 <sup>a</sup>	1.68 <sup>ab</sup>	1.94 <sup>a</sup>	1.60 <sup>ab</sup>	1.69 <sup>ab</sup>	1.27 <sup>b</sup>
<b>viridiflorol</b>	<b>1598</b>	-	-	-	-	-	0.32 <sup>a</sup>	0.36 <sup>a</sup>	0.26 <sup>a</sup>	0.31 <sup>a</sup>	0.36 <sup>a</sup>	0.30 <sup>a</sup>	1.05 <sup>a</sup>	0.98 <sup>a</sup>	1.08 <sup>a</sup>	0.83 <sup>a</sup>	0.97 <sup>a</sup>	0.80 <sup>a</sup>
<b>monoterpenes</b>		88.45	88.63	87.83	88.78	88.06	95.97	95.56	96.09	95.68	95.46	95.72	93.02	93.64	92.89	94.82	93.96	94.81
<b>sesquiterpenes</b>		1.78	1.50	1.77	1.48	1.68	3.57	3.76	3.23	3.69	3.72	3.67	6.05	5.44	6.13	4.93	5.31	4.31
<b>compounds &lt;1%</b>		1.01	0.90	1.03	0.94	0.89	3.79	5.00	5.25	5.02	5.54	4.94	8.15	7.63	7.85	6.79	6.84	7.50
<b>total</b>		91.23	91.03	90.63	91.20	90.62	99.19	99.04	99.23	99.27	99.18	99.38	99.08	99.08	99.02	99.76	99.29	99.13

Values are presented as Mean. <sup>1</sup> Retention indices. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%). Compounds that reached at least 1% GC area percentage are shown.



**Figure 20** Principal component plot analysis on the essential oil composition of peppermint

The content of proazulene in the EO of yarrow over three years presented in Table 18 shows a positive correlation between the EO content and the proazulene ratios of this species. The elicitors could not significantly enhance the proazulene' accumulation in any of the experiments; in fact, most of them resulted in a significant decrease in 2020 and 2021. The highest drop, by 26%, was observed with the SA 2 treatment in 2020, followed by MeJa 2 and MeJa 1 treatments (-21 and -17% changes, respectively). In 2021, the highest decreases were registered in the EO of MeJa 1, SA 1, and MeJa 2 treated yarrow plants, by 38, 25, and 18%, respectively. On the other hand, the proazulene content in the last year was slightly enhanced by the elicitors; it ranged from 0.18% in the control plants to 0.20% in MeJa 1 and SA 2 treated plants. However, these changes were not statistically significant.

**Table 18** Proazulene content (% DW) of yarrow between 2020-2022.

Treatment	2020	2021	2022
C	0.23±0.00 <sup>a</sup>	0.16±0.02 <sup>a</sup>	0.18±0.04 <sup>a</sup>
MeJa 1	0.19±0.00 <sup>c</sup>	0.10±0.00 <sup>b</sup>	0.20±0.02 <sup>a</sup>
MeJa 2	0.18±0.00 <sup>cd</sup>	0.13±0.04 <sup>b</sup>	0.19±0.03 <sup>a</sup>
SA 1	0.21±0.01 <sup>b</sup>	0.12±0.00 <sup>b</sup>	0.19±0.02 <sup>a</sup>
SA 2	0.17±0.00 <sup>d</sup>	0.15±0.03 <sup>ab</sup>	0.20±0.02 <sup>a</sup>

Values are presented as Mean ± SD. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%). C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA.

#### 4.1.4 Total phenolic content

The results of elicitation on the TPC of our target species in the open field studies over three consecutive years, presented in Figure 21 and the appendix 3 Tables 1 to 5 in the appendices, demonstrate that phenolics are not only affected by the treatment and its concentration but also by the year. The values of phenolics ranged from 90.61 mg to 286.45 GAE g<sup>-1</sup> DW in the control plants of 2021 and SA 1 treated plants in 2020, respectively.

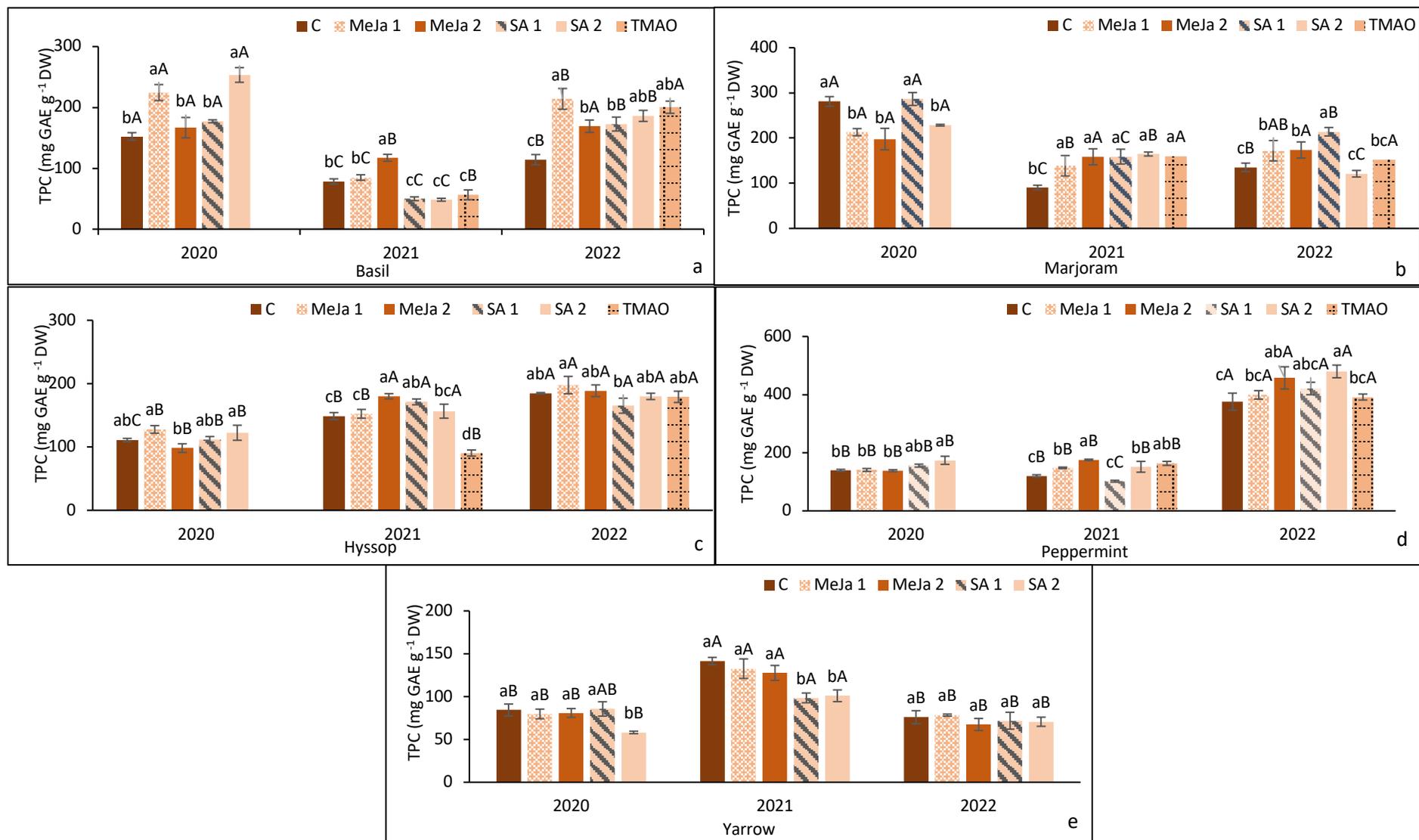
In regard to basil, the 2021 plantation seems to yield the lowest TPC compared to the other years. For instance, the SA 2 treated plants in 2020 demonstrated more than a 4-fold higher TPC than in 2021. In the first year, all treatments showed stimulation in the TPC, among which MeJa 1 and SA 2 were significant with increases of 47 and 66%, respectively. The same trend was shown in 2022; elicited basil leaves had higher content of phenolics in all cases than the control, with significant differences. The highest response was achieved by MeJa 1 treatment with an 88% increase. Moreover, SA and TMAO treatments were also able to enhance the studied parameter, while that was not the case in 2021, when decreases of 36, 38, and 25% were measured with treatments SA 1, SA 2, and TMAO, respectively. On the other hand, MeJa 2 resulted in an elevation of TPC by almost 50% in this second year.

Variations in the phenolic production were also observed between the years and treatments in hyssop plants. The lowest values were obtained by the treatment TMAO in 2021, measuring 90.46 GAE g<sup>-1</sup> DW, while the highest values were obtained by MeJa, reaching 197.71 GAE g<sup>-1</sup> DW in 2022. The effect of elicitors in the first and last years had the same tendencies. The highest TPC accumulation was attained by MeJa 1, with 15 and 8% increases in 2020 and 2021, respectively. However, they were not statistically significant, as well as the other treatments in these two years. The only significant variations in TPC of hyssop were registered in the 2021 trial. MeJa 2 and SA 1 enhanced the phenolic production by 21 and 15%. While TMAO suppressed their accumulation by approximately 40%.

In the first year, the TPC of marjoram significantly decreased with MeJa treatments, approximately by 24 and 30%, respectively, with the lower and higher concentrations, and by 19% with SA 2. However, this negative effect was not observed in subsequent years. In 2021, the TPC of marjoram increased with all treatments. MeJa 2, SA 1, and TMAO all elevated it by around 75%, with the highest increase recorded with the SA 2 treatment at 82%. The same treatment, along with TMAO, showed no significant changes in the last year. Meanwhile, MeJa 1, MeJa 2, and SA 1 significantly elevated phenolic accumulation by 27, 28, and 58%, respectively.

The effect of the plantation year was more pronounced in peppermint plants, where the TPC were somewhat similar in the first and second year. While the last year showed significantly higher values in all treatments. Referring to elicitors, the only treatment that led to a change in the phenolic production of peppermint in 2020 was SA 2, with a significant increase of approximately 25%. Similarly, SA 2 was able to enhance the phenolics in 2021 and 2022 by 26 and 28%, respectively. In 2021, other treatments such as TMAO, MeJa 1, and MeJa 2 increased the TPC by 36, 23, and 46%, respectively, with the latter elicitor resulting in the highest increase in that year. In the last year, besides the eliciting effects of SA 2, MeJa 2 was also able to significantly enhance the accumulation of phenolics by 22%.

In the case of yarrow, the year 2021 had the highest TPC across all treatments compared to the other years. Whereas the treatments failed to enhance phenolic levels in any of the trials. Instead, only negative effects were observed. In particular, SA 2 decreased the content in all years by 31, 29, and 7% in 2020, 2021, and 2022, respectively, with the latter effect not being statistically significant. The other concentration of SA in 2021 also had detrimental effects on TPC, resulting in a 30% reduction. The rest of the treatments resulted in no remarkable changes.



**Figure 21 TPC of the five plant species between 2020-2022.** Data are expressed as means  $\pm$  SD; Different letters are for significantly different groups. Capital letters to differentiate between the experimental years under fixed elicitation treatments and small letters are used to differentiate elicitors effects under fixed years. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO.

#### 4.1.5 Antioxidant capacity

AOC was evaluated across experimental trials to determine the effect of elicitors on its potential in our plants (Appendix 3 Tables 1 to 5, and Figure 22). The results reveal significant differences between the treatments and the effect of year across treatments depending on the species studied.

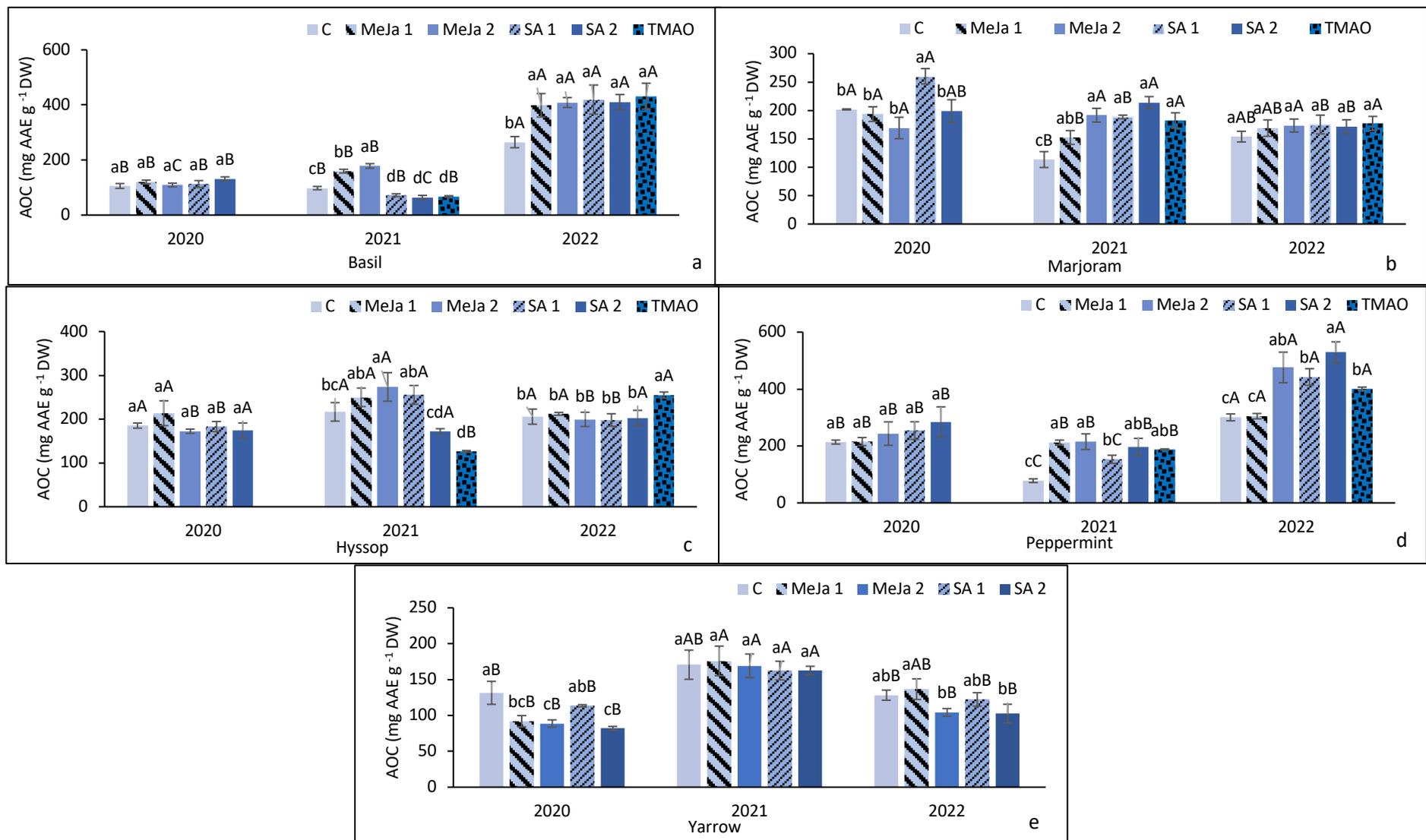
Significant elevations in AOC were registered in basil in the last year in general, compared to former trials. In this year the AOC ranged between 264.91 and 431.90 mg AAE g<sup>-1</sup> DW for control and TMAO treated plants, respectively, while previously the values varied from 63.37 to 178.26 mg AAE g<sup>-1</sup> DW. The assessment of the AOC revealed no significant differences between the treated plants in the first year, conversely to the other years, where all treatments changed the AOC of our extracts. In the last year, an evident increase was observed due to elicitation, with TMAO showing the highest stimulation by 63%, followed by SA 2 and SA 1 with 58 and 55% increases, respectively. Surprisingly, these three elicitors had suppressing effects on the AOC in 2021 by 31, 35, and 26%, respectively. However, MeJa treatments could reverse this effect; both concentrations were able to enhance the activity by 63 and 82% with 0.1 and 2 mM, respectively.

The values of the AOC in hyssop did not vary greatly between the years. Noteworthy differences were observed only in the MeJa 2 and SA 1 treated plants, which showed significantly higher values in 2021 compared to other years. Specifically, AOC ranged between 126.26 and 273.57 mg AAE g<sup>-1</sup> DW, obtained from TMAO and MeJa 2 treated plants in the same year (2021). No significant changes were observed in the first experimental year. The impact of elicitors was highlighted mostly in 2021; increases of 15, 26, and 16% were obtained, respectively, by treatments MeJa 1, MeJa 2, and SA 1. While SA 2 and TMAO decreased the activity by 21 and 42%, respectively. Notably, only the effects of MeJa 2 and TMAO were justified statistically significant. Conversely, the TMAO did not decrease the AOC in 2022, but it elevated it significantly by 24%.

In marjoram, the AOC varied from 113.60 mg AAE g<sup>-1</sup> DW in non-treated plants in 2021 to 260.23 mg AAE g<sup>-1</sup> DW in samples treated with SA 1 in the first year. Leaf extracts of marjoram show significant elevations in their AOC in 2021 under all treatments, where the highest differences were obtained by the concentration 2 mM of both MeJa and SA that reached a 69 and 88% increase, respectively. These effects were also manifested in the 2022 experiment; all elicitors enhanced the activity, particularly TMAO (by 15%), however, according to the statistical analysis, their effects were not significant in this year. On the other hand, the 2020 trial revealed that SA 1 was the only treatment effective in increasing the AOC significantly by approximately 29%.

Similarly to basil, peppermint extracts from 2022 exhibited higher AOC compared to the other years. For instance, treated peppermint plants with 2 mM SA showed a 6-fold increase in AOC compared to the non-treated plants from 2021. No significant differences in the AOC were observed between treatments in the first year. This contrasts with subsequent experimental years, which revealed significant elevations in the antioxidant activity of peppermint with all treatments, except MeJa 1 in the last year. In 2021, the most substantial increases were observed with MeJa 1 and MeJa 2, resulting in a 172 and 176% elevation of the parameter, respectively. However, in 2022, the highest effects were seen with MeJa 2 and SA 2, achieving increases of only 58 and 76%, respectively.

