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Physiological and Phytochemical Responses of Rosemary (*Rosmarinus officinalis* L.) Plant on *in vitro* Callus Formation

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Authors' contributions

This work was carried out in collaboration between all authors. Author MMEZ designed the study and performed the tissue culture work. Author GTMD performed the phytochemical analysis. Author IMAMS performed the statistical analysis, managed the analyses of the study results, the literature searches, wrote the first draft of the manuscript, corrected the manuscript and approved the final submission. All authors read and approved the final manuscript.

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ABSTRACT

Two experiments were carried out to determine the physiological and phytochemical responses in *Rosmarinus officinalis* on *in vitro* callus formation/induction using natural and synthetic auxin, different cytokinin types and concentrations, different phenylalanine concentrations and varying incubation periods. The first one was conducted to study the effect of different auxin types; IAA or

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NAA combined with cytokinins; BAP or TDZ. Second was done to examine the interaction between phenylalanine and incubation period. The results indicated that callus induction was completely inhibited in the absence of both auxin and cytokinin. Different ratios of auxin to cytokinin significantly affected the physiological callus responses. The treatment of $NAA_{0.125}$ + $BAP_{1.0}$ gave the highest callus induction and growth. Rosmarinic acid was slightly influenced growth regulators. Maximum callus fresh and dry weight and rosmarinic acid biosynthesis was recorded in (Phe_{150} + D_{42}) treatment. $Phe_{0.0}$ + D_{42} achieved the highest value of callus growth index (CGI), while the lowest CGI was found in the treatment of (Phe_{150} + D_{28}). The minimum value of relative growth rate (RGR) was resulted with (Phe_{150} + D_{56}), while ($Phe_{0.0}$ + D_{28}) achieved the best RGR.

Keywords: Rosmarinus officinalis; growth regulators; callus induction; phenylalanine; incubation period; rosmarinic acid.

1. INTRODUCTION

Rosemary (*Rosmarinus officinalis* L.) is one of the most important medicinal species of Lamiaceae family, endemic to the Mediterranean regions, but is now grown worldwide. It contains volatile oils, flavonoids, diterpenes, phenolic compounds and rosmarinic acid [1]. Plant tissue culture has been used as a biotechnological tool for the conservation and rapid micropropagation of medicinal plants and also for providing a source of secondary metabolites [2], also to overcome the limitations of extracting useful metabolites from limited natural resources, significant climatic variations, risks from pathogens and is independent of soil conditions [3]. Murashige & Skoog (MS) Medium [4] is very popular because most of the plants react to it favorably. Plant growth regulators are one of the most important factors affecting cell growth, differentiation, and metabolite production [5]. Type and concentration of plant growth regulators like auxins and cytokinins are known to be crucial components in callus induction and plant regeneration [6]. Although auxin and cytokinin were often regarded as an antagonist, their simultaneous use sometimes has a synergistic effect on physiological processes [7]. Callus induction depending upon the concentration and combination of hormones, but the choice of hormones is more important for callus induction than their concentrations [8]. Physiological responses of callus to the combination between auxin and cytokinin using different types and concentrations have been observed in many plants as *Lippia multiflora* [3], *Zingiber officinale* Roscoe [9], *Satureja avromanicais* [10], *Eustoma grandiflorum* [11], *Barringtonia racemosa* [12]. Rabie et al. [13] reported that a significant increment was achieved in callus growth of *Echinacea purpurea* with an increase in the incubation period. Bioconversion using an exogenous supply of biosynthetic precursor is believed to improve the accumulation of desired metabolite compounds [14]. Phenylalanine at 50, 100 and 150 mg/l showed a negative effect on both callus fresh and dry weight of *Zingiber officinale* Roscoe compared to control [9]. Rosmarinic acid ($C_{18}H_{16}O_8$) is a polyphenolic phytoconstituent found in many herbs of *lamiacea* species like rosemary, mint, thyme, basil, oregano. It exhibits a wide array of beneficial and pharmacological properties including antioxidant, anti-microbial and anti-inflammatory. The biosynthesis of rosmarinic acid starts with the amino acids phenylalanine and tyrosine. [15]. Rosmarinic acid concentration in rosemary leaves (3.3 µg/ml) was less than that in callus (3.7 µg/ml) by 12.12% [16]. The aim of the study was to determine the physiological and phytochemical responses in Rosmarinus officinalis on *in vitro* callus formation/induction using natural and synthetic auxin, different cytokinin types and concentrations, different phenylalanine concentrations and varying incubation periods.

2. MATERIALS AND METHODS

The present research was conducted at the Tissue Culture and Phytochemistry Labs. (Department of Medicinal Plants and Natural Products), Applied Research Center of Medicinal Plants (ARCMP), National Organization for Drug Control and Research (NODCAR) Giza government, Egypt during the period 2013-2016. Reagents and general chemicals (analytical grade) were purchased from either Sigma-Aldrich (Saint Louis, USA) or El-Gomhouria (Cairo, Egypt).

2.1 Plant Materials

Terminal cuttings of *Rosmarinus officinalis* L. (6 months old) were collected from growing plants

in the field of the Applied Research Center of Medicinal Plants (ARCMP). They were planted in controlled greenhouse at 27 ± 1℃ for three months (stock plants, Fig. 1). Shoot tips of stock plants were used as explants for all experiments because it possesses several axillary buds, has more survival chances and grows rapidly (George and Sherrington [17] and Rasool et al. [18].

2.2 Explants Preparation and Sterilization

Shoot tips of 0.8 -1.0 cm length were used as a source of explants in this study. These explants were kept in antioxidant solution (100 mg/l ascorbic acid + 100 mg/l citric acid + 100 mg/l polyvinyl pyrrolidone) for 2 hours to avoid browning that caused by oxidation of polyphenolic substances during start of culture, and washed several times with tap water. Then the explants were rinsed with a small amount of liquid soap for 5 minutes, and rinsed again under running tap water for 30 minutes to remove all the remaining detergent. All the steps of the sterilization were done under complete aseptic conditions in the laminar air flow. Explants were immersed in 95% ethanol for 2 Sec; surface disinfected with 0.1% mercuric chloride ($HgCl_2$) solution for 3-5 minutes. After surface sterilization, approximately 2 mm was removed from the cut ends of the explants, and they were washed three times with autoclaved distilled water for 5 min duration each.

