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In-Vitro Investigation for Improving Secondary Metabolites in *Origanum Vulgare* Plants Using Tissue Culture Technique at Taif Governorate, KSA.

Darwesh Hadeer YA[1,2]*, Nour El-Deen AH[3,4], and Fayad Eman M[1].

[1]Biotechnology Dept., Faculty of Science, Taif University, Saudi Arabia.
[2]Medicinal and Aromatic Plants Institute, Agriculture Research Center, Egypt.
[3]Biology Dept., Faculty of Science, Taif University, Saudi Arabia.
[4]Nematology Research Unit, Agricultural Zoology Dept., Faculty of Agriculture, Mansoura University, Egypt.

ABSTRACT

The main goal of this experiment has been aimed to achieve a method for improving secondary metabolites such as phenolics beside photosynthetic pigments content of *Origanum vulgare* (family: Lamiaceae) callus plants. This investigation has been studied the usage of two bioelicitors as thiamine and coconut water. Our results revealed that the different concentrations of thiamine and coconut milk affected callus fresh weight (g) of *O. vulgare*. Both treatments increased callus fresh weight than the control. They are also affecting photosynthesis pigments but, the increment in case of (coconut milk) almost was not significant compared with thiamine which absolutely increased fresh and dry weight, total phenolics and photosynthetic pigments content by the level of 0.5 g/L.

Keywords: *In vitro*, callus, thiamine, coconut milk, *Origanum vulgare*

Corresponding author

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INTRODUCTION

The attention of several investigators since the beginning of this century, and become of important value only during recent decades. They are now established as potent research tool having a lot of values: The most important one of them is tissue cultures can be used for feeding experiments in conjunction with label compounds, and are also useful for the determination of the site of synthesis of particular compounds. Accordingly, the authors thought that the applications of this branch of science on medicinal plants are of great [1]. They grow on limestone and calcareous rocks and slopes need very little water for their growth and maintenance. Sweet scent from leaves is admired since long times and the intensely aromatic leaves are prized to make their great potential for use in urban landscaping and as ornamental border plant in rock gardens. It is also used as aromatic plants, since ancient times for their preservative and medicinal attributes, as well as to impart flavor to foods [2].

Origanum vulgare L. is a member of the Lamiaceae family (Labiatea), commonly named oregano, wild marjoram, marzanjosh or mardaqoush, which grows abundantly on stony slopes and in rocky mountain areas at a wide range of altitude [3]. The *Origanum* species, which are rich in essential oils, have been used for thousands of years as spices and as local medicines. Arial parts of *Origanum vulgare* are used in respiratory tract disorders such as cough or bronchial catarrh (as expectorant and spasmolitic agents), in gastrointestinal disorders (as choleretic, digestive, eupeptic and spasmolitic agents) as oral antiseptic, in urinary tract disorders (as diuretic and antiseptic) and modulates blood [4].

The current research was performed to study the effect of adding thiamine and coconut milk in different levels to the culture media aiming to determine its optimum doe which in turn could be used for the commercial production of secondary metabolites such as phenol compounds, photosynthetic pigments content and callus fresh and dry weights (g).

MATERIALS AND METHODS

This study was carried out in the Plant Tissue Culture Laboratory, Faculty of Science, Taif University, Saudi Arabia during the season of 2014.

Sterilization method:

Seeds of *Origanum vulgare* were subjected to surface sterilization by washing in tap water containing soap and small drops of tween 20, 1 min. in ethanol 70%, washed with sterile distilled water and immersed in 15% commercial Clorox solution (1% Sodium hypochlorite) for 10 min. Seeds were washed three times with sterile distilled water in laminar air flow hood to remove the residuals.

Culture media:

Sterilized seeds were cultured in full strength of Murashige and Skoog (MS) media [5] as illustrated in Fig. (1). After germination, seedlings about 1-1.5 cm were cultured also in full strength of (MS) medium supplemented with 2 mg./l. kinetin combined with 2 mg./l. 2,4-D (dichlorophenoxy acetic acid), agar was used at 8 g/L, and sucrose at 30 g/L. for obtaining the adequate callus mass. The obtaining callus were cultured in (MS) medium supplemented with thiamine 0.0 and 0.5 g/L and coconut milk 0.0 and 2.5 ml/L, agar was used at 8g/L, and sucrose at 30 g/L. The media were distributed into clean jars. Each of which contained 30 ml of nutrient media. The media was adjusted to pH 5.7-5.8 before autoclaving for 15 min. at 121 ºC, 1.5 kg/cm^3. All treatments were incubated in the growth chamber at 26±2 ºC and exposed to 16 hr. light/day photoperiod under constant fluorescent light of 1500 Lux.

Experimental treatments:

The experiment consisted of three treatments; each treatment included ten jars (each contained three explants). The following combination treatments were carried out:

1- Thiamine 0.0 g/l + coconut milk 0.0 ml/l (control)

2- Thiamine 0.0, 0.5 g/L
3- Coconut milk 2.5 ml/l.