The AOC of yarrow extracts significantly increased in the year 2021 across all treatments. The highest value was achieved through the application of MeJa 1 in 2021, reaching 175.97 mg AAE g<sup>-1</sup> DW, while the lowest activity was recorded in SA 2 treated samples in 2020, amounting to 81.99 mg AAE g<sup>-1</sup> DW. Similarly to the EO and phenolics production, our treatments could not enhance the AOC in yarrow plants. Throughout each year, extracts from SA 2 treated plants consistently exhibited the lowest AOC values. This treatment resulted in a reduction of activity by 38, 5, and 20%, respectively, in 2020, 2021, and 2022 compared to their control. However, the observed effects in the last two years were not considered statistically significant. Additionally, MeJa 1 and MeJa 2 had a diminishing effect in the first year; a decrease of 30 and 33%, respectively, was observed. In the other experiments, none of the treatments could alter the AOC significantly.



**Figure 22** AOC of the five plant species between 2020-2022. Data are expressed as means  $\pm$  SD; Different letters are for significantly different groups. Capital letters to differentiate between the experimental years under fixed treatments and small letters are used to differentiate elicitors effects under fixed years. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO.

## 4.2 Elicitation under well-watered and non-irrigated conditions

### 4.2.1 Morphological traits

The results of the morphological traits assessment, summarized in Table 19 and in Appendix 3 Table 6, reveal that the fresh weight of basil plants was only affected significantly by irrigation, whereas both cultivation year and irrigation had significant effects on dry weight and plant height. For instance, in 2020, the fresh biomass ranged between 103.22 and 119.11 g plant<sup>-1</sup> in the non-irrigated plots with SA 2 and MeJa 2, respectively. While in the irrigated plots, the values ranged from 132.11 to 146.22 g plant<sup>-1</sup> with MeJa 1 and SA 1, respectively. The lack of irrigation in the non-irrigated plots negatively affected these parameters in both years; a decrease of 22, 8, and 16% in fresh weight, dry weight, and height, respectively, in 2020 was observed in the control plants. Similarly, in 2022, there was a decrease of 30, 33, and 11% in fresh and dry biomass and height, respectively.

On the other hand, despite the lack of remarkable effects from phytohormones on any of the parameters across both years, certain trends remained consistent. Regarding the height, the elicitors could not enhance this parameter in irrigated plots in either 2020 and 2022, whereas MeJa 1 and TMAO could slightly mitigate the negative effects of water stress and enhance the height by 6 and 2%, respectively, in 2020 and 2022. As for the biomass (fresh and dry), the highest values of irrigated basil plants were obtained with treatments SA 1 consistently in 2020 and 2022, while the highest elevation in the non-watered plants was achieved by MeJa 2 treatments in both years.

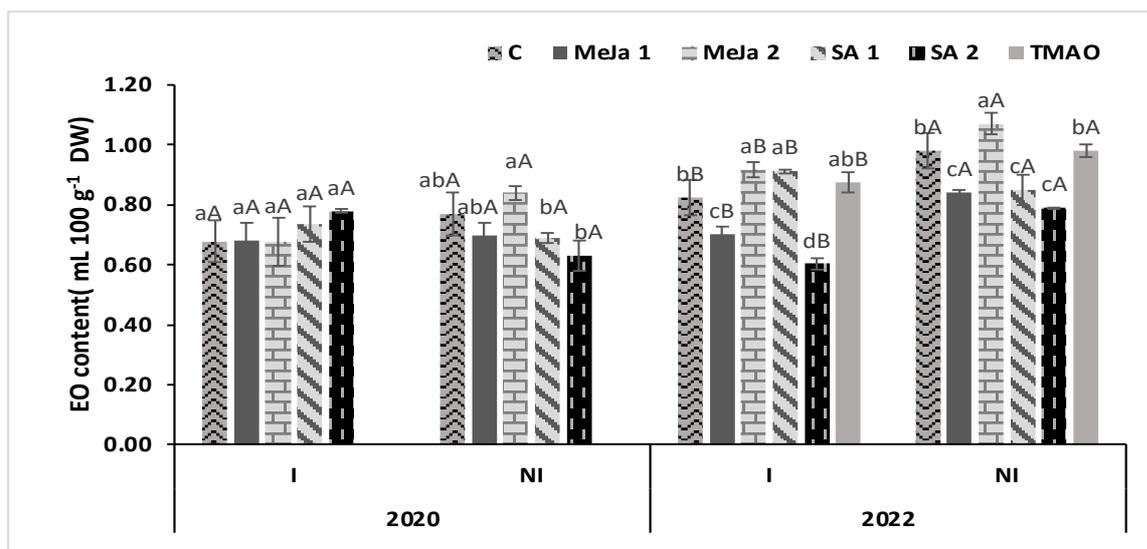
**Table 19 Fresh weight, dry weight, and height of irrigated (I) and non-irrigated (NI) basil under elicitation in 2020 and 2022.**

		2020					2022					
		C	MeJa 1	MeJa 2	SA 1	SA 2	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO
<b>Height (cm)</b>	I	69.7±1 .240 <sup>aA</sup>	55.8±1 2.01 <sup>aA</sup>	60.8±1 2.39 <sup>aA</sup>	64.8±7 .31 <sup>aA</sup>	58.7±1 1.05 <sup>aA</sup>	47.4±3.1 1 <sup>aA</sup>	44.5±4 .90 <sup>aA</sup>	44.9±3 .00 <sup>aA</sup>	44.7±2.7 1 <sup>aA</sup>	44.4±2 .44 <sup>aA</sup>	46.5±5 .4 <sup>aA</sup>
	NI	59.0±9 .82 <sup>aA</sup>	62.5±1 0.67 <sup>aA</sup>	60.8±1 0.45 <sup>aA</sup>	55.3±8 .29 <sup>aA</sup>	52.2±8 .04 <sup>aA</sup>	42.2±2.4 8 <sup>aB</sup>	41.3±3 .33 <sup>aB</sup>	42.2±3 .06 <sup>aB</sup>	39.7±3.5 5 <sup>aB</sup>	40.0±2 .47 <sup>aB</sup>	43.0±2 .65 <sup>aB</sup>
<b>Fresh weight (g/plant)</b>	I	133.4± 24.52 <sup>a</sup> A	132.1± 14.28 <sup>a</sup> A	136.0± 10.17 <sup>a</sup> A	146.2± 21.20 <sup>a</sup> A	143.4± 21.19 <sup>a</sup> A	163.7±2 3.17 <sup>aA</sup>	144.0± 12.49 <sup>a</sup> A	150.1± 27.95 <sup>a</sup> A	183.0±1 6.37 <sup>aA</sup>	135.7± 8.5 <sup>aA</sup>	150.7± 16.8 <sup>aA</sup>
	NI	109.6± 13.49 <sup>a</sup> A	106.7± 8.82 <sup>aA</sup>	119.1± 21.55 <sup>a</sup> A	110.3± 11.85 <sup>a</sup> A	103.2± 5.01 <sup>aB</sup>	113.9±5. 21 <sup>aB</sup>	109.6± 15.87 <sup>a</sup> A	126.9± 27.55 <sup>a</sup> A	109.2±1 5.28 <sup>aB</sup>	102.9± 14.98 <sup>a</sup> A	102.1± 18.57 <sup>aB</sup>
<b>Dry weight (g/plant)</b>	I	18.5±0 .30 <sup>aA</sup>	18.6±2 .09 <sup>aA</sup>	18.9±0 .87 <sup>aA</sup>	20.4±2 .68 <sup>aA</sup>	19.8±3 .42 <sup>aA</sup>	29.7±4.1 5 <sup>aA</sup>	24.7±1 .88 <sup>aA</sup>	25.9±4 .60 <sup>aA</sup>	32.5±5.7 5 <sup>aA</sup>	25.1±1 .49 <sup>aA</sup>	27.3±3 .3 <sup>aA</sup>
	NI	16.9±1 .19 <sup>aA</sup>	15.4±0 .92 <sup>aB</sup>	18.1±3 .59 <sup>aA</sup>	17.3±0 .73 <sup>aA</sup>	17.3±0 .66 <sup>aA</sup>	19.8±1.1 0 <sup>aB</sup>	19.7±4 .95 <sup>aA</sup>	22.1±7 .03 <sup>aA</sup>	19.0±4.2 0 <sup>aB</sup>	19.0±6 .16 <sup>aA</sup>	20.8±5 .9 <sup>aA</sup>

Values are presented as Mean ± SD. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA. Different letters are for significantly different groups. Capital letters are used to differentiate between the irrigated and non-irrigated plots under fixed treatments, while lower case letters are used to differentiate the effects of elicitors under fixed years and plots.

#### 4.2.2 Essential oil content

The effects of irrigation and elicitors on the EO content of basil portrayed in Figure 23 and Appendix 3 Table 6 indicate that neither elicitors nor irrigation significantly affected EO accumulation in the first year. However, in 2022, in addition to treatments, the absence of irrigation also positively impacted the EO content. It ranged between 0.79 and 1.07 mL 100 g<sup>-1</sup> DW in contrast to the irrigated group, where the contents ranged between 0.60 and 0.92 mL 100 g<sup>-1</sup> DW. *On the irrigated plots* in the first year, no significant differences were observed with the treatments while in 2022, basil oil content was enhanced significantly with MeJa 2 and SA 1 by 12 and 11%, respectively. In parallel, the treatments MeJa 1 and SA 2 caused opposite effects, and their application led to a decrease of 15 and 27%. In *non-irrigated* plants, MeJa 2 increased the EO content by 9% in both years, while MeJa 1, SA 1, and SA 2 decreased the content by 9%, 10%, and 18%, respectively, in 2020, and by 14%, 13%, and 19%, respectively, in 2022. However, it is worth noting that none of the described changes in the former year were statistically significant, in contrast to the latter year. Additionally, the TMAO treatment in 2022 maintained the same accumulation compared to the control in the non-irrigated plots.



**Figure 23** Essential oil content of irrigated and non-irrigated basil under elicitation in 2020 and 2022. Data are expressed as means  $\pm$  SD; C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA. Different letters are for significantly different groups. Capital letters are used to differentiate between the irrigated and non-irrigated plots under fixed treatments, while small letters are used to differentiate the effects of elicitors under fixed years and plots.

#### 4.2.3 Essential oil composition

The EO composition of irrigated and non-irrigated basil plants was examined in 2020 and 2022, as illustrated in Table 20. The findings indicate significant variations in the proportions of various components depending on the experimental year, irrigation status, and the type and concentration of elicitor used.

Concerning the treatments, in all experiments, the application of SA 2 was the only treatment resulting in shifting the ratio of the main component linalool significantly, but this is only in the *irrigated plots*. The elicitor increased the compound by 17% in 2020, while in 2022, the treatment inhibited the accumulation of linalool by 14%. In the *non-irrigated* plants, the changes of linalool were insignificant.

1,8-cineole, another compound present in higher ratios in the oils, showed a decrease only in *non-irrigated* plots, in 2020 due to SA 1 and in 2022 due to MeJa 1. Among sesquiterpenes, proportions of  $\tau$ -cadinol were also dropped due to SA 2 treatment in *irrigated* plants in 2020 and as a result of MeJa 2 and TMAO spraying in non-irrigated plants in 2022. Besides, other components also showed significant changes, but it was established that they are not consequent in the two years.

**Table 20 Essential oil composition (GC area %) of irrigated and non-irrigated basil under elicitation in 2020 and 2022**

	2020												2022										
	I						NI						I					NI					
	RI <sup>1</sup>	C	MeJa 1	MeJa 2	SA 1	SA 2	C	MeJa 1	MeJa 2	SA 1	SA 2	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO	C	MeJa 1	MeJa 2	SA 1	SA 2	TMAO
<b>1,8-cineole</b>	1034	6.76 <sup>a</sup>	7.27 <sup>a</sup>	5.21 <sup>a</sup>	6.51 <sup>a</sup>	7.22 <sup>a</sup>	9.31 <sup>a</sup>	6.64 <sup>ab</sup>	7.86 <sup>ab</sup>	4.76 <sup>b</sup>	7.75 <sup>ab</sup>	9.04 <sup>a</sup>	9.14 <sup>a</sup>	7.27 <sup>a</sup>	7.73 <sup>a</sup>	7.85 <sup>a</sup>	8.54 <sup>a</sup>	8.97 <sup>a</sup>	7.39 <sup>b</sup>	7.90 <sup>ab</sup>	8.61 <sup>ab</sup>	9.14 <sup>a</sup>	8.94 <sup>a</sup>
<b>linalool</b>	1097	51.60 <sup>b</sup>	48.13 <sup>b</sup>	49.55 <sup>b</sup>	51.21 <sup>b</sup>	60.09 <sup>a</sup>	46.61 <sup>a</sup>	50.01 <sup>a</sup>	53.77 <sup>a</sup>	46.01 <sup>a</sup>	51.03 <sup>a</sup>	41.29 <sup>a</sup>	38.73 <sup>b</sup>	41.20 <sup>a</sup>	43.23 <sup>a</sup>	35.72 <sup>c</sup>	38.27 <sup>b</sup>	37.86 <sup>a</sup>	37.07 <sup>a</sup>	38.41 <sup>a</sup>	35.56 <sup>a</sup>	37.31 <sup>a</sup>	33.99 <sup>a</sup>
<b><math>\alpha</math>-terpineol</b>	1189	1.07 <sup>a</sup>	1.09 <sup>a</sup>	0.94 <sup>a</sup>	0.87 <sup>a</sup>	0.81 <sup>a</sup>	0.90 <sup>a</sup>	0.98 <sup>a</sup>	1.10 <sup>a</sup>	1.09 <sup>a</sup>	1.14 <sup>a</sup>	1.34 <sup>a</sup>	1.33 <sup>a</sup>	1.16 <sup>a</sup>	1.30 <sup>a</sup>	1.23 <sup>a</sup>	1.46 <sup>a</sup>	1.41 <sup>a</sup>	1.38 <sup>a</sup>	1.18 <sup>a</sup>	1.48 <sup>a</sup>	1.37 <sup>a</sup>	1.48 <sup>a</sup>
<b>iso-bornyl acetate</b>	1281	1.83 <sup>ab</sup>	1.89 <sup>a</sup>	1.19 <sup>c</sup>	1.48 <sup>bc</sup>	1.17 <sup>c</sup>	1.69 <sup>ab</sup>	1.55 <sup>b</sup>	1.98 <sup>a</sup>	1.69 <sup>ab</sup>	1.12 <sup>c</sup>	2.02 <sup>bc</sup>	2.53 <sup>a</sup>	1.81 <sup>c</sup>	1.92 <sup>bc</sup>	2.25 <sup>ab</sup>	2.23 <sup>ab</sup>	2.06 <sup>c</sup>	2.21 <sup>bc</sup>	1.50 <sup>d</sup>	2.84 <sup>a</sup>	2.39 <sup>b</sup>	2.15 <sup>bc</sup>
<b>eugenol</b>	1361	2.74 <sup>b</sup>	2.85 <sup>b</sup>	4.05 <sup>a</sup>	2.39 <sup>b</sup>	2.24 <sup>b</sup>	2.11 <sup>a</sup>	2.94 <sup>a</sup>	2.55 <sup>a</sup>	3.02 <sup>a</sup>	3.17 <sup>a</sup>	4.77 <sup>b</sup>	5.77 <sup>b</sup>	5.69 <sup>b</sup>	4.79 <sup>b</sup>	5.05 <sup>b</sup>	6.77 <sup>a</sup>	6.81 <sup>a</sup>	6.76 <sup>a</sup>	7.19 <sup>a</sup>	5.39 <sup>a</sup>	4.87 <sup>a</sup>	6.35 <sup>a</sup>
<b><math>\beta</math>-elemene</b>	1391	0.75 <sup>b</sup>	0.85 <sup>ab</sup>	1.02 <sup>a</sup>	1.04 <sup>a</sup>	0.50 <sup>c</sup>	0.75 <sup>b</sup>	0.99 <sup>ab</sup>	0.72 <sup>b</sup>	1.19 <sup>a</sup>	0.83 <sup>ab</sup>	0.71 <sup>a</sup>	0.63 <sup>a</sup>	0.51 <sup>a</sup>	1.06 <sup>a</sup>	1.01 <sup>a</sup>	0.71 <sup>a</sup>	0.84 <sup>a</sup>	0.91 <sup>a</sup>	0.86 <sup>a</sup>	0.74 <sup>a</sup>	0.78 <sup>a</sup>	0.86 <sup>a</sup>
<b>trans-<math>\alpha</math>-bergamotene</b>	1437	5.13 <sup>a</sup>	4.31 <sup>a</sup>	5.39 <sup>a</sup>	2.87 <sup>b</sup>	5.14 <sup>a</sup>	5.69 <sup>ab</sup>	3.51 <sup>c</sup>	4.73 <sup>bc</sup>	7.00 <sup>a</sup>	5.38 <sup>ab</sup>	6.17 <sup>ab</sup>	6.89 <sup>a</sup>	6.00 <sup>ab</sup>	6.37 <sup>a</sup>	4.90 <sup>b</sup>	4.79 <sup>b</sup>	5.37 <sup>c</sup>	7.90 <sup>a</sup>	7.23 <sup>ab</sup>	6.54 <sup>b</sup>	5.03 <sup>c</sup>	7.71 <sup>a</sup>
<b>germacrene D</b>	1482	2.30 <sup>b</sup>	2.59 <sup>ab</sup>	3.08 <sup>a</sup>	2.96 <sup>ab</sup>	2.71 <sup>ab</sup>	2.37 <sup>b</sup>	2.77 <sup>ab</sup>	2.30 <sup>b</sup>	3.51 <sup>a</sup>	2.65 <sup>ab</sup>	3.67 <sup>b</sup>	3.56 <sup>b</sup>	3.72 <sup>b</sup>	3.74 <sup>b</sup>	5.07 <sup>a</sup>	3.99 <sup>b</sup>	4.54 <sup>a</sup>	3.89 <sup>a</sup>	3.95 <sup>a</sup>	4.29 <sup>a</sup>	4.49 <sup>a</sup>	4.50 <sup>a</sup>
<b>bicyclogermacrene</b>	1497	0.76 <sup>b</sup>	1.05 <sup>a</sup>	1.10 <sup>a</sup>	1.23 <sup>a</sup>	0.79 <sup>b</sup>	0.64 <sup>b</sup>	1.06 <sup>a</sup>	0.54 <sup>b</sup>	1.10 <sup>a</sup>	0.80 <sup>ab</sup>	0.75 <sup>b</sup>	0.68 <sup>b</sup>	0.89 <sup>b</sup>	0.70 <sup>b</sup>	1.28 <sup>a</sup>	1.03 <sup>a</sup>	0.91 <sup>ab</sup>	0.74 <sup>b</sup>	0.96 <sup>a</sup>	0.75 <sup>b</sup>	0.86 <sup>ab</sup>	0.90 <sup>ab</sup>
<b>trans-<math>\beta</math>-guaiene</b>	1499	0.16 <sup>b</sup>	2.46 <sup>a</sup>	0.21 <sup>b</sup>	0.21 <sup>b</sup>	-	0.10 <sup>a</sup>	0.11 <sup>a</sup>	0.06 <sup>a</sup>	0.17 <sup>a</sup>	0.06 <sup>a</sup>	-	-	-	-	-	-	-	-	-	-	-	-
<b><math>\alpha</math>-bulnesene</b>	1506	2.21 <sup>b</sup>	-	3.03 <sup>a</sup>	2.92 <sup>a</sup>	2.49 <sup>ab</sup>	2.15 <sup>b</sup>	2.83 <sup>ab</sup>	2.22 <sup>b</sup>	3.18 <sup>a</sup>	2.46 <sup>ab</sup>	3.02 <sup>b</sup>	3.04 <sup>b</sup>	2.89 <sup>b</sup>	2.65 <sup>b</sup>	4.07 <sup>a</sup>	3.23 <sup>b</sup>	3.49 <sup>ab</sup>	3.14 <sup>b</sup>	3.49 <sup>ab</sup>	3.34 <sup>ab</sup>	3.64 <sup>a</sup>	3.56 <sup>a</sup>
<b>cis-<math>\gamma</math>-cadinene</b>	1515	2.61 <sup>b</sup>	2.92 <sup>ab</sup>	3.32 <sup>a</sup>	2.85 <sup>ab</sup>	2.67 <sup>b</sup>	2.93 <sup>ab</sup>	3.01 <sup>a</sup>	2.18 <sup>b</sup>	3.33 <sup>a</sup>	2.94 <sup>ab</sup>	2.68 <sup>a</sup>	2.86 <sup>a</sup>	3.14 <sup>a</sup>	2.81 <sup>a</sup>	3.08 <sup>a</sup>	2.98 <sup>a</sup>	3.04 <sup>abc</sup>	2.83 <sup>bc</sup>	2.69 <sup>c</sup>	2.93 <sup>abc</sup>	3.28 <sup>a</sup>	3.12 <sup>ab</sup>
<b>spathulenol</b>	1584	1.06 <sup>a</sup>	1.21 <sup>a</sup>	0.83 <sup>b</sup>	1.23 <sup>a</sup>	-	0.90 <sup>a</sup>	1.23 <sup>a</sup>	0.82 <sup>a</sup>	0.98 <sup>a</sup>	0.66 <sup>a</sup>	-	-	-	-	-	-	-	-	-	-	-	-
<b>1,10-di-epi-cubonole</b>	1621	1.29 <sup>a</sup>	1.35 <sup>a</sup>	1.32 <sup>a</sup>	1.35 <sup>a</sup>	0.84 <sup>b</sup>	1.35 <sup>a</sup>	1.30 <sup>a</sup>	1.12 <sup>a</sup>	1.36 <sup>a</sup>	1.20 <sup>a</sup>	1.14 <sup>a</sup>	1.23 <sup>a</sup>	1.25 <sup>a</sup>	1.23 <sup>a</sup>	1.35 <sup>a</sup>	1.30 <sup>a</sup>	1.16 <sup>bc</sup>	1.21 <sup>abc</sup>	1.12 <sup>c</sup>	1.32 <sup>ab</sup>	1.34 <sup>a</sup>	1.26 <sup>abc</sup>
<b><math>\tau</math>-cadinol</b>	1644	9.23 <sup>ab</sup>	9.74 <sup>a</sup>	9.53 <sup>a</sup>	9.65 <sup>a</sup>	8.36 <sup>b</sup>	9.32 <sup>a</sup>	9.42 <sup>a</sup>	8.04 <sup>a</sup>	9.61 <sup>a</sup>	9.30 <sup>a</sup>	8.06 <sup>a</sup>	8.49 <sup>a</sup>	8.59 <sup>a</sup>	8.76 <sup>a</sup>	9.14 <sup>a</sup>	8.87 <sup>a</sup>	8.33 <sup>bc</sup>	8.34 <sup>bc</sup>	7.73 <sup>c</sup>	9.08 <sup>ab</sup>	9.59 <sup>a</sup>	8.72 <sup>b</sup>
<b>compounds &lt;1%</b>		7.89	10.01	8.46	8.97	4.33	10.79	10.91	9.88	9.56	8.18	5.39	5.39	6.00	4.96	6.68	5.80	5.66	5.00	5.94	6.33	6.30	6.24
<b>monoterpenes</b>		66.72	65.18	63.36	65.65	73.67	66.29	66.51	72.32	59.31	67.35	61.51	60.63	59.87	61.70	55.28	60.69	59.94	57.28	59.73	57.65	58.51	56.44
<b>sesquiterpenes</b>		30.65	32.55	34.88	32.09	25.67	31.31	32.74	27.53	38.25	31.33	28.52	29.63	30.26	29.55	33.40	29.27	30.49	31.50	30.41	31.54	31.87	33.34
<b>total</b>		97.38	97.73	98.24	97.75	99.34	97.60	99.25	99.85	97.56	98.67	90.03	90.26	90.12	91.25	88.68	89.96	90.44	88.78	90.14	89.20	90.38	89.78