2.3 Medium Preparation

Shoot tips (explants) were cultured on modified MS medium [4] supplemented with 200 mg/l KNO_3, 1000 mg/l NH_4NO_3, 300 mg/l $CaCl_2.2H_2O$, 200 mg/l $MgSO_4.7H_2O$, 80 mg/l KH_2PO_4, 16.7 mg/l $FeSO_4.7H_2O$, 22.4 mg/l Na_2-EDTA, 10-50 mg/l adenine sulfate, 50 mg/l malt extract and 30g/l sucrose and solidified by 0.5% agar. The pH value was adjusted to 5.7-5.8 by adding a suitable amount of 0.1N HCL and 0.1N KOH by using the pH meter. The culture medium was distributed into culture 300 ml jars, and each jar contained a 35 ml medium. The culture jars were immediately capped with polypropylene closures, and they were autoclaved at 121℃ and 1.2 Kg/cm^2 for 20 min [19].

2.4 Design of Experiments

Two *in vitro* experiments were established to examine the physiological callus response and accumulation of rosmarinic acid as influenced by natural and synthetic auxin, cytokinin concentration, phenylalanine and incubation period of *Rosmarinus officinalis* L.

2.4.1 Experiment 1

Two types of cytokinin (BAP or TDZ) with different concentrations (0.125, 0.250, 0.500 and 1.000 mg/l) were combined with two sources of auxin (natural; IAA or its synthetic derivative; NAA). The shoot tip explants were immediately cultured in a sterile jars 300 ml containing 35 ml of modified MS supplemented with either natural auxin; indole-3-acitic acid (IAA) or synthetic auxin naphthaleneacetic acid (NAA) at constant concentration (0.125 mg/l) combined with cytokinins 6-benzylaminopurine (BAP) or 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ) at different concentrations (0.125, 0.250, 0.500 and 1.000 mg/l). The first experiment consisted of 17 treatments. The experiment was replicated five times. The culture jars were directly plugged with polypropylene closure cabs and incubated in a growth room at 27 ± 1℃ under light condition 1 Lux light using cool white fluorescent lamps under a light/dark cycle of 14/10 h. The cultures were periodically examined and visually observed for necrosis, bacterial and fungal contamination. Cultures were observed daily for eight weeks to record data.

2.4.2 Experiment 2

Different concentrations of phenylalanine (0.0, 50, 100, 150 and 200 mg/l) were combined with the incubation periods (14, 28, 42 and 56 days) to examine the influence of them on callus production (callus fresh and dry weight, g/jar) and the rosmarinic acid accumulation (% dry weight). The best treatment of the first experiment (modified MS medium supplemented with 0.125 mg/l NAA combined with 1.0 mg/l BAP) was used as the basal medium in all the cultures in the second experiment. The second experiment was contained 20 treatments. Ten explants were placed on the medium in every jar (0.80 - 0.85 mg/ jar explants), and the jars were replicated five times per treatment. The culture jars were incubated in the growth room at 27 ± 1℃ under light condition of 1 flux light using cool white fluorescent lamps for daylight period (14 h light: 10 h dark). The cultures were periodically examined and visually observed for necrosis, bacterial and fungal contamination. Cultures were observed every two weeks for eight weeks to record data.

2.5 Measurements

2.5.1 Physiological responses of callus

2.5.1.1 Callus induction (%)

The percentages of callus induction were calculated according to Holme and Petersen [20] using the following equation:

$$Calus\ induction\ (\%) = \left[\frac{Number\ of\ explants\ that\ produced\ calli}{Total\ number\ of\ used\ explants}\right] \mathrm{X}\ 10^2 \quad (1)$$

2.5.1.2 Callus size (degree)

The formed callus size was expressed in scores according to the method described by Pattino [21] as follows:

Dead explants (no growth) = 1, Size degree below average = 2, Medium sized of callus = 3, Size above average growth of callus = 4 and Maximum callus growth = 5

2.5.1.3 Callus fresh weight (g /jar)

After eight weeks of explant culture, callus fresh weight was calculated by the difference between the weight of the culture jar at the end of and at the beginning of the experiment (g/jar), determined with a sensitive balance.

2.5.1.4 Callus dry weight (g /jar)

Callus was removed from the culture medium and washed with tap water for 2 min to remove any traces of culture medium and dried with tissue paper at 25 ± 2℃ until constant then the dry weight was obtained.

2.5.1.5 Callus growth index (CGI)

Callus growth index was expressed as a percentage of the increase in fresh weight calculated according to Khater et al. [22] and Dung et al. [23] using the following equation:

$$CGI = \left[\frac{W_2 - W_1}{W_1}\right] \times 10^2 \quad (2)$$

2.5.1.6 Relative growth rate (RGR)

Relative growth rates (RGR; mass increase per unit time and mass) of callus based on a fresh weight were calculated according to Galiba et al. [24] using the following formula:

$$RGR = \left[\frac{ln\, W_2 - ln\, W_1}{t_2 - t_1}\right] \times 10^3 \quad (3)$$

Where

ln is the natural logarithm
W_1 is the initial weight of callus (at t_1 time) after two weeks (14 days),
W_2 is the final weight of callus (at t_2 time) after D_4, 28 days; D_6, 42 days and D_8, 56 days.
t_1 is the initial growth time (D_2, 14 days),
t_2 is the final growth time (D_4, 28 days; D_6, 42 days and D_8, 56 days)

2.6 Phytochemical Responses of Callus

Identification of rosmarinic acid by Thin Layer Chromatography (TLC) and determination of total hydroxycinamic derivatives as the rosmarinic acid was done according to British Pharmacopoeia [25]. The following equation was used to calculate total hydroxycinamic derivatives as rosmarinic acid.

$$Total\ hydroxycinamic\ \% = \left[\frac{A \times 2.5}{M}\right] \quad (4)$$

i.e., taking the specific absorbance of rosmarinic acid to be 400 at 505 nm.

where:

A, is the absorbance at 505 nm,
M, mass of test substance to be examined in grams.

2.7 Statistical Analysis

Experiments were based on a complete randomized design with five replicates per treatment. One-way (first experiment) analysis of variance (ANOVA) was used to test the significance of the effects of plant growth regulators, while two-way (second experiment) analysis of variance (ANOVA) was used to test the significance of the effects of phenylalanine and incubation period. All data of experiments were statistically analyzed using COSTAT 6311Win and the mean values were compared using the L.S.D method at 5% level of significance according to Gomez and Gomez [26].

