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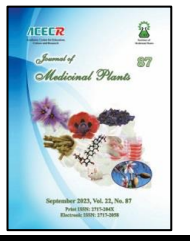

Research Article

Cell suspension culture of Lavender (*Lavandula angustifolia*) and the influence of Methyl Jasmonate and yeast extract on Rosmarinic Acid production

Mahboobeh Yazdi[1], Abdolreza Bagheri[1,*], Nasrin Moshtaghi[1], Fatemeh Keykha akhar[2], Azadeh Khadem[3]

[1] *Biotechnology and Plant Breeding Department, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*

[2] *Plant Biotechnology Department, Faculty of Agriculture, Jahrom University, Jahrom, Iran*

[3] *Horticultural Plants Biotechnology Department, Research Institute of Industrial Biotechnology, Academic Centre for Education, Culture and Research (ACECR)-Khorasan Razavi Branch, Mashhad, Iran*

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ABSTRACT

Background: Cell suspension culture and elicitation have been playing an important role in the synthesis of active secondary metabolites. Rosmarinic acid (RA) is one of the active compounds found in lavender essential oil that stands out due to its antioxidant and anti-inflammatory characteristics. **Objective**: This research was conducted to investigate the production efficiency of RA in *Lavandula angustifolia* suspension culture through elicitation with methyl jasmonate (MeJA) and yeast extract (YE). **Methods**: Cell suspension culture established in B5 liquid media supplemented with different combinations of Plant Growth Regulators (PGRs). The influence of different PGRs treatments on cell growth and accumulation of RA were analyzed. Then the effect of concentration and time course of elicitation with YE (0.1, 0.5, and 1 g/l after 1, 3 and, 6 days of elicitation) and MeJA (50, 100 and 200 µM after 1, 2 and 3 days of elicitation) separately and in combination with each other on cell growth and intracellular and extracellular content of RA were investigated. **Results**: HPLC analysis showed that the highest intracellular RA content (17.03 mg/g dry weight) was observed 24 hours after the addition of 50 µM MeJA in combination with 1 g/l YE, which was approximately 33% higher than that found in leaves. Furthermore, it was 9 and 11 times higher than cultures treated with MeJA and YE alone, respectively. In addition, both elicitors significantly affected the extracellular quantity of RA than control. **Conclusion**: Our results documented that the application of elicitors increased biomass and RA accumulation in *L. angustifolia* suspension cells.

Abbreviations: RA, Rosmarinic acid; MeJA, Methyl jasmonate; YE, Yeast extract; MS, Murashige and Skoog; BA, Benzyl adenine; PGR, plant growth regulator; DW, Dry weight; HPLC, High-performance liquid chromatography; LSD, Least Significant Difference

*Corresponding author: abagheri@um.ac.ir

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1. Introduction

Medicinal plants are renowned for their rich composition of secondary metabolites, also known as active substances [1]. These plants are important for discovering novel drugs to address various medical issues. Furthermore, they have gained significant attention in diverse industries, including perfumery, pharmaceuticals, and natural cosmetics [2]. Many of these products such as rosmarinic acid (RA) incorporate antioxidant compounds due to their beneficial properties [3].

Lavandula angustifolia Mill is widely recognized for its utilization in the extraction of RA. RA, an ester of caffeic acid and 3,4-dihydroxyphenyllacetic acid, was initially isolated from *Rosmarinus officinalis* L. This compound is commonly found in various genera of the Lamiaceae and Boraginaceae families. Within medicinal plants, RA serves as a defense compound [4]. Lavender has been extensively studied for its antiviral, antibacterial, antiallergic, and antioxidant activities [5]. These biological properties are primarily attributed to phenolic compounds, particularly RA [6]. Therefore, increasing the content of RA in lavender tissues through various methods is a subject of interest. In this regard, plant tissue culture has emerged as an effective approach for the production of pharmaceuticals and bioactive plant metabolites [7]. Plant cell suspension culture systems are particularly valuable for large-scale production of high-value secondary metabolites [1]. Numerous strategies have been developed to enhance plant metabolites in these systems, and elicitation has proven to be an effective approach for increasing metabolite accumulation [8].

Methyl jasmonate, a growth regulator in plants, plays a significant role in inducing various biological effects, including leaf abscission, senescence, stomatal closure, and growth inhibition [9, 10]. It has been successfully employed to stimulate RA production in suspension cultures of *Coleus blumei* [11], *Lavandula vera* [12], *Salvia miltiorrhiza* [13], *Mentha piperita* [14], *Satureja khuzistanica* [15], and *Salvia nemorosa* [16]. Yeast extract has also been extensively studied as a biotic elicitor for secondary metabolite production and cell growth in various plant species, including *Orthosiphon aristatus* [17], *Silybum marianum* [18], *Salvia miltiorrhiza* [13], *Ocimum sanctum* [19], *Agastache rugosa* [20], and *Thymus lotocephalus* [21]. These studies demonstrate that yeast extract is among the most efficient elicitors to increase the production of diverse secondary metabolites, including RA, in different plant species.

This study aims to investigate the impact of combining methyl jasmonate and yeast extract on the induction of RA accumulation in *L. angustifolia* suspension culture, relative to the RA content found in the leaves. Additionally, the study aimed to assess the influence of these elicitors on cell growth and the secretion of RA into the culture media.

2. Materials and methods

2.1. Callus induction

Young leaves of *L. angustifolia* were initially washed with tap water and then surface sterilized using 70 % ethanol for 1 minute. Subsequently, they were soaked in 1 % sodium hypochlorite for 20 minutes and thoroughly rinsed with sterile distilled water. They were then cut into 1 cm segments and placed on both solid B5 medium [22] and MS medium [23]. The media were supplemented with 30 g/l sucrose, 7 g/l agar, 2 mg/l 2,4-D, and 2 mg/l BA, with a pH of 5.75. The cultures were transferred to the growth room at a temperature

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of 25 ± 1 °C and exposed to different light conditions, including a 16/8-hour photoperiod and darkness. The explants were subcultured every 3 weeks onto the same medium under the same light condition.

