Effect of Jasmonic Acid and Salicylic Acid on Growth and Biochemical Composition of In-Vitro-Propagated Lavandula angustifolia Mill

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Abstract: This study assessed the effect of jasmonic acid (JA) and salicylic acid (SA) on the in vitro development and production of Lavandula angustifolia Mill. plant material, and the accumulation of polyphenols, chlorophylls, and carotenoids in explants. Results were compared with explants grown in control media and with in-vivo-grown mature and young L. angustifolia plants. After 21 days of incubation, all explants propagated on low-SA-concentration or elicitor-free media produced a greater number of shoots than explants cultivated on media with higher elicitor concentrations. Shoots grew taller when activated charcoal (AC) was added to the elicitor-supplemented media, while AC negatively affected or had no effect on the phytochemical composition of plants. Explants grown in the presence of elicitors had higher polyphenolic and chlorophyll content than the controls, demonstrating the beneficial impact of elicitors on the secretion of secondary metabolites. Lutein and β-carotene were the dominating carotenoids in all samples. Culture media supplemented with 0.5 mg/L JA and 1.5 mg/L SA + AC proved the most suitable to produce plant material with high polyphenol and carotenoid content, comparable with in-vivo-grown plants.

Keywords: Lavandula angustifolia Mill.; in vitro; development; chlorophylls; polyphenols; carotenoids

1. Introduction

Lavandula angustifolia Mill. is a well-known member of the Lamiaceae family. It is native to the Mediterranean region but is also grown in many other parts of the world [1]. The great interest associated with this plant relies on its ability to produce essential oils widely used in the perfume, cosmetic, flavoring, and pharmaceutical industries [2]. Essential oils are a mixture of chemical compounds, primarily monoterpenes and sesquiterpenes [3,4], that confer Lavandula its antibacterial, antifungal, and antioxidant properties [5–8]. L. angustifolia explants also produce polyphenols and carotenoids, which play an important role in human health through their therapeutic effect against several diseases, their antioxidant and antibacterial activities, and their regulation of metabolism [9–11]. The expression of phytochemicals produced by L. angustifolia is influenced by plant variety, plant age, geographical position, and in-vivo-applied nutrient solutions, as well as harvesting, transport, storage, and preparation techniques [7]. Metabolites can be isolated from naturally-grown plants, but their commercial production in this manner is limited due to environmental constraints [12]. The main advantage of tissue culture over conventional horticultural propagation is the production of large numbers of high-quality and uniform plants that are disease free and can be multiplied year-round,
regardless of weather conditions [13]. Secondly, this approach also reduces the pressure on forests, wetlands, or natural grasslands to be converted into agricultural land, while it does not have a negative impact on the area cultivated with food crops. Different in vitro systems have been successfully applied for the rapid multiplication of several plants with important bioactive compounds [14], but the optimization of these techniques is a continuous demand.

A widely-employed method of improving the in vitro production of secondary metabolites in several plant species is elicitation [15]. Elicitors stimulate stress responses in plants, enhancing the synthesis and accumulation of secondary metabolites or the induction of novel compounds [16]. Jasmonic acid (JA) and its related signal molecules jasmonates and salicylic acid (SA) are among the most-used elicitors [17]. These endogenous growth-regulating substances are produced in plants in response to abiotic stress or pathogen invasion and lead to accumulation of phenols, alkaloids, carotenoids, and chlorophylls [18,19]. They initiate signal transduction pathways that lead to transcriptional stimulation of various genes thereby inducing the synthesis of various molecules involved in defense or resistance of medicinal plants [20,21]. JA and jasmonates are cyclopentanone compounds derived from α-linolenic acid, a fatty acid which, under natural conditions, accumulates mostly in the leaves [22]. JA is involved in several physiological responses induced in plant tissues and cells which include activation of the antioxidant system [23], accumulation of amino acids (isoleucine and methionine) and soluble sugars [24], and regulation of stomatal opening and closing [25]. Jasmonates interact with other plant growth regulators (PGRs) (auxin, ethylene, and abscisic acid) [26] and transcription factors [27] and trigger the expression of JA-associated genes [28]. At optimal concentration, JA promotes petiole abscission, root formation, and ethylene and β-carotene synthesis [29]. It can also inhibit seed germination and hypocotyl growth and inhibit or stimulate callus growth, root growth, chlorophyll production, and pollen-grain germination [30].