3. RESULTS AND DISCUSSION

3.1 First Experiment

3.1.1 Physiological callus responses

3.1.1.1 Effect of the combination between auxins (IAA or NAA) and cytokinins (BAP or TDZ) on callus induction, callus fresh and dry weight and callus size of Rosmarinus officinalis L.

Table 1 shows the results of physiological callus responses to the combination between auxins (IAA or NAA) and cytokinins (BAP or TDZ). It was observed that the induction of callus was completely inhibited in the absence of both auxin and cytokinin (control) after eight weeks of culture. It was also shown that the ability to perform callogenesis (Fig. 1) varied depending on the type of auxins and cytokinins as well as the ratio of auxin to cytokinin. The combination between auxins (IAA or NAA) and cytokinins (BAP or TDZ) in different ratios of auxin to cytokinin (1:1, 1:2, 1:4 and 1:8) were significantly affected the physiological callus responses (callus induction, callus fresh weight, callus dry weight and callus size). In this concern, these results were in harmony with AL Masoody and Stanica [27] who reported that Rosemary callus growth expressed by callus volume, callus fresh and dry weight was significantly influenced by the combination of BA and NAA.

Also, in agreement with Arivalagan et al. [28] where they noted that there was a significant difference between the growth regulator concentrations in inducing callus of *Sauropus androgynous* (Sweet shoot). Neibaur et al. [6] reported that type and concentration of plant growth regulators like auxins and cytokinins are known to be crucial components in callus induction and plant regeneration.

The group-3 of treatments (Table 1) which contained modified MS supplemented with NAA at 0.125 mg/l + BAP at all its concentrations gave the best results of the physiological callus responses compared to the other treatments. Rasool et al. [18] found that the combined response of auxins and cytokinins increased the frequency of multiplication and resulted in callus formation. In this respect, by increasing the concentrations of BAP up to 1.0 mg/l, the physiological callus parameters increased.

Table 1. Effect of modified MS supplemented with auxin (natural, IAA or synthetic, NAA) and cytokinin (BAP or TDZ) on physiological callus responses (callus induction, callus fresh weight, callus dry weight and callus size)

Combination group	Modified MS supplemented with Auxin + Cytokinin, mg/l	Ratio of auxin to cytokinin	Physiological callus responses			
			Callus induction, %	Callus fresh weight, g/jar	Callus dry weight, g/jar	Callus size, degree
Control		----	00.00	00.00	00.000	1.00
Group 1	$IAA_{0.125} + BAP_{0.125}$	1:1	15.11	0.411	0.0457	1.68
	$IAA_{0.125} + BAP_{0.250}$	1:2	16.18	0.485	0.0539	1.88
	$IAA_{0.125} + BAP_{0.500}$	1:4	5.00	0.055	0.0012	1.14
	$IAA_{0.125} + BAP_{1.000}$	1:8	5.22	0.058	0.0013	1.15
Group 2	$IAA_{0.125} + TDZ_{0.125}$	1:1	2.00	0.021	0.0009	1.05
	$IAA_{0.125} + TDZ_{0.250}$	1:2	15.11	0.432	0.0041	1.65
	$IAA_{0.125} + TDZ_{0.500}$	1:4	13.33	0.405	0.0039	1.55
	$IAA_{0.125} + TDZ_{1.000}$	1:8	4.01	0.051	0.0011	1.09
Group 3	$NAA_{0.125} + BAP_{0.125}$	1:1	37.78	0.955	0.1026	2.75
	$NAA_{0.125} + BAP_{0.250}$	1:2	76.67	2.134	0.2361	4.28
	$NAA_{0.125} + BAP_{0.500}$	1:4	87.77	2.388	0.2653	4.89
	$NAA_{0.125} + BAP_{1.000}$	1:8	95.89	2.505	0.2683	5.00
Group 4	$NAA_{0.125} + TDZ_{0.125}$	1:1	37.11	0.872	0.0969	2.85
	$NAA_{0.125} + TDZ_{0.250}$	1:2	28.44	0.791	0.0879	2.43
	$NAA_{0.125} + TDZ_{0.500}$	1:4	51.55	1.237	0.1374	3.32
	$NAA_{0.125} + TDZ_{1.000}$	1:8	5.22	0.077	0.0015	1.11
LSD at 5%			----	0.103	0.0023	0.12

Fig. 1. Stock plants (rosemary) growing in the controlled greenhouse for three months

Fig. 2. Examples of callus formation from rosemary (shoot tips) in tissue culture laboratory

The ratio of NAA to BAP (1:8) gave the highest callus induction (95.89%), callus fresh weight (2.505 g/jar), callus dry weight (0.2683 g/jar) and callus size (5.0 degree) followed by ($NAA_{0.125}$ + $BAP_{0.50}$) and ($NAA_{0.125}$ + $BAP_{0.125}$). While the ratio of auxin to cytokinin that achieved the best of physiological callus parameters was (1:4) when TDZ was used instead of BAP (group-4).

In the case of using natural auxin IAA instead of NAA, the best results of all physiological callus response parameters were obtained with the ratio of (1:2) auxin to cytokinin with both cytokinins TDZ and BAP (group-1 and group-2).

These results may be due to the synergistic effect of auxin (NAA) with cytokinin (BAP) on callus formation with ratios (1:2, 1:4 and 1:8) of NAA to BAP. This synergistic effect turned to antagonism effect at the ratio (1:1) of NAA to BAP. The synergistic influence between natural auxin (IAA) and both cytokinins (BAP and TDZ) was evidently weaker than the synergistic influence between synthetic (NAA) and cytokinin (BAP). The most commonly used synthetic plant growth regulators with high auxin activity are NAA [29]. From these results, it became clear superiority of synthetic growth regulator (1-naphthaleneacetic acid, NAA) compared to natural (indole-3-acetic acid, IAA). This may be due to the replacement of the naphthyl (NAA) group with an indole (IAA) group enhance the physiological activity in the rosemary callus culture (Fig. 3).

The obtained results correspond to those obtained by Al Kaabi et al. [30] who found that NAA at every tested level was more effective than IAA at any tested concentration, regarding inducing the cultured explants to form roots. This was also in harmony with Aghaei et al. [31] in which they found that treatments with NAA achieved the best callus weight and growth rate of *Pistacia atlantica* plant. Seyyedyousefi et al. [32] mentioned that the medium containing 0.5 mg/l BAP combined with 1.0 mg/l NAA induced more callus formation (35.50%) on the explants of *Alstroemeria cv. Fuego*. The synergistic effect of auxins with cytokinins in callus induction was found to be greater in NAA [32]. Synthetic auxin (NAA) act by increasing the endogenous IAA concentrations either by promoting new synthesis or by inhibiting IAA conjugation or breakdown [33]. Moreover, NAA stimulates cell elongation at concentrations that were much lower than those required to stimulate cell division [34]. Also, from data in Table1 it highlighted that the effect of BAP (6-benzylaminopurine) was greater than TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea).