2.2. Cell suspension culture

To establish the cell suspension culture of *L. angustifolia*, 1.5 g of fresh weight from 7-week-old callus was added to 50 ml of liquid medium. The B5 medium was supplemented with 30 g/l sucrose and subjected to six different plant growth regulators (PGRs) treatments, utilizing two types of PGRs (2,4-D and BA). The PGR treatments were as follows: 1) 0.2 mg/l 2,4-D + 0.2 mg/l BA; 2) 1 mg/l 2,4-D + 0.2 mg/l BA; 3) 1 mg/l 2,4-D + 1 mg/l BA; 4) 2 mg/l 2,4-D + 0.2 mg/l BA; 5) 2 mg/l 2,4-D + 1 mg/l BA; 6) 2 mg/l 2,4-D + 2 mg/l BA.

The cell suspension cultures were maintained in 100 ml Erlenmeyer flasks placed on an orbital shaker operating at a speed of 110-120 RPM and incubated at room temperature under a 16/8-hour photoperiod.

2.3. Growth curve evaluation

The calli obtained from the six hormonal treatments were weighed and distributed into 24 individual flasks, with each treatment having four replicates. Each flask contained 50 ml of B5 medium supplemented with 30 g/l sucrose and the corresponding hormonal combinations. The flasks were then incubated at room temperature under a 16/8-hour photoperiod.

The initial evaluation of callus weight was conducted on the 10th day after inoculation, and subsequent evaluations were performed every 10 days thereafter, up to 50 days of culture [28].

2.4. Elicitor treatment

Methyl jasmonate (MeJA) and yeast extract (YE) were utilized as elicitors in this study. To prepare stock solutions, MeJA was dissolved in 96 % ethanol, while YE was dissolved in distilled water. The elicitors were applied to the cultures either individually or in combination with each other, at various concentrations and different durations of exposure as follows: Methyl jasmonate (MeJA) and yeast extract (YE), were applied to the individual flasks on the 20th day of culture, based on the growth curve of the cell suspensions. Final concentrations of 50, 100, and 200 µM MeJA [46] and 0.1, 0.5, and 1 g/l YE were used.

To determine the optimal combination of both elicitors, the treatment that exhibited the highest biomass growth and rosmarinic acid (RA) accumulation was selected and applied to the cell suspension cultures of lavender. All experiments were conducted based on a completely random block design in three replicates. The suspension cultures were incubated on a rotary shaker under the same conditions as described earlier.

Samples were harvested at 24, 48, and 72 hours after the addition of MeJA. For YE treatments, samples were collected at 24, 72, and 144 hours following elicitation.

2.5. Determination of dry weight

Cell growth was assessed by measuring the dry biomass of the suspension cultures. The cells in suspension cultures were filtered using Whatman no. 1 filter paper [50]. They were then dried at room temperature until a constant weight was achieved. The dry weight of the cells was then calculated and expressed as DW (dry weight).

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2.6. Extraction and purification of RA

The RA within the cells was extracted from 500 mg of powdered suspension biomass using 5 ml of 99 % methanol. The samples were then sonicated at 45 kHz and 70 % intensity for 7 minutes, followed by incubation at room temperature in the dark for 6 hours. Afterward, the mixture was centrifuged at 4000 rpm for 10 minutes and the supernatant was removed. The resulting extracts were collected and dried at room temperature for 24 hours. All samples were stored at 4 °C before analysis [47].

The standard of RA (98 % purity) purchased from Sigma-Aldrich (Germany), was used for reference. The stock standard solution was accurately prepared by dissolving 2 mg of RA in 2 ml of methanol with the aid of ultrasound radiation. Serial dilutions of RA were prepared at concentrations from 10 to 2000 mg/l, and their absorbance was measured at 330 nm against methanol as the reagent blank. The standard calibration curve of RA was constructed by plotting absorbance versus concentration. The concentration of extracellular (in the culture medium) and intracellular RA were calculated using the following equation derived from the standard RA calibration curve.

RA = Absorbance – 0.1153/0.0137 (1)

2.7. HPLC analysis

The RA content was quantified in suspension cells treated with both elicitors and leaf tissue from the parent plant using an Agilent 1260 series high-performance liquid chromatography (HPLC) system (USA). The extract solution was subjected to separation and analysis using a 250 × 4.6 mm Zorbax Rx C18 (5 µl) column [48]. The absorbance at 330 nm was then measured. The amount of RA was measured according to the calibration curve of the RA authentic standard.

2.8. Statistical analysis

Statistical analysis of all data was conducted in a completely randomized design with three replicates using JMP version 8 statistical software. To determine significant differences between treatment means, LSD (Least Significant Difference) test was employed at a significance level of 5 %.

3. Results

3.1. Effects of various media and photoperiod conditions on callus culture

The application of different media and photoperiod conditions had a significant impact on the percentage of callogenesis, the rate of callus production, and the fresh and dry weight of the callus. The highest percentage of callogenesis, rate of callus production, the fresh weight, and the dry weight of the callus were observed in explants cultured on B5 basal medium under normal photoperiod conditions (16 hours light and 8 hours dark). In contrast, callus was formed in only 50 % of explants cultured on MS basal medium under dark conditions (Table 1).

Furthermore, the color and tissue characteristics of the callus varied among the explants. The B5 medium resulted in the most friable callus, while the MS medium led to the formation of a compact callus. The color of the callus was also influenced by light conditions. Explants in the dark produced creamy-colored calli, whereas brown calli were produced under light conditions (Fig. 1).

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Table 1. Effects of different media and photoperiod conditions on callus induction of *L. angustifolia*

Medium	photoperiod condition	Callogenesis percentage (%)	Rate of callogenesis	D.W (mg)	F.W (mg)
B5	Light	100 ± 0[a]	101.8 ± 1.14[a]	300.8 ± 3.63[a]	6000 ± 163.29[a]
	Dark	85 ± 4.08[b]	86.6 ± 4.49[b]	246.9 ± 4.4[b]	3200 ± 219.69[b]
MS	Light	70 ± 8.16[c]	33.6 ± 1.38[c]	151.9 ± 3.26[c]	3823.3 ± 192.58[c]
	Dark	50 ± 4.08[d]	30.6 ± 5.68[c]	144.3 ± 3.83[c]	292 ± 12.19[d]

* Values are mean ± standard deviation. Numbers with the same letters do not exhibit significant difference based on LSD test. ($P \leq 0.05$)
D.W=Dry Weight, F.W= Fresh Weight

