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Increasing Amounts of Secondary Metabolites and Medicinal Compounds in Callus Culture of *Moringa Oleifera* (Lam.) Using Abiotic Elicitors

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Abstract

Moringa oleifera Lam. tree has often been considered a medicinal and economical plant for its nutritional and medicinal properties. This study is intended to use plant tissue culture and elicitation technologies to increase secondary metabolites in the callus cultures of the *M. oleifera*. Young nodal segments were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of plant growth regulators (PGRs). A combination of 1.5 mg/l NAA plus 0.5 mg/l TDZ resulted in a high callusing rate (70%) with the largest size. Various concentrations of Salicylic acid (SA) and Methyl jasmonate (MeJa) were individually added to the growth medium to evaluate their impact on the biomass of callus cultures, DPPH antioxidant activity, and accumulation of some flavonoids. Results indicated that all elicitors doses reduced weights and growth index of callus. While DPPH assay recorded high significant values at high concentrations of SA and MeJa compared to other treatments. HPLC analysis revealed that every concentration of SA and MeJa used led to rising flavonoids content, including Baicalin, Rutin, Hesperidin, Rosmarinic acid, Quercitrin, Quercetin, and Kaempferol. It should be noted that 20 mg/l MeJa gave the highest flavonoid content compared to the control and other treatments. In conclusion, the *in vitro* elicitation by SA or MeJa can improve beneficial biological substances like flavonoids and other antioxidant compounds in *Moringa* callus cultures.

Keywords: *Moringa oleifera*, flavonoids, abiotic elicitors, DPPH

زيادة كميات المستقلبات الثانوية والمركبات الطبية في مزرعة كالس المورينجا اوليفيرا باستخدام محفزات غير حيوية

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الخلاصة

غالبًا ما تُعتبر شجرة المورينجا أوليفيرا نباتًا طبيًا واقتصاديًا لخصائصها الغذائية والطبية. تهدف هذه الدراسة إلى استخدام تقنيتي زراعة الأنسجة النباتية والتحفيز لزيادة المستقلبات الثانوية في مزارع الكالس في *M. Oleifera* Lam. تم استزراع المقاطع العقدية الفتية على وسط موراشج وسكوج (MS) مع إضافة تراكيز مختلفة من منظمات نمو النبات PGRs. أدى الجمع بين 1.5 مجم / لتر NAA مضافا له 0.5 مجم / لتر TDZ إلى معدل عالٍ للكالس (70%) مع الحجم الأكبر. إضيفت تراكيز مختلفة من حامض الساليسيليك (SA) وميثيل جاسمونيت (MeJa) بشكل فردي إلى وسط النمو لتقييم تأثيرها على الكتلة الحيوية لمزارع الكالس، ونشاط مضادات الأكسدة DPPH، وتراكم بعض الفلافونويدات. أشارت النتائج إلى أن جميع جرعات المحفزات خفضت الأوزان ومؤشر نمو الكالس. بينما سجل اختبار DPPH قيم معنوية عالية بتركيزات عالية من SA و MeJa مقارنة بالمعالجات الأخرى. أظهر تحليل HPLC أن كل تركيز من SA و MeJa أدى إلى ارتفاع محتوى الفلافونويدات، بما في ذلك Quercitrin، Rosmarinic acid، Hesperidin، Rutin، Baicalin، Quercetin و Kaempferol. وتجدر الإشارة إلى أن 20 مجم / لتر MeJa أعطت أعلى محتوى من الفلافونويدات مقارنة بمجموعة السيطرة والمعاملات الأخرى. في الختام، يمكن أن يؤدي التحفيز في المختبر بواسطة SA أو MeJa إلى تحسين المواد البيولوجية المفيدة مثل الفلافونويدات والمركبات المضادة للأكسدة الأخرى في مزارع كالس المورينجا.

1. Introduction

Moringa species belong to the Moringaceae family and are native to Asia [1]. According to numerous scientific studies, *M. oleifera* distinguishes itself from other plants by containing large amounts of minerals that are beneficial to human health and serve as a good feed source for livestock [2]. The dried leaves contained 17 times the calcium of milk, 10 times the Vitamin-A of carrots, 15 times the potassium of bananas, and 25 times the Fe of spinach [3], 25.0% protein, 28.5% carbohydrates, with valuable content of β -carotene vitamin-B₁ and vitamin-C [4]. As a medicinal plant, every part of this tree has been utilized for numerous ailments in South Asian folk medicine [1]. Many studies have reported his activities in lowering blood pressure and lipids [5], Diabetes control [6], weight loss [6], hepatoprotective [7] and antioxidant benefits [8].