The lowest responses regarding callus induction (2.0%), callus fresh weight (0.021 g/jar), callus dry weight (0.0009 g/jar) and callus size (1.05 degree) were expressed on the modified MS medium supplemented with ($IAA_{0.125}$ + $TDZ_{0.125}$). The effect of auxin and cytokinin on cell induction and growth may be due to that hormonal regulation of auxin and cytokinin balance is a key factor in the control of cell division in tissue culture [35]. In the same context, auxin and cytokinin regulate cell division synergistically in callus cells and protoplasts as well as control of cell-cycle progression [36,37]. Auxin and cytokinin are considered to be key factors in controlling cell cycle progression in plants. This is achieved by regulating the expression and the activity of the Cyclin-Dependent Kinases (CDK) and mitotic cyclins. Both auxin and cytokinin were able to induce gene transcription of the CDK_s *cdc2aAt* in suspension-cultured cells [38,39]. It is also suggested that plant growth regulators can modify the synthesis of antioxidants and the activity of basic antioxidant enzymes, and some of these

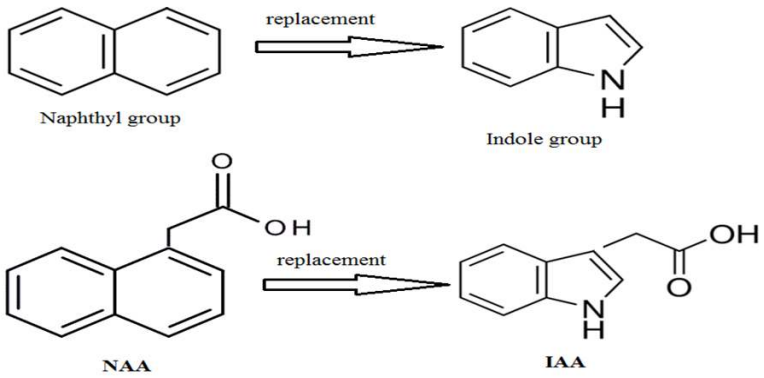

Fig. 3. Chemical structure of naphthyl group, indole group, NAA and IAA

enzymes are also implicated in phytohormone catabolism [40]. Auxin and H_2O_2 possess antagonistic effects on cell cycle progression and gene activation [41]. Auxins also, promote increases in the activity of antioxidant enzymes regulating [42,43]. Moreover, all auxins stimulated enzymatic (ascorbate peroxidase, catalase, superoxide dismutase) and non-enzymatic antioxidant (ascorbate, glutathione) systems in *Chlorella vulgaris*, and therefore, suppressed lipid peroxidation and hydrogen peroxide accumulation [44].

3.1.2 Phytochemical responses of callus

3.1.2.1 Identification of rosmarinic acid

Data shown in Table 2 indicated the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. It was identified rosmarinic acid (Fig. 4) appeared as an intense light blue fluorescent zone in the test solution.

3.1.2.2 Effect of the combination between auxins (IAA or NAA) and cytokinins (BAP or TDZ) on total hydroxycinamic derivatives as rosmarinic acid in Rosmarinus officinalis callus

The treatments that achieved the best results of physiological callus responses ($NAA_{0.125}$ + $BAP_{0.250}$), ($NAA_{0.125}$ + $BAP_{0.500}$) and ($NAA_{0.125}$ + $BAP_{1.000}$) were selected as well as shoot tip explant (control) to determinate the accumulation of total hydroxycinamic derivatives as the rosmarinic acid accumulation of air dried callus. The obtained results were illustrated in Fig. 5, it was indicated that there was a slightly positive significant effect of the modified MS supplemented with NAA and BAP on rosmarinic acid accumulation, especially with BAP (at 0.5 and 1.0 mg/l) compared with shoot tip explant (control).

The highest rosmarinic acid production (0.487% based on dry weight, DW) was obtained with $NAA_{0.125}$ + $BAP_{0.500}$. This result is in agreement with [45] who found that rosmarinic acid was readily accumulated in undifferentiated plant cell cultures.

Table 2. Identification of rosmarinic acid by TLC plate under UV at 365 nm

Top of the plate	
Caffeic acid: a light blue fluorescent zone	A pink fluorescent zone
Rosmarinic acid: a light blue fluorescent zone	A blue fluorescent zone of low intensity
Reference solution	An intense light blue fluorescent zone
	Test solution

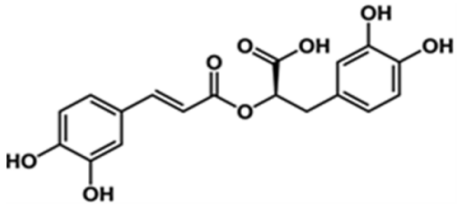

Fig. 4. Chemical structure of rosmarinic acid

They also found that some concentrations of rosmarinic acid were much higher than that in the plant itself. The findings in Fig. 5 revealed that there was no significant difference between the modified MS supplemented with ($NAA_{0.125}$ + $BAP_{0.\ 500}$) and ($NAA_{0.125}$ + $BAP_{1.00}$) treatments, also between ($NAA_{0.125}$ + $BAP_{0.250}$) and control. Results in Fig. 5 and Table 1 showed that cytokinin (BAP) promoted cell division or cytokinesis and then callus formation more than rosmarinic acid biosynthesis.