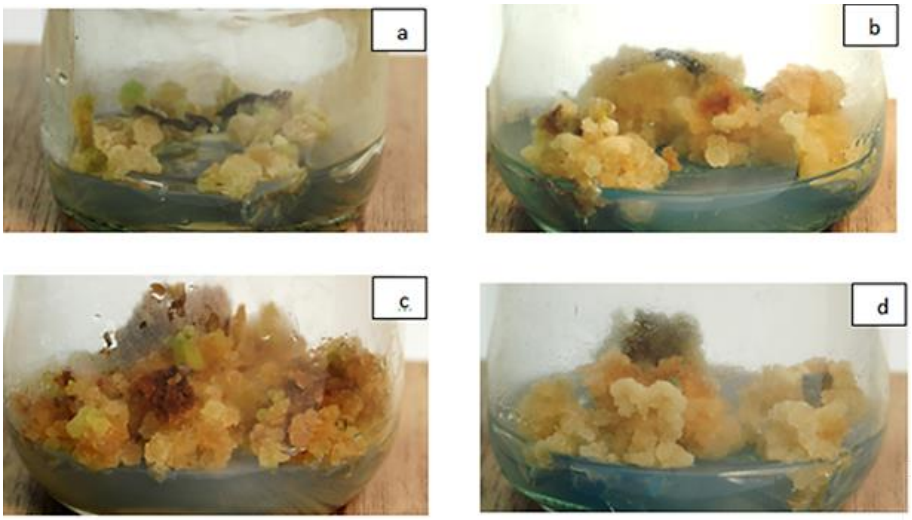

Fig. 1. Comparison of callus color and tissue in a) MS medium, b) B5 medium, c) light condition in B5 medium, d) dark condition in B5 medium. The color change in the culture medium is attributed to the blue pigment production.

3.2. Suspension culture of L. angustifolia and the effects of various hormonal treatments on rosmarinic acid production

The calli induced in B5 solid media were transferred to liquid B5 medium supplemented with different hormonal treatments under light conditions. In most treatments, except 2 mg/l 2,4-D + 2 mg/l BA, cell growth followed a logarithmic curve for up to 20 days. However, in the case of 2 mg/l 2,4-D + 2 mg/l BA, this trend continued for 30 days of cultivation. Afterward, the growth rate started to decline (Fig. 2).

Our findings indicate that the medium containing 0.2 mg/l 2,4-D + 0.2 mg/l BA or 1 mg/l 2,4-D + 1 mg/l BA resulted in the increased biosynthesis of rosmarinic acid (RA) (Table 2).

3.3. Effect of elicitors on the growth of L. angustifolia suspension culture

Both elicitors had a significant impact on cell growth and biomass accumulation in the samples ($P \leq 0.05$). Specifically, the application of 200 µM of MeJA resulted in the maximum biomass accumulation (Fig. 3).

Similar to MeJA, the addition of yeast extract (YE) also promoted the growth of *L. angustifolia* cell suspension (Fig. 3). A higher concentration of YE (1 $g.l^{-1}$) demonstrated greater effectiveness in biomass growth. Interestingly, the duration of exposure to YE had minimal impact on cell growth.

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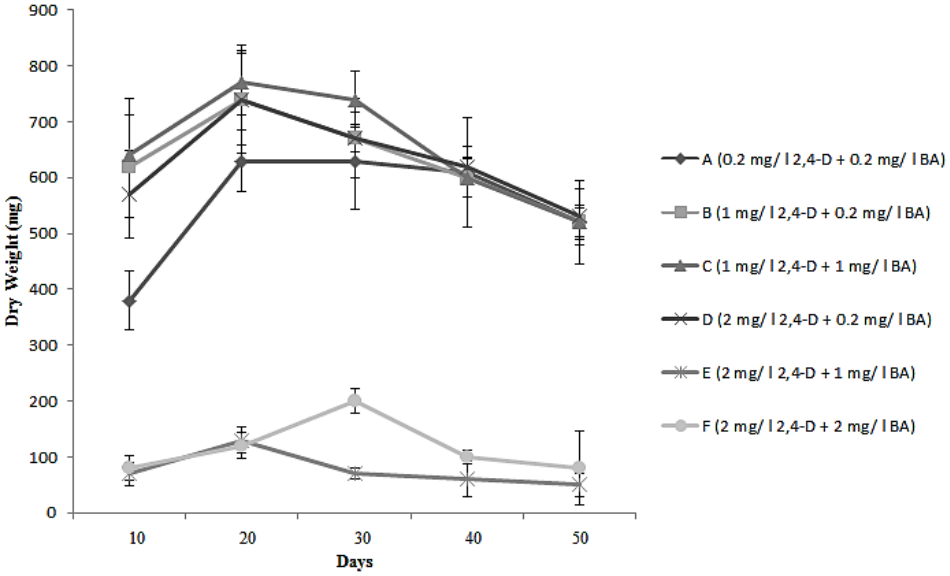
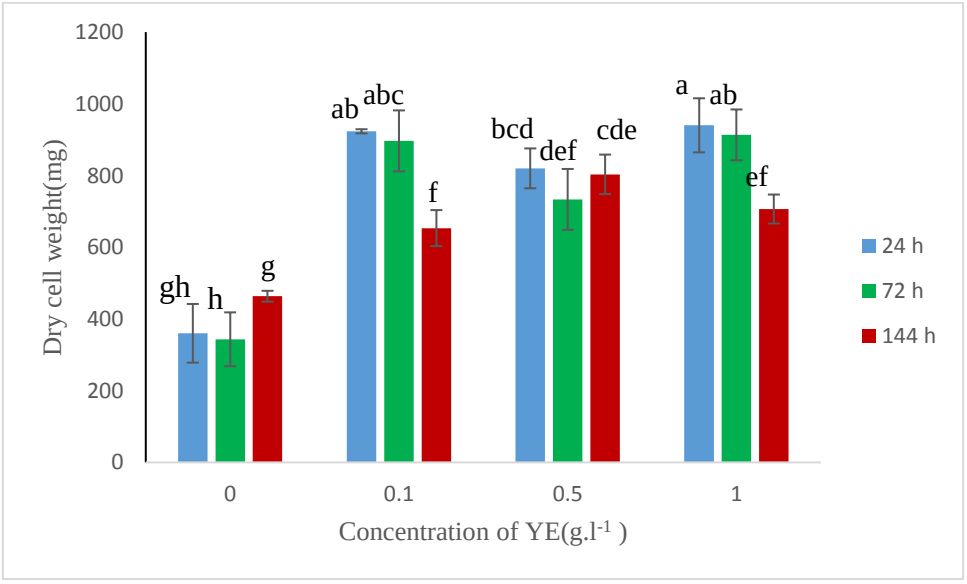


Fig. 2. Growth curve of lavender (*L. angustifolia*) suspension culture on B5 medium with various hormonal treatments. Values are mean ± standard deviation.