SA is widely distributed throughout the plant kingdom [31] and can influence a large number of physiological processes such as seed germination, thermogenesis, plant signaling or plant defense, and response to biotic and abiotic stress [32,33]. It has been proposed that exogenously-applied SA could reduce drought stress impact on plants [34]. Various levels of SA have been shown to protect the buds of Vitis genotypes during cryopreservation [35] and to stimulate flowering in Eleusine coracana L. [36] and fruit development in Fragaria x ananassa Duch. [37], Malus domestica Borkh. [38], and Mangifera indica L. [39]. Alone or in combination with other compounds, SA has been widely employed as an elicitor of secondary metabolites in various species such as: Vitis vinifera L. [40], Bacopa monnieri L. [40], Silybum marianum L. [41], Carthamus tinctorius L. [42], and Hypericum perforatum L. [43].

Previous research indicates that JA changes L. angustifolia growth and essential oil composition [44,45], but nothing is known about its influence on the polyphenol and carotenoid contents of the plant. Similarly, there is little information on how SA alters L. angustifolia development and synthesis of bioactive compounds [44,45]. The aim of this research was to assess the effect of JA and SA on in vitro shoot proliferation of L. angustifolia as well as on the production of bioactive compounds (polyphenols, chlorophylls, and carotenoids).

2. Materials and Methods

2.1. Plant Material and Culture Conditions

All macro- and micronutrients for tissue culture media, sucrose, agar, and PGRs were acquired from Duchefa Biochemie, (Haarlem, The Netherlands). Mature plants were field-grown at coordinates 47°20’18.6” N, 23°17’10.9” E and used as starting material to establish cultures in pots and in vitro.

To initiate in vitro culture, fragments of stems from mature field-growing plants were harvested at the end of June. Nodal segments with a length of 1–1.5 cm were sterilized by dipping in 70% ethanol for 30 s, immersion in a 10% solution of commercial bleach for 10 min and rinsing in sterile water. The culture medium employed for this stage was Murashige and Skoog medium (MS) [46] supplemented with 30 g/L sucrose, solidified with 6 g/L agar and pH was adjusted to 5.8 before
autoclaving at 121 °C for 20 min. After sterilization explants were placed in MS supplemented with 2 mg/L 6-benzyladenine (BA) and kept at 20–25 °C under an 18/6 h light/dark regime provided by cool white fluorescent lamps with a light intensity of 40 µmol/m²/s. Cultures were transferred to fresh culture medium without PGRs every four weeks until enough stock material was available to perform further experiments.

Around 10–14 lavender shoots were transferred, under sterile conditions, into 720 glass jars, with a diameter of 9 cm and a height of 13.5 cm, supplied in advance with 100 mL of culture medium. A total of fourteen experiments were carried out using the following culture media supplemented with different concentrations of JA and SA with or without activated charcoal (AC) (2 g/L): 0.1 mg/L JA, 0.5 mg/L JA, 1 mg/L JA + AC, 0.5 mg/L JA + AC, 1 mg/L JA + AC, 1.5 mg/L SA, 5 mg/L SA, 15 mg/L SA, 1.5 mg/L SA + AC, 5 mg/L SA + AC, and 15 mg/L SA + AC. Two control media were used, one without elicitors (control) and another one containing AC (control + AC). The in vitro cultures were incubated in the growth room for 21 days under the same conditions as mentioned above. At the end of the incubation period, the number of shoots, shoot height (cm), plant tissue fresh weight (g), and the number of explants that resumed growth (explant response) was recorded. These measurements were used to calculate the number of shoots per explant, mean height of all the shoots, average height of the main shoot, the quantity of plant tissue produced by each explant, and response of explants. Bioactive compounds (total phenols, chlorophylls, and carotenoids) of the explants from each experiment were determined. For comparison, mature lavender plants cultivated in vivo in field conditions and young plant grown in vivo in pots were also analyzed.

2.2. Total Phenolic Content

For all chemical analyses reagents and solvents used in extractions and sample preparation were of analytical, HPLC grade and were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany).