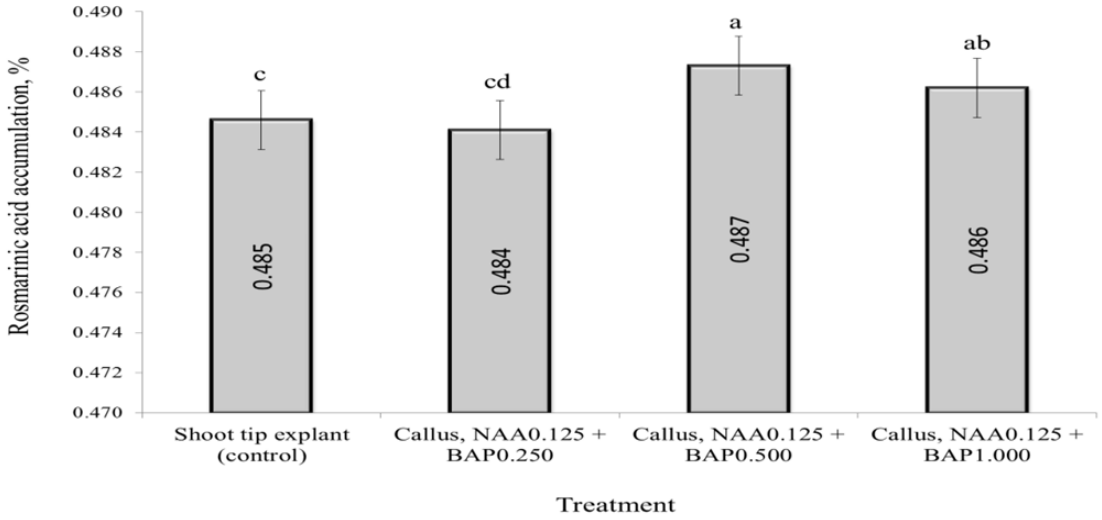

Fig. 5. Effect of modified MS supplemented with auxin (NAA) and cytokine (BAP) on rosmarinic acid accumulation (% based on dry weight) in callus compared with shoot tip explant (control)

3.2 Second Experiment

3.2.1 Physiological callus responses

3.2.1.1 Callus fresh and dry weight

Figs. 6 and 7 demonstrated the effect of phenylalanine concentration, incubation period and the interaction between them on callus fresh and dry weight of *Rosmarinus officinalis*. The results showed that the physiological callus response callus regarding callus fresh and dry matter accumulation was significantly affected by phenylalanine concentration, incubation period and the interaction between them. Callus weight (fresh and dry) was increased proportionally with an increase in phenylalanine level up to 150 mg/l, while with more than this concentration callus weight was reduced. It was also revealed that $Phe_{0.0}$ (medium without phenylalanine) gave the lowest mean of callus weight compared to the other phenylalanine concentrations, while the highest callus weight was observed at 150 mg/l phenylalanine. The results in Figs. 6 and 7 observed that incubation period affected significantly on callus (fresh and dry) weight.

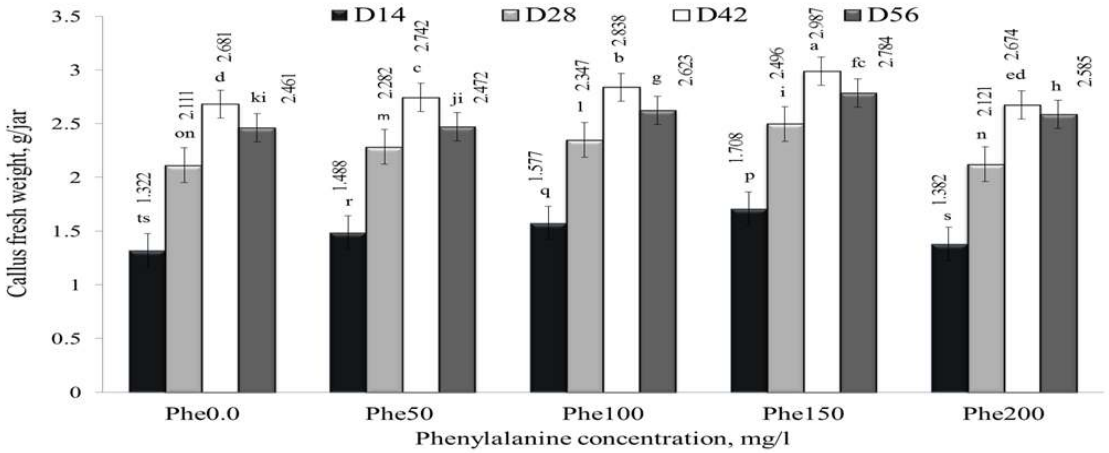

Fig. 6. Effects of the interaction between phenylalanine concentration and incubation period on the fresh weight of *Rosmarinus officinalis* callus culture in the best modified MS medium (with $NAA_{0.125}$ + $BAP_{1.000}$) incubated at 27 ± 1℃

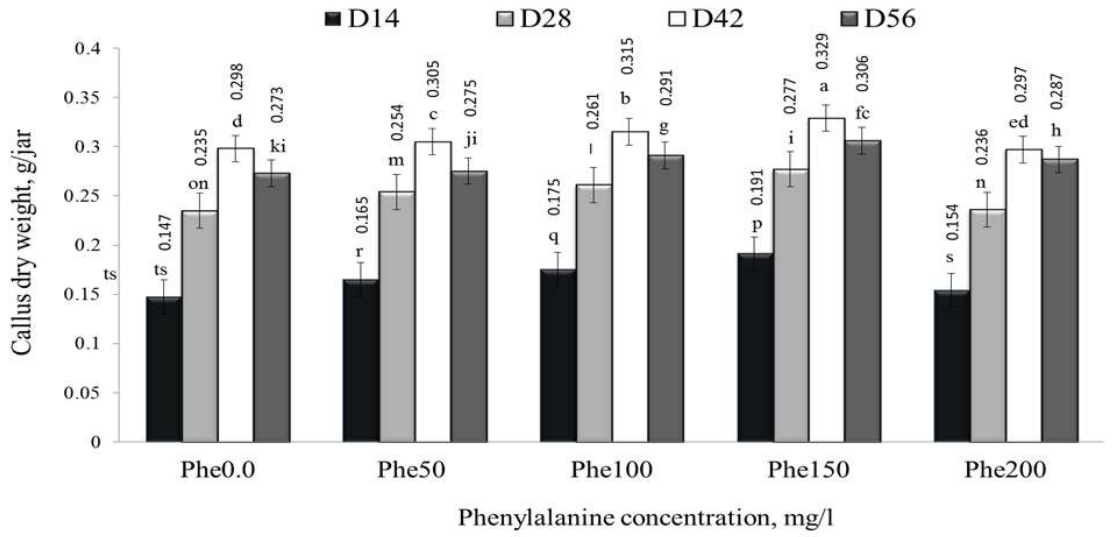

Fig. 7. Effects of the interaction between phenylalanine concentration and incubation period on callus dry weight of *Rosmarinus officinalis* callus culture in the best modified MS medium (with $NAA_{0.125}$ + $BAP_{1.000}$) incubated at 27 ± 1℃