Tissue culture techniques successfully encourage the biosynthesis and growth of many secondary metabolic compounds in plant tissues, so they have been considered the most promising technologies in the pharmaceutical industry [9]. The callus cultures of *M. oleifera* were successfully established from various explants such as leaves [10], cotyledon [11], seed [12], shoots, and roots [13]. Many abiotic stimulators like temperature, saline, and salicylic acid were applied to increase the biosynthesis of many metabolic compounds in the *in vitro* cultures of *M. oleifera*, including phenolic acids and flavonoid compounds [10]. In general, signalling molecules such as salicylic acid (SA) and methyl jasmonate (MeJa) have been frequently employed to promote the accumulation of bioactive molecules in plant cells and organ cultures [14]. For example, exogenous application of SA and MeJa leads to a significant increase in phenolic compounds and flavonoid content in *Thevetia peruviana* (Schum) cell cultures [15]. Additionally, these elicitors also enhanced the Withanolide compounds in *Withania somnifera* (Dunal) [16]. Moreover, rutin content and total flavonoids were also elevated after treating *Gardenia jasmonides* (Ellis.) callus cultures with SA [17].

In this investigation, the callus cultures of *M. oleifera* were treated individually with various concentrations of (SA) and (MeJa) to increase the activation and production of secondary metabolic compounds to provide the pharmaceutical supplement industry with high-quality and quantity of natural products.

2-Methodology

1.1 Plant Material and sterilization

Young nodal segments of *M. oleifera* were collected from the garden of the Biotechnology Research Center, AL Nahrain University, in Baghdad, Iraq. The surface sterilization was achieved by immersion nodes in ethanol 70% for 1 min, then in NaOCl 20% for 10 min. Finally, sterilized nodes were thoroughly washed four times with autoclaved distilled water [18]. All disinfectant steps were done under a laminar airflow cabinet.

1.2 Callus Induction

For callus initiation, the disinfectant nodes were cultured in jars containing basal Murashige and Skoog (MS) medium [19] (HI media, India) augmented with different concentrations (0, 0.5, 1.0, 1.5) mg/l of Naphthalene acetic acid (NAA) alone or combined with 0.5 mg/l of Benzylaminopurine (BAP) or Thidiazuron (TDZ) (all PGRs were provided by Sigma, USA). After adding 30 g/l sucrose to the medium, the pH was adjusted to 5.7–5.8. The solidifying agent was 7 g/l agar (HI media, India). For 30 days, the cultured jars were incubated at 25 1 °C with a 16 h light/24 h dark cycle.

1.3 Callus culture maintenance and elicitation

According to the obtained results, the callus culture was induced and maintained on a medium supplemented with 1.5 mg/l NAA and 0.5 mg/l TDZ. The abiotic stress included (0, 5, 10, 15) mg/l salicylic acid (SA) and (0, 10, 20, 30) mg/l methyl jasmonate (MeJa) were sterilized by a 0.22 µm syringe Millipore filter and added to the autoclaved maintenance medium. Approximately 150 mg of callus tissue was transplanted onto the elicited medium.

1.4 Calculation of callus growth after the elicitation period

Data analysis was performed after 30 days of elicitation. All the replicates' fresh and dry weights at each treatment were measured using an analytical balance (KERN, Germany) and presented as average values. While the growth index G.I. of elicited callus was calculated according to Pandey *et al.* [20] as the formula below:

$$G.I (%) = \frac{(\text{Final fresh weight} - \text{Initial fresh weight})}{\text{Initial fresh weight}} \times 100$$

1.5 Assessment of the free radical scavenging activity of DPPH

Preparation of samples to achieve free radical scavenging activity via DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay was performed according to Ahmad *et al.* [21]. Concisely, 10 mg of dried callus was suspended overnight in 5 ml of methanol 95% and then filtered through a Whatman 1 filter paper and a 0.22 µm syringe Millipore filter. 0.0039 g of DPPH was dissolved in 100 ml of methanol 95% by Vortex (Stuart) to create a solution with a concentration of 1 mM DPPH. Afterward, 1 ml of each extract was added to the 1 ml of DPPH solution with well mixing by Vortex, and then incubated for 30 min in darkness at room temperature. The absorbance at 517nm was carried out by a spectrophotometer (OPTIMA SP-300 VIS). The percentage of DPPH antioxidant activity was applied as the equation [22]:

$$DPPH (%) = \frac{Ab - As}{Ab} \times 100$$

Where *Ab* = Absorbance of blank (without extract), *As* = Absorbance of the sample solution (sample + DPPH).