Table 2. Effect of various hormone concentrations on RA production in suspension culture of *L. angustifolia*

Hormonal treatments	RA concentration (mg/g DW)
0.2 mg/l 2,4-D + 0.2 mg/l BA	0.123 ± 0.034[a]
1 mg/l 2,4-D + 0.2 mg/l BA	0.043 ± 0.007[b]
1 mg/l 2,4-D + 1 mg/l BA	0.124 ± 0.052[a]
2 mg/l 2,4-D + 0.2 mg/l BA	0.066 ± 0.07[ab]
2 mg/l 2,4-D + 1 mg/l BA	0.059 ± 0.008[ab]
2 mg/l 2,4-D + 2 mg/l BA	0.057 ± 0.031[ab]

* Values are mean ± standard deviation. Numbers with the same letters do not exhibit significant difference based on LSD test. ($P \leq 0.05$)



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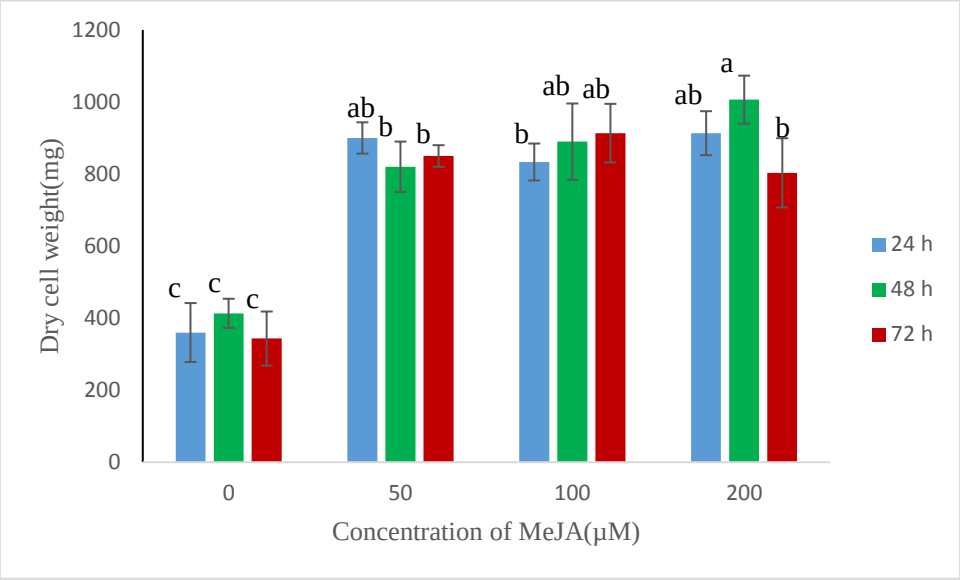

Fig. 3. Effect of MeJA and YE treatments on the Growth of *L. angustifolia* Suspension Cultures. Values are mean ± standard deviation. Numbers with the same letters do not exhibit significant difference based on LSD test. ($P \leq 0.05$).

3.4. Effect of elicitors on the accumulation of intracellular and extracellular rosmarinic acid

Both elicitors influenced the biosynthesis and secretion of RA. When treating with MeJA, the most effective concentration for severely impacting intracellular RA accumulation was found to be 50 µM within 24 hours (Table 3). However, as the concentration of MeJA and the duration of exposure increased, the accumulation of intracellular RA steadily decreased. Additionally, rosmarinic acid was released into the culture medium. Compared to the control cells, MeJA treatment increased in extracellular RA accumulation. The highest concentration of RA in the culture medium was observed 72 hours after introducing 50 µM MeJA to the cell cultures (Table 3).

Table 3. Effect of MeJA concentrations and exposure time on intracellular and extracellular rosmarinic acid in *L. angustifolia* suspension cultures.

Concentrations of MeJA (µM)	Time of MeJA exposure (hour)	Intracellular RA concentration (mg/g DW)	Extracellular RA concentration (mg/l medium)
0	24	0.32 ± 0.075^{cde}	48.7 ± 19.2^{f}
	48	0.26 ± 0.058^{de}	63.2 ± 23.6^{f}
	72	0.24 ± 0.13^{ef}	29.5 ± 7.04^{f}
50	24	0.55 ± 0.057^{a}	322.6 ± 82.1^{b}
	48	0.42 ± 0.055^{abc}	185.9 ± 33.2^{cd}
	72	0.41 ± 0.019^{bc}	391.8 ± 46.9^{a}
100	24	0.32 ± 0.17^{cde}	91 ± 15.7^{ef}
	48	0.46 ± 0.074^{ab}	165.16 ± 26.6^{cd}
	72	0.42 ± 0.04^{abc}	181 ± 21.9^{cd}
200	24	0.39 ± 0.028^{bcd}	220.8 ± 57.4^{c}
	48	0.37 ± 0.027^{bcd}	135.4 ± 20.9^{de}
	72	0.13 ± 0.044^{f}	141.7 ± 32.2^{de}

* Values are mean ± standard deviation. Numbers with the same letters do not exhibit significant difference based on LSD test. ($P \leq 0.05$)

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The addition of YE had a significant impact on RA production, as shown in Table 4. The highest concentration of YE (1 $g.l^{-1}$) significantly increased RA production after 72 hours.

RA secreted into the culture medium following elicitation with YE was confirmed. The strongest effect on RA secretion was observed 72 hours after adding 1 g/l YE, and it was significantly higher than that of control cells (Table 4).

Table 4. Effect of YE concentrations and exposure duration on intracellular and extracellular rosmarinic acid content in *L. angustifolia* suspension cultures.