Callus weight increased with an incubation period up to 42 days (D_{42}) which this period of incubation gave the maximum result of callus weight, while the period of incubation, 14 days (D_{14}) achieved the minimum call our weight. The effect of interaction between phenylalanine and incubation period on callus (fresh and dry) weight of *Rosmarinus officinalis* observed a wide range of variation in average weight of callus. It was appeared that the interaction between phenylalanine at all concentrations (0.0, 50, 100, 150 and 200 mg/l) and the incubation period of 42 days (D_{42}) promoted and gave the optimal callus weight. In contrast, the minimum of physiological callus response was achieved with medium supplemented with phenylalanine at all its concentrations with 14 days incubation period (D_{14}). The best callus growth regarding fresh and dry weight was recorded in the treatments of interaction between phenylalanine concentration and incubation period, namely, (Phe_{150} + D_{42}) followed by (Phe_{100} + D_{42}), (Phe_{150} + D_{56}) and (Phe_{50} + D_{42}). The explants cultured on medium without amino acid for two weeks (14 days) incubation period (Phe0.0 + D14) gave the lowest callus fresh and dry weight (1.322 g/jar and 0.147 g/jar) respectively, compared with the other treatments. On the other hand, there was no significant difference between every pair of the following combination treatments; ($Phe_{0.0}$ + D_{14}) and (Phe_{200} + D_{14}), ($Phe_{0.0}$ + D_{28}) and (Phe_{200} + D_{28}), (Phe_{150} + D_{56}) and (Phe_{50} + D_{42}), (Phe_{200} + D_{42}) and ($Phe_{0.0}$ + D_{42}) regarding callus weight.

The results were in agreement with Urmantsva et al. [46] who found that none of the tested amino acids enhanced biomass production in cell cultures of *Thalictrum minus*. A similar observation was found by El-Nabarawy et al. [9] who found that phenylalanine up to 100 mg/l (Phe100) had a significant effect on the callus growth of *Zingiber officinale*. The obtained results also in agreement with Bosila et al. [47] who reported that 10 and 50 mg/l of phenylalanine recorded a significant increasing value of *Hyoscyamus muticus* callus weight, while the higher levels (100 and 200 mg/l) recorded very weak values in callus fresh weight. Rabie et al. [13] found that all phenylalanine concentrations significantly increased callus fresh weight of *Silybum marianum* over the control. Amino acids are fundamental ingredients in the process of Protein Synthesis. Many studies have verified that amino acids can directly or indirectly control the physiological actions of the plant [48]. Amino acids provide plant cells with a source of organic nitrogen that is easily assimilated by the tissues and cells that faster than inorganic nitrogen sources [49].

3.2.1.2 Callus growth index (CGI, %)

The results of callus growth index (CGI, %) were observed in Fig. 8. It was shown that CGI decreased gradually with an increase in the phenylalanine concentration up to 150 mg/l.

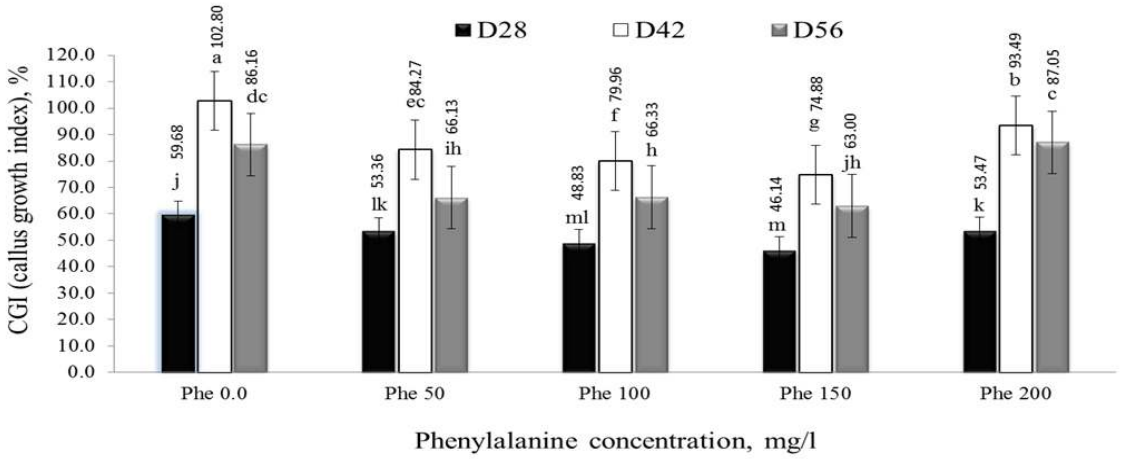

Fig. 8. Effects of the interaction between phenylalanine concentration and incubation period on CGI of *Rosmarinus officinalis* callus culture in the best modified MS medium (with $NAA_{0.125}$ + $BAP_{1.000}$) incubated at 27 ± 1℃

This trend may be due to increasing both the initial weight of callus after two weeks (W_1) and the final weight of callus after 28 days and 42 days (W_2) with phenylalanine concentration (equation, 2), but also phenylalanine enhanced callus fresh weight formation in W_1 (at the age of 14 days old) more than in W_2 (at the age of 28, 42 and 56 days old). Data in Fig. 8 also showed that callus growth index was increased with increasing the incubation period up to D_{42}.

The results of overlap between different phenylalanine concentrations and incubation period revealed that there were diverse impacts on the callus growth index. It also appeared that the shoot tip explants were cultured in medium without phenylalanine amino acid and were incubated for 42 days; $Phe_{0.0}$ + D_{42} achieved the highest value of CGI (102.80%), while the lowest CGI (46.14%) was found in the treatment of (Phe_{150} + D_{28}). In this respect, the effect of phenylalanine on CGI was in contrast to Kawthar et al. [50] in which they found that phenylalanine concentrations significantly increased callus growth index of *Silybum marianum* up to 10 mg/l, while the rest of treatments decreased callus growth index. However, these results were in agreement with the same researchers regarding the impact of incubation period on CGI.

