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Salvia officinalis L.'in *İn Vitro* Kallus Kültürü ve Bazı Amino Asitlerin Rosmarinik Asit Birikimi Üzerine Etkisi

Rağbet Ezgi DURAN[1*]

[1]Süleyman Demirel Üniversitesi, Fen Edebiyat Fakültesi, Biyoloji Bölümü, 32260, Isparta

[1]https://orcid.org /0000-0003-0853-3680
*Sorumlu yazar: ezgiduran@sdu.edu.tr

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ÖZ

Bu çalışmada *Salvia officinalis* (adaçayı) bitkisinin çeşitli eksplantlarının *in vitro* kallus oluşturma yeteneği ve oluşan kalluslardaki rosmarinik asit üretimi araştırılmıştır. Kallus oluşumu için genç yapraklar (<1-1.5 cm), yapraklar (>1-1.5 cm), nodlar ve gövde parçaları olmak üzere 4 çeşit eksplant 0.8 mg/L 2.4-D, 0.5 mg/L NAA ve 2.0 mg/L BAP içeren MS ortamında kültüre alınmıştır. En yüksek kallus oluşum oranı (%100) genç yaprak eksplantlarından elde edilmiştir. 4 hafta sonra oluşan kalluslar 0.1 mg/L NAA+1.0 mg/L BAP içeren MS ortamına aktarılmış, ayrıca *in vitro* rosmarinik asit üretimini artırmak için besin ortamına L-tirozin (10 mg/L) ve L-fenilalanin (10 mg/L) eklenmiştir. Kalluslar bu ortamlarda 1 ve 2 ay boyunca kültüre alınmış ve adaçayı kalluslarındaki rosmarinik asit üretimi HPLC ile analiz edilmiştir. Amino asitlerin ortama eklenmesi kalluslarda rosmarinik asit üretimini önemli ölçüde arttırmıştır. Ayrıca sonuçlar amino asitler ve kallus kültür süreleri bakımından önemli ölçüde farklılıklar göstermiştir. Tirozinin adaçayı kalluslarında rosmarinik asit üretimini artırmada daha etkili olduğu gözlenmiştir. Hem tirozin hem de fenilalanin ile desteklenmiş ortamlarda 2 ay boyunca kültüre alınan kallusların, yalnızca 1 ay boyunca kültüre alınan gruba göre daha yüksek miktarda rosmarinik asit ürettiği belirlenmiştir.

In Vitro Callus Culture of *Salvia Officinalis* L. and the Effect of Some Amino Acids on Rosmarinic Acid Accumulation

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ABSTRACT

This study investigated the *in vitro callus* formation ability of various explants obtained from the *Salvia officinalis* (common sage) and the production of rosmarinic acid in the resulting calli. Four types of explants, including young leaves (<1-1.5 cm), older leaves (>1-1.5 cm), nodes, and stem pieces, were cultured on MS medium containing 0.8 mg/L 2.4-D, 0.5 mg/L NAA, and 2.0 mg/L BAP to induce callus formation. The highest callus formation (100%) was achieved from young leaf explants. After 4 weeks, the formed calli were transferred to an MS medium containing 0.1 mg/L NAA+1.0 mg/L BAP, and in vitro rosmarinic acid production was enhanced by adding L-tyrosine (10 mg/L) and L-phenylalanine (10 mg/L) to the nutrient medium. The calli were cultured in these media for 1 and 2 months, and the production of rosmarinic acid in sage calli was analyzed using HPLC. The addition of amino acids to the medium significantly increased rosmarinic acid production in the calli. Furthermore, the results varied significantly with different amino acids and callus culture durations. Tyrosine was observed to be more effective in increasing rosmarinic acid production in sage calli. Moreover, calli cultured for 2 months in both tyrosine- and phenylalanine-

supplemented media exhibited higher rosmarinic acid production compared to the control group cultured for only 1 month.

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1. Introduction

The genus *Salvia* consists of approximately 1000 species distributed worldwide (Kahraman et al., 2011), with many of them being utilized for herbal teas, food flavoring, cosmetics, perfumery, and pharmaceuticals. There are 99 *Salvia* species in Turkey (Tursun, 2019), more than half of which are found nowhere else (57 species) (Kahraman et al., 2011; Gökdoğan and Bürün, 2022). The well-known herbal plant with antioxidant properties is sage, which is used as a common folk remedy for alleviating various ailments such as antispasmodic and antiseptic (Hamidpour, 2014).

