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OPTIMIZATION OF SECONDARY METABOLITES PRODUCTION BY THE APPLICATION OF ELICITORS

Doctoral (Ph.D.) thesis

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1. Introduction

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 (Bachheti & Bachheti, 2023). These compounds- called secondary metabolites (SMs)- are synthesized after a plant's exposure to various stresses, whether biotic or abiotic, or during specific developmental stages. They have long been employed for various purposes, including human therapeutic use, as well as flavor and fragrance enhancement in foodstuffs and cosmetics (Ahmad et al., 2018; Garagounis et al., 2021; Wu et al., 2023).

Several biotechnological approaches have been implemented to meet the burgeoning demand for these metabolites, 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 as they can 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 by protecting their cell contents against damage, scavenge radicals, adjust osmotic pressure, and stabilize protein structures (Ghosh et al., 2021; Jogawat, 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 (Kandoudi & Németh-Zámboriné, 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). 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.

2. Objectives

The main goal of this research was to optimize the accumulation of SMs, notably the essential oil (EO) 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:

1. How do MeJa, SA, and TMAO influence the accumulation of volatile compounds in the essential oil 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:

2. How do elicitor concentrations, treatment frequency, and exposure duration differences impact the observed plant characteristics?
3. Could we maintain an acceptable dry matter production (height, biomass, drug ratio) in parallel with changing active compounds' accumulation?
4. Are the effects of the studied elicitors similar to each other?
5. Are the responses exhibited by the five model species uniform to each other?
6. 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.

3. Materials and methods

3.1 Experimental site and plant material

Multiple experiments were conducted under three different growing conditions (open field, greenhouse, and climatic chamber). The open-field experiments were conducted at the Experimental Station of the University of Agricultural and Life Sciences (MATE) in Soroksár (Pest County, Hungary) over the summer of four consecutive years (2020–2023). Semi-controlled environment experiments were carried out in a greenhouse for two consecutive years (2021 and 2022) during the spring at the MATE Buda campus, and controlled environment experiments were carried out in a climatic chamber 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). All propagation material originated from the gene bank of the MAP Department, except for the first greenhouse experiment (2021), which used basil and marjoram seedlings purchased from Zöldpont Kft (Albertirsa, Hungary).

Table 1 Genotypes and age of the experimental plants during the study years

Species	Growing environment	2020	2021	2022	2023
Achillea collina	**Open field**	'Azulenka' (1st year)	'Azulenka' (2nd year)	'Azulenka' (1st year)	-
Hyssopus officinalis	**Open field**	Meran gene bank accession (3rd year)	'Sophie' (1st year)	'Sophie' (2nd year)	-
Mentha x piperita	**Open field**	'Mexian' (4th year)	'Mexian' (1st year)	'Mexian' (2nd year)	-
	Climatic chamber		'Mexian' (1st year)		
Ocimum basilicum	**Open field**	'Genovese' (1st year)	'Genovese' (1st year)	'Genovese' (1st year)	'Genovese' (1st year)
	Climatic chamber			'Genovese' (1st year)	
	Greenhouse		'Commercial variety' (1st year)		
Origanum majorana	**Open field**	'Magyar' (1st year)	'Magyar' (1st year)	'Magyar' (1st year)	'Magyar' (1st year)
	Greenhouse		'Commercial variety' (1st year)	'Magyar' (1st year)	

3.2 Treatments

3.2.1 Open field elicitation

The open-field study evaluated the effects of elicitors (MeJa, SA, and TMAO) on the above-mentioned species over three consecutive years under consistent conditions. Treatments were applied two weeks before each species' optimal harvest stage, with MeJa and SA tested at two concentrations (0.1 and 2.0 mM) and TMAO introduced in 2021 at 2.0 mM, dissolved in 0.3% ethanol. Control plots received only water and ethanol. Solutions were sprayed uniformly on aboveground shoots twice (50 mL plant^{-1}), one week apart, using a hand sprayer. Samples were collected one week after the second treatment. The experiment followed a completely randomized block design with three replications per treatment. Details for each species are summarized in Table 2.