These results were in contrast with Rabie et al. [13], who reported that all phenylalanine concentrations (10, 20, 30 and 40 mg/l) increased significantly callus growth index of *Silybum marianum* over the control, also with Mathur and Goswami [51] who investigated the effect of the interaction between various concentrations of β-phenylalanine (25, 50, 75 and 100 mg/100 ml) and callus age (2, 4, 6, 8, 10 and 12 weeks) on callus growth index of *Maytenus emarginata* and found that GI of callus was increased with an increase phenylalanine concentration. On the other hand, our result in agreement with Rabie et al. [13] with *Silybum marianum*, Mathur and Goswami [51] with *Maytenus emarginata*, where they found that CGI was increased with the incubation period.

3.2.1.3 Relative growth rate (RGR)

Relative growth rate (RGR) is the fundamental parameter, which provides one of the most ecologically significant and useful indices of plant growth [52]. Fig. 9 presents the results of the effect of phenylalanine concentration, incubation period and the interaction between them on this parameter of *Rosmarinus officinalis* callus.

Data in this figure showed that RGR was significantly influenced by the overlap between various concentrations of phenylalanine and incubation periods. RGR of callus as affected by different concentrations of amino acid phenylalanine differed completely from that of callus fresh weight. It was decreased gradually with the phenylalanine concentration in the culture medium up to 150 mg/l (Phe_{150}). The highest RGR was obtained by using culture medium without phenylalanine ($Phe_{0.0}$). On the other hand, relative growth rate (RGR) was decreased with the incubation period. The maximum RGR was recorded with 28 days of the

incubation period (D_{28}), while incubation period of 56 days (D_{56}) gave the minimum value of relative growth rate. The interaction between phenylalanine concentration and incubation period (Phe_{150} + D_{56}) stated the lowest value of RGR (11.63 mg/g.day), while the treatment of ($Phe_{0.0}$ + D_{28}) achieved the best result of relative growth rate (33.43 mg/g.day). It was recorded that there was no significant difference between the following treatments (Phe_{50} + D_{56} and Phe_{100} + D_{56}), ($Phe_{0.0}$ + D_{56} and Phe_{200} + D_{56}) and (Phe_{50} + D_{28} and Phe_{200} + D_{28}).

3.2.2 Phytochemical responses of callus

Fig. 10 shows the effect of phenylalanine concentration, incubation period and the combination between them on total hydroxycinamic derivatives as the rosmarinic acid in *Rosmarinus officinalis* callus.

Results in this figure showed that there are significant differences between different levels of phenylalanine, the incubation period and their interaction for an accumulation of rosmarinic acid. According to the results presented in the Fig. 10, all treatments of phenylalanine concentrations caused enhancement in rosmarinic acid biosynthesis compared with non-supplemented callus with phenylalanine ($Phe_{0.0}$). It was also observed that increasing the phenylalanine level up to 50 mg/l (Phe_{50}) led to rising the content of rosmarinic acid, which gave the highest accumulation of rosmarinic acid. The findings were in harmony with Nabila et al. [53] who investigated the effect of phenylalanine amino acid on *Salvia officinalis* cell culture, and they found that phenylalanine enhanced the rosmarinic acid accumulation in its callus. Also Hakkim et al. [54] found that the addition of phenylalanine into agar medium improved in rosmarinic acid yield in *Ocimum sanctum* cell cultures. Data also showed that the accumulation of rosmarinic acid significant gradually increased with an incubation period up to D_{42} (42 days). The lowest rosmarinic acid content was observed in 14 days incubation period (D_{14}). These results were in harmony with that obtained by Hakkim et al. [54] who found that the content of rosmarinic acid gradually increased with incubation period 3, 6, 9, 12, and 15 days after phenylalanine is treated. From the interaction between phenylalanine concentrations and incubation periods, there was a real significant effect of these interactions on rosmarinic acid (Fig. 10).

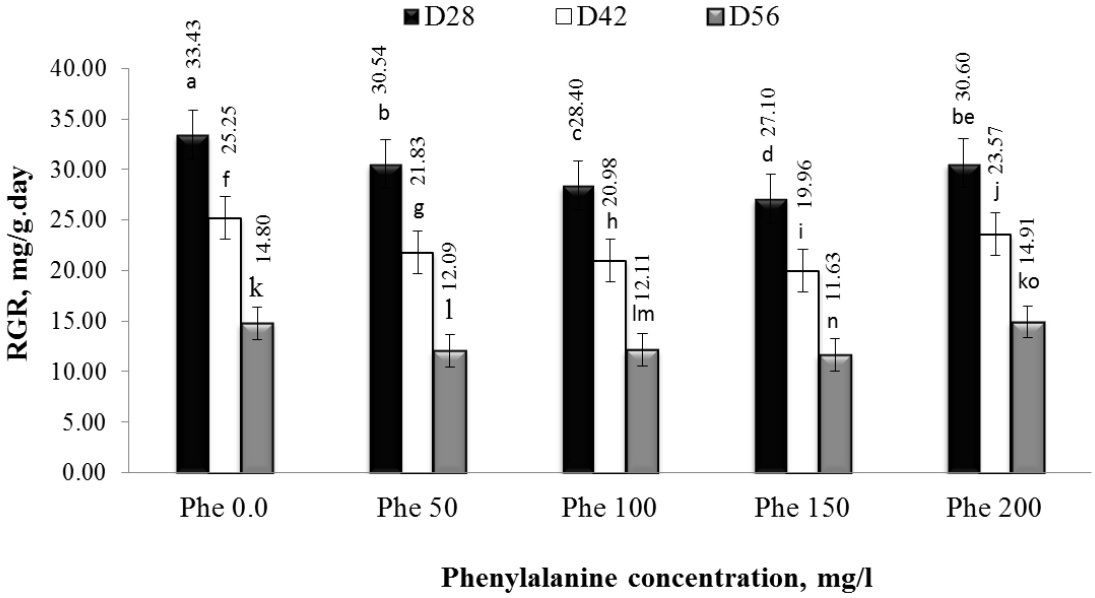

Fig. 9. Effects of the interaction between phenylalanine concentration and incubation period on RGR of *Rosmarinus officinalis* callus culture in the best modified MS medium (with $NAA_{0.125}$ + $BAP_{1.000}$) incubated at 27 ± 1℃