1.6 Extraction of flavonoid compounds and HPLC analysis

The flavonoid extraction method was performed according to Obouayeba *et al.* [23] with minor modifications. For each sample, the dried callus was crushed to create a fine powder. In

20 ml of ethanol and deionized water (80:20 v/v), 100 mg of each sample was suspended. Vigorously, all samples were shaken and sonicated by ultra-sonication bath at 60% duty cycles for 20min at 25 °C. Centrifugation was performed at 7500 rpm for 15min. Each sample's supernatant was exposed to charcoal before evaporation under a vacuum to remove the pigments. The dried samples were resuspended with a vortex in 1 ml of methanol and filtered through a 0.22 µm syringe Millipore filter. 20 µl of prepared standard solution and samples were injected into the HPLC system (All standard solutions were purchased from Sigma-Aldrich, Germany). The HPLC system consists of an LC-10A Shimadzu pump with (SPD-UV-Vis 10A) detector and a C18-DB column (3µm particle size, 50 x 4.6 mm I.D). The mobile phase consists of a linear gradient of solvent A (0.05% Ttrifluoroacetic acid (TFA) in deionized water) and solvent B (0.05% TFA in methanol). The gradient program started from 0% B and ended with 100% B for 15min. The pH was 2.5, the flow rate was 1.1 ml/min, and UV at 355 nm was used to detect it.

1.7 Statistical Analysis

The *in vitro* experiments were performed in 20 replicates, with three replicates each of HPLC and DPPH analysis assessments. The data was presented in the form of an average. Statistical analysis was conducted using the ANOVA test at $P \leq 0.05$, followed by the Duncan's Multiple Range test using SPSS 26 software.

2 Results

2.1 Callus induction

The nodal explants had the potential to dedifferentiate and form callus tissue. Various concentrations of NAA (0.5, 1, and 1.5) mg/l resulted in a low percentage of callus induction. The highest callogenesis rate was achieved at medium supplemented with a 1.5mg/l NAA combined with 0.5mg/l TDZ, to give a 70% callusing rate with a large size of callus tissue compared to other treatments (**Table 1, Figure 1**). Most treatments were found to yield yellow and friable callus. It is noteworthy that the control treatment without PGRs did not give any stimulation for callus induction.

Table 1: The effect of different concentrations of NAA alone or combined with BAP and TDZ on the callus induction of *M. oleifera* from nodal explants

| Media code | PGRs (mg/l) | | | Callus induction | | | |
|------------|-------------|-----|-----|------------------|------|--------------|-----------|
| | NAA | BAP | TDZ | Callogenesis (%) | Size | Color | Texture |
| M1 | 0.0 | 0.0 | 0.0 | 0 | - | - | - |
| M2 | 0.5 | 0.0 | 0.0 | 40 | + | Yellow | Friable |
| M3 | 1.0 | 0.0 | 0.0 | 50 | + | Yellow | Friable |
| M4 | 1.5 | 0.0 | 0.0 | 50 | + | Yellow-brown | Friable |
| M5 | 0.5 | 0.5 | 0.0 | 50 | ++ | Yellow | Semi sold |
| M6 | 1.0 | 0.5 | 0.0 | 50 | + | Yellow | Friable |
| M7 | 1.5 | 0.5 | 0.0 | 60 | + | Yellow | Friable |
| M8 | 0.5 | 0.0 | 0.5 | 60 | + | Yellow | Semi sold |
| M9 | 1.0 | 0.0 | 0.5 | 70 | ++ | Yellow | Friable |
| M10 | 1.5 | 0.0 | 0.5 | 70 | +++ | Yellow | Friable |

Where NAA= Naphthalene acetic acid, BAP= Benzylaminopurine, TDZ= Thidiazuron, - = No response, + = Small, ++ = Medium, +++ = Large.