Values are presented as Mean. <sup>1</sup> Retention indices. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO. Values within rows in each year with the same letters (a,b,c) are not significantly different (significance level at 5%). Compounds that reached at least 1% GC area percentage are shown.

#### 4.2.4 Total phenolic content and antioxidant capacity

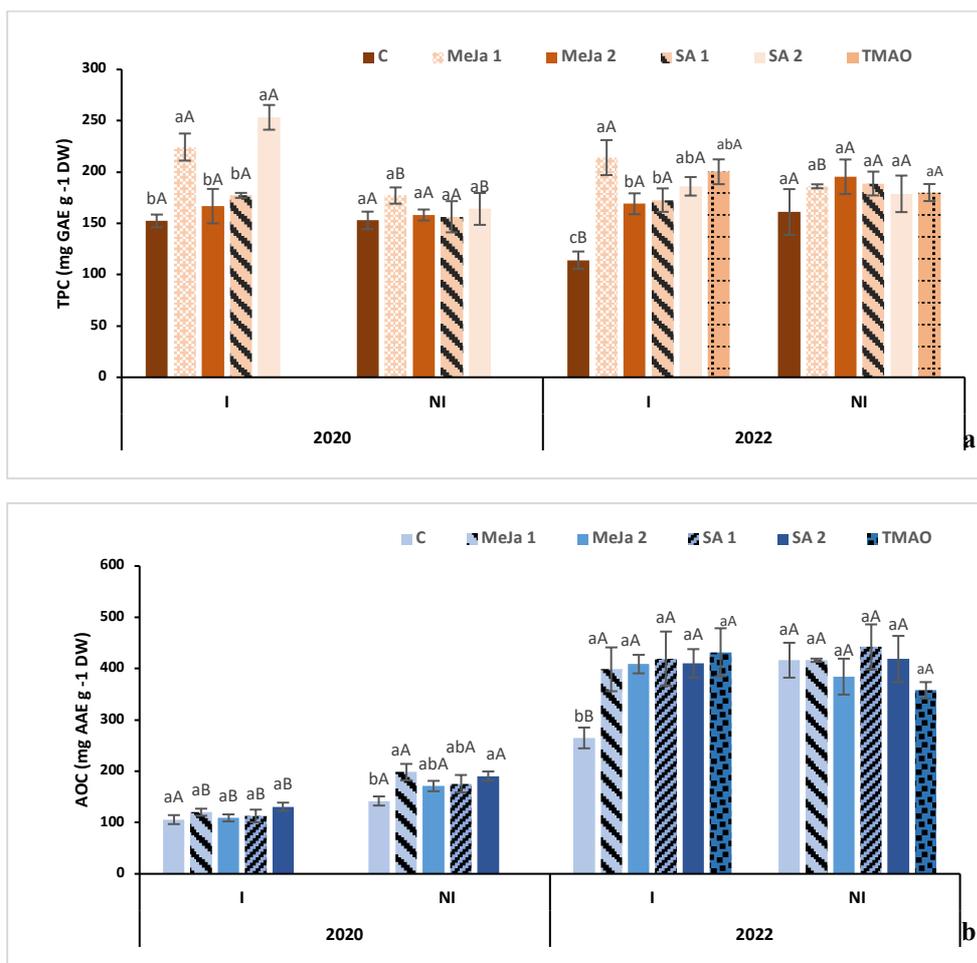
The analysis of TPC and AOC of irrigated and non-irrigated basil plants under elicitation across 2020 and 2022 is portrayed in Figures 24 and Appendix 3 Table 6.

In the *irrigated plots*, significant increases were demonstrated with MeJa 1 and SA 2 in both years, reaching 47 and 66% elevations in 2020, respectively, and by 88 and 63%, respectively, in 2022. The other treatments were also able to enhance the accumulation of TPC; however, the changes were only significant in the second year, reaching 48, 51, and 75% elevations with MeJa 2, SA 1, and TMAO, respectively.

As for the *non-irrigated plots*, none of the treatments were successful at enhancing the TPC to a significant level; the highest elevation in 2020 was detected in the MeJa 1 (+16%), while a maximum 21% increase was achieved with the higher concentration of MeJa in 2022. None of the treatments resulted in any decrease of the TPC of the samples in either year.

Concerning the AOC, there were significant differences from the first-year experiment between the irrigated and non-irrigated groups with all treated samples. The activity ranged from 105.70 to 130.94 mg AAE g<sup>-1</sup> DW in the former group and from 142.00 to 198.25 mg AAE g<sup>-1</sup> DW in the latter group. While in 2022, the AOC did not differ due to irrigation differences, except for the control plants from the non-irrigated group that portrayed higher values by 57%.

The effect of treatments on the AOC correlated with the TPC results in most cases. In the *irrigated plots*, MeJa 1 and SA 2 elevated the AOC by 14 and 24% in the first year, respectively, and by 50 and 55% in the second year, respectively. Moreover, the other treatments could enhance this parameter as well, with TMAO resulting in the highest increase (+63%) in 2022. Statistical analysis revealed that all the previously mentioned variations in 2020 were insignificant. In the *non-irrigated plots* of 2020, the same elicitors discussed above, MeJa 1 and SA 2, significantly enhanced the AOC by 40 and 34%, respectively. However, in the non-irrigated basil, no significant changes could be detected in the second year.



**Figure 24** Total phenolic content (a) and antioxidant capacity (b) of irrigated and non-irrigated basil under elicitation in 2020 and 2022. Data are expressed as means  $\pm$  SD; C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA. Different letters are for significantly different groups. Capital letters are used to differentiate between the irrigated and non-irrigated plots under fixed treatments, while small letters are used to differentiate the effects of elicitors under fixed years and plots.

### 4.3 Effect of increased elicitor concentration

The investigation of the effects of elevated concentrations of MeJa and SA was conducted on peppermint plants grown in both an open field and a controlled environment in a climatic chamber in 2022.

#### 4.3.1 Essential oil content and composition

The results presented in Table 21, detailing the EO content and its volatile spectrum obtained via GC/MS analysis of peppermint plants grown under open field conditions, indicate that treatment with 10 mM MeJa led to a significant 19% increase in EO content. In contrast, treatments with SA at the same concentration did not produce a significant change in EO accumulation, although a modest increase of 5% was observed. Regarding the volatile compounds, the treatments MeJa 3 and SA 3 both substantially increased the levels of the major compounds menthone and menthol.

Specifically, menthone increased by 41 and 22% under MeJa 3 and SA 3 treatments, respectively. Menthol levels rose by 11% with MeJa 3 and by 20% with SA 3. Conversely, significant reductions were observed in isomenthol and isomenthyl acetate following treatment with MeJa 3, with decreases of 95 and 30%, respectively. Treatment with SA 3 did not significantly impact isomenthyl acetate, but an 18-fold decrease in isomenthol content was noted compared to untreated plants. Unfortunately, treatments with both SA and MeJa led to increases in the adverse compounds menthofuran and pulegone, with respective increases of 59 and 86%. Additionally, the proportions of sesquiterpenes such as germacrene D and viridiflorol were significantly inhibited by SA 3, resulting in appr. a 25% decrease. This reduction contributed to a notable decrease in total sesquiterpene content in samples treated with SA 3.

**Table 21 Essential oil content (mL 100g<sup>-1</sup> DW) and composition (GC area %) of peppermint under high concentration elicitation treatments grown in open field**

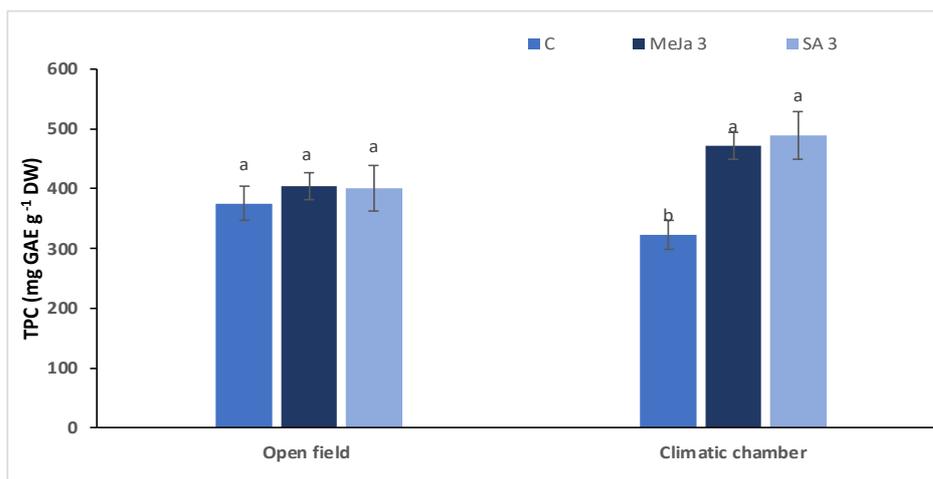
Compounds	RI <sup>1</sup>	C	MeJa 3	SA 3
limonene	1029	6.53 <sup>a</sup>	6.54 <sup>a</sup>	6.24 <sup>a</sup>
1,8-cineole	1034	5.84 <sup>a</sup>	5.01 <sup>ab</sup>	4.73 <sup>b</sup>
<i>trans</i> -sabinene-hydrate	1070	1.56 <sup>a</sup>	1.39 <sup>a</sup>	1.36 <sup>a</sup>
menthone	1158	18.87 <sup>c</sup>	26.65 <sup>a</sup>	23.08 <sup>b</sup>
menthofuran	1158	0.86 <sup>b</sup>	1.26 <sup>ab</sup>	1.37 <sup>a</sup>
neomenthol	1159	5.26 <sup>a</sup>	5.29 <sup>a</sup>	5.69 <sup>a</sup>
isomenthone	1168	3.04 <sup>a</sup>	3.40 <sup>a</sup>	3.13 <sup>a</sup>
menthol	1171	24.74 <sup>b</sup>	27.36 <sup>a</sup>	29.59 <sup>a</sup>
isomenthol	1182	7.76 <sup>a</sup>	0.37 <sup>b</sup>	0.42 <sup>b</sup>
pulegone	1236	1.41 <sup>b</sup>	2.62 <sup>a</sup>	2.16 <sup>ab</sup>
piperitone	1249	1.87 <sup>ab</sup>	2.05 <sup>a</sup>	1.77 <sup>b</sup>
<i>iso</i> -menthyl acetate	1291	10.21 <sup>a</sup>	7.20 <sup>b</sup>	11.35 <sup>a</sup>
germacrene D	1482	1.90 <sup>a</sup>	1.83 <sup>ab</sup>	1.45 <sup>b</sup>
viridiflorol	1598	1.05 <sup>a</sup>	0.89 <sup>ab</sup>	0.79 <sup>b</sup>
compounds < 1 %		8.15	7.21	6.71
monoterpenes		93.02	93.51	95.41
sesquiterpenes		6.05	5.26	4.44
total		99.08	99.03	99.85
EO content mL 100 g <sup>-1</sup> DW		3.03 <sup>b</sup>	3.61 <sup>a</sup>	3.17 <sup>b</sup>

Values are presented as Mean. <sup>1</sup> Retention indices. C: control, MeJa 1: 0.1 mM of MeJa, MeJa 2: 2 mM of MeJa, SA 1: 0.1 mM of SA, SA 2: 2 mM of SA, TMAO: 2 mM of TMAO. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%). Compounds that reached at least 1% GC area percentage are shown.

### 4.3.2 Total phenolic content and composition

The TPC and phenolic composition of peppermint plants grown in two different environments, open field and a growth chamber, under elicitation with MeJa 3 and SA 3, are shown in Figure 25 and Table 22. The elicitors were able to enhance the accumulation of phenolics in both experiments. In the open field, both treatments increased the content by 8%; however, this slight increase was not statistically significant compared to the control plants. Whereas in the phytotron, MeJa 3 and SA 3 treatments resulted in a significant elevation of TPC by 46 and 52%, respectively.

As for the phenolic composition, interesting results were found. Although the TPC in peppermint grown in the controlled environment was relatively comparable to that in the open field, the actual phenolic components were notably less numerous. In the open field trial, the main flavonoids, eriocitrin, luteolin glucoside, and hesperidin, were significantly reduced by 6%, 15%, and 20%, respectively, following MeJa 3 treatment, and by 8%, 19%, and 33%, respectively, after SA 3 treatment. In contrast, the production of the flavonoids in peppermint plants grown in the climatic chamber reacted differently to the elicitors. Eriocitrin exhibited a significant increase of 26% with both treatments, while luteolin glucoside showed increments of 53 and 32% with MeJa 3 and SA 3 treatments, respectively. On the other hand, hesperidin levels remained relatively unchanged following treatment of peppermint leaves. Another notable flavonoid, diosmin, also exhibited significant growth in response to MeJa 3 elicitation in both experiments: in the open field by 39% and in the phytotron by 116%. SA 3 also contributed to the accumulation of diosmin, resulting in increases of 4 and 63% in the open field and controlled environment experiments, respectively, although the increase was not statistically significant in the former trial. Opposite effects on the accumulation of phenolic acids were also observed between our experiments with MeJa 3. The accumulation of rosmarinic acid, salvionolic acids A and B, and ferulic acid was significantly stimulated by 28, 25, and 34%, respectively, in the open field trial. Whereas in the phytotron, MeJa 3 decreased the acids by 46, 38, and 35%, respectively. SA 3, on the other hand, did not cause any significant changes in phenolic acids in both experiments, except for a 35% elevation in the salvionolic acids A and B in the open field peppermint.