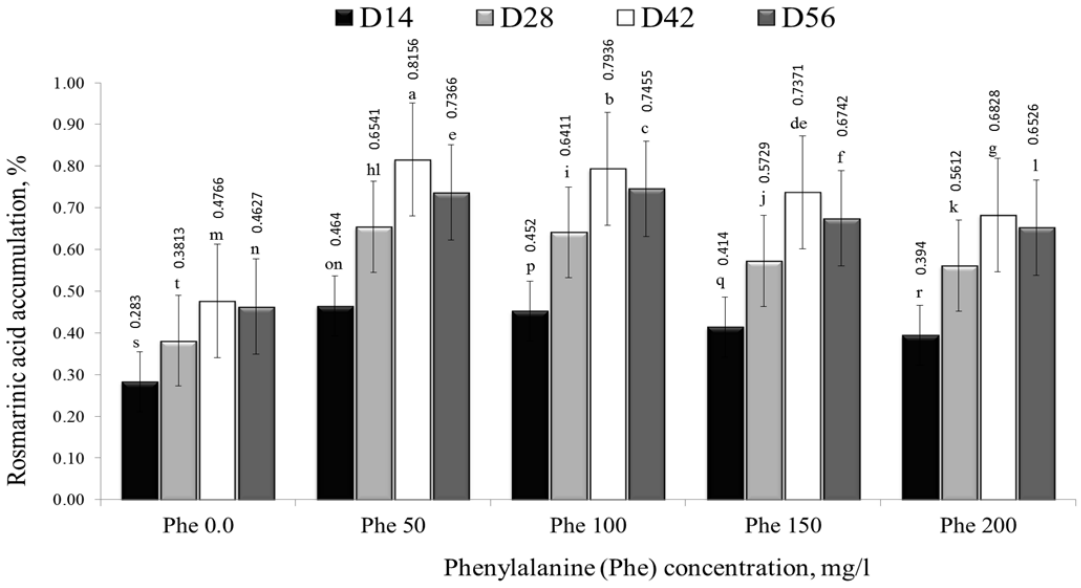

Fig. 10. Effects of the interaction between phenylalanine concentration and incubation period on rosmarinic acid accumulation of *Rosmarinus officinalis* callus culture in the best modified MS medium (with $NAA_{0.125}$ + $BAP_{1.000}$) incubated at 27 ± 1℃

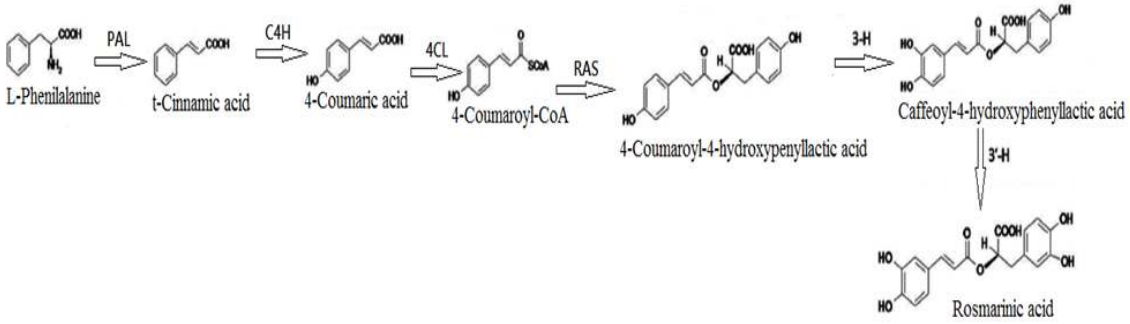

Fig. 11. The biosynthetic pathway leading to rosmarinic acid with some side reactions. PAL phenylalanine ammonia lyase, C4H cinnamic acid 4-hydroxylase, 4CL 4-coumarate: CoAligase, RAS rosmarinic acid synthase

The results showed that there is wide variation in rosmarinic acid (%) as affecting by the interaction between phenylalanine concentrations and incubation periods. The highest biosynthesis of rosmarinic acid was obtained when we cultured the shoot tip explant on medium supplemented with 50 mg/l (Phe_{50}) of phenylalanine incubated for 42 days (D_{42}). In this respect, it was observed that this treatment (Phe_{50} + D_{42}) produced the amount of rosmarinic acid (0.8156% DW) more than that in shoot tip explant (0.485% DW) by 68.25%.

The results are in harmony with that obtained by Park et al. [15] who reported that Plant cell cultures, e.g. from *Coleus blumei* or *Salvia officinalis*, accumulate rosmarinic acid in amounts much higher than in the plant itself (up to 36% of the cell dry weight). While the treatment of ($Phe_{0.0}$ + D_{14}) gave the lowest accumulation of rosmarinic acid (0.2833% DW). On the other hand, there was no significant difference between ($Phe_{0.0}$ + D_{56} and Phe_{50} + D_{14}), also between (Phe_{50} + D_{28} and Phe_{200} + D_{56}), and (Phe_{150} + D_{42} and Phe_{50} + D_{56}). The positive effect of phenylalanine on the biosynthesis of rosmarinic acid may be due to the role of phenylpropanoid pathway that is one of the most important secondary metabolism pathways of plants, which yields a variety of

phenolics with different structural and defense-related functions [55,48]. Al-Jibouri et al. [56] reported that the addition of different concentrations of amino acids as a precursor adding separately to the tissue culture medium led to raising the accumulation levels of phenolic compounds in callus tissue. Phenylalanine is the substrate of phenylalanine ammonia-lyase (PAL) that catalyzes the reductive de-amination of L-phenylalanine into trans-cinnamic acid as the first step of the biosynthesis of plant phenolic compounds [57]. Rosmarinic acid is a phenolic compound which is found in many genera of Labiatae and exhibits important biological activities [58]. Phenylalanine plays a vital role (precursor or starter) in the biosynthesis of rosmarinic acid [59,15]. Petersen et al. [60] suggested that the phenylpropanoid pathway is involved in the biosynthesis of rosmarinic acid in plants (Fig. 11).

4. CONCLUSION

Results obtained in the present study indicated that callus induction was completely inhibited in the absence of both auxin and cytokinin. The combination between auxins and cytokinins in different ratios of auxin to cytokinin was significantly affected the physiological callus responses. Also, modified MS supplemented with plant growth regulators auxins and cytokinins especially NAA and BAP gave rosmarinic acid content almost equal to that obtained from shoot tip explants, while addition of phenylalanine (50 mg/l) to this medium and incubation for 42 days (D_{42}) achieved a positive increasing of rosmarinic acid content more than that in shoot tip explant by 37.64 and 44.56%, respectively. The interaction between phenylalanine and incubation period (Phe_{50} + D_{42}) showed a really positive significant increasing of rosmarinic acid content; it was 68.25% more than that in shoot tip explant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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