The total phenolic content (TPC) of the plantlets was determined using the Folin–Ciocalteu method. Fresh plant samples (2 g) were crushed and homogenized in a mortar with pestle and extracted with acidified methanol (0.3%, 15 mL) for 24 h. Extracts were filtered, and each extract sample (1 mL) was diluted with distilled water (60 mL). Folin–Ciocalteu reagent (5 mL) was then added and the sample was kept in the dark for 2 h. Following the addition of sodium carbonate solution (7.5%, 15 mL), the absorbance was read at 750 nm using a V-530 UV-VIS spectrophotometer (Jasco, Eason, MD, USA). A blank sample was prepared in the same way but replacing the extract with ethanol solution (40%, 1 mL). Concentrations were calculated from a calibration curve using gallica acid as the standard (0–0.25 mg/mL) and expressed as mg gallic acid equivalents (GAE)/g sample. Parameters of the calibration curve: \(a = 2.364, b = 0.0649\), and \(R = 0.9909\).

2.3. Chlorophyll Content

Chlorophyll content was determined using a published method [47]. Fresh plant samples (0.2 g) were homogenized and extracted with 90% acetone in a round-bottom flask using a magnetic stirrer. After 2 h, the solution was filtered, and the extraction procedure was repeated until the residue was uncolored. Absorbance was read at 645 and 663 nm using the V-530 UV-VIS spectrophotometer. Chlorophyll a (Chl a) and chlorophyll b (Chl b) concentrations were quantified using the following formulas:

\[
\text{Chl a (mg/g)} = \left(11.75 \times \frac{A_{663}}{A_{645}} - 2.35 \times \frac{A_{663}}{A_{645}}\right) \times \frac{V}{g}
\]

\[
\text{Chl b (mg/g)} = \left(18.61 \times \frac{A_{645}}{A_{663}} - 3.96 \times \frac{A_{663}}{A_{645}}\right) \times \frac{V}{g}
\]

where \(A_{645}\) and \(A_{663}\) represent the optical density at specific wavelength, \(V\) represents the volume of the extract (mL), and \(g\) represents samples weight (mg).
2.4. Carotenoid Content

Extraction of carotenoids from homogenized fresh explants (1 g) was carried out using a mixture of methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v) as described previously [48]. Extraction was repeated until the residue became colorless. The combined extracts were partitioned in a separation funnel with diethyl ether and saturated saline solution. The organic phase was separated and evaporated to dryness under reduced pressure, using a rotary evaporator. The residue was dissolved in diethyl ether (10 mL) and treated with an equal volume of potassium hydroxide (30% methanolic solution) for saponification (for 2 h). The samples were transferred in a separation funnel and washed with saline solution until neutral pH of the aqueous phase was reached. The organic phase was dried over anhydrous sodium sulfate and evaporated under vacuum. Samples were stored at −20 °C until further use. Total carotenoid content was estimated spectrophotometrically from saponified samples, at 450 nm, using a Shimadzu UV-2102 PC scanning spectrophotometer.

For the quantification of individual carotenoids, extracts were diluted with ethyl acetate, filtered through membrane filter (PTFE, 0.45 μm pore size, Millipore, Germany) and analyzed on a Shimadzu HPLC system equipped with LC-20 AT binary pump (Prominance), degasser DGU-20 A3 (Prominance), and photodiode array detector SPD-M20 (HPLC-PDA). The carotenoids were separated on YMC C30 column (24 cm × 4.6 mm, 5 μm particle size) using a mixture of two solvents as mobile phase at 0.8 mL/min flow rate. Solvent A: methanol/tert-butyl methyl ether/water (81:15:4, v/v/v) and solvent B: tert-butyl methyl ether/methanol/water (90:7:3, v/v/v). Gradient elution started with 1% B at 0 min and increased to 100% B by 160 min. The carotenoids identification was realized by comparison of the UV–VIS spectra and retention time of sample peaks with those of the standards (neoxanthin, violaxanthin, lutein, and β-carotene).

2.5. Statistical Analysis

For elicitation of lavender explants, the experimental design was completely randomized with four replications for each of the 14 treatments and two jars for each replication. The data for total phenolic content, chlorophyll content, and carotenoid profile are presented as the mean of three replicates. One-way analysis of variance (ANOVA) was done for all treatments to investigate whether the differences in morphological parameters and content of bioactive compounds between in vitro shoots were affected by the presence of elicitors and activated charcoal. Post hoc testing for the ANOVAs was carried out using the Tukey test with $p < 0.05$ being considered statistically significant. Statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software (San Diego, CA, USA).