**Figure 25 Total phenolic content of peppermint extracts under high concentration elicitation.** C: control, MeJa 3: 10 mM of MeJa, SA 3: 10 mM of SA. Different letters are for significantly different groups.

**Table 22 Phenolics composition of peppermint under high concentration (10mM) elicitation**

Phenolic compound	Open field			Climatic chamber		
	C	MeJa 3	SA 3	C	MeJa 3	SA 3
Luteolin-7- <i>O</i> -rutinoside	1.32 <sup>a</sup>	1.54 <sup>a</sup>	1.35 <sup>a</sup>	0.29 <sup>a</sup>	0.22 <sup>a</sup>	0.26 <sup>a</sup>
Eriocitrin	42.42 <sup>a</sup>	39.84 <sup>b</sup>	39.14 <sup>b</sup>	5.02 <sup>b</sup>	6.34 <sup>a</sup>	6.33 <sup>a</sup>
Luteolin-7- <i>O</i> -glucoside	14.59 <sup>a</sup>	12.39 <sup>b</sup>	11.77 <sup>b</sup>	0.88 <sup>b</sup>	1.35 <sup>a</sup>	1.16 <sup>a</sup>
Quercitrin-glucosides	4.13 <sup>a</sup>	3.52 <sup>b</sup>	4.07 <sup>a</sup>	-	-	-
Luteolin-7-galactoside	2.05 <sup>a</sup>	1.71 <sup>b</sup>	1.63 <sup>b</sup>	0.23 <sup>a</sup>	0.14 <sup>b</sup>	0.20 <sup>a</sup>
Naringin-7- <i>O</i> -glycoside	3.62 <sup>a</sup>	3.25 <sup>b</sup>	3.56 <sup>a</sup>	0.59 <sup>b</sup>	1.03 <sup>a</sup>	0.99 <sup>a</sup>
Apigenin-7- <i>O</i> -Glucoside	1.49 <sup>a</sup>	1.27 <sup>b</sup>	1.23 <sup>b</sup>	0.31 <sup>a</sup>	0.28 <sup>a</sup>	0.35 <sup>a</sup>
Hesperidin	8.64 <sup>a</sup>	6.89 <sup>b</sup>	5.76 <sup>b</sup>	0.63 <sup>a</sup>	0.65 <sup>a</sup>	0.71 <sup>a</sup>
Dicaffeoylquinic acid	1.01 <sup>a</sup>	1.06 <sup>a</sup>	1.14 <sup>a</sup>	0.09 <sup>b</sup>	0.16 <sup>a</sup>	0.12 <sup>ab</sup>
Rosmarinic acid	23.80 <sup>b</sup>	30.47 <sup>a</sup>	25.58 <sup>b</sup>	5.01 <sup>a</sup>	2.71 <sup>b</sup>	4.25 <sup>a</sup>
Salvionolic acid A & B	2.63 <sup>b</sup>	3.28 <sup>a</sup>	3.54 <sup>a</sup>	0.45 <sup>a</sup>	0.28 <sup>b</sup>	0.39 <sup>a</sup>
Ferulic acid	1.89 <sup>b</sup>	2.53 <sup>a</sup>	2.07 <sup>b</sup>	1.40 <sup>a</sup>	0.91 <sup>b</sup>	1.33 <sup>a</sup>
Diosmin	6.84 <sup>b</sup>	9.48 <sup>a</sup>	7.10 <sup>b</sup>	1.26 <sup>b</sup>	2.73 <sup>a</sup>	2.05 <sup>a</sup>
Phenolic acids	29.15	37.26	32.03	6.87	3.89	5.97
Flavonoids	86.10	80.93	76.73	9.92	13.59	12.80

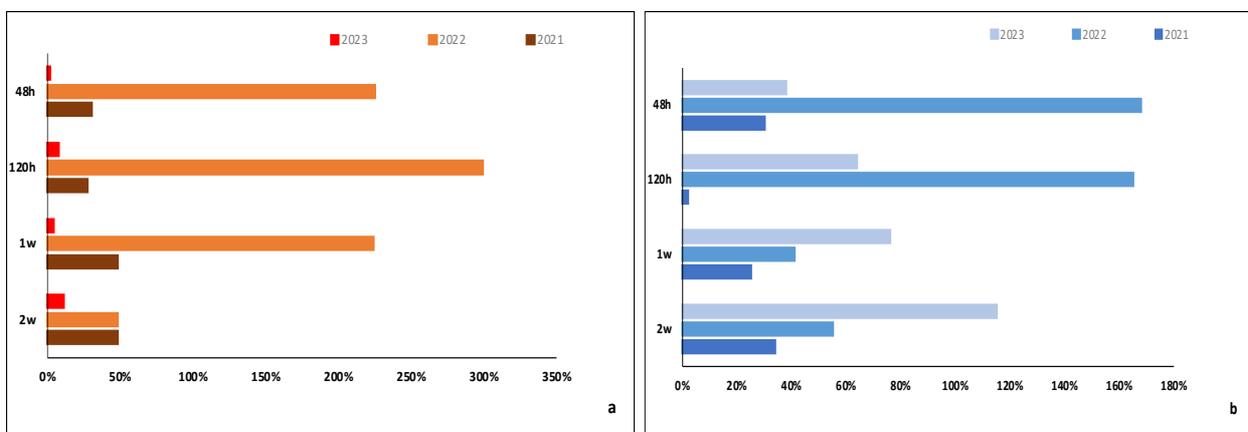
Values (mg g<sup>-1</sup> DW) are expressed as mean. C: control, MeJa 3: 10 mM of MeJa, SA 3: 10 mM of SA. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%).

#### 4.4 Effect of elicitation duration

Investigating the time-dependent dynamics of elicitor responses constituted a pivotal aspect of our research aimed at optimizing our elicitation strategy. For this purpose, we selected MeJa 2 as the elicitor and marjoram as a model species to assess the temporal impact on phenolic production and the AOC across three distinct experimental trials.

Figure 26 summarizes the changes of TPC and AOC resulting from the foliar application of MeJa 2 on marjoram, harvested at four different times. The data includes a commercial variety grown in a greenhouse in 2021 and the ‘Magyar’ variety grown in a greenhouse in 2022 and in an open field in 2023. The results of 2022 represent the largest changes and reflect an optimum curve. The TPC in treated marjoram reached the maximum increase after 5 days of elicitation, resulting in a 3-fold growth compared to non-elicited plants. Still, after one week, the effect was strong, maintaining a 224% advantage. However, the TPC dropped 2 weeks after the spraying considerably. In the 2021 greenhouse and in 2023 open field experiments, the highest TPC was accumulated after one week, and the higher level remained practically the same (2021) or was even elevated (2023) until the harvest after 2 weeks.

Similarly to TPC, the AOC of treated samples throughout the experiments demonstrated an increase with varying degrees, depending on the elicitation time, the variety used, and the experimental conditions. The 2022 experiment had the highest increases in the AOC, where the plants reached these capacities already after 2 days (168% increase). To some extent differently, in the 2021 greenhouse and 2023 open field experiments, the maximum elevation of AOC was found in samples 2 weeks after elicitation treatments. The rise in these two trials amounted to 34 and 115%, respectively.



**Figure 26 Percentage change in Total Phenolic Content (a) and Antioxidant Capacity (b) of marjoram after MeJa 2 application compared to control harvested at different timings in three experiments. 48h: harvested after 2 days of elicitation, 120h: harvested after 5 days of elicitation, 1w: harvested after one week of elicitation, 2w: harvested after two weeks of elicitation.**

## 4.5 Effect of repeated elicitor treatment

The impact of varying treatment frequencies of MeJa on basil and marjoram plants, conducted separately in both open fields and controlled environments for each species, is discussed in this chapter.

### 4.5.1 Production parameters

The effect of treatment frequency of MeJa 2 on the biomass of basil and marjoram grown under open field and controlled environment conditions (Table 23) indicate that the fresh and dry mass of basil did not exhibit significant changes following MeJa 2 elicitation in either the open field or phytotron experiments, regardless of the number of treatments. In contrast, significant differences were observed in the fresh and dry mass of marjoram with varying treatment frequencies in the open field. One treatment elevated the biomass by 67 and 69% and in parallel, the mass of the drug, respectively, while a repeated treatment already dropped them again.

We found that, although not justified statistically, tendency-like changes show that a single treatment was in some other cases more favorable, resulting in higher values of both fresh and dry masses of both species than the control or the repeated spraying.

**Table 23 The effect of treatment application of MeJa 2 on the biomass of basil and marjoram**

Species	Growing environment	Fresh weight g plant <sup>-1</sup>			Dry weight g plant <sup>-1</sup>		
		C	1 treatment	2 treatments	C	1 treatment	2 treatments
Basil	Climatic chamber	18.9±3.13 <sup>a</sup>	22.3±1.67 <sup>a</sup>	22.8±2.79 <sup>a</sup>	3.1±1.28 <sup>a</sup>	3.5±1.23 <sup>a</sup>	3.7±0.98 <sup>a</sup>
	Open field	115.5±10.30 <sup>a</sup>	121.3±8.12 <sup>a</sup>	112.8±5.23 <sup>a</sup>	17.8±4.03 <sup>a</sup>	20.0±4.02 <sup>a</sup>	18.4±1.60 <sup>a</sup>
Marjoram	Greenhouse	34.8±5.74 <sup>a</sup>	34.9±5.68 <sup>a</sup>	36.5±6.89 <sup>a</sup>	4.7±1.14 <sup>a</sup>	4.7±0.87 <sup>a</sup>	4.9±1.89 <sup>a</sup>
	Open field	11.7±4.29 <sup>b</sup>	19.6±3.85 <sup>a</sup>	12.3±1.33 <sup>b</sup>	3.1±1.01 <sup>b</sup>	5.2±0.65 <sup>a</sup>	3.3±0.45 <sup>b</sup>

Values are presented as Mean ± SD. C: control, 1 treatment: a singular treatment by 2 mM of MeJa, 2 treatments: repeated treatments with one week interval by 2 mM MeJa. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%).

### 4.5.2 Essential oil content and composition

Table 24 shows that MeJa elicitation induced substantial compositional changes in marjoram's EO profile, with varying effects between the commercial and 'Magyar' varieties grown in greenhouse and open field conditions, respectively. In greenhouse conditions, MeJa treatments caused dramatic reductions in several compounds; however, only after two treatments: sabinene decreased by 97%, and  $\gamma$ -terpinene by approximately 67%. Notably, the compounds *cis*-sabinene hydrate and linalool dropped (non-significantly) with one treatment but exhibited large increases of 148 and

190%, respectively, after the two sprayings. In general, the direction of changes after the single treatment is more comparable with the effects detected in the open field.

Under open field conditions, repeated treatments were able to significantly alter the ratios of predominant compounds, resulting in a 46% increase in terpinene-4-ol and a 34% reduction in *cis*-sabinene hydrate and linalool. When treatments were applied only once, their ratios showed only slight, non-significant changes.

**Table 24 The effect of treatment application of MeJa 2 on the essential oil content and composition (GC area%) of marjoram**

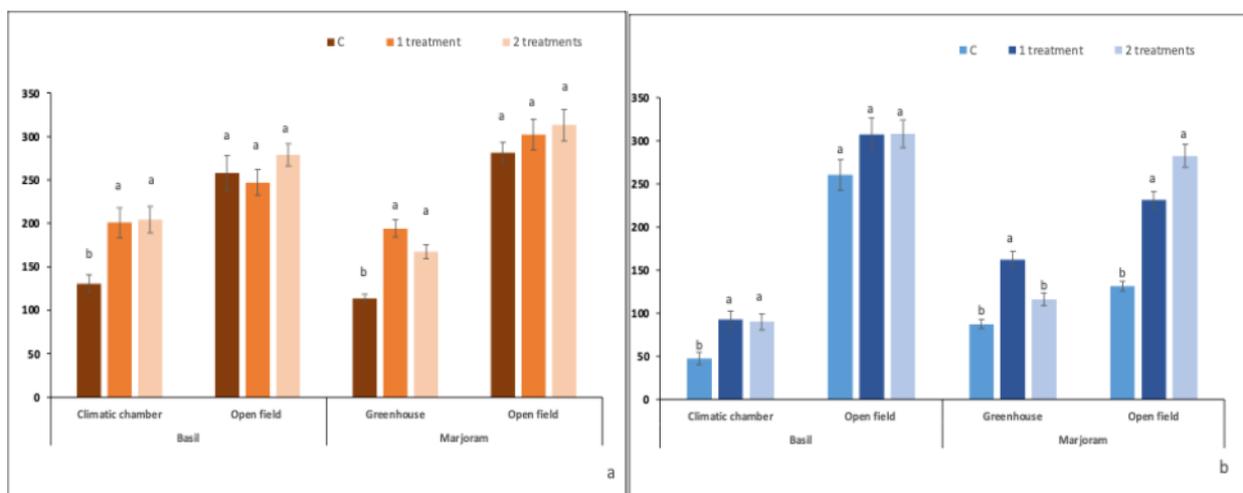
Compound	Greenhouse				Open field		
	RI <sup>1</sup>	C	1 treatment	2 treatments	C	1 treatment	2 treatments
sabinene	976	5.06 <sup>a</sup>	2.27 <sup>b</sup>	0.13 <sup>c</sup>	2.39 <sup>b</sup>	3.94 <sup>a</sup>	2.15 <sup>b</sup>
$\alpha$ -terpinene	1018	5.08 <sup>a</sup>	4.18 <sup>a</sup>	0.94 <sup>b</sup>	2.19 <sup>a</sup>	3.00 <sup>a</sup>	2.59 <sup>a</sup>
p-cymene	1026	2.82 <sup>a</sup>	3.36 <sup>a</sup>	0.37 <sup>b</sup>	1.10 <sup>a</sup>	1.80 <sup>a</sup>	2.38 <sup>a</sup>
$\beta$ -phellandrene	1029	2.27 <sup>a</sup>	1.62 <sup>a</sup>	0.29 <sup>b</sup>	1.62 <sup>a</sup>	2.08 <sup>a</sup>	1.38 <sup>a</sup>
$\gamma$ -terpinene	1056	10.27 <sup>a</sup>	10.58 <sup>a</sup>	3.40 <sup>b</sup>	5.22 <sup>a</sup>	6.52 <sup>a</sup>	6.61 <sup>a</sup>
<i>trans</i> -sabinene hydrate	1070	4.11 <sup>b</sup>	1.85 <sup>c</sup>	7.67 <sup>a</sup>	7.67 <sup>a</sup>	7.19 <sup>a</sup>	6.40 <sup>a</sup>
$\alpha$ -terpinolene	1085	1.98 <sup>a</sup>	1.91 <sup>a</sup>	0.74 <sup>a</sup>	1.14 <sup>a</sup>	1.45 <sup>a</sup>	1.32 <sup>a</sup>
<i>cis</i> -sabinene hydrate	1096	9.60 <sup>b</sup>	5.97 <sup>c</sup>	23.85 <sup>a</sup>	23.39 <sup>a</sup>	19.85 <sup>ab</sup>	15.48 <sup>b</sup>
linalool	1097	4.12 <sup>b</sup>	2.56 <sup>c</sup>	11.94 <sup>a</sup>	16.95 <sup>a</sup>	14.39 <sup>ab</sup>	11.22 <sup>b</sup>
dehydrosabinaketone	1120	1.02 <sup>a</sup>	0.95 <sup>a</sup>	1.58 <sup>a</sup>	1.28 <sup>a</sup>	1.49 <sup>a</sup>	1.32 <sup>a</sup>
terpinene-4-ol	1175	29.25 <sup>b</sup>	40.27 <sup>a</sup>	27.99 <sup>b</sup>	18.65 <sup>b</sup>	20.13 <sup>b</sup>	27.21 <sup>a</sup>
$\alpha$ -terpineol	1189	2.48 <sup>b</sup>	2.83 <sup>b</sup>	4.25 <sup>a</sup>	4.27 <sup>a</sup>	4.12 <sup>a</sup>	4.07 <sup>a</sup>
<i>trans</i> -sabinene hydrate acetate	1247	4.57 <sup>a</sup>	1.18 <sup>b</sup>	0.41 <sup>bc</sup>	1.68 <sup>a</sup>	1.70 <sup>a</sup>	1.77 <sup>a</sup>
linalyl-acetate	1250	6.85 <sup>b</sup>	8.42 <sup>a</sup>	6.34 <sup>b</sup>	3.42 <sup>ab</sup>	2.80 <sup>b</sup>	4.65 <sup>a</sup>
$\beta$ -caryophyllene	1420	3.44 <sup>a</sup>	4.85 <sup>a</sup>	4.13 <sup>a</sup>	3.21 <sup>a</sup>	3.08 <sup>a</sup>	3.85 <sup>a</sup>
bicyclogermacrene	1497	2.43 <sup>b</sup>	3.45 <sup>a</sup>	3.44 <sup>a</sup>	2.53 <sup>a</sup>	2.27 <sup>a</sup>	3.02 <sup>a</sup>
compounds <1%		3.93	3.28	2.36	2.40	3.20	3.11
MONOTERPENES		93.09	91.02	92.09	93.11	93.41	91.13
SESQUITERPENES		6.16	8.48	7.70	6.01	5.60	7.41
SABINENE COMPOUNDS		24.35	12.21	33.62	37.12	35.06	28.15
TERPINENE COMPOUNDS		49.06	59.76	37.31	31.48	35.22	41.8
<b>Total</b>		99.25	99.50	99.79	99.12	99.01	98.54
<b>EO content mL 100 g<sup>-1</sup> DW</b>		0.98 <sup>a</sup>	0.69 <sup>b</sup>	0.67 <sup>b</sup>	1.70 <sup>b</sup>	2.40 <sup>a</sup>	1.38 <sup>c</sup>

Values are presented as Mean. <sup>1</sup> Retention indices. C: control, 1 treatment: a singular treatment by 2 mM of MeJa, 2 treatments: repeated treatments with one week interval by 2 mM MeJa. Values within rows with the same letters (a,b,c) are not significantly different (significance level at 5%). Compounds that reached at least 1% GC area percentage are shown.