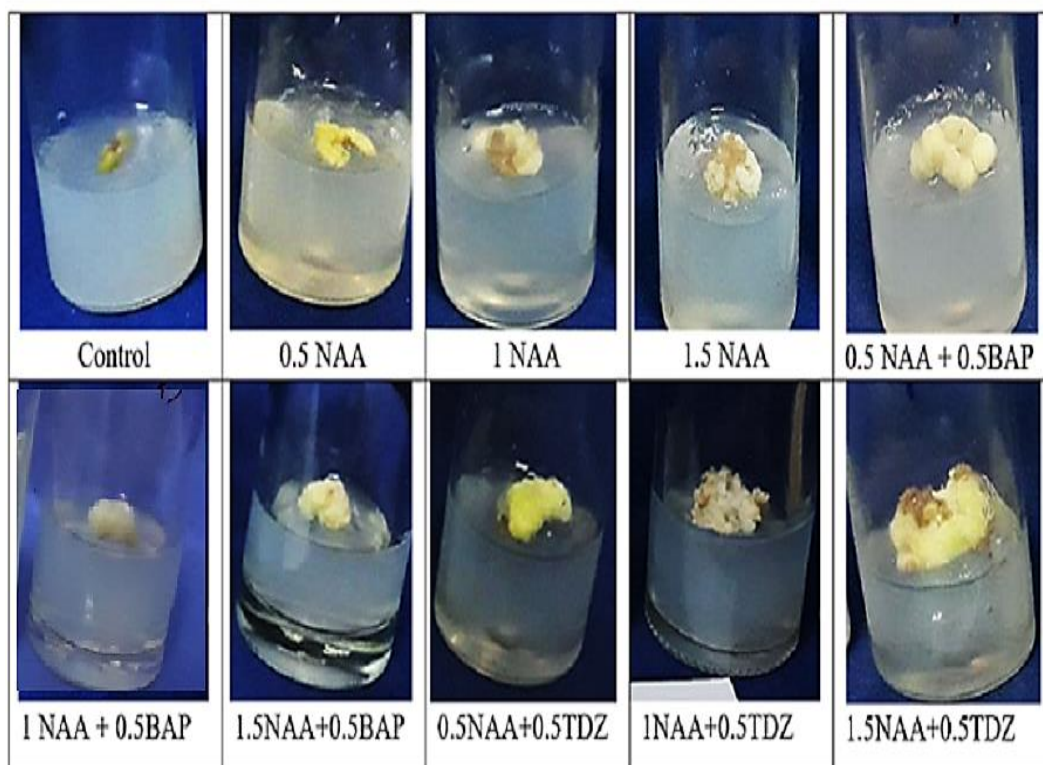


Figure 1: The phenotypic features of callus cultures formed on various combinations of plant growth regulators, where NAA= Naphthalene acetic acid, BAP= Benzylaminopurine, TDZ= Thidiazuron.

2.2 Effect of SA and MeJa on callus growth

As for the effect of the exogenous application of SA and MeJa on the biomass of callus culture, the data in **Table (2)** show that all treatments significantly decreased the fresh and dry callus weight compared to the control. Furthermore, the control treatment had the highest percentage of callusing growth index G.I. (74.6%), while all concentrations of SA and MeJa (especially high concentrations) caused significantly lower rates (20.4-9.1%).

Table 2: The influence of different concentrations of SA and MeJa on fresh and dry weights and the growth index of cultured callus on maintenance medium.

| | Con. of SA and MeJa (mg/l) | | | | | | |
|----------|----------------------------|---------|---------|--------|---------|---------|---------|
| | 0 | 5 SA | 10 SA | 15 SA | 10 MeJa | 20 MeJa | 30 MeJa |
| FW (mg) | 262.2 a | 180.6 b | 164.8 c | 163.7c | 167.4 c | 170.1c | 166.4 c |
| DW (mg) | 25.8 a | 13.9 b | 13.2 b | 12.3 b | 15.2 b | 15.7 b | 15.1 b |
| G.I. (%) | 74.6 a | 20.4 b | 9.8 c | 9.1 c | 11.6 c | 13.4 c | 10.9 c |

Where FW=fresh weight, DW=dry weight, GI=growth index, Con=concentration, SA= Salicylic acid, and MeJa= Methyl jasmonate. In same row, "different letters indicate significant differences at $P \leq 0.05$ "