Concentrations of YE ($g\ l^{-1}$)	Duration time of YE exposure (hour)	Intracellular RA concentration ($mg/g\ DW^{-1}$)	Extracellular RA concentration (mg/l medium)
0	24	0.26 ± 0.11^{bc}	32.2 ± 10.9^{d}
	72	0.27 ± 0.03^{bc}	40.3 ± 24.3^{d}
	144	0.24 ± 0.03^{bc}	29.5 ± 7.04^{d}
0.1	24	0.25 ± 0.06^{bc}	59.7 ± 10.6^{d}
	72	0.2 ± 0.05^{c}	156.9 ± 28.2^{b}
	144	0.35 ± 0.08^{b}	117.7 ± 20.4^{c}
0.5	24	0.24 ± 0.08^{bc}	59.5 ± 20.8^{d}
	72	0.26 ± 0.08^{bc}	192.6 ± 24.7^{b}
	144	0.28 ± 0.07^{bc}	46.6 ± 30.4^{d}
1	24	0.28 ± 0.08^{bc}	103.9 ± 13.4^{c}
	72	0.76 ± 0.16^{a}	245.3 ± 38.3^{a}
	144	0.36 ± 0.14^{b}	38.1 ± 6.6^{d}

* Values are mean ± standard deviation. Numbers with the same letters do not exhibit significant difference based on LSD test. ($P \leq 0.05$)

3.5. HPLC profile of RA in L. angustifolia suspension culture after MeJA and YE treatment

The presence of rosmarinic acid (RA) in the tested samples was successfully confirmed using HPLC chromatograms, which were compared to its pure standard. Based on the HPLC analysis, the retention time of RA was determined to be 7 minutes (Fig. 4).

In terms of single elicitor treatments (MeJA and YE), there was no significant effect on RA accumulation in *L. angustifolia* suspension culture compared to the leaf tissue from mature plant (Fig. 5). However, when a combination of 50 µM MeJA and 1 g/l YE was applied to the cells for 24 hours, a significant increase in RA content was observed. Specifically, the RA concentration in the combined treatment was 13 times higher than in the leaf tissue and 9 times higher than in cultures treated with MeJA or YE alone (Fig. 5). However, after 72 hours of exposure to the combined elicitors, the RA concentration decreased considerably.

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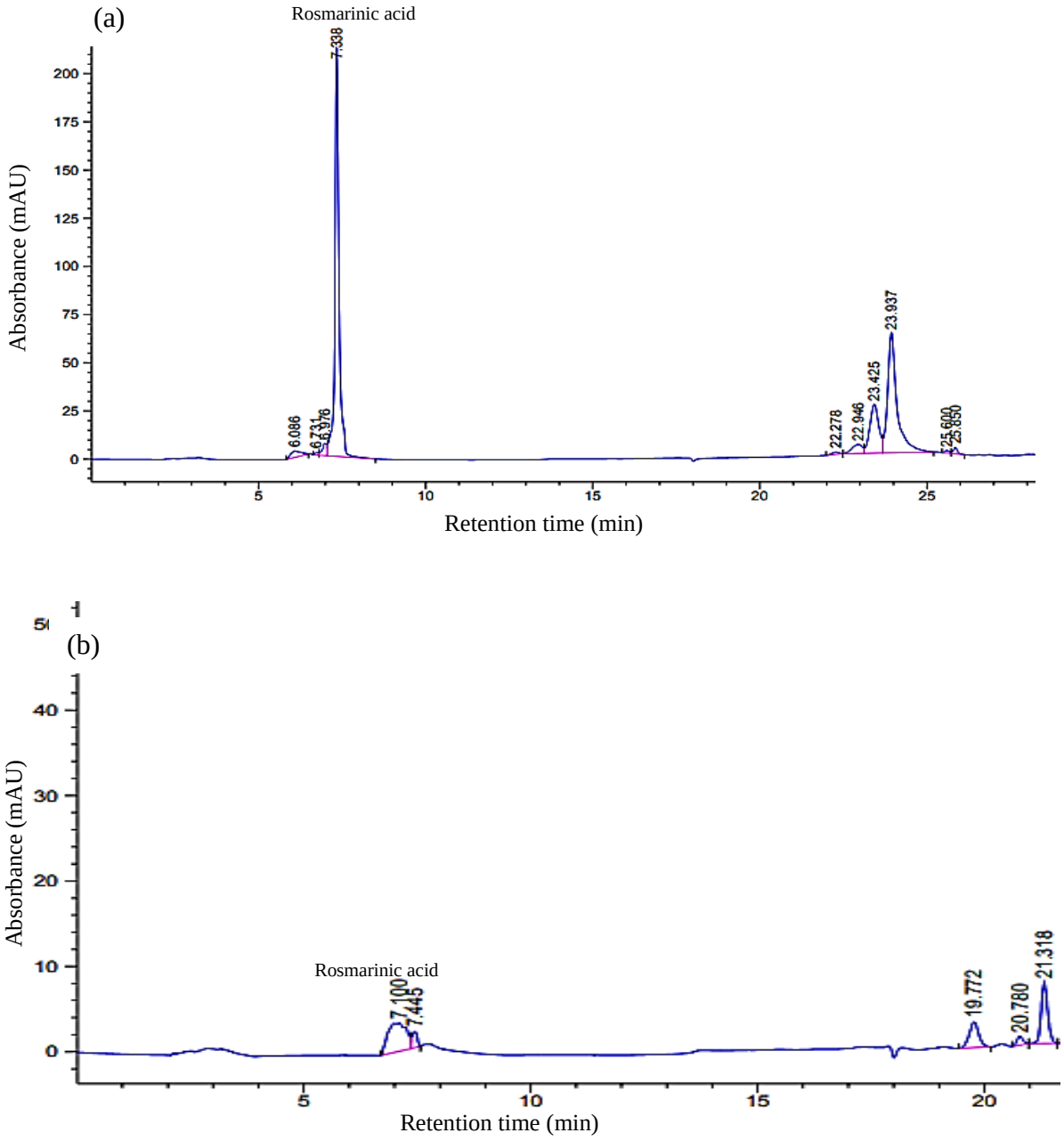