Table 2 Treatment and harvest timelines with corresponding phenological stages in open field experiments

		Basil	Hyssop	Marjoram	Peppermint	Yarrow
2020	**1st treatment**	29 July	12 June	26 June	26 June	14 July
	2nd treatment	5 August	19 June	2 July	2 July	21 July

	Harvest date	13 August	26 June	10 July	10 July	29 July
	Phenological stage	Full flowering	Full flowering	Budding	Full flowering	Full flowering
2021	**1st treatment**	28 June	12 July	28 June	28 June	7 June
	2nd treatment	5 July	19 July	5 July	5 July	14 June
	Harvest date	12 July	27 July	12 July	12 July	21 June
	Phenological stage	Beginning of flowering	Full flowering	Budding	Full flowering	Beginning of flowering
2022	**1st treatment**	1 July	6 June	1 July	20 June	13 June
	2nd treatment	8 July	13 June	8 July	28 June	20 June
	Harvest date	15 July	20 June	15 July	6 July	28 June
	Phenological stage	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) in 2020 and 2022. Basil plants were selected for this experiment. 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. Irrigated plots received water twice weekly, while non-irrigated plots relied solely on natural precipitation. The timeline for treatments and harvest was consistent with that used for basil plants in the elicitation experiment (Table 2).

3.2.3 Effect of increased elicitor concentration

Peppermint was chosen for experimentation in both open-field and controlled-environment settings with a 10 mM concentration of MeJa and SA. The treatments were applied as a foliar spray using a hand-pump sprayer, distributing approximately 20 mL plant^{-1} in the controlled environment and 50 mL plant^{-1} in the open-field experiments. Details of the experiment are provided in Table 3.

Table 3 Treatment and harvest timelines with corresponding phenological stages in high concentration elicitation experiments

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

3.2.4 *Effect of elicitation duration*

The experiments were conducted over three years with marjoram in a greenhouse and open field. Only one treatment was applied across all trials: 2.0 mM MeJa. The treatment conditions followed the above-mentioned protocol; however, the harvest was conducted at varying time intervals: 48 hours, 120 hours, 1 week, and 2 weeks after the treatment. The phenological stage at sampling and the timelines of treatments and harvests are provided in Table 4.

Table 4 Treatment and harvest timeline with corresponding phenological stages in time interval elicitation

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

3.2.5 *Effect of repeated elicitor treatment*

This study compared the effects of applying 2.0 mM MeJa either once or twice on two species, marjoram and basil, cultivated in different environments. In each experiment, 2.0 mM MeJa was applied, and the effects were assessed following a single treatment (with harvest after two weeks) and repeated treatments, similarly as in 3.2.1. For sampling, 10 individual plants were randomly selected from each group. Details of the experiment are provided in Table 5.

Table 5 Treatment and harvest timelines with corresponding phenological stages in repeated treatment experiments

	1st Treatment	2nd treatment*	Harvest	Phenological stage
Basil (climatic chamber)	11 November 2022	18 November 2022	25 November 2022	Budding
Basil (open field)	28 June 2023	5 July 2023	12 July 2023	Beginning of flowering
Marjoram (greenhouse)	28 April 2021	5 May 2021	12 May 2021	Budding
Marjoram (open field)	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 before sample collection, with 10 plants measured from ground level to the shoot tip in 2021 and 2022. Sampling involved cutting three plants 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 high plant density. In controlled and semi-controlled environments, plant height was similarly measured for 10 plants, and biomass was assessed by collecting three replications with three individuals per replicate. Fresh and dry masses (after drying at room temperature) were determined.

Glandular hair density

The glandular hair density 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^{nd} 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.

3.3.2 Biochemical analysis

Essential oil extraction

The entire aerial parts of hyssop and yarrow were used for EO extraction. For the other species, the leaves and flowers were separated from the stems, and dried stemless plant material from each sample was hydro-distilled to extract the EO by using a Clevenger-type apparatus following the method of the VII Hungarian Pharmacopoeia (Hungarica, 1986). The content was expressed as mL 100 g^{-1} DW (dry weight).

Essential oil composition

The composition of essential oils was determined using GC-FID and GC-MS analysis methods. An Agilent Technologies 6890N GC System equipped with an HP-5 capillary column (30 m length, 0.25 µm film thickness) was used for GC-FID analysis. A 0.2 mL sample was injected, and essential oil composition was calculated from GC peak areas.

For GC-MS analysis, the same instrument was coupled with an Agilent Technologies MS 5975 inert mass selective detector. Compounds were identified by calculating linear retention indices using the Van Den Dool and Kratz equation and matching mass spectra with the NIST MS Search 2.0 and Wiley 275 libraries (Adams, 2007; van Den Dool & Dec. Kratz, 1963).

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*). Proazulene content was expressed as chamazulene percentages using the following formula: (2.1 x A) /m where A is the absorbance measured in a Thermo Evolution 201 spectrophotometer at 608 nm and m is the mass of the sample in grams.