Experimental design and statistical analysis:

A complete randomize design was used throughout the research. The obtained data were subjected to analysis of variance and the treatment means were compared using L.S.D. test as outlined by [6].

Data recorded:

The following data were recorded after 4 weeks from culturing on media for:
1- Average callus fresh weight (g).
2- Average callus dry weight (g).

Physiological and biochemical measurements:

Photosynthetic pigments content

Contents of chlorophyll a (*Chl a*) and chlorophyll b (*Chl b*) were spectrophotometrically determined according to [7]:

Chlorophyll *a* (µg/mL) = $10.3 \times E_{663} - 0.918 \times E_{644}$
Chlorophyll *b* (µg/mL) = $19.7 \times E_{644} - 3.87 \times E_{663}$
Total carotenoid (µg/mL) = $4.2 \times E_{452} - \{(0.0264^*$ Chl *a*) + (0.426* Chl *b*)}.
Finally, these pigment fractions were calculated as mg/g fresh weight.

Total anthocyanin determination:

Total anthocyanin content in the extracts was determined according to the procedure described by [8] as follows: after extraction of anthocyanin with methanol supplemented with 1% HCl, and centrifugation, absorbance was measured at 530 and 657 nm. The formula $A = (A_{530} - 0.25 \times A_{657})$ was applied to take into account the contribution of chlorophyll and its degradation products to the absorption at 530 nm. The anthocyanin content was expressed as milligrams of Cyanidin-3-glucoside equivalent per mg fresh weight.

Total phenolics content:

Total phenolics were determined using Folin-Ciocalteau reagents [9]. Gallic acid standard solution (2.0 mg/ml) was prepared by accurately weighting 0.01 g and dissolving in 50 ml of distilled water. After standing 60 min at room temperature, absorbance was measured at 765 nm. Results are expressed as mg/g gallic acid equivalents (GAE).

RESULTS AND DISCUSSION

Fresh weight (g) of *Origanum vulgare* callus:

Data represented in Table (1) and Figure (2) showed the improving effect of different concentrations of (Thiamine) and (Coconut milk) on callus fresh weight (g) of *Origanum vulgare***.** Both treatments increased callus fresh weight than the control, in almost of them it was significant increment. The highest value of callus fresh weight (0.327 g) was obtained as a result of modifying MS medium with 0.5 mg/l (Thiamine) followed by (0.191 g) when (Coconut milk) combined with MS medium at 2.5 ml/L comparing with control as shown in figure (2) and the increment also was significant. Our data agreed with [10] who illustrated that application of thiamine significantly increased growth parameters. The highest values of results were obtained in gladiolus plants treated with 100 ppm thiamine. The increments effect on fresh and dry weight of leaves by 116.4 % and 129.6 %, respectively was obtained compared with control plants in the two seasons. Thiamine is a necessary ingredient for the biosynthesis of the coenzyme thiamine pyrophosphate; in this latter form it plays an important role in carbohydrate metabolism.

On the other hand, our data contrasted with [11] who reported that the medium contained 25% coconut water only supported viability, shooting, shoot elongation, rooting, root elongation and leaf formation. Coconut water as an organic supplement in culture media had significant effect on growth and shoots production. He also reported that the success achieved with the use of coconut water in tissue culture is 5 – 20 % and it is reasonably significant. [12] reported the presence of zeatin-O-glucoside and dihydrozeatin-O-glucoside, a cytokinin in coconut water. Data in Table (1) agree with [13] who found that coconut water could be used to initiate and maintain growth and tissue culture of several plants. Coconut water contains natural cytokinin, adding it to the medium often has the same effect as adding the compound cytokinin itself [14]. Part of the growth promoting property of coconut water is due to its myoinositol content [15].

Dry weight (g) of *Origanum vulgare* callus:

Data of callus grown on MS medium including two levels of growth regulators shown in the same table. It is obvious that the level of 0.5 g/l (Thiamine) significantly produced the highest value of *O. vulgare* callus dry weight (0.032 g). However, inclusion of 2.5 ml/L (Coconut milk) in the medium significantly decreased the callus dry weight to (0.012 g).

Effect of thiamine and coconut milk on photosynthesis pigments of *Origanum vulgare* callus:

Data in Table (2) illustrated that there was not significant increment between both treatments concerning chlorophyll A content of *O. vulgare* callus but, the treatment of coconut water gave a significant reduction of chlorophyll A content comparing with control. On the other wise, thiamine increased the content of chlorophyll B but it was insignificant increment. Whereas, adding coconut milk in the medium significantly decreased the content of chlorophyll B with value of (0.183 mg/g). In the present work, the results obtained from application of thiamine cleared that carotenoids content was increased to be (0.203 mg/g), whereas, the addition of coconut milk induces carotenoids content to (0.162 mg/g) when compared with control.