Fig. 4. HPLC Profiles of *L. angustifolia* Suspension Culture Extracts of MeJA+YE-Treated Cultures, (a) for 24 hours, (b) for 72 hours,

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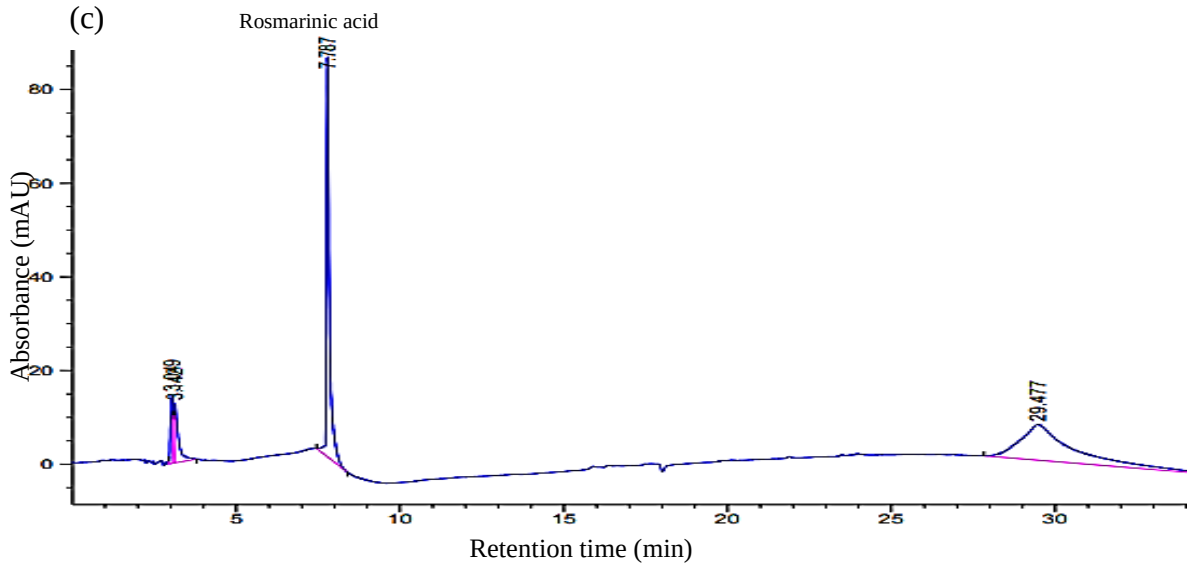

Fig. 4. HPLC Profiles of *L. angustifolia* Suspension Culture Extracts of MeJA+YE-Treated Cultures, (c) Leaf Tissue from Parent Plants.

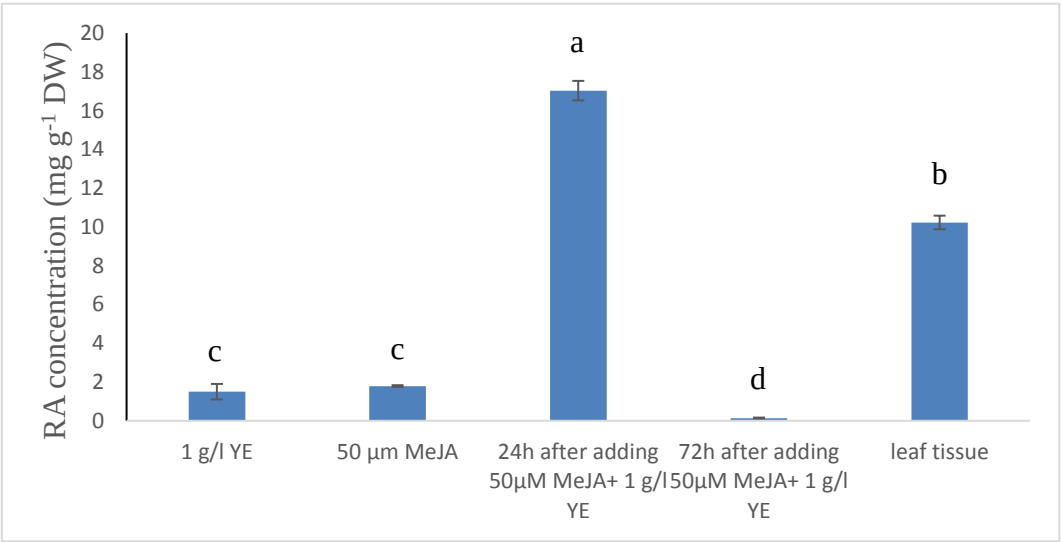

Fig. 5. RA production in suspension cell treated with MeJA and YE alone or in combination and leaf tissue of parent plants. Values are mean ± standard deviation Numbers with the same letters indicate no significant difference based on LSD test. ($P \leq 0.05$)

4. Discussion

Our results showed that application of B5 solid media under light condition have higher callus growth. Therefore, cell suspension culture established in this condition. The growth pattern of cells in cell suspension culture and measurement content of rosmarinic acid showed that combination of 1 mgl^{-1}2,4-D + 1 mgl^{-1}BA and 0.2 mgl^{-1}2,4-D + 0.2 mgl^{-1}BA reached the maximum cell growth and content of rosmarinic acid after 20 days of cultivation. Since rapid growth phase is the most suitable time for subculture and elicitor treatment, so elicitors add to the shake-flask culture on day 20 after culture initiation. Treatment of the cell cultures with methyl jasmonate and yeast extract

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increased cell growth compared to control cells. In addition to analysis of rosmarinic acid content in cell suspension culture showed that combination methyl jasmonate with yeast extract after 1 day of elicitation the rosmarinic acid content was 13 times higher than in the leaf tissue and 9 times higher than in cultures treated with MeJA or YE alone.

Various studies have shown that media and light conditions have a main effect on the callus growth of *L. angustifolia*. In a study, Keykha [44] observed that MS medium significantly enhanced the fresh and dry weight of callus in *L. angustifolia*. Previous studies have also reported that B5 is optimum for callus induction and growth [24, 25]. Bona [28] have suggested that light is necessary for callus induction of *L. angustifolia*, as they found that callus was formed in the explants subjected to light but not in the dark.