Secondary metabolites, formed through specific biosynthesis mechanisms, serve as a crucial part of the antioxidant system in plants, playing a central role in defending against environmental stressors. These specialized molecules are produced by plants in response to various signals, both internal and external, to ensure their survival and adaptation as relatively immobile organisms (Yeshi et al., 2022). The accumulation of these compounds within cells is often a consistent parameter in plant defense mechanisms, enhancing tolerance to different stresses. The genus Salvia is significant in this context, as it contains more than 160 identified phenolics and polyphenols. The type and quantity of phenolic acid derivatives may vary among Salvia species and different plant parts. The biosynthesis of secondary metabolites in *S. officinalis* is influenced by various genetic, environmental, and physiological factors. These specialized compounds, including rosmarinic acid (RA), caffeic acid derivatives, and terpenoids, play crucial roles in plant defense mechanisms, stress response, and ecological interactions. RA is a phenolic compound abundantly present in *S. officinalis*, where it serves as a major bioactive constituent contributing to the herb's therapeutic effects. Extensive research has demonstrated the diverse pharmacological activities of RA, ranging from antioxidant and anti-inflammatory actions to neuroprotective and anticancer properties (Noor et al., 2022). The biosynthetic pathway leading to RA involves the concerted action of enzymes within the phenylpropanoid and tyrosine-derived pathways, with regulatory mechanisms governing its production in plant tissues (Petersen et al., 2009).

Propagation of sage plants can be achieved through cuttings and seeds, but traditional propagation methods are seldom used due to low seed germination rates, poor seedling development, and slow growth. However, tissue culture techniques are recognized as one of the most effective methods for propagating sage plants (Avato et al., 2005), particularly for obtaining higher quantities of secondary metabolites. *In vitro* plant culture has been considered as an alternative subject to strict control, can generate the same valuable natural products (Espinosa‐Leal et al., 2018). In recent years, the establishment of callus culture systems has emerged as a promising strategy for the mass production of bioactive compounds from medicinal plants like *S. officinalis*. Callus cultures, consisting of

undifferentiated, rapidly proliferating cells, offer several advantages over conventional plant cultivation methods, including year-round availability, controlled growth conditions, and enhanced production of secondary metabolites.

Numerous studies have demonstrated the potential of *in vitro* cultures, including shoot, root, and callus cultures, as efficient platforms for rosmarinic acid production in sage. For instance, Grzegorczyk et al. (2005) found that diterpenoid production is strongly associated with shoot differentiation while rosmarinic acid remained consistent across callus, suspension cultures, and shoots. In another study, in vitro shoots of *S. officinalis* were established under various cytokinin supplementations, involving the culturing of nodal segments. It was observed that an increase in the concentration of kinetin (KIN) led to a decrease in the accumulation of rosmarinic acid (Santos-Gomes et al., 2002). However, studies demonstrating the effect of amino acids on the amount of rosmarinic acid produced in *Salvia* callus culture are quite limited in number.

RA is a natural antioxidant produced by cell cultures of sage, and its growth and production can be influenced by the type of culture medium (Khojasteh et al., 2020). The primary aim of this study is to investigate the potential of establishing callus cultures from various explants of sage, including leaf, nodes, and shoot explants, and to identify the most effective explant type for callus induction. Furthermore, we seek to assess and the compare the influence of tyrosine, phenylalanine and culture duration on the production of rosmarinic acid (RA), a key secondary metabolite with significant biological activities. By elucidating the optimal conditions for callus formation and RA production, this research aims to contribute to the development of efficient biotechnological approaches for the production of valuable bioactive compounds in sage plants.

2. Material and Methods

2.1. Plant Material and Sterilization

This research was carried out in the Plant Biotechnology Laboratory, Department of Biology, Faculty of Arts and Science, Suleyman Demirel University. Sage seedling were grown in pots which contain peat and sand (3:1) in a plant growth room. Light was supplied by lamps (10-15 klux m-2) on a photoperiod of 16h/8h day/night at 18 ± 20ºC and 51-54% humididty (PeakTech 3695).

The average diameter of 2-2.5 cm sage seedlings washed under running tap water for about 1 h and surface sterilized for 15 min in 15% sodium hypochloride solution, containing 1–2% Tween-80, then rinsed 5-6 times in sterilized distilled water. After this stage, seedlings were kept 15% ethanol for 1 minute and then rinsed 5-6 times in sterilized distilled water.