Total phenolic content (TPC)

The extraction process involved adding 100 mL of boiling distilled water to 1 g of powdered plant material. The extracts were filtered and finally stored in a freezer after soaking for 24 h.

The modified method of Singleton and Rossi (Singleton & Rossi, 1965) was used to quantify the total phenolic content (TPC). The results were expressed as mg of gallic acid equivalents per g of dry weight of extract (GAE $mg \cdot g^{-1}$ DW). 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 values of samples were calculated from the standard curve equation and expressed as mg ascorbic acid equivalent (AAE) g^{-1} of dry extract. The measurements were performed in six replications.

Composition of phenolic compounds

HPLC analysis was conducted at "Tudásközpont Laboratory" of MATE, Gödöllő. Phenolic compounds were extracted from 0.5 g of dried plant leaves using 50% ethanol in 2% ortho-phosphoric acid. The extract was shaken at 80°C, ultrasonicated, centrifuged, and filtered before injection into a Chromaster Hitachi HPLC system equipped with a diode-array detector (DAD).

The separation of phenolic compounds was performed on Ascentis phosphor-conditioned C18 with gradient elution of 1% ortho-phosphoric acid (A) and acetonitrile (B). The DAD detection was performed between 190 and 700 nm. Phenolic compounds were quantified by comparing peak areas to standard solutions, with tentative identification based on spectral and chromatographic data when standards were unavailable.

Phenylalanine ammonia-lyase activity (PAL)

PAL (phenylalanine ammonia-lyase) enzyme activity was measured at the Hun-Ren Research Institute, Martonvásár, using leaf samples from basil and marjoram treated with MeJa 2, harvested two weeks post-elicitation. Samples from at least five plants per treatment were flash-frozen in liquid nitrogen and stored until analysis. PAL activity was quantified spectrophotometrically at 290 nm, measuring trans-cinnamic acid formation, and expressed as enzyme units per gram of fresh weight (U g^{-1} FW), with one unit defined as an absorbance increase of 0.01 min^{-1}.

Lipoxygenases activity (LOX)

LOX (lipoxygenase) enzyme activity was measured at the Hun-Ren Research Institute, Martonvásár, using a similar sample preparation method as for PAL. LOX activity was determined using linoleic acid as a substrate, following Axelrod et al. (1981) and was quantified using a molar extinction coefficient of 25,000 M^{-1} cm^{-1}.

Statistical analysis

IBM SPSS version 29 software 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.

All the measurements and treatments carried out are summarized in Table 6.

Table 6 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) SA (0.1 & 2.0 mM)		X	X			
		Hyssop			X	X			
		marjoram			X	X			
		Peppermint (1st cut)			X	X			
		Peppermint (2nd cut)			X				X
		Yarrow			X	X			
2021	Open field	Basil	MeJa (0.1 & 2.0 mM) SA (0.1 & 2.0 mM) TMAO (2.0 mM)	X	X	X			
		Hyssop		X	X	X			
		Marjoram		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) SA (0.1 & 2.0 mM) TMAO (2.0 mM)	X	X	X			
		Hyssop		X	X	X			
		Marjoram		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) SA (0.1 & 2.0 mM)	X	X			X	X
		Marjoram		X	X			X	

4. Results and discussion

Effect of elicitors on the yield characteristics

Our results showed that the elicitors rarely affected the biomass of our species in a significant manner; in fact, most variations were 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, no effect was registered. In peppermint, some treatments even increased the yield. These changes, however, were observed exclusively in one of the two years. The height of the majority of our experimental species was also not altered by the treatments, except for the MeJa 2 that significantly enhanced the height of marjoram in the second trial year by 17%.

Our experiments involving the exogenous application of elicitors to enhance drought tolerance in basil revealed that drought conditions generally had a negative impact on the plant's fresh and dry biomass, as well as its height. However, none of the applied elicitors significantly mitigated the adverse effects of water deficit stress on basil growth and yield.

As for the trials where single and repeated applications of MeJa were compared, we observed that a single treatment led to a slight increase in yield for basil and a significant increase for marjoram. The effect of two treatments was not significant. Given that jasmonates are known to slow the growth in many plant species (Heinrich et al., 2013; Li et al., 2018), the timing of the treatments may have a role. In our open field experiments, the time period between the phytohormone applications and harvest was only two weeks before flowering, when intensive plant growth had already ended, a severe reduction in these traits was not observed.