### 4.5.3 Total phenolic content and antioxidant capacity

Figure 27 illustrates the TPC and AO of basil and marjoram under MeJa 2 elicitation. The results of TPC indicate that the effects of elicitation on the plants grown under controlled and semi-controlled environmental conditions were more pronounced than in open fields for both species. TPC of basil was increased by 54 and 56% with MeJa 2 applied once and twice, respectively. As for marjoram, elevations of 71 and 48% in TPC were measured in samples treated once and repeated two times, respectively. On the other hand, the open field experiments revealed no statistically significant differences in the TPC after spraying the MeJa 2 treatments. Thus, in the TPC, there were no significant differences between the frequency of the treatments in either of our experiments.

Regarding the AOC, in basil, the AOC was remarkably enhanced in the phytotron experiment by 94 and 90% when MeJa 2 was applied once and twice, respectively. However, in open field basil, both treatments of MeJa 2 led to an 18% rise in AOC, though this increase wasn't statistically significant compared to the control plants. Conversely, marjoram plants exhibited a greater sensitivity to the number of elicitor applications. In the greenhouse, marjoram plants experienced a significant 86 % increase with one application of MeJa 2 and a 33% increase with two applications. While, in the case of open field-grown marjoram plants, the highest accumulation was achieved with two applications of MeJa 2, reaching a 115% elevation, compared to only a 76% increase when the treatment was sprayed once.



**Figure 27** The effect of treatment application on the Total Phenolic Content (a) and Antioxidant Capacity (b) following MeJa 2 application on basil and marjoram. C: control, 1 treatment: a singular treatment by 2 mM of MeJa, 2 treatments: repeated treatments with one week interval by 2 mM MeJa. Different letters are for significantly different groups.

## 4.6 Enzymatic and anatomical responses to elicitor treatments: phenylalanine ammonia-lyase activity, lipoxygenase activity, and glandular hair density

### 4.6.1 Phenylalanine ammonia-lyase activity

PAL -one of the key enzymes in SA biosynthesis- activity data in basil shown in Table 25 reveal distinct patterns across different experimental conditions over three years. The greenhouse experiment in 2021 with the commercial variety showed high initial activity levels in both treated and non-treated samples with no significant changes. While the PAL activity of the ‘Genovese’ variety grown under controlled and open field conditions was considerably lower and significantly affected by SA 2 elicitor, showing reductions of 62 and 42% in 2022 and 2023, respectively. The enzymatic activity of marjoram plants was also significantly inhibited by SA 2 elicitation.

**Table 25 PAL activity of basil and marjoram under SA 2 elicitation in different trials**

Species	Year	Growing conditions	PAL (Unit g <sup>-1</sup> F W)	
			C	SA 2
Basil	2021	Semi-controlled	43.03±11.60 <sup>a</sup>	43.38±10.10 <sup>a</sup>
	2022	Controlled	0.84±0.34 <sup>a</sup>	0.32±0.82 <sup>b</sup>
	2023	Open field	24.78±1.90 <sup>a</sup>	14.36±1.94 <sup>b</sup>
Marjoram	2023	Open field	18.08±5.25 <sup>a</sup>	12.08±3.49 <sup>b</sup>

Values are presented as Mean ± SD. Different letters within the rows are for significantly different groups C: control, SA 2: 2 mM

### 4.6.2 Lipoxygenase activity

The results of LOX -one of the key enzymes in jasmonates synthesis- activity in basil plants indicate that the highest levels were observed in the control samples (Table 26), however, MeJa 2 treatment significantly reduced LOX activity by nearly 96%. Similarly, in the open field experiment, the treatment led to a 40% decrease in LOX activity. The tendency of the change in the climatic chamber experiment is the same, although the data here do not show significant differences. In marjoram, to the contrary of the aforementioned data in basil, the MeJa 2 treatment resulted in an increase of LOX activity by 67% after two weeks.

**Table 26 Lox activity of basil and marjoram under MeJa 2 in different trials**

Species	Year	Growing conditions	LOX (nkatal)	
			C	MeJa 2
Basil	2021	Semi-controlled	0.392±0.006 <sup>a</sup>	0.016±0.005 <sup>b</sup>
	2022	Controlled	0.010±0.002 <sup>a</sup>	0.007±0.003 <sup>a</sup>
	2023	Open field	0.015±0.008 <sup>a</sup>	0.009±0.004 <sup>b</sup>
Marjoram	2023	Open field	0.006±0.001 <sup>b</sup>	0.009±0.004 <sup>a</sup>

Values are presented as Mean ± SD. Different letters within the rows are for significantly different groups C: control, MeJa 2: 2 mM

### 4.6.3 Glandular hair density

Table 27 illustrates the impact of different elicitors on the glandular hair density of basil, marjoram, and peppermint. With MeJa, the results were similar in all three species: the lower concentration elevated the number to a lower extent, while the higher one led to significant rises in the number of glands. In basil, MeJa 2 resulted in 203% more glands; in marjoram, the rise was 40%; and in peppermint, 20%. However, the effectiveness of SA differed in this regard. While it increased EO gland density in basil by approximately 44% with the lower concentration, its impact on the other two species was negative.

Comparing the number of glands and the determined EO content of the same plants (Appendix 3 Table 9), we established a high and positive correlation in the case of basil, a lower but positive correlation for marjoram, and surprisingly a negative correlation in peppermint.

**Table 27 Elicitation effect of the applied plant hormone on the glandular hair density of basil, marjoram and peppermint leaves (Glandular hair density 100 mm<sup>-2</sup>)**

Plant species	C	MeJa 1	MeJa 2	SA 1	SA 2	EO-glandular hair correlation (r)
<b>Basil</b>	122±12 <sup>b</sup>	194±33 <sup>b</sup>	370±47 <sup>a</sup>	181±43 <sup>b</sup>	177±10 <sup>b</sup>	0.94
<b>Marjoram</b>	189±35 <sup>b</sup>	205±32 <sup>b</sup>	265±61 <sup>a</sup>	152±30 <sup>b</sup>	203±41 <sup>b</sup>	0.60
<b>Peppermint</b>	509±28 <sup>ab</sup>	576±66 <sup>ab</sup>	611±26 <sup>a</sup>	392±20 <sup>b</sup>	429±39 <sup>b</sup>	-0.55

Values are presented as Mean. Different letters within the rows are for significantly different groups C: control, SA 2: 2 mM

## V. Discussion

### **Effect of elicitors on the yield characteristics**

The exogenous application of jasmonates has been described in many studies to have a retardant effect on the growth of many plant species under different environmental conditions (Cappellari et al., 2019a; Heinrich et al., 2013; C. Li et al., 2018). Yet, the underlying reasons behind the growth inhibition may include different mechanisms. MeJa can interact antagonistically with other plant growth regulators, such as gibberellins, by inhibiting their transcription genes or by interfering with the gibberellin signaling cascade, thus negatively affecting plant growth and development (Heinrich et al., 2013; D. L. Yang et al., 2012). Another mechanism includes the allocation and redistribution of carbon resources, where jasmonates can redirect the plant's energy to the biosynthesis of SMs at the expense of growth ( Arnold & Schultz, 2002; Gould et al., 2021).

Our results, on the other hand, showed that the elicitors rarely affected the biomass of our species in a significant manner; in fact, most variations were mostly observed between the plantation years rather than among the treatments. In basil and marjoram, only TMAO treatment resulted in a significant drop of the biomass, while in hyssop, other treatments had similar effects, and in peppermint, some treatments even increased the yield. These changes, however, were observed exclusively in one of the two years, indicating a significant influence of external factors. The inconsistent effectiveness of the elicitors may be partly explained by the impact of environmental conditions. Nevertheless, under the given circumstances, there was only a slight variation in climatic factors during the two weeks between treatment applications and harvesting. In 2021, maximum and minimum average daily temperatures ranged between 27-39°C and 11-19°C, respectively. While the following year experienced slightly cooler conditions, with the maximum average daily temperatures ranging between 25-37°C and minimum temperatures between 6-18°C.

In former studies, contradictory results have been published in connection with plant yield. Application of jasmonates (up to 0.1 mM) did not cause any changes in the fresh mass of marjoram (Farsi et al., 2019; Złotek, 2017), as well as fresh and dry shoot weight of two different cultivars of basil (0.5 mM) (Moghaddam and Talebi, 2016). Further on, studies on the foliar application of SA showed promoting effects on the growth parameters of several medicinal plants; a concentration of 0.1 mM SA seems to be effective in enhancing the fresh and dry mass of marjoram and basil grown in Egypt and peppermint grown in India. However, these treatment conditions cannot really be compared with our trials. Not only concerning the weather and soil conditions, the variety but also the number of treatments was different: the plants were sprayed four times and five times in the mentioned two studies, respectively (Ahmad, Jaleel, et al., 2018; Gharib, 2006).

On the other hand, there are references about adverse effects of phytohormones, too. Manchurian crab apple (*Malus mandshurica* (Maxim.) Kom.) sprayed 14 times with 10 mM of MeJa caused significant decreases in leaf fresh weight of (Janoudi & Flore, 2003). Similarly, tomato plants (*Solanum lycopersicum* L.) sprayed 7 times with 2 mM of SA exhibited significant fruit yield reduction compared to lower concentrations of the treatment (Santana Aires et al., 2022). We assume that the observed negative effects in these and other experiments may not be attributed just to the elicitor concentration but could also result from factors such as the frequency of application, the application method, and the plant's growth phase at the time of treatment.

Let us mention our trials where single and repeated applications of MeJa were compared, and we presented that one treatment resulted in a slight (basil) or even a significant (marjoram) yield elevation. However, two treatments decreased the biomass and drug mass. Unfortunately, the role of repeated application cannot be ascertained from our open field trials, where, although mass measurements were carried out on each of the 5 species, we always applied 2 treatments. Furthermore, we may also consider the timing of the treatments as a factor. In our open field experiments, the time period between the sprayings with the phytohormones and the sampling/harvest in these experiments was altogether 2 weeks. This period (before the beginning of flowering) occurred when the plants' intensive growth had typically already finished. Therefore, a severe reduction in development and biomass was unlikely to be achieved, regardless of the concentrations applied.

In connection to morphological traits, MeJa and SA did not alter the height of the majority of our experimental species, except for the MeJa 2 that significantly enhanced the height of marjoram in the second trial year. Thus, our findings are in accordance with Ramos Melo et al. (2023), demonstrating that 1 mM of SA failed to enhance the height of wild sage (*Varronia curassavica* Jacq) even after four repeated treatments. In contrast, the same concentration (1 mM of JA), when applied twice and thrice, stimulated the height of Madagascar periwinkle (*Catharanthus roseus* L.) and lavender (*Lavandula angustifolia* L.), respectively (Ali-Huqail & Ali, 2021; El-Ziat et al., 2023). Interestingly, the latter study also revealed that even a low concentration of MeJa at 0.25 mM significantly increased lavender height compared with JA.

Our experiments involving the exogenous application of elicitors to improve drought tolerance in basil revealed that drought conditions negatively impacted the plant's fresh and dry biomass, as well as its height, in most cases. These findings were supported by Mulugeta et al. (2023) and Asghari et al. (2023). However, none of the applied elicitors could mitigate the adverse effects of water deficit stress on the growth and yield of basil significantly. Similar results were shown in a

study by Chungloo et al. (2023), where exogenous MeJa treatments could not significantly affect the height, fresh, and dry weight of *Andrographis paniculata*. Our results were also in harmony with Zulfiqar et al. (2021), who reported that the inhibition of fresh and dry shoot masses of basil caused by drought was not improved by SA elicitation. However, the impact of the phytohormones and TMAO has been reported in other studies to have beneficial effects on the morphological traits of various plants, including *Dracocephalum kotschyi* Boiss (Shirani Bidabadi & Sharifi, 2021), thyme species *Thymus vulgaris* L. and *Thymus kotschyanus* Boiss. & Hohen. (Mohammadi et al., 2019), and tomato (Catalá et al., 2021). Existing literature has proven that elicitors may act as messengers and activate a cascade of complex reactions such as osmotic adjustments, increasing the water use efficiency, and hormonal changes, ultimately leading to the mitigation of the detrimental effects of abiotic stresses like drought (Forouzandeh et al., 2023; Khanam & Mohammad, 2018; Ma et al., 2014). Limitations in our experimental design (unexpected precipitation) might have hindered achieving results comparable to previous studies. Abdi et al. (2019) found in their study that MeJa affected peppermint plants only under mild water deficit stress but could not compensate for the loss of fresh weight under moderate drought stress.

#### **Effect of elicitors on the EO accumulation: concentration**

The results of our open field studies revealed that enhancing the production of EO varied significantly depending on the plant species, the type of elicitor and its concentration, as well as the experimental year. MeJa seems to be the most effective elicitor for promoting the EO accumulation in our species in a dose-dependent manner, especially in basil, marjoram, and peppermint. Similar results were reported in the case of other Lamiaceae species in *in vivo* studies: the production of EO was enhanced in anise hyssop (*Agastache foeniculum* L.) (Fard et al., 2012) and savory (*Satureja khuzistanica* L.) (Saadatfar & Hossein Jafari, 2023), as well as in *Lippia alba* (Mill) N.E. Brown (Silva-Santos et al., 2023) and fennel (Peymaei et al., 2024). Jasmonates are key compounds associated with the defense reactions of plants and involved in SMs biosynthesis, including EO. Their exogenous application has been proven to stimulate the SMs by altering a large number of control points at biochemical and molecular levels (Giri & Zaheer, 2016). Another hypothesis suggests a shift in resource allocation, resulting in increased availability of carbon sources for terpene biosynthesis, triggered by stress-like conditions induced by elicitors (Sangwan et al., 2001). Loomis (1932, 1953) first mentioned this phenomenon and predicted that plants allocate resources between differentiation-related processes such as secondary metabolism and growth-related processes in different environmental conditions. They found that the carbon skeletons are redirected to the plant's defense mechanisms under moderate stress conditions.

Similar outcomes were observed in peppermint, fennel, and sage (*Salvia officinalis* L.) under abiotic stresses (Ben Taarit et al., 2009; Charles et al., 1990; Gholami Zali & Ehsanzadeh, 2018). Besides the above, recent findings indicate that the background of elicitation with jasmonates may increase the density of the special structures, trichomes, and/or their size while also upregulating the genes involved in the pathways responsible for the biosynthesis of volatiles (Giri & Zaheer, 2016; Maes et al., 2011; Maes & Goossens, 2010). Our investigations on the EO gland density ascertained the mentioned phenomenon in marjoram and basil while -interestingly- the applied elicitors were ineffective in influencing the gland number of peppermint. Based on this, we assume that the enhancement of EO volatiles through elicitation with MeJa may be -at least partially- manifested by enhancing the formation of glandular trichomes in some plants. Whether the difference between species is based on the genetic or molecular physiological background factors or on the speed of development of the target plant individuals remains a question still. Namely, it has been demonstrated for several decades that EO glands are developing during the juvenile phase of leaf life. Previous studies have reported that exogenous treatments with MeJa and SA phytohormones could increase the trichome density on peppermint leaves and other species (Cappellari et al., 2019b; C. Li et al., 2018). Unfortunately, these references did not describe the age and position of the examined leaves.

The variable results in the literature and our field studies might also be attributed to differences in the growth dynamics among plants grown in open fields in different years or under varying growth conditions. Our findings with peppermint support this theory. In the first year, we had an old plantation in relatively poor condition, and none of the elicitors showed any effects. In the second year, we had a new, annual plantation of high vitality, and all of the elicitors demonstrated advantageous effects on the EO accumulation. However, the following year, when the second-year-old stand suffered during the hot and dry weather and showed only limited growth, again, only some treatments were effective. Therefore, we can conclude that the proportion of younger, just-developing plant organs, where treatments may induce more significant changes, might have been different.

In parallel, we observed that under our conditions, MeJa was much less effective in the other two experimental species—hyssop (except for 2022) and yarrow. No elicitation effect on EO production was detected in the latter species in any of the years. Whether this is due to structurally distinct EO gland types requires further investigation.

Treatments with SA, on the other hand, did not significantly affect the EO content of the studied species in most trials. As an exception, we must highlight the results with hyssop, where this

elicitor—although at different concentrations in different years—led to increased EO content in the shoots. In yarrow, similar to MeJa, SA was ineffective in any of the measurements. In other species, only sporadic increase in EO content could be observed. Thus, the effect of SA is less likely to manifest itself through the enhancement of EO gland formation but through other physiological processes in connection with the defense reactions of the plants.

Our species seem to show different sensitivities towards the applied plant hormone elicitors, and each plant might have a tolerance threshold. In basil, the lower concentration could enhance the EO content significantly, in contrary to the higher concentration that decreased it in the last year. Similarly, in peppermint, SA 1 was more successful in enhancing the EO content than the higher concentration. While marjoram was practically not influenced by this elicitor, both concentrations negatively impacted EO synthesis in yarrow, with SA 2 causing a more pronounced decrease. Thus, the controversial effect of higher concentrations was more pronounced in the case of SA, although in some cases (marjoram 2022, hyssop, 2021, yarrow 2020) it could also be observed for MeJa. This suggests that the concentration of SA 2 may have been too high, as SA toxicity can result in severe oxidative damage, particularly with prolonged exposure, ultimately lowering plant yield and disrupting secondary metabolite production (A. Ali et al., 2024; Hayat & Ahmad, 2007; Jumali et al., 2011; Yuan & Lin, 2008).

Nevertheless, another explanation may also be reasonable, concluding from the data of our measurements on biosynthetic enzymes. We could explain the adverse influence of the higher elicitation concentrations by possible negative feedback to the synthesis of the enzymes (PAL, LOX) responsible for producing these hormones. This reaction would show that the concentration of MeJa and SA might have a limit in the plant body and could not be increased above a certain level by external spraying.

All these facts indicate that each species' concentration during elicitation must be determined separately. For example, studies have reported optimal concentrations of exogenous SA to range from 0.1 to 0.5 mM for most plants (Hara et al., 2012; Yuan & Lin, 2008). However, in our study, peppermint exhibited tolerance to high SA concentrations, showing no toxicity symptoms even at levels up to 10 mM. Nevertheless, this concentration did not enhance EO content. Conversely, 10 mM of MeJa significantly increased EO content without negatively affecting plant growth. Similarly, 8 mM of MeJa significantly enhanced the EO content and yield of lemon balm (*Melissa officinalis* L.) plants, unlike lower concentrations such as 2 and 0.5 mM (Medeiros et al., 2024).