2.2. Callus Induction and Culture Condition

Murashige and Skoog (MS) (Murashige and Skoog 1962) (Sigma-M0404) basal medium solidified with 0.7% agar and supplemented with 3% sucrose, 0.8 mg/L 2.4-D, 0.5 mg/L 1-naphthaleneacetic acid (NAA) and 2.0 mg/L 6-benzylaminopurine (BAP) as plant growth regulators was used for callus

induction. Callus growth medium was prepared in the same way as the callus induction medium. However, plant growth regulators were added to this medium at a rate of 0.1 mg/L NAA and 1.0 mg/L BAP, and also 10 mg/L L-Tyrosine and 10 mg/L L-Phenylalanine amino acids were added. The pH of the culture medium was adjusted with 1 M NaOH and 1 M HCl to 5.7 ± 0.1 before addition of bacto agar for both media and autoclaved for 20 min at 121°C. The culture medium was dispensed into 9-cm Petri dishes in amounts of about 20 to 25 mL.

Young leafs (<1-1.5 cm), leafs (>1-1.5 cm) nodes and shoots of sage seedlings was used as expant source. Explant pieces, 0.3–0.5 cm long, were cut into pieces with sterile scalpel and inoculated onto culture media for callus induction. Each petri dish contains approximately 15 samples. The cultures were maintained darkness and at 25 ± 2°C. After 4 weeks incubation time, callus was transferred to culture medium containing amino acids and maintained under a darkness and at 25 ± 2°C.

2.3. HPLC Analysis of Rosmarinic Acid

The analysis of rosmarinic acid (RA) in the samples was conducted at the Innovative Technologies Application and Research Center, Suleyman Demirel University. For the determination of RA content, 100 mg of dried callus samples were ground and then extracted with 10 ml of methanol for a period of 14 hours at a controlled temperature of 15±2 °C. The resulting extracts were subsequently decanted, passed through a 0.45-mm acetate filter, and separated using a Shimadzu High-Performance Liquid Chromatography (HPLC) system equipped with a Diode Array Detector (DAD) with a maximum wavelength (λmax) of 278 nm, an SIL–10AD vp autosampler, and an LC-10ADvp pumping system. The chromatographic column employed was an Agilent Eclipse XDB-C18 (250x4.60 mm) with a flow rate of 0.8 ml/min. The mobile phase consisted of two solvents, methanol, and 3% acetic acid, which were utilized in a gradient elution process. Each extract was injected in a 20-ml volume and subjected to measurement.

2.4. Statistical analysis

The trials were conducted using the Randomized Block Design pattern, with each trial repeated at least three times. Data analysis involved performing a One-way ANOVA using the SPSS 23.0 software package for variance analysis, followed by the Duncan Multiple Comparison Test to compare the means.

3. Results and Discussion

3.1. Callus Induction

Callus formation was induced from four different types of explants, resulting in a total of 128 calli (71.91%) out of 178 explants. The formation of calli typically occurred within 2-3 weeks, with leaf explants demonstrating a relatively shorter formation time compared to other explant types. All explants produced friable callus; however, many of them exhibited the development of brown necrotic

areas (refer to Figure 2b), which, in some instances, led to the decline and death of the explants. Nevertheless, it was observed that if the browning did not affect the entire tissue, it did not appear to inhibit callus induction. The ability to induce callus formation varied significantly among the four types of explants ($P<0.05$). The highest percentage of callus induction (100%) was achieved with young leaf explants, followed by 85.45% from regular leaf explants, 60% from nodes, and 7.69% from shoots (Figure 1).