3. Results

3.1. Effect of Elicitors on the Morphological Characteristics of In Vitro Explants

After 21 days of culture in MS media supplemented with either JA or SA, alone or in combination with AC, growth parameters were analyzed (Table 1). Shoot production per explant was relatively high when explants were cultured in the presence of SA and was comparable with controls. Conversely, shoot production was significantly lower in JA-containing media, except for 0.1 mg/L JA + AC. The highest number of shoots was obtained in 1.5 mg/L SA medium followed by the AC-free control and 1.5 mg/L SA + AC media. The tallest shoots were recorded for explants propagated in 1.5 mg/L SA + AC and 5 mg/L SA + AC media. Shoot height followed the same trend as shoot production per explant being stimulated by the presence of low (1.5 mg/L) and medium (5 mg/L) concentrations of SA in combination with AC. Calculation of tissue weight/explant showed that 1.5 mg/L SA + AC and 5 mg/L SA + AC media permitted the formation of significantly-larger amounts of plant material, followed by 1.5 mg/L SA. The lowest quantity resulted when 15 mg/L SA was added to the medium, which was not enough to analyze bioactive compounds of this sample. Measurements corresponding to 15 mg/L SA were consistently placed among the smallest for all parameters.
AC was added to the culture medium. This suggests that supplementation with AC had a positive effect irrespective of JA and SA concentration. The Chl b content of the explants showed a similar tendency of JA and SA media with AC resulted in similar or a slightly lower Chl a content than in controls, except for the 0.5 JA + 1.5 SA combination. The Chl a content was 1.45 ± 0.25 mg/g in the young lavender plants and in almost all in vitro media than in the field-grown mature plants, compared to mature lavender plants cultivated in the field (Figure 1). Chlorophyll content tended to be higher in young in vivo plants and in almost all in vitro media than in the field-grown mature plants, except for the 0.5 JA + AC medium. Among in-vitro-propagated shoots, 0.5 mg/L JA medium resulted in the highest Chl a concentration (1.05 ± 0.9 mg/g), followed by 1.5 mg/L SA + AC (0.74 ± 0.1 mg/g), and 1.5 mg/L SA (0.73 ± 0.1 mg/g). The Chl a content was 1.45 ± 0.3 mg/g in the mature lavender plants collected from field and 0.89 ± 0.2 mg/g in the young lavender plants from pots. The supplementation of JA and SA media with AC resulted in similar or a slightly lower Chl a content than in controls, irrespective of JA and SA concentration. The Chl b content of the explants showed a similar tendency.