Besides concentration, environmental variations may strongly influence the accumulation of volatiles and potentially explain the inconsistency of our experimental results during the trials. Basil and marjoram exhibited the highest EO content in 2021, compared with the other years,

presumably attributed to optimal weather conditions during their cultivation. Over the final two weeks before harvest, the average temperature was approximately 24°C, with humidity levels around 60%. In contrast, 2020 experienced excessive rainfall in the same period, resulting in humidity up to 88% on certain days. In the last year, the minimum daily temperatures dropped significantly, reaching as low as 6°C on some nights. Rezaie et al. (2020) observed that cold stress conditions (4°C) negatively impact the content of EO in basil. We assume that if the weather conditions do not favor the production of oil volatiles, the effect of the elicitors would be, unfortunately, diminished.

The significance of weather conditions was ascertained in the drought-stressed basil trial. Although more frequent rainfall in 2020 limited the drought stress in the experimental plots, the 2022 experiment clearly showed that water deficit in non-irrigated plants led to a notable increase in EO content. This result aligns with previous research reporting the stimulating effects of drought on the production of EO in multiple MAPs, including *Thymus daenensis* Celak., chamomile (*Matricaria recutita* L.), black cumin (*Nigella sativa* L.), tarragon (*Artemisia dracuncululus* L.), and lavender (Baghalian et al., 2011; Bahreininejad et al., 2013; Bayati et al., 2020; Gorgini Shabankareh et al., 2021; Mumivand et al., 2021). In this second year, all elicitors except TMAO significantly affected the EO production. Interestingly, the response to SA 1 elicitor was notably distinct between irrigated and non-irrigated plots. Whereas SA 1 increased the EO production in irrigated plants, it decreased its synthesis in non-irrigated plants. This suggests that the additive stress of water deficit and SA 1 treatment might have exceeded the basil's tolerance threshold, resulting in a downregulation in EO volatile biosynthesis pathways. As for the rest of the treatments, the responses to elicitors were similar between irrigated and non-irrigated plants. Notably, MeJa 2 consistently enhanced EO production in both groups, regardless of the irrigation levels, in agreement with previous research demonstrating the stimulatory impact of MeJa on basil EO accumulation under various abiotic stress conditions, including drought and salinity (Malekpoor et al., 2015; Sorial et al., 2010; Talebi et al., 2018).

Besides weather conditions, in some other cases in our row of experiments, developmental and/or intraspecific differences could contribute to the varying results in consecutive years. The varying health and age status of the peppermint plantations have been mentioned previously. A similar pattern is observed in yarrow, where in 2020 and 2022, the elicitation trials were conducted on first-year stands, while in 2021, the plantation was a second year stand already in a deteriorated condition. Intraspecific genotypic differences may also be reflected in hyssop, where—due to organizational constraints—another accession was used in the first year compared to the second

and third years. This finding highlights the need for further trials focusing on intraspecific differences in MAP responses to elicitation treatments.

It seems likely that even for the same species, the endogenous (genotype, growth and development dynamics, internal hormone balance) and exogenous (environmental) circumstances should be stabilized to achieve a stable effect through elicitation *in vivo*.

### **Effect of elicitors on the composition of EO volatiles**

The chemical analysis of the oils showed that the elicitation treatments did not generate significant qualitative changes in the composition. However, some compounds did change quantitatively. Linalool, the major constituent of basil EO, exhibited significant fluctuations influenced by both the experimental year and the treatments applied. For instance, in 2020, the SA 2 treatment significantly increased the compound's levels, but in 2022, it led to a decrease in the linalool ratio. In parallel, significant elevations of sesquiterpenes were detected. This observed trend aligns with the findings of Senji & Mandoulakani (2018), demonstrating significant decreases in linalool alongside increases in germacrene D and  $\gamma$ -cadinene in basil plants under cold stress. MeJa treatment, however, played a role in altering the ratios of several minor compounds, like the decrease of iso-bornyl acetate with the increase of eugenol content. Notably, MeJa 1 appeared to stimulate *trans*- $\beta$ -guaiene formation in the first year, which occurred at the expense of its related compound,  $\alpha$ -bulnesene (formerly known as  $\delta$ -guaiene), which was missing in the treated samples. Previous studies exploring the impact of jasmonates on basil EO composition have shown significant and cultivar-specific alterations, particularly in major components. For example, research by Złotek, Michalak-Majewska, et al. (2016) demonstrated that JA, when applied to the *Crispum* cultivar of basil, led to notable shifts in the levels of key compounds such as methyl eugenol, eugenol, and 1.8-cineole. The effects of jasmonates appear to be highly dependent on the basil cultivar (Talebi et al., 2018).

The use of elicitors in combination with drought stress resulted in some changes in basil's EO composition, especially due to the SA treatments. However, given the contradictory findings from the two experimental years (2020 and 2022), further detailed studies are needed to explain the compositional deviations observed.

In the case of hyssop, the TMAO elicitor resulted in the highest number of significant changes in the oil, especially in the last year. Interestingly, the direction of changes is the opposite in many samples compared to the MeJa and SA-treated ones. Nevertheless, in hyssop, we also found considerable differences between vegetation years. The most characteristic effect is the decrease of the ratio of the total sesquiterpenes and the increase of the percent of the total monoterpenes. It

appeared after all the first and third year treatments. These are the years when hyssop plants were perennial ones, while in the young plantation in 2021, the changes are less numerous.

In marjoram EO, sabinenes are known for carrying the main aroma profile of marjoram as a spice, while terpinenes represent a higher pharmaceutical character of the oil (Németh-Zámbori, 2020). Most of the elicitation treatments resulted in either a significant or tendency-like decrease in sabinenes and an increase in terpinenes. Sabinenes are favored if marjoram oil is used in food flavoring but terpinene-4-ol is especially important if the oil is used for disinfection/medicinal purposes.

The required proportions of the major compounds of peppermint EO are announced in the Ph Eur. Considering these requirements, we could establish that the changes of menthol and menthone were in several cases unfavorable as the elicitors, especially SA, decreased the former compound while increasing the latter one. 1,8-cineole, limonene, and menthol isomers also changed in some cases, but characteristically, the differences and their directions were not uniform in the consecutive years. Therefore, we assume that the growth of the plants and the ratio of younger or older leaves on the shoots might have a significant impact on the composition, too. Unfortunately, the ratios of menthofuran and pulegone also increased in some treatments, such as MeJa 2 (2020), SA 2 (2021), and both concentrations of SA in 2022. Despite these increases, the resulting ratios remained within the safety limits established by the European Pharmacopoeia. However, this trend should be carefully monitored before the practical application of these elicitors in peppermint, as the concentrations of the above-mentioned compounds are restricted in food and flavor products due to the potential risk of hepatotoxicity (Commission, 2008; HMPC, 2020).

Yarrow seems to show the lowest sensitivity against the elicitors used. Proazulene accumulation did not change significantly in any experiment, except for some decreases in 2020 and 2021. We have to note that according to the used Ph.Eur. method, the proazulene is calculated for the drug and not as a % of the oil; thus, it is in connection also with the concentration of the oil, which showed similar tendencies due to our treatments.

As for the complex changes in EO composition in samples of elicited plants, the figures (Figures 17-20) on the results of multivariate statistical analysis demonstrate that these changes in the complex composition of the EO are only exceptionally considerable. In these cases, mostly SA and TMAO were the elicitors, which induced larger changes like SA 2 in basil (2020, 2022), SA 1 (2020), and TMAO (2021) in marjoram, SA 1 (2022) and SA 2 (2020) in peppermint, and SA 1 (2020) and TMAO (2021, 2022) in hyssop. However, these figures also represent quite well that the differences among samples from different years are more significant than the differences (distances) among treatments in the same year.

### **Effect of elicitors on TPC and AOC**

We established that similarly to EO content, the TPC and AOC changed following elicitor treatments, but the effects varied depending on the experimental year, the type of elicitor and its concentration, and the plant species. In peppermint, in all experimental years, SA 2 treatments successfully enhanced both the TPC and AOC of the extracts. This is consistent with previous findings by Skrypnik et al. (2022) and (Figuroa Pérez et al., 2014), who demonstrated that SA could boost TPC and AOC in peppermint. Interestingly, when a higher concentration of SA (10 mM) was applied, no improvement in TPC was observed, which may be in connection with the fact of negative feedback to the endogenous production - as mentioned above.

In peppermint, the composition of the phenolic fraction was analyzed both in a climatic chamber and in an open field. The open field data revealed a reduction in key flavonoids, including eriocitrin, luteolin 7-*O*-glycoside, and hesperidin. A similar effect was observed with MeJa 3 treatments, which was also associated with a significant increase in phenolic acids such as rosmarinic acid, salvianolic acids, and ferulic acid. Significant increases in the main phenolic acids were also observed in peppermint (Mehdizadeh et al., 2024), lemon balm (Kianersi et al., 2022), and coneflower (*Echinacea purpurea* L.) (Mohebbi et al., 2021). Plants are continuously exposed to various environmental stimuli throughout their life cycle. These environmental and stress conditions strongly influence the production of phenolic compounds (Cohen & Kennedy, 2010). This relationship is reflected in our study (Table 22), where plants grown in the open field exhibited significantly higher levels of phenolic compounds, whereas peppermint plants cultivated under quasi-optimal conditions in a climatic chamber, produced appr. 5-6-times lower levels. In open fields, the elicitation treatments with MeJa and SA showed characteristic decreases in flavonoid accumulation (eriocitrin, luteolin-7*O*-glucoside, luteolin -7-galactoside, hesperidin, etc.) and in parallel a significant increase of phenolic acids, mainly rosmarinic acid, which has been demonstrated as one of the most important AO compounds in numerous plants (Bulgakov et al., 2012).

As for the other species, concerning TPC, we can summarize that there is a species-specific feature in the level of phenolic accumulation for each year, as well as a species-specific response to elicitation treatments. In basil, MeJa spraying frequently stimulated TPC, whereas, in peppermint and marjoram, SA was more often responsible for enhancing phenolic content. At the same time, hyssop and yarrow showed less sensitivity towards SA in connection with phenolic accumulation. In several cases, even adverse effects were observed, like MeJa treatments in marjoram in 2020, SA treatments in basil and yarrow in 2021, and TMAO treatments in basil and hyssop in 2021.

In the comparison trial of irrigated and drought-stressed basil plants, we demonstrated that non-irrigated basil plants resulted in slightly elevated TPC levels compared to the irrigated ones, especially in 2022, when the water deficiency was more pronounced (as mentioned above). However, while the effect of all the elicitors was significant on the irrigated plots, they could not boost any changes in the non-irrigated ones. Here we could conclude that presumably the drought stress had already stimulated sufficient phenolic production for the plant's defense on the non-irrigated plantations. This can be explained by the well-documented role of drought stress in sweet basil, which induces the accumulation of SMs, particularly phenolics. This increase is associated with the upregulation of expressed genes involved in the biosynthesis of phenolic compounds, which serve as a defense mechanism under adverse conditions. Phenolic compounds play a critical role in protecting plants from oxidative damage caused by ROS generated during environmental stress, thereby ensuring the plant's survival and resilience in challenging environments (Abdollahi Mandoulakani et al., 2017; Al-Huqail et al., 2020; Luna et al., 2015; Mulugeta et al., 2023; Rahimi et al., 2023).

The AOC exhibited a pattern in many cases that was very similar to the changes observed in TPC due to the applied treatments. This is not surprising, as phenolics are well-known for their general antioxidant capacity. In addition to their roles as free radical inhibitors, reducing or scavenging molecules, phenolic compounds are involved in critical functions related to plant growth and development, including reproduction, photosynthesis, seed germination, and signal transduction, especially when plants are under stress (A. Sharma, Shahzad, Rehman, et al., 2019; Tanase et al., 2019; Tuladhar et al., 2021). Certain modifications in the polyphenolic spectrum may occur in order to meet the plant's demands, potentially diminishing their reducing power. For instance, a discrepancy was observed in the hyssop and peppermint samples in 2022, where the TMAO treatment did not significantly affect the TPC but resulted in a significant enhancement of AOC. Additionally, we observed varying changes in TPC and AOC due to elicitation in marjoram (2022), basil (2020), and yarrow (2020). These variations are probably due to the diverse polyphenolic composition of individual species and the differing responses to treatments responsible for the variations in AOC. From a methodological point of view, there is an inherent dilemma. The FRAP assay measures the ferric reducing power of extracts, reflecting antioxidant capacity. However, compounds besides polyphenols—such as vitamins, volatile compounds, and other bioactive substances—may also contribute to this reducing power in plant extracts (Fernandes et al., 2016; Javanmardi et al., 2003). Weak correlations between TPC and AOC have also been observed in some studies. For example, neither hyssop root extracts nor aerial parts of wild thyme (*Thymus serpyllum*), pretreated with 50 mM of salt and 1 mM of SA, respectively, have shown significant

changes in TPC, although they exhibited a significant enhancement in AOC when the DPPH method was used (Skrypnik, Golovin, et al., 2022; Soheilikhah et al., 2021). Assays like DPPH and FRAP are useful for preliminary estimations and quality assessment of extracts. However, for a more comprehensive understanding of antioxidant activity, it may be advisable to combine multiple assays that assess different aspects of antioxidant function (Granato et al., 2018), which, however, exceeded the capacity of the present study.

In our trials focusing on the duration of the stimulus, phenolic accumulation began as early as 2 days after treatment with MeJa 2 in marjoram. However, we observed that this response varies depending on the growing conditions, as in other experimental settings, the onset was delayed. Consequently, the maximum level of TPC was typically reached between days 5 and 14 (with the final sampling conducted 2 weeks after treatment). These experiments revealed that the initiation of phenolic production stimulation by elicitors can vary based on environmental conditions—potentially linked to the homeostatic status of the target plant. They rapidly trigger a multitude of signal transduction pathways through which the plant responds to multiple stimuli. Subsequently, after these immediate responses have been activated, such phytohormones are quickly inactivated or their concentration reduced to reestablish the homeostasis within the plant system (Gasperini & Howe, 2024). The positive effects of MeJa elicitation on phenolic accumulation have been documented in various species. In sage and lilac sage (*S. verticillate* L.), for example, phenolic levels peaked at 8 hours and 4 hours post-treatment, respectively (Pesaraklu et al., 2021). In contrast, red sage (*S. miltiorrhiza* Bunge) hairy roots showed a much later peak, with the highest TPC occurring 6 days after MeJa application (Xing et al., 2018). The findings of Yousefian et al. (2020) indicated a selective response in spearmint (*Mentha spicata* L.) hairy roots, where rosmarinic acid reached its maximum after 6 hours, while chlorogenic acid and caffeic acid took 3 days to reach their peak levels following MeJa elicitation. However, a limitation of these studies is that they were carried out in an artificial environment *in vitro* and did not investigate longer elicitation periods, with the maximum duration being only 4 days.

Marjoram proved to be a suitable experimental subject for also comparing the effects of single versus repeated applications of MeJa 2. Similar to several previously discussed experiments, a single treatment often resulted in more favorable outcomes, including higher fresh and dry masses as well as EO content, compared to repeated spraying, which tended to decrease these values. Compositional changes were also more pronounced with double applications. The repeated application appears to be closely linked to the phenological phase and developmental stage of the target plants. However, for TPC, no significant differences were observed between the frequencies

of treatments in either of our experiments. This might seem surprising, but considering the duration of the elicitation effect discussed earlier, it is possible that at the time of sampling, we were still measuring the influence of the first application. In addition, the elicitation effect is likely affected by the plant's current condition.

### **Effect of elicitors on the enzymatic parameters**

In the enzymatic studies, we found that the treated basil samples demonstrated increased TPC and higher AOC (Appendix 3 Table 7-8) without elevated LOX activity. It might support the negative feedback theory mentioned above or suggest that there is an alternative metabolic pathway for SMs production independent of LOX activity and its substrates in this plant. Qiu et al., (2020) reported that exogenous MeJa applications inhibited the accumulation of endogenous jasmonates by reducing LOX activity in citrus (*Citrus reticulata* × *sinensis*), as well as inhibited key enzymes in jasmonates synthesis, such as 12-oxo-phytodienoic acid reductase. These LOX enzymes belong to a family of non-heme iron-containing proteins that catalyze the oxidation of polyunsaturated fatty acids like linoleic and linolenic acids, resulting in the production of signaling molecules such as jasmonates, which are crucial in plant growth, development, and responses to biotic and abiotic stresses (Viswanath et al., 2020). While studies have shown that LOX activity typically upregulates under various stresses, including wounding (Prasad et al., 2017), cold and chilling injuries (Cao et al., 2009; Zhu et al., 2018), salt and drought stresses (Lim et al., 2015; Sofu et al., 2004; X. Y. Yang et al., 2012), and biotic stresses (Bruinsma et al., 2010; Hwang & Hwang, 2010; Woldemariam et al., 2018), our results indicate no LOX stimulation from any treatments across different environments. Although jasmonates are major signals in the production of SMs, the process is not regulated by a single pathway. Instead, it typically involves multiple signaling pathways that collaboratively regulate their production (Zhao et al., 2005).

The situation is similar with the other enzyme PAL. It has been found positively correlating with phenolic production in several plant species, such as Indian pennywort (*Centella asiatica* L.) (Hafiz Ibrahim et al., 2017), service tree (*Sorbus domestica* L.) (Rutkowska et al., 2020), Syrian mesquite (*Prosopis farcta* (Banks & Sol.) J.F. Macbr.) (Zafari et al., 2016), common self-heal (*Prunella vulgaris* L.) (Tang et al., 2023), *Dracocephalum kotschy* Boiss. (Kahromi & Khara, 2021), and yarrow (Gorni et al., 2021). Nevertheless, our findings indicate that while TPC and AOC were significantly enhanced in both the controlled environment and open field experiments, the PAL activity decreased.

## VI. Conclusion and recommendations

*In vivo* elicitation holds high potential for enhancing the SMs in MAPs, such as EO accumulation and phenolic production. However, the effects are driven by many factors: environmental conditions, species, and elicitor-specific dynamics.

Our study showed that treatments of MeJa, SA, and TMAO rarely affected biomass for the studied species. Other morphological traits, like plant height, were also minimally impacted, with the only exception of MeJa 2, which significantly increased marjoram height during the second year of trials. These results suggest that our treatments did not cause a permanent stress state in our plants, as no observable morphological shift linked to the activation of their defense mechanism was noted. It is possible that the applied concentrations, up to 2 mM of elicitors, were still lower than the threshold leading to adverse effects concerning biomass production.