The tissue characteristics of the callus were found to be an important factor in its growth. For instance, homogeneous and friable callus is considered suitable for cell suspension culture. Keykha [44] reported a similar observation in lavender (*L. angustifolia*) callus culture, where the number of creamy calli was lower under light conditions compared to brown calli. The browning of callus tissue under light conditions is likely attributed to the production of phenolic compounds that can be toxic and hinder growth. Also under light conditions, the activity of oxidative enzymes increases, resulting in darker callus tissue compared to dark conditions [26, 27].

The growth curve of lavender (*L. angustifolia*) suspension culture on B5 medium with various hormonal treatments showed a declining trend in the last days of cell growth. This deceleration in growth may be attributed to the depletion of nutrients in the medium, leading to cell death and ultimately resulting in a reduction in growth. Additionally, as the growth of cells progressed, physical area and air availability within the flask became limited, hampering gas exchanges between the cells and the medium [28, 29]. Therefore, exchanging the medium every 20 days appeared to be suitable for maintaining the logarithmic growth phase. This time interval was also determined as the optimal period for elicitor treatment [30, 31].

Growth regulators play a key role in callus induction and improve cell growth. Among them, auxins and cytokinins have shown significant effects on secondary metabolite production. They are crucial producing and accumulating these bioactive compounds. The ratio, type, and concentration of auxins and cytokinins have a considerable impact on both the growth and the production of bioactive metabolites in cell suspension culture. So, it is worth selecting the appropriate hormones and optimizing their concentrations based on the specific requirements of each plant species for callus induction and secondary metabolite production [32]. In a study conducted by Xu [45], the highest production of RA in the cell suspension culture of *Agastache rugosa* was achieved in liquid B5 medium supplemented with 2 mg/l 2,4-D and 0.1 mg/l BA. Other studies have also reported the effects of various hormone concentrations on the biosynthesis of secondary metabolites in suspension culture, such as podophyllotoxin production in *Linum album* and alkaloid production in *Catharanthus roseus* cell culture [33, 34].

Different studies have demonstrated the positive effects of MeJA on the growth of various in vitro cell cultures. For instance, in suspension cultures of *Lavandula vera* MM, biomass increased after 4 hours of MeJA treatment. However, from 8 hours of elicitation until the end of the growth phase, the biomass accumulation in the cells decreased compared to

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the control [12]. In contrast, Krzyzanowska [14] investigated the effect of MeJA on *M. piperita* suspension culture and reported that the highest reduction in cell growth occurred with 200 µM of MeJA. The variation in responses could be attributed to the different sensitivity levels of plant species to elicitors [35, 36]. Similar to MeJA, YE as an elicitor promoted the growth of *L. angustifolia* cell suspension especially in higher concentrations. But, the duration of exposure to YE had minimal impact on cell growth. Zhao [13] reported a gradual reduction in cell growth in *Salvia miltiorrhiza* suspension culture from the initiation of YE elicitation until 6 days thereafter. In contrast, Hakkim [19] found that in *Ocimum sanctum* suspension culture, cells exhibited steady growth and reached their maximum level at 18 days across all YE concentrations. These variations in response may also be attributed to the specific characteristics and sensitivity of different plant species to YE elicitation.

Exogenous application of MeJA has been shown to stimulate the biosynthesis of secondary metabolites in various plant species [37-39]. Therefore, the signaling pathway through MeJA is an essential signal for the accumulation of valuable plant compounds. [40]. Consistent with our findings, rosmarinic acid (RA) biosynthesis in *Lavandula vera* cell suspension cultures containing 50 µM MeJA significantly increased. At this concentration, the RA content in elicited cells was approximately 2.4 times higher than non-elicited cells. Additionally, trace amounts of RA were detected in the culture medium. In this study, the highest level of RA was observed when 150 µM MeJA was applied to the culture medium [12]. However, it is worth noting that high extracellular concentrations of RA can inhibit cell growth. In response, cells produce the peroxidase enzyme as a defense mechanism which rapidly degrades rosmarinic acid [15]. Therefore, the amount of this phenolic acid is influenced by two concurrent processes: the secretion of rosmarinic acid into the culture medium and its subsequent degradation by antioxidant enzymes. Interestingly, the application of elicitors affected both processes [12]. Krzyzanowska [14] reported an approximately 1.5-fold increase in rosmarinic acid production under MeJA elicitation compared to the control in a *Mentha piperita* cell suspension culture. Their results indicated that application of 100 µM MeJA after 24 hours stimulated high production of RA. While a small amount of RA was secreted into the culture medium after MeJA elicitation, it did not significantly affect extracellular RA levels compared to the control culture.

In our study, YE had a significant impact on RA production. Similar findings were reported in *Agastache rugosa* suspension cultures, where the addition of 500 mg/l YE for 72 hours increased RA accumulation in treated cells [20]. Furthermore, in *Ocimum sanctum* suspension cultures, the addition of 0.5 g/l YE to the culture medium considerably enhanced RA content, 7-fold compared to the control treatment [19].

Our results confirmed RA secretion into the culture medium after elicitation with YE. RA secretion into the culture medium commenced 24 hours after elicitation and gradually increased over the cultivation period until 72 hours but the accumulation of this antioxidant decreased with longer exposure times, such as 144 hours. A similar phenomenon was observed in *Solenostemon scutellarioides* after YE application, where the formation of rosmarinic acid reached its peak on the first day after YE addition and decreased thereafter. According to their findings, the activity of enzymes involved in the

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biosynthetic pathway of RA, including tyrosine aminotransferase, was affected by elicitors. The maximum activity of this enzyme was observed one day after yeast extract treatment and substantially decreased after three days, which was correlated with the production of RA [41].

In the present study, HPLC analysis showed that the highest intracellular RA content was observed 24 hours after the addition of 50 µM MeJA in combination with 1 g/l YE that was significantly higher than that in the leaf tissue of the parent plant. A study conducted by Miclea et al. Investigated the effect of elicitors (Jasmonic acid and Salicylic Acid) on biochemical accumulation in *L. angustifolia*. Their results showed that elicitation with JA and SA had a significant effect in polyphenol and carotenoid content of in vitro explants compared to mature lavender plants cultivated in vivo [49].