### Table 1. Effects of elicitors on shoot multiplication parameters in Lavandula angustifolia Mill.

| Treatment     | Number of Shoots/Explant | Overall Shoot Height (cm) | Main Shoot Height (cm) | Tissue Weight/Explant (mg) | Explant Response Rate (%) |
|---------------|--------------------------|---------------------------|------------------------|---------------------------|---------------------------|
| C             | 4.70 ± 0.95              | 1.35 ± 0.08               | 2.96 ± 0.34            | 127.38 ± 4.40             | 100.00 ± 0.00             |
| C + AC        | 3.97 ± 0.72              | 1.62 ± 0.11               | 2.90 ± 0.26            | 102.45 ± 5.58             | 100.00 ± 0.00             |
| 0.1 JA        | 1.69 ± 0.49              | 1.18 ± 0.40               | 2.31 ± 0.18            | 127.60 ± 10.12            | 71.51 ± 2.03              |
| 0.5 JA        | 2.73 ± 0.33              | 1.57 ± 0.31               | 3.01 ± 0.77            | 154.15 ± 6.19             | 100.00 ± 0.00             |
| 1 JA          | 0.00 ±                   | 0.13 ± 0.09               | 2.94 ± 0.83            | 112.43 ± 3.78             | 100.00 ± 0.00             |
| 0.1 JA + AC   | 4.18 ± 1.33              | 1.30 ± 0.09               | 2.94 ± 0.83            | 112.43 ± 3.78             | 100.00 ± 0.00             |
| 0.5 JA + AC   | 2.38 ± 0.85              | 1.49 ± 0.40               | 2.75 ± 0.27            | 64.63 ± 1.66              | 80.00 ± 2.83              |
| 1 JA + AC     | 1.72 ± 0.78              | 1.46 ± 0.30               | 2.39 ± 0.35            | 63.08 ± 4.16              | 70.00 ± 2.94              |
| 1.5 SA        | 5.76 ± 1.68              | 1.43 ± 0.33               | 2.98 ± 0.71            | 164.90 ± 8.26             | 95.83 ± 4.82              |
| 5 SA          | 3.33 ± 1.05              | 1.18 ± 0.31               | 2.96 ± 0.57            | 85.28 ± 3.41              | 79.02 ± 2.44              |
| 15 SA         | 1.50 ± 0.68              | 0.86 ± 0.05               | 2.48 ± 0.12            | 27.39 ± 1.15              | 30.00 ± 0.00              |
| 1.5 SA + AC   | 4.41 ± 0.74              | 1.95 ± 0.56               | 4.41 ± 0.89            | 223.55 ± 5.39             | 100.00 ± 0.00             |
| 5 SA + AC     | 3.88 ± 0.93              | 1.88 ± 0.05               | 3.41 ± 0.25            | 222.30 ± 6.36             | 93.94 ± 8.57              |
| 15 SA + AC    | 3.29 ± 0.50              | 1.04 ± 0.23               | 2.54 ± 0.13            | 93.00 ± 3.01              | 73.47 ± 8.62              |

Values represent the mean ± standard deviation. In the same column, means not sharing any letter (a to i) are significantly different (p < 0.05). n = 4.

The percentage of shoots producing explants was 100% in elicitor-free media and media with reduced concentrations of JA and SA, particularly if AC was also added. A significantly lower value for explant response was observed for 15 mg/L SA (30.00%), but the frequency increased to 73.47% if AC was added to the culture medium. This suggests that supplementation with AC had a positive effect on shoot multiplication. For the highest elicitor concentrations (1 mg/L JA, 15 mg/L SA) the addition of AC consistently improved growth parameters. No shoot production could be observed in medium supplemented with 1 mg/L JA which was the highest employed JA concentration. Therefore, no assays for bioactive compounds were performed for this treatment. It is also worth mentioning that no roots developed during the growth period.

### 3.2. Effect of Elicitors on the Total Phenolic and Chlorophyll Contents of In Vitro Explants

Elicitors had considerable effect on the secretion of polyphenols in in-vitro-grown lavender explants (Figure 1). TPC tended to be highest in explants grown on the medium supplemented with 0.5 mg/L JA (22.5 ± 1.7 mg/g). This value was significantly lower than TPC obtained in the control medium (13.9 ± 2.5 mg/g) and comparable with the values obtained in SA and SA + AC media and in field-grown mature plants (25.0 ± 2.5 mg/g) or pot-grown young plants (23.0 ± 4.6 mg/g). Explants from other JA and JA + AC media had lower TPC, although differences were not statistically significant. The addition of AC to JA-containing media did not induce any notable increase in TPC as compared with the AC-free media. Conversely, all three SA + AC media induced higher TPC (17.2 ± 2.0 – 20.0 ± 2.5 mg/g) than the control (13.9 ± 2.5 mg/g).

The total chlorophyll content was enhanced by the addition of elicitors to the culture media as compared to mature lavender plants cultivated in the field (Figure 1). Chlorophyll content tended to be higher in young in vivo plants and in almost all in vitro media than in the field-grown mature plants, except for the 0.5 JA + AC medium. Among in-vitro-propagated shoots, 0.5 mg/L JA medium resulted in the highest Chl a concentration (1.05 ± 0.9 mg/g), followed by 1.5 mg/L SA + AC (0.74 ± 0.1 mg/g), and 1.5 mg/L SA (0.73 ± 0.1 mg/g). The Chl a content was 1.45 ± 0.3 mg/g in the mature lavender plants collected from field and 0.89 ± 0.2 mg/g in the young lavender plants from pots. The supplementation of JA and SA media with AC resulted in similar or a slightly lower Chl a content than in controls, irrespective of JA and SA concentration. The Chl b content of the explants showed a similar tendency.
when compared to the Chl a profile, except for shoots that developed on 0.5 mg/L JA + AC medium. In this latter case, Chl b value (1.30 ± 0.5 mg/g) was significantly above the values detected in any other sample, except for the field-grown lavender (0.91 ± 0.1 mg/g). Explants grown in JA- and SA-supplemented media tended to produce higher concentrations of Chl b than the AC-free control. When AC was added to the media, results were similar to those obtained in both controls, except for 1.5 SA + AC and 0.5 JA + AC, as mentioned above.