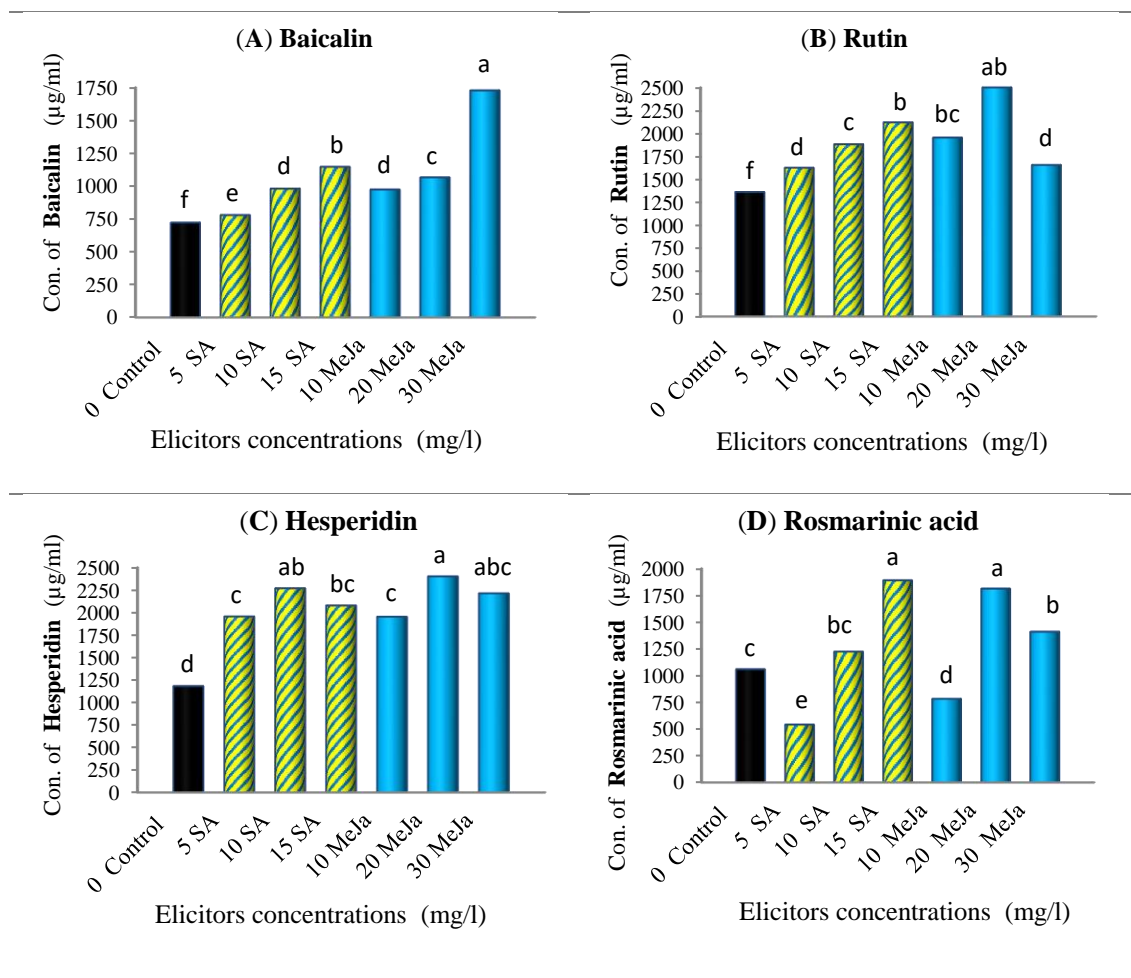
2.3 The Influence of SA and MeJa on flavonoids Productivity

Various concentrations of SA and MeJa dosages were particularly effective in the production of *M. oleifera* active compounds. In comparison to the control, all callus cultures showed a considerable increase in flavonoids concentration. Results indicated the highest concentration of Baicalin (1728.0 $\mu\text{g/ml}$) was found in 30 mg/l MeJa, whereas, the maximum accumulation of Rutin, Hesperidin, Rosmarinic acid, Quercitrin, Quercetin, and Kaempferol (2562.0, 2400.6, 1812.0, 1497.4, 1456.9, and 784.2) $\mu\text{g/ml}$, respectively, were recorded at 20 mg/l MeJa (Figure

2, B-G). In contrast, the control treatment (untreated group) showed lower content of Baicalin, Rutin, Hesperidin, Quercetin, and Kaempferol (722.1, 1358.2, 1182.4, 422.6, and 520.2) $\mu\text{g/ml}$, respectively, than the other treatments (Figure 3). Finally, we compared the sum content of the studied flavonoids to determine the best elicitor and their concentration for accumulating these compounds. The results showed that the influence of MeJa at 20 mg/l resulted in a maximum value of 11578.8 $\mu\text{g/ml}$ of total flavonoids, which was 94.8% higher than the control. While 15 mg/l SA gave 10526.2 $\mu\text{g/ml}$ of flavonoids with a 77% increase rate (Figure 4).

2.4 The Influence of SA and MeJa on the antioxidant activity (DPPH)

The results in **Figure (5)** indicated that 20mg/l MeJa had the highest significant value of DPPH activity (60.9 %). Generally, high SA or MeJa concentrations resulted in a considerable rate of DPPH activity compared to the control treatment, which displayed the least degree of antioxidant activity (54.4%).