Unlikely to the effects of the treatments on the biomass production and growth of the plants, more considerable differences were registered due to variations in vegetation years. These findings highlight the pivotal influence of external conditions on plant responses to elicitation. Although some previous data mention a stress-mitigating effect of elicitors under unfavorable weather conditions, we could not ascertain that. The biomass of our model species, basil, was negatively affected by drought conditions, particularly in 2022, when the drought intensity was highest. However, the elicitors could not alleviate the drought stress effects, underlining the limitations of their application under such conditions. It seems that the severity of abiotic stress is also a critical factor influencing the efficacy of exogenous elicitor applications in mitigating its adverse effects. Under different environmental conditions, maybe different concentrations or application frequencies might be needed. Therefore, it is crucial that all factors influencing the outcome of elicitation should be taken into consideration when developing effective elicitation strategies.

EO production, a major focus of this study, revealed strong species-, environment-, and condition-specific dependencies. MAPs cope with stresses and challenges frequently by altering their biochemical defense processes, such as the accumulation of EO. *In vivo* elicitation seems to be a simple method to induce the production of EO volatiles; however, due to the involvement of many factors, the elicitation outcome must be carefully planned, and all the possible circumstances standardized (Kandoudi & Németh-Zámboriné, 2022). This statement has been ascertained by the present series of studies in the frame of the doctoral work.

MeJa seems to be the most effective for promoting the EO accumulation in a dose-dependent manner, especially in the *Lamiaceae* species basil, marjoram, and peppermint. However, MeJa

was less effective in hyssop and in the *Asteraceae* species yarrow. Treatments with SA did not significantly affect the EO content of the studied species in most of the trials except hyssop. Thus, with our systematic experiments, we demonstrated that although both jasmonates and SA are widespread plant hormones, their effects in stimulating SM formation may be manifested as species-specific.

We established that elicitation treatments did not generate significant qualitative changes in the typical EO composition of the species. However, quantitative (proportional) changes of some components may occur, which, in most cases, are within the limits of the characteristic and acceptable oil quality. These changes may, however, be even more important in the case of species where the composition or at least the proportion of some major compounds is defined as a quality parameter.

In peppermint, the pharmaceutical quality of the essential oil is determined by the pharmacopoeia, which specifies the required ratios of the main components, menthol and menthone. Additionally, minimizing the ratios of menthofuran and pulegone is considered critical. In marjoram, however, small quantitative changes, particularly between the *cis*-sabinene hydrate and terpinene-4-ol components, hold practical significance. Depending on the production goal—whether for aromatic or pharmaceutical oil—the application of MeJa or SA to elicit their production in marjoram can be evaluated either positively or negatively. In the future, these treatments may even serve as tools for optimization. In some cases, we also found that the EO composition may be in connection with the year (weather) or plant developmental phase, which most likely interacts with the effects of elicitors. We suggest paying attention to that and clarifying this aspect more in detail. Thus, it would be worth studying in the future the sensitivity of hyssop to the elicitors in connection to plant age or that of peppermint in connection to plant stand condition.

Our results show that the five species exhibit different sensitivities towards the applied elicitors, and each plant may have a tolerance threshold. This might be in connection with negative feedback on the biosynthetic enzymes catalyzing the indigenous production of the corresponding hormones in the plant and/or a toxic effect of them. Our results also emphasize the importance of optimizing elicitor concentration and the frequency of its application, as higher concentrations might occasionally lead to adverse effects, likely due to oxidative damage or feedback inhibition of biosynthetic enzymes. Therefore, the concentration should be determined for each species separately.

Most likely, the background mechanism of EO changes can be traced back to both the activation of volatile compounds' biosynthesis and the stimulation of trichome formation, but their actual

contribution is not known and might even be species-specific. In any event, it seems that replication of the treatments may be of great importance in reaching a larger proportion of juvenile parts of the plants, where new glands are just starting to develop. Developmental and structural characteristics of the plants are at the same time obviously also in connection with the environmental conditions and agrotechnology. This suggests that under different conditions (plant variety, cultivation conditions, plant care, harvesting technology, etc.), maybe fine-tuning of the elicitation technology is needed in the future.

Comparing the results of our *Lamiaceae* species and the results on yarrow (*Asteraceae* family), it may also be concluded that species from different plant families forming different accumulation structures of volatiles may react differently to the same elicitation treatment. Based on that, further research is suggested to focus on different species with different accumulation structures, which would also contribute to a deeper understanding of the mechanisms.

In connection with EO composition, we also have to note that for a comprehensive search of background mechanisms underlying compositional shifts in volatile compounds, GC area percentages are not the most suitable methodology due to their relative nature. Other calculations, using internal or external standards, would provide a more accurate representation of the actual changes in individual components.

TPC and AOC were influenced by elicitors in a highly variable and context-dependent manner. Factors such as the experimental year, plant species, elicitor type, concentration, and environmental conditions collectively determine the outcomes of phenolic and antioxidant responses. 2 mM of SA was able to consistently enhance the TPC and AOC in peppermint in all experiments, while higher concentrations were less effective due to potential feedback inhibition. MeJa treatments modulated phenolic fractions, increasing phenolic acids like rosmarinic acid while reducing flavonoids, particularly in open-field experiments. Growing conditions greatly affected phenolic levels, where open-field cultivation favored higher TPC compared to those grown under quasi-optimal conditions in climatic chambers, which reflects the influence of stress on SMs production and potentially on the elicitor effect. Moreover, drought stress in basil naturally elevated TPC, reducing the efficacy of additional elicitors.

The elicitation dynamics regarding the timing and frequency of elicitor application further showed the complexity of phenolic accumulation. Single application of MeJa often outperformed repeated treatments (especially the yield and EO outcomes in marjoram as described above), while TPC responses remained stable.

In an attempt to understand a part of the elicitation background, LOX and PAL enzymatic activities were assessed; our findings reveal that while these key enzymes are pivotal in stress signaling and SM biosynthesis, their activities are not always directly correlated with SM accumulation. LOX activity, despite having a critical role in the biosynthesis of jasmonates and stress responses, showed no significant stimulation by elicitor treatments in basil under different growing environments. Instead, it showed a consistent suppression under MeJa 2 treatments. The accumulation of phenolics and the enhanced AOC in these treated samples provide evidence that SM synthesis can be mediated by LOX-independent pathways. Similarly, the activity of PAL presented variability influenced more by environmental conditions than by treatments with SA 2. PAL activity of samples grown under controlled environments with minimal stress conditions exhibited lower levels, while greater variability was exhibited in semi-controlled and open-field conditions. The lack of consistency in PAL stimulation despite enhanced TPC and AOC in several experiments may support the fact that phenolic biosynthesis is governed by a network of enzymes and regulatory pathways rather than a singular one. Furthermore, species- and cultivar-specific responses add another layer of complexity, as can be seen from the different basil cultivars used in our experimental setups.

Similarly to the EO, we found that TPC accumulation as response to plant hormone elicitation is species-specific and compound-specific, too. Thus, the accumulation of total phenolic compounds can be elicited primarily with MeJa in basil while SA is more effective in peppermint and marjoram. Hyssop and yarrow showed less sensitivity to elicitation in connection with phenolic accumulation. In some cases, also negative effects (decrease of TPC) may be manifested. Therefore, we have to conclude that it is not solely the weather factors or vegetation year that determine phenolic accumulation, but rather the overall status of the plantations (age, nutrient supply, health) and their species-specific responses to the weather conditions that may play a significant role in the accumulation of phenolics. In practice, this implies that the optimization and achievement of stable elicitation of phenolic production appears to be even more complex and thus uncertain compared to the enhancement of EO.

Based on our comprehensive studies, we declared that *in vivo* elicitation has significant potential to improve SMs in MAPs. However, it seems impractical to establish a uniform experimental design suitable for all MAPs. This calls for a tailored approach that involves stabilization of both endogenous factors involving genotype, growth dynamics, and hormonal balance, and exogenous factors such as environmental and technological conditions, elicitor type and concentration, application timing, and frequency. Such a strategy will ensure consistent and effective results.

Therefore, in the near future, hormonal elicitation under controlled conditions, such as indoor, regulated environments, seems to have a higher potential. Furthermore, customizing each elicitation strategy to target specific SMs is critical. For instance, repeated treatments appear to influence the EO volatile compound accumulation more significantly than phenolic accumulation. Therefore, research in this direction may yield more flourishing results.

The influencing factors should be taken into careful consideration in order to optimize the elicitation strategies, and the treatment protocols should be adapted for specific cultivation conditions and objectives. Finally, future research should focus on exploring the mechanism of elicitor responses, beyond just the anatomical and enzymatic pathways, at molecular and physiological levels, for the advancement of knowledge in elicitation dynamics and the improvement of its application in the cultivation of MAPs.

## VII. New scientific findings

1. We demonstrated that elicitation of SMS as volatiles and phenolic compounds is possible by plant hormones without resulting in consistent significant reduction of the plant biomass. Reduction was observed only 2 out of 50 cases
2. It was established that the effects of MeJa, SA, and TMAO are species-specific, as the five species exhibit varying sensitivities to the applied elicitors. Therefore, the optimal concentration should be determined individually for each species.
3. MeJa was the most effective elicitor in promoting EO accumulation in a concentration-dependent manner, particularly in the *Lamiaceae* species basil, marjoram, and peppermint, leading to increases of up to 24%, 22%, and 33%, respectively. However, MeJa exhibited lower efficacy in hyssop and the *Asteraceae* species yarrow.
4. The elicitation treatments with MeJa, SA, and TMAO did not induce significant qualitative alterations in the typical EO composition of the species. However, quantitative variations may occur in certain cases, which should be considered if EO quality is subject to regulatory standards (e.g., increased proportions of menthone and pulegone in peppermint in 2021).
5. We demonstrated a positive correlation between the number of EO glands and EO content following MeJa treatment in marjoram and basil, with correlation coefficients of  $r = 0.60$  and  $r = 0.94$ , respectively.
6. The effects of elicitation differed between TPC accumulation and EO content. In basil, TPC accumulation was primarily enhanced by MeJa, with an increase of up to 88%, whereas in peppermint and marjoram, SA was more effective, leading to increases of up to 82%. In contrast, hyssop and yarrow exhibited lower sensitivity to elicitation in terms of phenolic accumulation.

## VIII. Summary

Elicitation is a method used to enhance the accumulation of secondary metabolites (SMs) in plants by the application of chemicals or biological factors in a controlled manner. Elicitors mimic stress conditions and trigger a cascade of responses that lead to increased production of targeted SMs within the plant. However, research on *in vivo* elicitation and its effects on medicinal and aromatic plants (MAPs) is limited and the results of individual studies are often contradictory. Therefore, the goal of our research was to effectively optimize strategies and gain insight into how to achieve the desired effects.

We have conducted a comprehensive study over 4 years (2020-2023) aimed to optimize SMs accumulation, particularly volatiles and phenolics, through foliar application of the phytohormones methyl jasmonate (MeJa) and salicylic acid (SA), and the osmolyte Trimethylamine N-oxide (TMAO) in five MAP species: basil (*Ocimum basilicum* L.), hyssop (*Hyssopus officinalis* L.), marjoram (*Origanum majorana* L.), peppermint (*Mentha piperita* L.), and yarrow (*Achillea collina* Becker). Key objectives included understanding the influence of MeJa, SA, and TMAO on plant morphology and SM production, identifying species-specific and shared responses, comparing the effects of phytohormone elicitors and the osmolyte, and evaluating the impact of environmental conditions and drought stress. The study also explored how variations in elicitor concentration, frequency, and exposure affect outcomes, while investigating the roles of glandular trichomes and enzymatic activities, specifically phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX), in biochemical responses to elicitation.

In open field settings, 2 concentrations from each phytohormone, 0.1 mM and 2 mM, were sprayed on the upper parts of plants over three consecutive years (2020-2022), and one concentration of TMAO (2 mM) was foliarly applied over two years (2021-2022) on the five species. Additionally, basil plants were subjected to an additional abiotic stress: plantation from 2020 and 2022 was divided into irrigated and non-irrigated plots, where the latter relied only on precipitations. The treatments were sprayed twice with an interval of one week before the harvest. The results demonstrated that elicitors had minimal effects on the biomass and height of each species, with environmental factors such as drought, in case of basil, and plantation year having a more pronounced influence. TMAO treatments notably reduced biomass in basil and marjoram (2021), while MeJa increased marjoram height under specific conditions. Moreover, remarkable increases in the biomass were registered in a newly established peppermint plantation (2021) with all elicitors, while in case of basil plants under no irrigation regime, the elicitors could not mitigate the negative effects of drought; in fact, they reduced slightly the fresh weight of plants in both

years, except for 2 mM of MeJa, although the change was not statistically significant. These trends suggest that the plant health status has a critical role in the outcome of elicitation.

MeJa was consistently the most effective elicitor for increasing essential oil (EO) content, particularly in basil, marjoram, and peppermint, likely due to its stimulation of volatile biosynthesis. However, it seems that the regulation mechanism is species-specific. In case of basil and marjoram MeJa enhances significantly the glandular hair density, suggesting that MeJa stimulates the volatiles accumulation through this process, while in peppermint the data from glandular hair density and EO content correlated negatively, which is why, at this stage, we cannot rule out the involvement of other mechanisms. SA showed a clear efficacy only in elevating the volatile production of hyssop while in case of yarrow the applied elicitors in the applied concentrations were mostly ineffective.

The effects of the treatments on EO composition were mostly quantitative, altering the ratio of some compounds. The effect was varying by species, genotype, environmental conditions, and treatment year. For instance, linalool levels in basil fluctuated across years under elicitor treatments, while compounds like *trans*-pinocamphone and  $\beta$ -phellandrene in hyssop showed species-specific shifts. While TMAO rarely affected significantly the concentration of EO, however, it significantly influenced the volatile composition, particularly in the case of hyssop, by altering the entire chemical profile. This effect was evident in the Principal Component Analysis, where samples treated with TMAO were distinctly separated from their respective clusters.

Additional studies on the impact of treatment frequency were conducted on marjoram in greenhouse and open field. The findings showed that the frequency of application may significantly affect the volatile accumulation. In the open field trial, a single treatment of MeJa increased the EO content, whereas after repeated treatments, it was reduced in both experiments. Furthermore, a single treatment notably enhanced the production of terpinene compounds at the expense of sabinene compounds, while samples subjected to two treatments displayed more balanced terpinene-to-sabinene ratios.

Concerning the total phenolic content (TPC) and antioxidant capacity (AOC), our studies reveal a highly variable response, even more so than the volatiles, with the results being context-dependent. For instance, both concentrations of MeJa suppressed phenolic production and AOC during the experimental year 2020, whereas the same treatments enhanced both parameters in the subsequent years. Similar trends were observed in other species in most cases, possibly due to the strong connection between phenolics and environmental conditions, which was particularly evident under

the open-field conditions. This was also portrayed in case of peppermint, where open-field experiments using a high concentration of phytohormones (10 mM) failed to enhance TPC, while plants grown under controlled environments in climatic chambers showed a significant increase in response to the elicitors. However, compositional analysis by HPLC revealed that variations were more pronounced in the open-field experiments, where MeJa 3 promoted the synthesis of phenolic acids and resulted in lower levels of flavonoids.

We also investigated the timing of the stimulus on phenolic accumulation in marjoram following MeJa 2 treatment. We found that the increase of TPC and AOC began as early as 2 days post treatment regardless of the cultivar and growing conditions. However, the time required to reach maximum levels varied. The commercial variety in greenhouse conditions (2021) and the 'Magyar' variety in open field conditions (2023) required up to 2 weeks for peak elevations. Conversely, the 'Magyar' variety grown in a greenhouse in 2022 responded rapidly, achieving increases of nearly 300% in TPC and 165% in AOC within just 5 days before levels declined again. This result highlights that, in certain conditions, MeJa stimulates phenolic production through rapid activation of signaling pathways, followed by the plant's regulation to restore homeostasis.

Combined abiotic stress and elicitor treatments in basil plants had minimal significant effects on EO content, its composition, and the phenolic production. Although drought effects alone naturally elevated phenolic levels, which might have diminished the impact of elicitor applications.

The experimental data on enzyme activity suggest that LOX and PAL activities respond differently to elicitor application based on species, concentration, and duration. These enzymatic changes do not consistently correlate with SM accumulation, highlighting the complex regulatory mechanisms underlying secondary metabolism in response to stress.

Our results show that *in vivo* elicitation is a complex and challenging strategy. Nevertheless, it holds significant promise for enhancing the accumulation of desired SMs when tailored protocols are developed to align with specific cultivation goals and environmental conditions. We emphasized, that it seems impractical to establish a uniform experimental design for all MAPs. Future research should focus on a tailored approach that involves stabilization of both endogenous and exogenous factors. It seems that in the near future hormonal elicitation under controlled (indoor) conditions may have a higher potential. Furthermore, customizing each elicitation strategy to target specific SMs is critical. Influencing volatile compound accumulation seems to be more effective than that of phenolic accumulation. Investigation of species with different accumulation structures (organelles) would also be desirable for optimization.

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## Appendices

### Appendix 1- References

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1. **Kandoudi, W.**, Radácsi, P., Gosztola, B., and Zámboriné Németh, É. (2022). Elicitation of medicinal plants in vivo—Is it a realistic tool? The effect of methyl jasmonate and salicylic acid on Lamiaceae species. *Horticulturae*, 8(1), 5. <https://doi.org/10.3390/horticulturae8010005>
2. **Kandoudi, W.**, and Nemeth-Zamboriné, E. (2022). Stimulating secondary compound accumulation by elicitation: Is it a realistic tool in medicinal plants in vivo? *Phytochemistry Reviews*, 1-19. <https://doi.org/10.1007/s11101-022-09822-3>
3. **Kandoudi, W.**, Radácsi, P. and Zámboriné Németh, É. (2023). Regulation of secondary metabolites of basil (*Ocimum basilicum* L.) by the application of elicitors in vivo. *Acta Hort.* 1358, 229-234. <https://doi.org/10.17660/ActaHortic.2023.1358.30>
4. **Kandoudi, W.**, Tavaszi-Sárosi, S., Németh-Zámboriné, É. (2023). Inducing the Production of Secondary Metabolites by Foliar Application of Methyl Jasmonate in Peppermint. *Plants*, 12, 2339. <https://doi.org/10.3390/plants12122339>
5. Zámboriné Németh, É., **Kandoudi, W.**, Radácsi, P., Tavaszi-Sárosi, S. (2025). The complexity of plant responses to hormonal treatments in vivo – A case study with basil (*Ocimum basilicum* L.) and marjoram (*Origanum majorana* L.) *Industrial Crops and Products* 226,120705. <https://doi.org/10.1016/j.indcrop.2025.120705>.