![Graph showing total phenolic, total chlorophyll, and chlorophyll a and b contents of the in-vitro-cultured Lavandula angustifolia Mill. plantlets.](image)

**Figure 1.** Total phenolic, total chlorophyll, and chlorophyll a and b contents of the in-vitro-cultured Lavandula angustifolia Mill. plantlets. FGL, field-grown mature lavender plants; PGL, young lavender plants cultivated in pots; C, control; AC, activated charcoal; JA, jasmonic acid; and SA, salicylic acid. Error bars represent standard deviations. Different letters denote significant differences (p < 0.05). 

3.3. Effect of Elicitors on the Carotenoid Profile of In Vitro Plantlets

The highest total carotenoid concentration was detected in the mature lavender plants collected from the field (86.03 ± 9.97 µg/g) (Figure 2). Among in vitro samples, the greatest carotenoid concentrations were obtained in shoots grown in 0.5 mg/L JA (64.19 ± 7.02 µg/g) and 1.5 mg/L SA + AC media (55.00 ± 5.56 µg/g). The carotenoid concentration was similar to that measured in the plants grown in pots (68.81 ± 7.56 µg/g). Explants cultivated on other JA or JA + AC media had carotenoid content close to the controls and slightly lower than those propagated in SA and SA + AC media.

The concentrations of lutein and β-carotene were much higher than the concentrations of other carotenoids, in all samples. Comparatively-high neoxanthin and violaxanthin concentrations were detected in explants propagated on 5 mg/L SA and 1.5 mg/L SA + AC, respectively.

Among in vitro plant tissue, both lutein and β-carotene concentrations were highest in explants developed in the 0.5 mg/L JA and 1.5 mg/L SA + AC media (Figure 2). These concentrations were lower than in the in-vivo-grown mature plants, almost the same as in the in-vivo-grown young plants, and much higher than in the shoots propagated on controls or other JA-supplemented media. Although cis-β-carotene content was much lower than β-carotene content in all samples, its variation
followed the same trend. The highest value was obtained in the 0.5 mg/L JA and in media supplemented with SA or SA + AC, while the lowest were obtained in other JA-containing media (with or without AC).

Figure 2. Carotenoid composition of *Lavandula angustifolia* Mill. explants grown in elicitor-enriched culture media. FGL, field-grown mature lavender plants; PGL, young lavender plants cultivated in pots; C, control; AC, activated charcoal; JA, jasmonic acid; and SA, salicylic acid. Error bars depict standard deviation. Different letters denote significant differences (*p* < 0.05). *n* = 3.

Highest neoxanthin concentration was found in shoots grown on the 5 mg/L SA medium (6.17 ± 0.95 µg/g), significantly higher than in any other plant material, including mature plants collected from the lavender field (2.22 ± 0.98) or young plants cultivated in pots (1.29 ± 0.25). Neoxanthin content of shoots propagated in JA and JA + AC media tended to be higher than that of shoots developed in the AC-free control. The neoxanthin concentrations of the 5 mg/L SA + AC and 15 mg/L SA + AC samples were much lower than in any other treatments, including controls.

Violaxanthin concentration was greatest in the young in vivo plants from pots (7.20 ± 0.98 µg/g) and in vitro sample from 0.5 mg/L JA (7.00 ± 0.67 µg/g) and 1.5 mg/L SA + AC (6.08 ± 0.44 µg/g) media, while the concentration in mature plants cultivated on the field was 1.11 ± 0.45 µg/g. All other in vitro samples had violaxanthin content comparable with the controls.