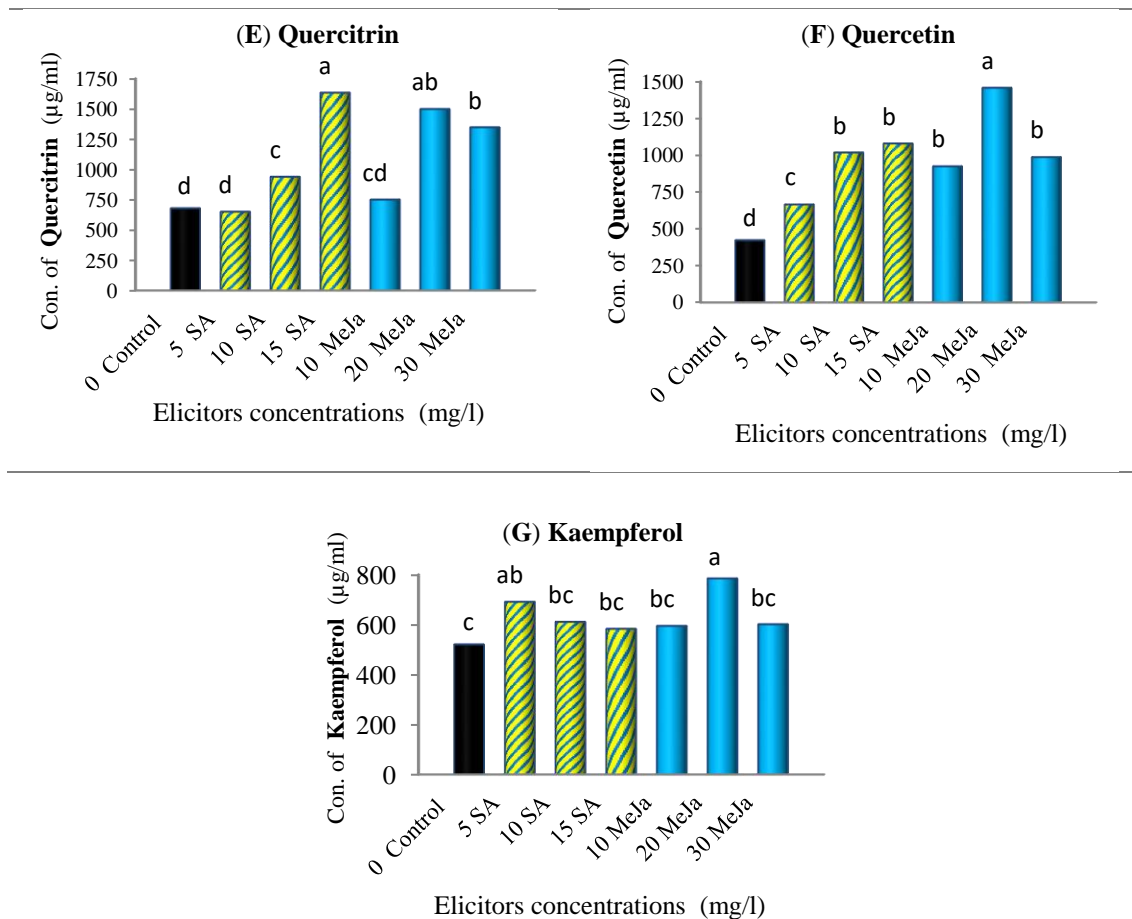


Figure 2: Accumulation of some flavonoid compounds (µg/ml) in callus cultures of *M. oleifera* elicited with different concentrations of Salicylic acid (SA) and methyl Jasmonat (MeJa) using HPLC analysis: (A) Baicalin, (B) Rutin, (C) Hesperidin, (D) Rosmarinic acid, (E) Quercitrin, (F) Quercetin, (G) Kaempferol.

"Bars denoted by the same letter are not significantly different at $P \leq 0.05$ "