In our study, after 72 hours of exposure to the combined elicitors, the RA concentration decreased considerably. This suggests that prolonged exposure to the combined elicitors has inhibitory effects on RA accumulation in *L. angustifolia* suspension culture. Interestingly, Cheng [42] found that the application of combined elicitors promoted the accumulation of cryptotanshinone, indicating synergistic effects on tanshinone accumulation. Therefore, the effect of elicitation on secondary metabolite production may vary depending on the specific target compounds, the concentration and combination of elicitors, and the duration of elicitor exposure [42].

The application of combined elicitors has demonstrated effectiveness in stimulating tanshinone production in *Salvia miltiorrhiza* cell suspension culture [13]. Firouzi [43] also reported a strong enhancement in silymarin content in *Silybum marianum* suspension cells when treated with a combination of MeJA and YE. Based on the findings of this study, the application of elicitors appears to be an impressive approach to improve the production of rosmarinic acid (RA) in *L. angustifolia* cell suspension culture.

5. Conclusion

Several strategies have been utilized to increase secondary metabolite production. Elicitors are key factors in inducing defense responses and promoting the accumulation of secondary metabolites in plant cells. Therefore, in this study, the growth of *L. angustifolia* suspension culture and the production of rosmarinic acid (RA) upon elicitation was investigated.

The addition of MeJA and YE effectively stimulated RA production in the cell suspension culture compared to the control. Interestingly, the combined application of elicitors showed a greater effect than individual elicitors alone. The maximum amount of RA (17.1 mg/g DW) was observed 24 hours after adding the combined elicitors (MeJA+YE). This value was significantly higher than that in the leaf tissue of the parent plant. Furthermore, it was 13 and 9 times greater than the RA content in cells treated with MeJA or YE alone, respectively. However, it was observed that 72 hours after elicitation with both elicitors (MeJA+YE), the RA content in the cells significantly decreased. This suggests that the combination of elicitors exhibits antagonistic effects with prolonged exposure time, resulting in a significant decline in RA production. RA was predominantly detected in the cells, while only trace amounts were secreted into the culture medium, similar to the control medium cultures.

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Conflicts of interest

All authors declare that they have no conflict of interest.

Author contributions

MY: Investigation, Formal analysis, writing original-draft; AB: Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition; NM: Resources, Writing - Review & Editing. FK: Methodology, Resources, Writing - Review & Editing. AK: Writing - Review & Editing, Data interpretation.

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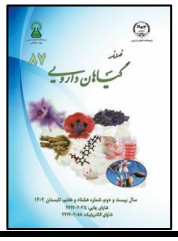

مقاله تحقیقاتی

کشت سوسپانسیون سلولی اسطوخودوس (*Lavandula angustifolia*) و تأثیر متیل جاسمونات و عصاره مخمر بر تولید رزمارینیک اسید

محبوبه یزدی[۱]، عبدالرضا باقری[۱،*]، نسرین مشتاقی[۱]، فاطمه کیخا آخر[۲]، آزاده خادم[۳]

[۱] گروه بیوتکنولوژی و به‌نژادی گیاهی، دانشکده کشاورزی، دانشگاه فردوسی مشهد، ایران
[۲] گروه بیوتکنولوژی گیاهی، دانشکده کشاورزی، دانشگاه جهرم، ایران
[۳] گروه بیوتکنولوژی گیاهان باغبانی، پژوهشکده بیوتکنولوژی صنعتی، جهاد دانشگاهی واحد خراسان رضوی، مشهد، ایران

اطلاعات مقاله

گل‌واژگان:
محرک
اسطوخودوس
متیل جاسمونات
رزمارینیک اسید
کشت سوسپانسیون
عصاره مخمر

چکیده

مقدمه: کشت سوسپانسیون سلولی و محرک نقش مهمی در سنتز متابولیت‌های ثانویه فعال دارد. رزمارینیک اسید (RA) یکی از ترکیبات فعال موجود در اسانس اسطوخودوس با خواص آنتی‌اکسیدانی و ضد التهابی است. **هدف:** این تحقیق به منظور بررسی کارایی تولید RA در کشت سوسپانسیون *Lavandula angustifolia* از طریق تیمار با محرک متیل جاسمونات (MeJA) و عصاره مخمر(YE) انجام شد. **روش بررسی:** کشت سوسپانسیون سلولی در محیط مایع B5 با ترکیبات هورمونی مختلف استقرار یافت و تأثیر آن‌ها بر رشد سلولی و تجمع RA مورد بررسی قرار گرفت. سپس تأثیر غلظت و مدت زمان تیمار با YE (۰/۱، ۰/۵ و ۱ میلی‌گرم در لیتر، ۱، ۳ و ۶ روز پس از تیمار) و MeJA (۵۰، ۱۰۰ و ۲۰۰ میکرومولار، ۱، ۲ و ۳ روز پس از تیمار) جداگانه و ترکیبی بر رشد سلولی و میزان رزمارینیک اسید درون سلولی و خارج سلولی مورد بررسی قرار گرفت. **نتایج:** نتایج HPLC نشان داد که بیشترین مقدار RA درون سلولی (۱۷/۰۳ میلی‌گرم بر گرم وزن خشک) ۲۴ ساعت پس از افزودن ۵۰ میکرومولار MeJA در ترکیب با ۱ گرم در لیتر YE مشاهده شد که تقریباً ۳۳ درصد بیشتر از مقدار این ماده در برگ بود. همچنین، تیمار ترکیبی به ترتیب منجر به ۹ و ۱۱ برابر افزایش رزمارینیک اسید در مقایسه با بکارگیری این دو محرک به تنهایی شد. همچنین، هر دو محرک به طور قابل‌توجهی بر مقدار RA خارج سلولی نسبت به شاهد تأثیر گذاشتند. **نتیجه‌گیری:** کاربرد محرک‌ها باعث افزایش تجمع زیست توده و RA در سلول‌های سوسپانسیون *L. angustifolia* می‌شود.

مخفف‌ها: RA، رزمارینیک اسید؛ MeJA، متیل جاسمونات؛ YE، عصاره مخمر؛ MS، موراشیگ و اسکوگ، BA، بنزیل آدنین؛PGR ، تنظیم‌کننده رشد گیاهی؛ DW، وزن خشک؛ HPLC، کروماتوگرافی مایع با کارایی بالا؛ LSD، حداقل اختلاف معنی‌دار

* نویسنده مسؤول: abagheri@um.ac.ir

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