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 appears to be the most effective elicitor for promoting EO accumulation in our species in a concentration-dependent manner, particularly in basil, marjoram, and peppermint. Notably, MeJa 2 resulted in EO increases of 33% in peppermint (2021), 24% in basil (2022), and 22% in marjoram (2020). To assess the role of glandular hairs in EO accumulation, we examined EO gland density. Our findings confirmed the phenomenon stating that jasmonates may increase the density of the special structures, particularly in marjoram and basil, whereas, interestingly, the applied elicitors had no direct effect on peppermint's gland number. This suggests that MeJa-induced enhancement of volatiles may be—at least partially—linked to increased glandular trichome formation in certain species. However, whether these species-specific differences arise from genetic and molecular physiological factors, or the developmental speed of the plants, remains uncertain.

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. 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. We could conclude that the proportion of younger, developing plant organs, where treatments may induce more significant changes, might also be an influencing factor. 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 accumulation was detected in the latter species in any of the years. Whether this is due to structurally distinct EO gland types in this Asteraceae species, 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 hyssop, where SA—although at different concentrations—led to increased EO content. In yarrow, similar to MeJa, SA was ineffective in any of the measurements. In other species, increase in EO content could rarely be observed. 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 and peppermint, SA 1 was more successful in enhancing the EO accumulation than the higher concentration, while marjoram was practically not influenced by this elicitor. The controversial effect of higher concentrations was more obvious in the case of SA, although in some cases (marjoram 2022, hyssop, 2021, yarrow 2020) it could also be observed for MeJa.

Besides the concentration, environmental variations may strongly influence the biosynthesis of EOs 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. We assume that if the weather conditions do not favor the production of EO, 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, reaching 1.07 mL 100 g^{-1} DW in non-irrigated basil treated with MeJa 2.

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 by 16%, but in 2022, it led to a decrease in the linalool ratio by 13%. In parallel, significant elevations of sesquiterpenes were detected. 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*-β-guaiene formation in the first year, which occurred at the expense of its related compound, α-bulnesene (formerly known as δ-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 (Talebi et al., 2018; Złotek et al., 2016).

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 proportions of oil constituents, 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, 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 oil, 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. Therefore, selecting an elicitation strategy should take into consideration the intended use of the plant.

In peppermint, 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. The ratios of menthofuran and pulegone also increased in some treatments, such as MeJa 2 (2020), SA 2 (2021), and both dosages of SA in 2022. Despite these changes, the resulting spectrum remained within the safety limits established by the Ph Eur. However, this trend should be carefully monitored before the practical application of these elicitors in peppermint.

Yarrow showed the lowest sensitivity against the elicitors used. Proazulene accumulation did not change significantly in any experiment, except for some decreases in 2020 and 2021.

As for the complex changes in EO composition in samples of elicited plants 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, the analysis also represents 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 accumulation, 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, SA 2 treatments successfully enhanced both the TPC and AOC of the extracts in all experimental years. Interestingly, when a higher dosage of SA (10 mM) was applied, no improvement in TPC was observed, which might be in connection with negative feedback to the endogenous production.

This relationship of environmental and stress conditions with accumulation of phenolics is reflected in one of our studies 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-7O-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, phenolic accumulation varies in a species-specific manner each year, and each species also exhibits a distinct 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 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.

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. However, 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.

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 (day of the final sampling conducted 2 weeks after treatment).

Marjoram proved to be a suitable experimental subject for also comparing the effects of single versus repeated applications of MeJa 2. The single treatment resulted in more favorable outcomes, including higher fresh and dry masses as well as EO content, compared to repeated spraying. However, for TPC, no significant differences were observed between the frequencies of treatments in either of our experiments.

Effect of elicitors on the enzymatic parameters

In the enzymatic studies, we found that the treated basil samples demonstrated increased TPC and higher AOC without elevated LOX and PAL activities. Moreover, no LOX or PAL stimulation from any treatments across different environments was observed. Although LOX enzymes have a critical role in the biosynthesis of jasmonates and stress responses leading to the production of SMs, the process might have not been regulated by a single pathway. Moreover, 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.

5. Conclusion and recommendations

Based on our comprehensive studies, we declared that *in vivo* elicitation has significant potential to improve SMs such as volatile compounds and polyphenols 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 dosage, application timing, and frequency. Such a strategy will ensure consistent and effective results. In the near future, hormonal elicitation under indoor controlled conditions seems to have a higher potential. Furthermore, customizing each elicitation strategy to target specific SMs is critical. For instance, repeated treatments appear to influence volatile compound accumulation in the EO more effectively, than that of phenolic accumulation.

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.

6. 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.

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