However, anthocyanin content increased to (0.003 mg/g) when thiamine was 0.5 g/L but the increment was not significant. The presence of coconut milk at 2.5 ml/L gave the same result of control (0.002 mg/g). These results are in agreement with the findings reported by [16] on Dahlia plant who found that thiamine at 100 ppm significantly increased photosynthetic pigments content, which recorded high levels of ch a, b and (a+b) and total carotenoids compared with other treatments. These results are in agreement with the findings report of [17] on Syngonium, they recorded that the highest values of ch (a), (b) and total carotenoids were obtained when plants treated with 50 ppm thiamine. In addition, [10] found that application of 1000 ppm thiamine gave the best results on chlorophylls content than the other treatments and control plants of gladiolus.

Total phenolics content (mg/g) of *Origanum vulgare* callus:

Table (2) cleared the highest significant increment of phenolics content which averaged (8943.3 mg/g) when cultured medium supplemented with 0.5 g/L thiamine comparing with all other treatments followed by (3093.3 mg/g) which obtained when cultured medium supplemented with coconut milk at 2.5 ml/L, in this case the increment was not significant.

Table 1: Effect of biotic elicitors on growth performance of *Origanum vulgare* callus.

Treatments	*Fresh weight (g)	Dry weight (g)
2.0 mg/l Kin.+2.0 ml/l 2, 4-D (Control)	0.142 c	0.015 b
Thiamine (0.5 g/L)	0.327 a	0.032 a
Coconut milk (2.5 ml/L)	0.191 b	0.012 b
L.S.D at 5%	0.036	0.0047

*Each value is the mean of ten replicates.

Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple- rage test.

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Table 2: Effect of biotic elicitors on pigments and total phenolics contents of *Origanum vulgare* callus.

Treatments	*Chlorophyll A (mg/g)	Chlorophyll B (mg/g)	Carotenoids (mg/g)	Anthocyanin (mg/g)	Total phenolics content (mg/g)
2.0 mg/l Kin.+2.0 ml/l 2, 4-D (Control)	0.141 ab	0.232 b	0.146 b	0.002 b	2843.3 b
Thiamine (0.5 g/L)	0.155 a	0.271 a	0.203 a	0.003 a	8943.3 a
Coconut milk (2.5 ml/L)	0.108 b	0.183 c	0.162 ab	0.002 ab	3093.3 b
L.S.D at 5%	0.038	0.030	0.050	4.314	1013.3

*Each value is the mean of ten replicates.

Means in each column followed by the same letter (s) did not differ at P< 0.05 according to Duncan multiple- rage test.

Figure 1: Germinated seedlings cultured in full strength of Murashige and Skoog (MS) medium

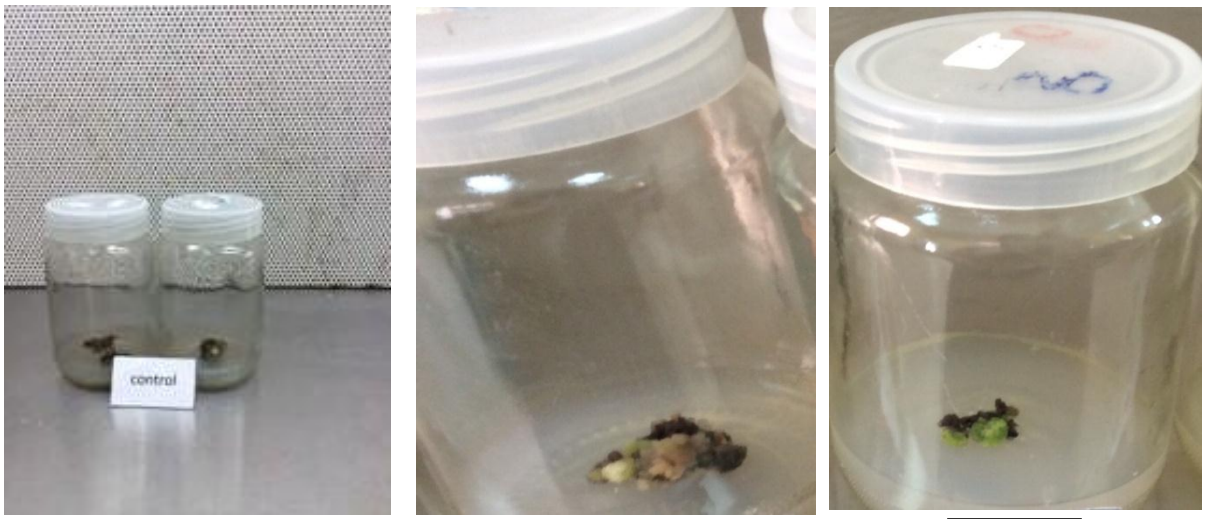

Figure 2: The effect of thiamine and coconut water on callus fresh weight of *Origanum vulgare*

CONCLUSION

The present study concluded that adding thiamine at 0.5 mg/l to MS media gave the highest value of callus fresh and dry weights of *Origanum vulgare*. Thiamine treatment was the best for increasing all

photosynthesis pigments and phenolics contents with significant differences when compared with other treatments. On the hand, adding coconut milk in the medium decreased the content of chlorophyll A and B, whereas, the addition of coconut milk increased carotenoids and total phenolics contents comparing with control.

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