4. Discussion

It is well-documented in the literature that the composition of the culture medium highly influences the micropropagation of lavender and determines the development, morphological parameters, and phytochemical composition of the plant tissue [3,4,45]. This study evaluated the effect of different concentrations of JA and SA, alone or in combination with AC, on the proliferation of *L. angustifolia* explants, their morphological parameters and phytochemical composition in terms of phenolic, chlorophyll, and carotenoid content.

In-vitro-cultured explants produced more shoots in the presence of small concentrations of SA or in the absence of elicitors, while shoots grew slightly taller if AC was added to the 1.5 mg/L
SA or 5 mg/L SA media. As a level of SA above 1 mM is considered high and likely to negatively regulate plant development and growth [49], the markedly negative influence of 15 mg/L SA on the multiplication rate, shoot formation, and quantity of plant tissue in our study could not be unexpected. This was also supported by in vitro experiments on *Thymus membranaceus* Boiss. explants which had drastically-decreased viability after growing in the presence of 1000 ÂµM SA. At the same time, 10 ÂµM and 100 ÂµM SA had no significant effect on plant survival and growth [50]. Nevertheless, the in vitro application of optimal concentrations of SA to several species—*Matricaria chamomilla* L. [51], *Oryza sativa* L. [52], and *Zea mays* L. [53]—showed beneficial effects on plant growth particularly under abiotic stress conditions such as halostress. For *Hibiscus acutosella* Welw. and *Hibiscus moscheutos* L. the addition of 0.5 or 1 mM SA to the in vitro culture medium improved shoot growth and multiplication both in the presence and the absence of NaCl [54]. SA has enhanced callus growth in *Carthamus tinctorius* [42] and biomass accumulation in *Bacopa monnieri* [55].

In several cases, SA allowed for better development than JA and the production of a larger amount of tissue. The highest concentrations of JA (1 mg/L) killed all explants. These effects followed the same trend but were more radical than those obtained by other researchers who used JA in *L. angustifolia* culture medium [45]. In their experiment, high JA concentrations (1 and 1.5 mg/L) induced growth inhibition and decreased plant height and weight but did not cause explant death. In the same investigation, plant height was similar to what we observed but explants regenerated a smaller number of shoots. The antioxidant activity of plants propagated on JA media (regardless of concentration) was higher than that of the control plants, suggesting that the addition of JA to culture medium may contribute to the production of plant tissues with higher antioxidant activity [45].

In a trend similar to the one we observed, elicitation with high JA concentrations (50, 100, and 200 ÂµM) suppressed growth of *Mentha x piperita* L. cell suspension cultures [56]. JA has been used not only for elicitation but also propagation in various other species with contrasting results. High JA (0.5, 1, and 2 mg/L) concentrations inhibited the formation and growth of shoots and roots in Pyrdwarf pear rootstock and Gisela 6 cherry rootstock while a low concentration (0.2 mg/L) encouraged these processes [57]. At the same time, medium with JA (0.5, 1, and 2.0 ÂµM) stimulated the development of potato plants [58]. In vivo elicitation of *Ocimum basilicum* L. had no negative effect on growth and yield parameters [59].

In several studies, the proliferation of *L. angustifolia* explants was successfully performed in MS medium supplemented with other PGRs such as benzyladenine (BA) [60,61], 6-benzyaminopurine (BAP), thidiazuron (TDZ), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, gibberellic acid (GA3) [62–67], or zeatin [68]. In most studies, the large increase of the PGR concentration had a negative effect on shoot multiplication. While we observed a positive effect of AC on the growth parameters of plants, in a previous study, the addition of AC to the culture media (0.2 g/L) slightly inhibited the growth of the shoots [62]. Conversely, in another study, the use of 1 g/L AC in Lloyd and McCown medium (WPM) significantly enhanced the growth of shoots, while AC concentration above 2.5 g/L inhibited their proliferation [69]. All these data demonstrated the importance of the AC use at optimal concentration. The efficiency of PGRs to induce shoot multiplication was also demonstrated for other lavender species—*L. dentata* L. [70–72], *L. vera* DC. [73,74], *L. latifolia* Medik. [75], *L. pedunculata* Mill. [76], *L. multifida* L. [77], and *L. viridis* L’Hér. [78,79].