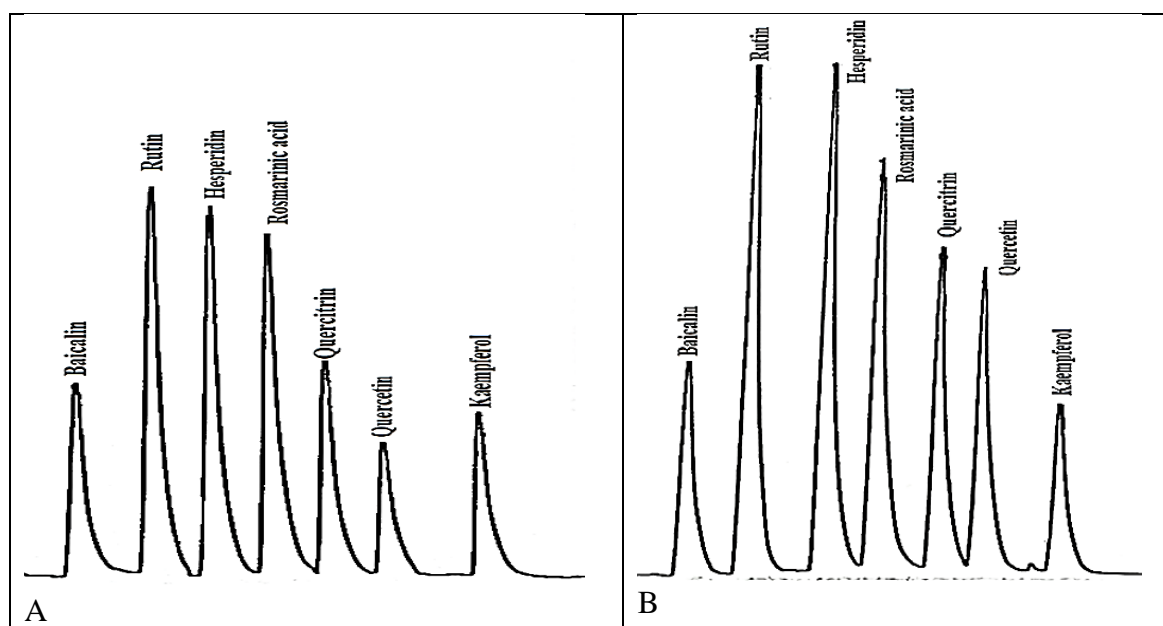
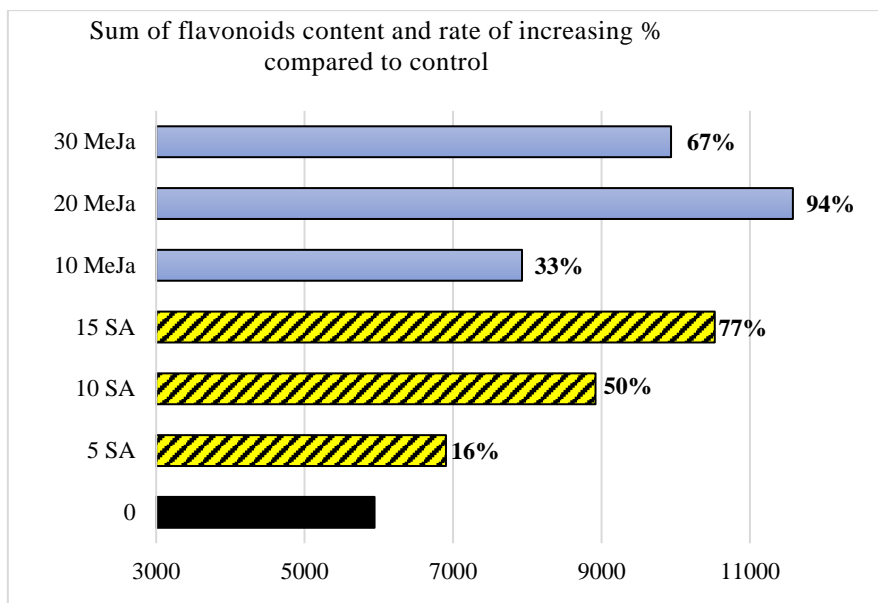


Figure 3: The HPLC profiles display peaks of some flavonoid compounds in *Moringa oleifera* callus cultures; (A) in control treatment, and (B) in 20 mg/l MeJa treatment.



Sum of flavonoids (µg/ml)

Figure 4: Sum of flavonoids content in callus cultures elicited with different concentrations of salicylic acid (SA) and methyl jasmonat (MeJa), and rate of increasing % compared to the control treatment.

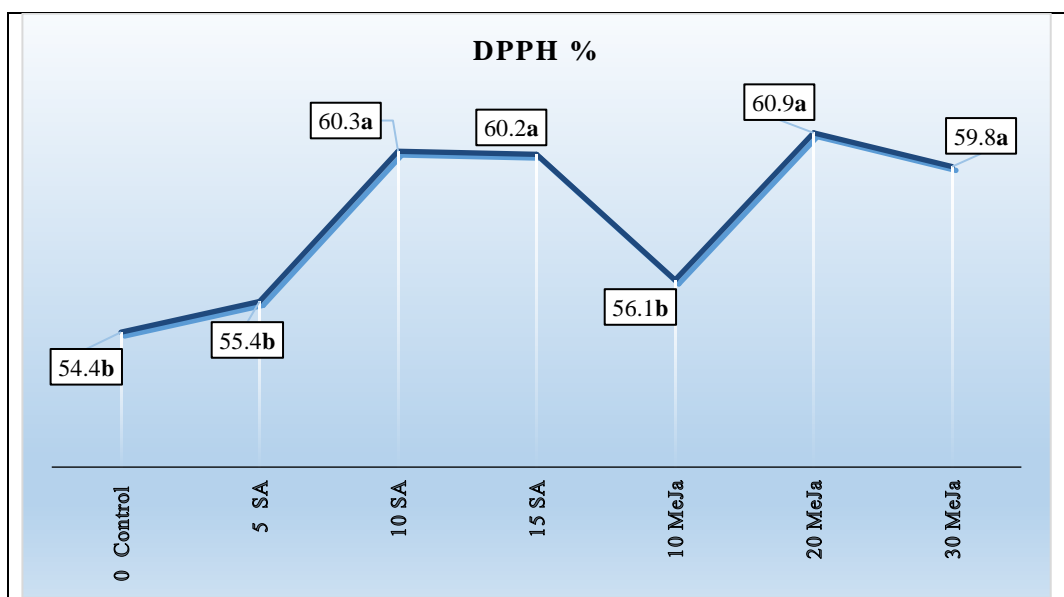


Figure 5: The DPPH scavenging activity (%) of methanolic extract of *M. oleifera* callus cultures elicited with different concentrations of Salicylic acid (SA) and Methyl Jasmonat (MeJa).