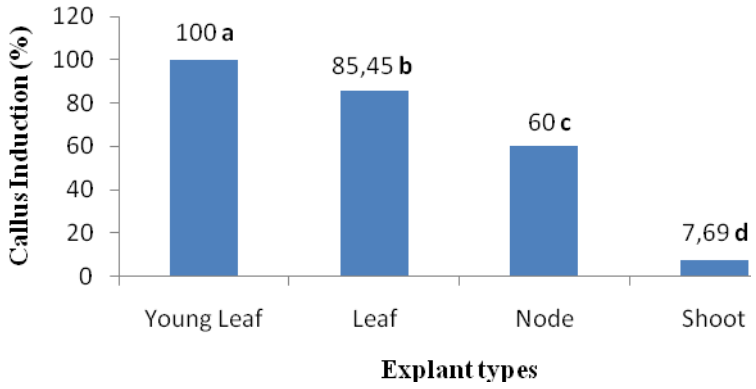

Figure 1. Callus induction capability of various explants. Values with identical letters following them are not statistically different at a significance level of 0.05

Numerous studies have focused on establishing callus cultures in *Salvia* species, highlighting the pivotal role of plant growth regulators like 2,4-D and BAP. Hemmati et al. (2020) found that *Salvia tebesana* exhibited maximum callus formation from shoot apical meristem on MS medium supplemented with 0.5 and 1.5 mg/L 2,4-D + 1 mg/L BAP, emphasizing the significant role of these regulators in callus induction and secondary metabolite production in Salvia species. Bano et al. (2022) reported a similar induction success rate in *S. moorcroftiana* L. leaf explants using 1 mg/L 2,4-D and 1.0 mg/L BAP. Our study employed a combination of 0.8 mg/L 2,4-D, 0.5 mg/L NAA, and 2.0 mg/L BAP, achieving 100% callus formation from young leaf explants and 85.45% from regular leaf explants, paralleling the high induction rates observed in previous research. Notably, Jafari et al. (2017) documented a 95% callus induction rate from node-type explants in *S. officinalis* using 2.0 mg/L NAA and 0.5 mg/L BAP, while Mederos-Molina (2004) achieved high induction rates using a higher ratio of NAA to BAP. Our findings of 60% induction from node-type explants and 7.69% from shoot-type explants offer valuable insights into the nuanced responses of different explant types to growth regulator combinations.

In our study, the distinctive combination of growth regulators not only facilitated a high callus induction rate but also underscored the variability in response among different *Salvia* explants. This variability is echoed in the literature, where the optimal hormonal balance for callus induction appears species and explant-specific. For instance, Modarres et al. (2018) developed an efficient procedure for cell suspension culture in *S. leriifolia*, optimizing growth regulators and sucrose concentrations for phenolic acids production. Similarly, Revutskaya et al. (2019) identified the most effective hormonal

combinations for callogenesis in *S. hispanica*, highlighting the impact of growth regulator concentration on callus growth and morphogenic ability.

The literature corroborates our findings that the specific combinations and concentrations of growth regulators play a critical role in the success of callus induction in *Salvia* species. By comparing our results with those in the literature, we demonstrate the complexity of optimizing callus culture conditions, which is influenced by the choice of explant and the specific hormonal balance. This comparison not only validates our experimental approach but also contributes to the growing body of knowledge on in vitro tissue culture techniques in *Salvia* species.

After a 4-week period, the callus was transferred to a callus growth medium containing 10 mg/L of L-Tyrosine and 10 mg/L of L-phenylalanine amino acids. The addition of L-tyrosine (10 mg/L) to the culture medium resulted in a reduced occurrence of necrotic symptoms compared to L-phenylalanine (Figure 2b-c).

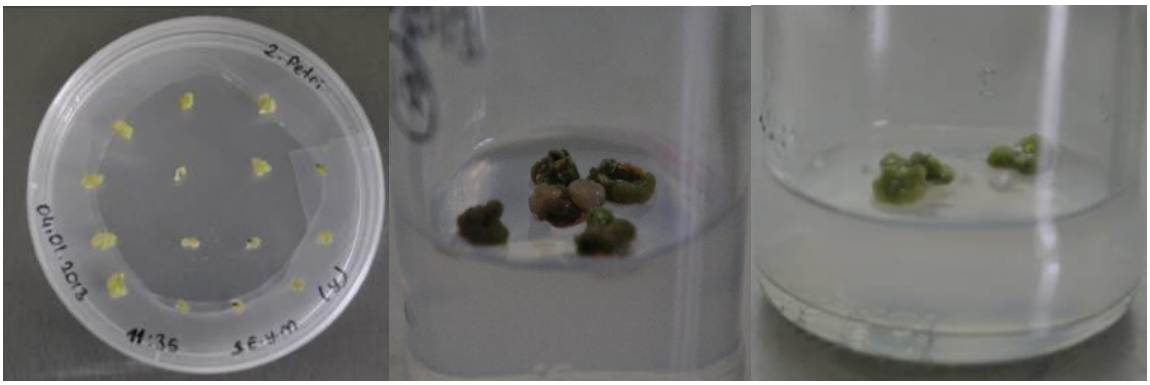

Figure 2. Callus appearance 30 days post-inoculation: b) Callus cultured in a medium containing L-phenylalanine amino acid with a slight browning tint c) Callus cultured in a medium containing L-tyrosine amino acid