In our study, most explants grown in the presence of elicitors tended to have higher TPC and Chl a and b contents than explants developed in elicitor-free control medium (Figure 1), demonstrating the beneficial impact of elicitors on the secretion of secondary metabolites. This effect has previously been confirmed by several studies [71,72,78,80]. Essential oil concentration of in-vitro-propagated lavenders resembled the content in field-grown plants [76,81]. In one study, monoterpens had even higher concentrations in the in vitro *L. viridis* plants than in field-cropped plants [82]. The essential oil profile, however, differed between in-vitro-propagated and field-grown *L. angustifolia* plants [61]. Plants developed in culture media supplemented with silver or gold nanoparticles contained several
new compounds as compared to the control. In both media, lower molecular weight compounds decreased and higher molecular weight compounds increased [83].

Increasing the concentration of JA led to the accumulation of phenolic compounds. While 0.1 mg/L JA did not have any effect on the polyphenolic content and was comparable with the control media, 0.5 mg/L JA induced the highest TPC (22.5 mg/g), similar to the concentrations measured in the in vivo plants (25.0 mg/g (field-grown plants), 23.0 mg/g (pot-grown plants)). The same effect was reported by Andrýs et al. In that study, the addition of JA to the culture medium increased the polyphenol content in plant material compared to the control; the highest concentration being obtained in plants propagated on 1.5 mg/L JA medium (43.9 mg/g) [45]. All shoots propagated in the presence of JA (0.2, 0.5, 1, and 1.5 mg/L) had higher polyphenol content than the control [45]. In another report, *L. angustifolia* plants had polyphenol content of 12.4–18.2 mg/g [84]. The majority of explants grown under in vitro conditions had similar TPC and total chlorophyll content, comparable with controls, indicating the poor effect of elicitors on the accumulation of these secondary metabolites. However, significant differences were observed in the pattern of Chl a and Chl b content. Increasing concentration of JA slightly increased the Chl a and b content, while the opposite trend was observed for the SA-supplemented media.

The total carotenoid content of all in vitro samples was below the carotenoid content of field-grown mature plants, but higher than in the elicitor-free control. Overall, explants grown on SA and SA + AC media tended to express higher amount of carotenoids than those developed on JA and JA + AC media, though the highest values were typically obtained in JA-supplemented medium. The culture media supplemented with 0.5 mg/L JA provided the best results for almost all individual carotenoids. Lutein and β-carotene were the dominating carotenoid in all samples.

While the AC-supplemented control medium that did not contain any other PGRs and gave better results than the AC-free control in most analyses, the addition of AC to in vitro media supplemented with JA tended to inhibit the accumulation of secondary metabolites, probably due to the partial adsorption of elicitors [85]. This behavior was less remarkable in SA-supplemented media.

The lower content in secondary metabolites for in-vitro-propagated shoots might have been due to the different growth conditions and stages of plant development, which are connected to juvenility of plant tissue in in vitro conditions [86]. Further research is needed to improve in vitro shoot multiplication, especially the synthesis of bioactive compounds in *L. angustifolia*. This could prove timely as the widespread lavender fields that have been established in the Mediterranean basin are facing serious problems because of *Rhizoctonia* and *Fusarium* fungal infections [87,88]. In vitro tissue culture could be a source of healthy, pathogen-free plants that would continue producing secondary metabolites, and also be a way of maintaining biodiversity among lavender species and cultivars.

5. Conclusions

In the present study, elicitation of in-vitro-grown *L. angustifolia* explants with JA (0.5 mg/L) and SA (1.5 mg/L, 5 mg/L) enhanced growth. In the case of SA this could be further increased by adding AC to the culture medium. Increasing concentrations to 1 mg/L for JA and 15 mg/L for SA was detrimental to shoot development but in the case of SA this effect could be mitigated by the presence of AC. Concentrations of total phenolics, chlorophylls, and carotenoids were higher in explants grown in elicitor-supplemented media as compared with controls. Elicitors were effective at low concentrations, with high polyphenol and carotenoid content being obtained for explants propagated in culture media supplemented with 0.5 mg/L JA and 1.5 mg/L SA + AC. The most potent elicitor was JA at 0.5 mg/L. The presence of AC in the media had a beneficial effect on plant growth, particularly in combination with SA, but in many cases, it reduced or did not affect the phytochemical composition of explants.

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