## CONFERENCE AND CONGRESS PARTICIPATIONS

1. **Kandoudi, W.**, Zámboriné Németh, É. (2021, November 12-14). The effect of methyl jasmonate and salicylic acid on the essential oil of peppermint (*Mentha piperita*) and marjoram (*Origanum majorana*) [Oral Presentation]. 51<sup>st</sup> International Symposium on Essential oils. Online.
2. Németh-Zámbori, É., **Kandoudi, W.** (2022, February 22), Beeinflussung der Produktion von Majoran und Basilikum mit pflanzlichen Hormonen [Oral Presentation]. 32. Bernburger Winterseminar Medicinal and Spice Plants. Online.
3. **Kandoudi, W.**, Zámboriné Németh, É. (2022, August 14-20). Regulation of secondary metabolites of basil (*Ocimum basilicum* L.) by the application of elicitors in vivo [Oral Presentation]. International symposium on medicinal and aromatic plants: domestication, breeding, cultivation and new perspectives, International Horticultural Congress. Angers, France.
4. Zámboriné Németh, É., Tavaszi-Sárosi, S., **Kandoudi, W.** (2022, September 4-7), Inducing volatile production by plant hormones in marjoram and peppermint [Oral Presentation]. 52<sup>nd</sup> International symposium on Essential Oils. Wrocław, Poland.
5. Zámboriné Németh, É., **Kandoudi, W.**, Tavaszi-Sárosi, S. (2023, September 13-16). Changes in accumulation and spectrum of volatiles in peppermint as result of elicitation [Poster Presentation]. 53<sup>rd</sup> International Symposium on Essential Oils. Milazzo, Messina, Italy.

## Appendix 2

**Appendix 2 Table 1 EO composition of different basil cultivars**

	Chemotype	Example	Cultivar used	Geographic location	Reference
1	Linalool type	1,8-cineole (5.0%), linalool (76.2%), $\tau$ -cadinol (3.9 %)	Little green	Italy	(Marotti et al., 1996)
2	Linalool/ <i>trans</i> - $\alpha$ -bergamotene	1,8-cineole (10.0%), fenchone (7.0%), linalool (17.0%), <i>trans</i> - $\alpha$ -bergamotene (17.5%), $\beta$ -caryophyllene (7.5%)	Wild growing purple type	Iran	(Yavari et al., 2011)
3	Linalool/methyl chavicol	methyl chavicol (52.4%), linalool (20.1%), <i>epi</i> - $\alpha$ -cadinol (5.9%), <i>trans</i> - $\alpha$ -bergamotene (5.2%)	Purple	Iran	(Sajjadi, 2006)
4	Linalool/ <i>trans</i> -methyl cinnamate	linalool (16.4%), methyl cinnamate (59.6%)	Purpurascens	Bangladesh	(Mohiuddin et al., 2012)
5	Methyl chavicol	1,8-cineole (2.3%), linalool (1.7%), methyl chavicol (85.5%)	Wild growing plants	Togo	(Koba et al., 2009)

**Appendix 2 Table 2 EO content and composition of hyssop from different geographical locations**

Plant material	EO content	Main components	Origin	Reference
Flowering shoots	1.5 mL 100g <sup>-1</sup> DW	$\beta$ -pinene (16.0-19.1%), pinocarvone (6.9-8.9%), <i>cis</i> -pinocamphone (51.8-55.9%)	Hungary	(Németh-Zámbori, Rajhárt, et al., 2017)
Flowering shoots	0.60% (v/w)	$\beta$ -pinene ( $\approx$ 21%), isopinocamphone ( $\approx$ 40%), pinocamphone ( $\approx$ 11%), elemol, ( $\approx$ 4%)	Iran	(Ahmadi et al., 2020)
Flowering shoots	<0.36%	$\beta$ -pinene (8.5%), $\beta$ -phellandrene (3.6%), pinocarvone (28.1%), isopinocamphone (15.5%), germacrene D (3.8%), hedycaryol (4.2%).	Lithuania	(Bernotienė & Butkienė, 2010)
Flowering shoots	0.65 mL 100 g <sup>-1</sup> DW	$\beta$ -pinene (15.8%), limonene (23.8%), <i>trans</i> -pinocamphone (8.3%), <i>cis</i> -pinocamphone (14.7%), methyl eugenol (28.3%)	Montenegro	(Mićović et al., 2021)
Leaves	0.24% (v/w)	$\alpha$ -pinene (70.9%), $\beta$ -pinene (10.9%), limonene (2.7%).	Nigeria	(Ogunwande et al., 2011)
Flowering shoots	0.60% (v/w)	$\beta$ -pinene (19.6%), 1,8-cineole (36.4%), isopinocamphone (15.3%)	Serbia	(Džamić et al., 2013)
Flowering shoots	0.70%	limonene (5.8%), 1,8-cineole (15.5%), limonen-10-yl- acetate (67.9%).	Italy	(Guerrini et al., 2021)
Flowering shoots	0.50%	$\beta$ -pinene (17.8%), 1,8-cineole (5.9%), <i>trans</i> -pinocamphone (5.0%), pinocarvone (23.4%), <i>cis</i> -pinocamphone (20.3%)	Himalayan region (India)	(Stappen et al., 2015)
Flowering shoots	0.21%	$\beta$ -pinene (3.8%), isopinocamphone (70.7%), myrtenol (5.7%),	Russia	(Plugatar et al., 2023)

**Appendix 2 Table 3 Factors influencing the phytochemical fractions of marjoram.**

<b>Plant material habitat</b>	<b>Compound group</b>	<b>Part used</b>	<b>Extraction method</b>	<b>Main bioactive compounds</b>	<b>Geographical region</b>	<b>Reference</b>
<b>Collection from the wild</b>	Volatiles	Dried aerial parts	Hydro distillation	$\alpha$ -pinene (7.9%), camphene (13.4%), $\delta$ -2-carene (20.1%), <i>cis</i> -sabinene hydrate (5.4%), terpinen-4-ol (29.6%).	Italy	(Pepa et al., 2019)
<b>Collection from the wild</b>	Volatiles	Dried aerial parts	Steam distillation	sabinene (5.2%), $\alpha$ -terpinene (9.5%), $\beta$ -phellandrene (4.4%), $\gamma$ -terpinene (13.7%), Terpinene-4-ol (26.7%), $\alpha$ -Terpineol (6.6%).	Egypt	(Ragab et al., 2019)
<b>Cultivation</b>	Volatiles	Dried aerial parts	hydro distillation	p-cymene (16.3%), $\gamma$ -terpinene (7.3%), terpinene-4-ol (36.2%), $\alpha$ -terpineol (5.4%)	Iran	(Khanavi et al., 2010)
<b>Cultivation</b>	Volatiles	Dried aerial parts	Hydro distillation	$\gamma$ -terpinene (6.2%), <i>trans</i> -sabinene hydrate (5.0%), <i>cis</i> -sabinene hydrate (27.7%), terpinene-4-ol (29.1%), geranyl acetate (7.1%)	Tunisia	(Sellami et al., 2009)
<b>Cultivation</b>	Volatiles	Dried Leaves	Hydro distillation	p-cymene (6.8%), terpinen-4-ol (33.0%), $\alpha$ -terpineol (6.7%), spathulenol (6.0%), caryophyllene oxide (11.9%)	China	(Jiang et al., 2011)
<b>Collection from the wild</b>	Volatiles	Fresh aerial parts	Hydro distillation	sabinene (4.9%), p-cymene (7.0%), $\gamma$ -terpinene (6.9%), <i>cis</i> -sabinene hydrate (15.0%), terpinen-4-ol (38.4%), $\alpha$ -terpineol (4.9%)	Reunion Island	(Vera & Chaneming, 1999)
<b>Collection from the wild</b>	Volatiles	Dried aerial parts	Solvent extraction	sabinene (14.1%), $\alpha$ -terpinene (8.9%), $\gamma$ -terpinene (10.2%), <i>cis</i> -sabinene hydrate (11.8%), <i>trans</i> -sabinene hydrate (16.0%), terpinen-4-ol (5.8%), $\alpha$ -terpinyl acetate (10.0%).	Yemen	(Al-Fatimi, 2018)
<b>Collection from the wild</b>	Volatiles	Fresh leaves and stems	Hydro distillation	p-cymene (12.6%), $\beta$ terpineol (5.5%), <i>trans</i> -4-thujanol (24.6%), terpinen-4-ol (29.1%), $\alpha$ -terpineol (9.1%),	Morocco	(Ouedrhiri et al., 2016)
<b>N.A*</b>	Phenolics	Seeds	Alcoholic extraction	cinnamic acid (63.02 $\mu$ g/ml), ascorbic acid (9.11 $\mu$ g/ml), catechol (1.76 $\mu$ g/ml), gallic acid (0.89 $\mu$ g/ml)	India	(Dhull et al., 2016)

<b>Cultivation</b>	Phenolics	Dried leaves	Aqueous extraction	salvianolic acid A (3.4 mg/g), salvianolic acid B (5.4 mg/g), rosmarinic acid (52.4 mg/g), luteolin derivative (6.9 mg/g), apigenin-6,8-di-C-hexoside isomer I (7.3 mg/g).	Portugal	(Gomes et al., 2020)
<b>Collection from the wild</b>	Phenolics	Aerial parts	Aqueous extraction	catechin, chlorogenic acid, vanillic acid, caffeic acid, vanillin, <i>trans</i> -ferulic acid, rutin, o-coumaric acid, luteolin, cinnamic acid.	Morocco	(Makrane et al., 2018)
			Alcoholic extraction	vanillic acid, <i>trans</i> -ferulic acid, rutin, o-coumaric acid.		

\*Not applicable

**Appendix 2 Table 4 EO composition of peppermint from different geographical locations**

	<b>India</b>	<b>China</b>	<b>Morocco</b>	<b>Slovakia</b>	<b>Hungary</b>	<b>USA</b>
<b>limonene</b>	0.50	1.76	3.01	4.32	5.51	1.58
<b>1,8-cineole</b>	6.00	2.91	6.06	nd*	4.28	5.62
<b>menthone</b>	31.20	14.51	7.42	14.49	35.05	21.80
<b>menthofuran</b>	t**	nd	13.18	nd	7.70	2.08
<b>neomenthol</b>	4.30	9.26	4.79	nd	nd	4.19
<b>menthol</b>	43.70	30.69	46.32	70.08	27.79	38.45
<b>pulegone</b>	t	4.36	nd	nd	1.96	0.91
<b>menthyl acetate</b>	1.00	12.86	12.10	3.76	4.57	3.90
<b>β-caryophyllene</b>	t	2.52	0.55	2.96	nd	2.87
<b>germacrene D</b>	0.9	1.13	nd	t	1.78	3.24
<b>References</b>	(Padalia et al., 2011)	(Sun et al., 2014)	(Marwa et al., 2017)	(Camele et al., 2021)	(Kandoudi et al., 2021)	(Wu et al., 2019)

\* not detected, \*\* traces (<0.1%)

### Appendix 3

**Appendix 3 Table 1 The mean square and significance levels of morpho-chemical traits of basil in open field (2020-2022)**

Traits	Source of variations		
	Elicitors	Years	Elicitors x Years
Fresh biomass	2016.59**	6248.89**	449.12 ns
Dry biomass	74.11***	6.67 ns	23.16 ns
Height	25.48 ns	357.93***	76.04*
EO content	0.02***	0.24***	0.03***
TPC	1206.02***	74006.58***	4911.98***
AOC	5983.71***	444000.88***	7444.27***

ns: non-significant ( $p>0.05$ ); \*: significant ( $P<0.05$ ); \*\*significant ( $P<0.01$ ); \*\*\*: significant ( $P<0.001$ )

**Appendix 3 Table 2 The mean square and significance levels of morpho-chemical traits of hyssop in open field (2020-2022)**

Traits	Source of variations		
	Elicitors	Years	Elicitors x Years
Fresh biomass	370.35**	1595.56***	210.66*
Dry biomass	21.73**	146.78***	8.31 ns
Height	10.62 ns	-	-
EO content	0.06***	0.53***	0.03***
TPC	1572.34***	21786.11***	1149.54***
AOC	2618.19***	5436.26***	5026.11***

ns: non-significant ( $p>0.05$ ); \*: significant ( $P<0.05$ ); \*\*significant ( $P<0.01$ ); \*\*\*: significant ( $P<0.001$ )

**Appendix 3 Table 3 The mean square and significance levels of morpho-chemical traits of marjoram in open field (2020-2022)**

Traits	Source of variations		
	Elicitors	Years	Elicitors x Years
Fresh biomass	75.92*	520.13***	43.59 ns
Dry biomass	5.69*	11.86*	4.88 ns
Height	5.17 ns	555.84***	35.57**
EO content	0.15***	0.36***	0.04*
TPC	3243.43***	40457.59***	3491.59***
AOC	3803.95***	6007.22***	1608.83***

ns: non-significant ( $p>0.05$ ); \*: significant ( $P<0.05$ ); \*\*significant ( $P<0.01$ ); \*\*\*: significant ( $P<0.001$ )

**Appendix 3 Table 4 The mean square and significance levels of morpho-chemical traits of peppermint in open field (2020-2022)**

Traits	Source of variations		
	Elicitors	Years	Elicitors x Years
Fresh biomass	301.51*	32380.00***	335.93 ns
Dry biomass	21.93*	186.51**	30.70 ns
EO content	0.18***	2.00***	0.15***
TPC	3971.80***	435250.47***	2082.89***
AOC	22424.37***	255606.81***	8367.64***

ns: non-significant ( $p>0.05$ ); \*: significant ( $P<0.05$ ); \*\*significant ( $P<0.01$ ); \*\*\*: significant ( $P<0.001$ )

**Appendix 3 Table 5 The mean square and significance levels of morpho-chemical traits of yarrow in open field (2020-2022)**

Traits	Source of variations		
	Elicitors	Years	Elicitors x Years
Fresh biomass	266.13 ns	4428.17**	643.28 ns
Dry biomass	35.76 ns	58.02 ns	63.03 ns
Height	20.17 ns	1212.96**	17.13 ns
EO content	0.00**	0.17***	0.00*
Proazulene content	0.00*	0.00***	0.00**
TPC	821.16***	10192.19***	369.74***
AOC	1129.70**	17983.53***	451.04*

ns: non-significant ( $p>0.05$ ); \*: significant ( $P<0.05$ ); \*\*significant ( $P<0.01$ ); \*\*\*: significant ( $P<0.001$ )

**Appendix 3 Table 6 The mean square and significance levels of morpho-chemical traits of basil in open field drought experiment (2020-2022)**

Traits	Source of variations						
	Elicitors	Years	Irrigation	Irrigation x Elicitors	x Elicitors	x Elicitors x Years	
				Years	Years	Irrigation	x Irrigation
Fresh biomass	485.53 ns	1463.56 ns	21415.95***	770.42 ns	239.64 ns	395.80 ns	235.33 ns
Dry biomass	6.87 ns	514.59***	403.99***	96.44**	13.43 ns	6.20 ns	19.60 ns
Height	78.55 ns	8019.68***	661.14**	9.08 ns	42.99 ns	75.48 ns	66.43 ns
EO content	0.05***	0.26***	0.07***	0.04***	0.03***	0.02**	0.02***
TPC	4841.26*	40.69 ns	3418.08***	7227.49***	1114.20**	2504.08***	409.80 ns
	**						
AOC	5291.88*	939287.29**	16039.10***	2359.62 ns	1893.05 ns	4930.07***	4850.61**
	**	*					

ns: non-significant ( $p>0.05$ ); \*: significant ( $P<0.05$ ); \*\*significant ( $P<0.01$ ); \*\*\*: significant ( $P<0.001$ )

**Appendix 3 Table 7 Essential oil content (mL 100 g<sup>-1</sup> DW), total phenolic content (mg GAE mg<sup>-1</sup> DW), and antioxidant capacity (mg AAE mg<sup>-1</sup> DW) of basil**

	Growing conditions	Semi-controlled (2021)	Controlled (2022)
<b>EO</b>	<b>C</b>	0.16	0.23
	<b>MeJa 2</b>	0.31	0.23
	<b>SA 2</b>	0.19	0.39
<b>TPC</b>	<b>C</b>	76.95	130.66
	<b>MeJa 2</b>	142.63	204.09
	<b>SA 2</b>	73.1	225.03
<b>AOC</b>	<b>C</b>	84.72	47.67
	<b>MeJa 2</b>	156.55	90.08
	<b>SA 2</b>	81.88	101.67

Values are presented as Mean. C: control, MeJa 2: 2 mM, SA 2: 2 mM

**Appendix 3 Table 8 Essential oil content (mL 100 g<sup>-1</sup> DW), total phenolic content (mg GAE mg<sup>-1</sup> DW), and antioxidant capacity (mg AAE mg<sup>-1</sup> DW) of basil and marjoram grown in open field (2023)**

	Species	Basil	Marjoram
<b>EO</b>	<b>C</b>	0.74	1.7
	<b>MeJa 1</b>	1.54	2.04
	<b>MeJa 2</b>	2.13	1.38
	<b>SA 1</b>	1.77	1.49
	<b>SA 2</b>	2.25	1.91
<b>TPC</b>	<b>C</b>	257.7	281.53
	<b>MeJa 1</b>	207.38	248.94
	<b>MeJa 2</b>	256.62	312.99
	<b>SA 1</b>	242.12	263.82
	<b>SA 2</b>	257.37	280.72
<b>AOC</b>	<b>C</b>	260.5	131.52
	<b>MeJa 1</b>	243.78	179.2
	<b>MeJa 2</b>	308.1	180.81
	<b>SA 1</b>	334.21	194.03
	<b>SA 2</b>	354.32	218.24

Values are presented as Mean. C: control, MeJa 1: 0.1 mM, MeJa 2: 2 mM, SA 1: 0.1 mM, SA 2: 2 mM

**Appendix 3 Table 9 Essential oil content (mL 100 g<sup>-1</sup> DW) of basil, marjoram, and peppermint grown in open field**

	<b>Basil</b>	<b>Marjoram</b>	<b>Peppermint</b>
<b>C</b>	0.74	1.72	3.83
<b>MeJa 1</b>	1.54	2.06	3.38
<b>MeJa 2</b>	2.13	1.97	3.45
<b>SA 1</b>	1.77	1.64	3.80
<b>SA 2</b>	2.25	1.76	3.47

Values are presented as Mean. C : control, MeJa 1: 0.1 mM, MeJa 2: 2 mM, SA 1: 0.1 mM, SA 2: 2 mM