3.2. Rosmarinic Acid Content

The quantity and quality of growth regulators, along with the presence of sucrose in the culture or different elicitors, exerted evident effects on both culture growth and RA accumulation. Our study aimed to elucidate the accumulation of rosmarinic acid (RA) in *S. officinalis* callus following the addition of two precursor molecules, namely tyrosine and phenylalanine. Upon harvesting callus samples at 1 and 2 months of incubation, we quantified the RA content using HPLC. The inclusion of amino acids significantly enhanced RA synthesis in the callus (Figure 3). Hakkim et al. (2011) reported that RA production reached a maximum when the culture medium was supplemented with 5.0% sucrose, 0.25 g/L phenylalanine, and the elicitor MeJA in *Ocimum sanctum* cell suspension cultures. Ibrahim (1987) found that incorporating phenylalanine into agar-based medium improved rosmarinic acid yield in *Coleus blumei* cell cultures. However, Sahraroo et al. (2018) reported that 3.0 mM phenylalanine led to the highest yield of rosmarinic acid in *Satureja khuzistanica* cell suspension culture, and Khoshsokhan et al. (2023) observed a significant increase in rosmarinic acid content with 0.5 g/L phenylalanine in *Salvia nemorosa* cell suspension culture.

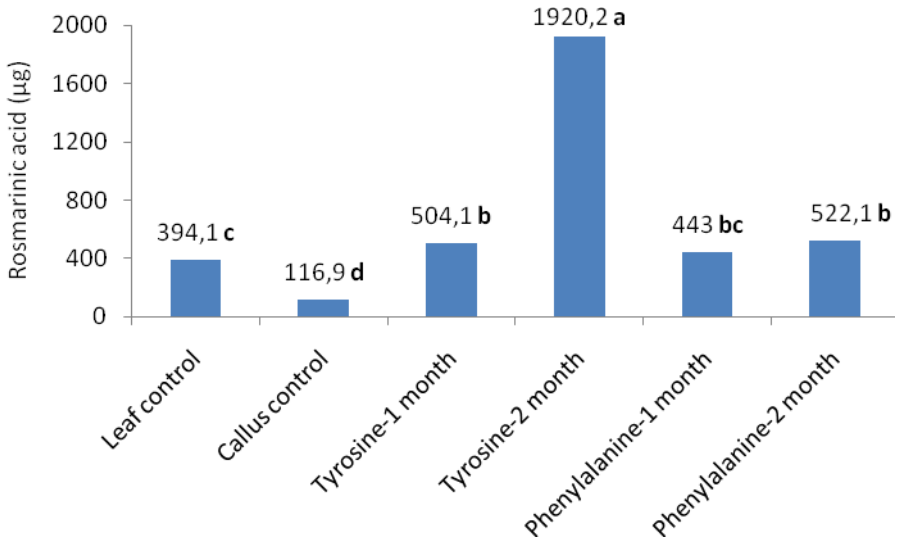

Figure 3. RA production callus using different amino acids and incubation times. Values with identical letters following them are not statistically different at a significance level of 0.05

Significant variations in RA production were observed based on different amino acids and the duration of callus culture in this study. Regardless of the culture duration, significant increases in RA production were observed in both amino acids compared to both leaf control and callus control groups. However, RA accumulation in sage callus cultured in tyrosine-enriched media was higher than in phenylalanine-enriched media, indicating that both amino acids did not elicit similar responses in enhancing RA production. The highest accumulation of RA was observed in callus cultured for 2 months and 1 month in the presence of tyrosine, with 1920.2 µg/g and 504.1 µg/g of RA in the dry callus tissue, respectively (Figure 3). Chaturvedi and Chowdhary (2013) demonstrated the enhancement of kaempferol content in callus cultures using different precursors, among which tyrosine proved to be the most effective precursor. Our study corroborates previous findings regarding the efficacy of tyrosine as a precursor for the enhancement of secondary metabolite production.