"Means followed by the same letter are not significantly different at $P \leq 0.05$ "

3 Discussion

The presented results indicate that implanting axillary nodal explants on MS medium supplemented with appropriate PGRs is a suitable method for callus induction of *M. oleifera*. These observations agree with Stephenson and Fahey [24], who demonstrated the efficiency of the MS medium in stimulating callus induction of the *Moringa* plant using axillary nodal explants. The selection of the appropriate combination of growth regulators has a great impact on directing the callogenesis in terms of the time required for emergence, its texture, color, and

its sustainability [25]. The lack of any growth in the control group indicates the explant's weak content of endogenous hormones. The majority of the physiological activities in the plant are controlled by internal hormones [26]. The high incidence of callus development in treatments containing TDZ with NAA demonstrated that the synergistic interaction between auxin and cytokines often leads to beneficial consequences. The capacity of TDZ to enhance the synthesis of endogenous purine cytokinins or hinder their degradation has been used to successfully induce callus development in various plant species [27]. Additionally, it is believed that TDZ can modify the ratio of endogenous auxins to cytokinins [28]. Moreover, cytokinin can reduce the unfavorable effects of auxin's oxidative activity [29]. Furthermore, when cytokinin and auxin are combined, the callus' fresh weight may increase; this could be because cytokinins accelerate cell proliferation, particularly in meristematic cells, which leads to an increase in the size of plant tissues when grown *in vitro* [30, 31].

Elicitation is among plants' most effective strategies for increasing secondary metabolite biosynthesis [32]. But most biotic or abiotic stresses often have a detrimental effect on biomass growth in most agricultural systems. The reduction of callus yield by MeJa was observed in *Malus domestica* (Borkh.) cultures [33] and *Centella asiatica* (L.) suspension cultures [34]. Another investigation reported that SA at various dosages caused a decrease in the biomass of cell cultures in the *Nicotiana tabacum* plant by inhibiting cell respiration and ATP formation [35]. Despite the undesirable effects of stimuli on cell growth, they support the biosynthesis of secondary metabolites in various plant genera. Some explanations have been provided to understand the mechanism of action of these compounds. SA, for example, may stimulate enzymatic activities such as phenylalanine ammonium lyase (PAL), which increases phenolic substance accumulation in plant tissues [36]. SA can also increase "reactive oxygen species ROS" scavenging capacity, as well as interact with other signal molecules (e.g., jasmonates and NO) to regulate phytochemical biosynthesis expression genes [37, 38]. In different cultures, SA was found to enhance the production of withanolides in *Withania somnifera* [39], Dicentrine in *Stephania venosa* (Spreng.) [40], phenolic compounds in *Thevetia peruviana* (Schum.) [15], and Rutin in *Gardenia jasmonides* [17]. According to Kamińska [41], jasmonates also contribute to the signalling pathways that promote the enzymes essential for biochemical transformations involved in secondary metabolism. The exogenous application of MeJa increased the catechin production in cell suspension cultures of *Taxus cuspidata* [42], enhanced terpene production (5-fold) in *Lepechinia caulescens* (Ortega.) [43], and increased Asiaticoside content (69-fold) in *Centella asiatica* [34].

The DPPH assay has been extensively utilized to assess the capacity of compounds to function as hydrogen donors or free radical scavengers; thus, the antioxidant activity is estimated using this ability [22]. In this regard, Fitriana *et al.* [44] demonstrated that a methanolic extract of *Moringa* leaves had a high value of DPPH scavenging activity in comparison to other solvents, and they attributed this finding to the potency of methanol in the successful extraction of phenolic compounds known for their anti-oxidation properties. Moreover, Zanella *et al.* [10] confirmed the correlation between high antioxidant activity and high phenolic content produced under various abiotic stresses in *M. oleifera* callus cultures. Substantially, many investigations regarded that using exogenous abiotic elicitors are very important to improve the valuable formation of secondary metabolites in medicinal plants [45, 46].

4 Conclusion

This research aimed to see how different concentrations of SA and MeJa affected *M. oleifera* callus culture for enhancement production of active and medicinal constituents.

Generally, MeJa showed the highest flavonoids content compared to the SA. Thus, plant biotechnologies could improve *M. oleifera*'s potential for scavenging free radicals and boost human health by protecting it from oxidative stress.

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