It has been observed that calli cultured for 2 months were more successful in RA production, a finding applicable to both amino acids. RA accumulated in 1 and 2 month tyrosine growth callus nearly 1.5-fold to 5-fold and 4.5-fold to 16.5-fold higher than leaf control and callus control, respectively. This value increased approximately 1.1-fold to 1.3-fold and 4-fold to 4.5-fold for 1 and 2 month old phenylalanine callus, respectively. Karam et al. (2003) found that RA production for *S. fruticosa* callus reached its maximum after 5 weeks of culture. Additionally, Kintzios et al. (1999) and Khoshsokhan et al. (2023) obtained similar results at 1-2 weeks and 4-5 weeks after culture for *S. officinalis* callus. Several studies have reported varying levels of rosmarinic acid or other secondary metabolite accumulation in callus lines, which were either positively or inversely related to the tissue growth rate (Bauer et al., 2004; Castro et al., 2016; Duran et al., 2019).

Previous research has indicated that the supplementation of amino acids enhances also various secondary metabolite productions in plant cell cultures (Masoumian et al., 2011; Indu et al., 2013).

Taha et al. (2009) reported that the highest values of mass cell cultures and indole alkaloid production in *Catharanthus roseus* were achieved with a modified MS medium containing 300 mg/L of either L-glutamine for mass cell induction or L-tryptophan for the enhancement of total indole alkaloids. Additionally, Ahmed et al. (2000) demonstrated that the indole alkaloid content of callus tissue from *Catharanthus roseus* was increased through amino acid supplementation. The impact of proline on thymol production in *Origanum vulgare* and on hyoscyamine and scopolamine in callus culture of *Hyoscyamus niger* has been investigated by Al-Jibouri et al., (2012a-b), revealing that proline enhanced the production of secondary metabolites in callus tissue. Moreover, Roy and Mukhopadhyay (2012) demonstrated that the application of excess amino acids increased tissue growth, elevated carbon levels, and enhanced phenol accumulation, possibly by stimulating the key enzyme Phenylalanine Ammonia Lyase activity. Similar increases in the contents of secondary metabolites have been observed in callus and regenerated plants of various species (Arya and Patni, 2013; Chakraborty et al., 2013; Samani et al., 2019).

However, some studies have shown that also different elicitors affect the amount of rosmarinic acid in *Salvia* species. Supporting our findings, Ejtahed et al. (2015) showed that the application of salicylic acid led to an up-regulation of PAL gene expression, albeit without a direct positive correlation to RA accumulation in *Salvia* species, suggesting the involvement of complex regulatory mechanisms in RA biosynthesis. Similarly, Su et al. (2020) highlighted the potential of optimizing extraction conditions, including the use of enzymatic methods, to enhance RA yield from *S. officinalis*, pointing towards biotechnological approaches as effective means to increase RA availability. This aligns with our observation of significant RA production enhancement following amino acid supplementation, suggesting a promising avenue for targeted genetic interventions to optimize RA yields.

Moreover, the study by Pirooz et al. (2022) on the synergistic effects of silicon and nitric oxide in modulating secondary metabolism under stress conditions in *S. officinalis* further supports the potential of elicitation strategies in improving RA and other secondary metabolite productions. These findings collectively underscore the complexity of RA biosynthesis and the potential for biotechnological and genetic strategies to enhance RA production in medicinal plants.

4. Conclusion

In conclusion, among the studied explants of sage, the young leaf explant was found to have the highest callus induction capacity. It was observed in the present investigation that both of amino acid but specially L-tyrosine could be effective in rosmarinic acid production in callus cultures of sage. Particularly, the rosmarinic acid levels in calli cultured for 2 months, which applies to both amino acids, were higher than those cultured for 1 month. The observed variation in RA accumulation between L-tyrosine and L-phenylalanine treatments underscores the importance of precursor selection in optimizing secondary metabolite yields. These results suggest potential avenues for further investigation into the metabolic pathways involved in RA biosynthesis and the optimization of callus

culture conditions for enhanced RA production. The cultivation conditions of plants from which rosmarinic acid and other secondary metabolites are obtained, due to their lengthy growth processes and seasonal limitations, *in vitro* culture will offer significant advantages in many sectors such as cosmetics and pharmaceuticals.

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Conflict of interest

The author declare that have no competing interests.

Consent for publication

The author declares that she has contributed 100% to the article.

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