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ORIGINAL ARTICLE

Assessment of Callus Growth and Bio-production of Diosgenin in Callus Culture of *Trigonella foenum-graecum* L.

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ABSTRACT
Diosgenin have various effects, such as a hypocholesterolemic action, an antioxidant activity and anticancer effects against a wide variety of tumor cells. This paper studies the effects of different media and various concentrations of NAA, 2,4-D and Kin hormones on the induction, callus growth and level of diosgenin in callus culture of Trigonella foenum-graecum leaf. The results showed that the MS medium supplement with 1.5 mg/l 2,4-D and 0.5 mg/l Kin hormones is the best medium for callus induction and growth and diosgenin accumulation. The amount of diosgenin detected in leaf callus at 60 days was more than to 20, 40 and 80 days after culture. The data showed that 2,4-D hormone compared with NAA hormone caused 32% increase in diosgenin content.
Keywords: Callus growth, NAA, 2,4-D, Kin, Diosgenin

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INTRODUCTION

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world's population. Bioactive compounds currently extracted from plants are used as food additives, pigments, dyes, insecticides, cosmetics and perfumes and fine chemicals [17]. Secondary metabolites plays a major role in the adaptation of plants to their environment, but also represent an important source of pharmaceuticals [17]. Fenugreek (*Trigonella foenum-graecum* L.) is an annual crop with trifoliate leaves, branched stem, white flowers, roots bearing nodules and golden yellow seeds [14]. Fenugreek is also known as one of the oldest medicinal plants recognized in recorded history [14]. Steroidal saponins are secondary metabolites that widely distributed in vascular plants [12]. The importance of saponins in anti-fungal defense systems in plants has been well documented. A derivative of tobacco saponin B showed hemolytic and fungicidal activity [8]. Traditionally, saponins have attracted attention owing to their pharmacological activities. Many reports have described the chemical structures of saponins and their bioactivities, including anti-inflammatory, anti-cancer, and antiherpes effects [20]. Fenugreek seed is an important source of steroidal sapogenins such as diosgenin which are used extensively by both pharmaceutical and nutraceutical industries [23]. Diosgenin, is a steroidal sapogenin belonging to the group of triterpenes and an aglycon of dioscin. Diosgenin is often used as a raw precursor for the production of steroidal drugs and hormones such as testosterone, glucocorticoids, progesterone, prednisolone, dexamethasone, norethisterone and metenoloneetc [14]. Plant drugs and herbal formulation are frequently considered to be less toxic and more free from side effects than synthetic one [16]. Diosgenin have various effects, such as a hypocholesterolemic action, an antioxidant activity and anticancer effects against a wide variety of tumor cells including colorectal cancer, breast cancer, osteosarcoma and leukemia. It also plays an important role in the control of variation in the lipoxygenase activity of human erythroleukaemia cells and responsible for morphological and biochemical variations in megakaryocyte cells [6]. Diosgenin is performed as basic material for synthesis of the oral contraceptives, sex hormones and the other steroids [6]. It is thought that steroidal sapogenins are biosynthesized from cholesterol via a series of oxygenation and hydroxylation steps, and that they are then glycosylated to form steroidal saponins [12]. In previous studies, using plants of *Trigonella foenum-graecum* L., it is observed that the maximum levels of this steroidic sapogenin are reached in young leaves, lower levels

being detected in stems and roots [20]. It also shown that, as in the mother plant, the levels of diosgenin produced in *Trigonella foenumgraecum* leaf calli are greater than in stem or root calli.
Also the effect of ethylene on diosgenin biosynthesis in *T. foenum-graecum* seedlings reported by De and Dy (2003) before. Advances in biotechnology methods for culturing plant cells, should provide new means for the commercial processing of plants and the chemicals. The *in vitro* production of this secondary compound, diosgenin, perhaps being an alternative way to obtain this important sapogenin. Some studies at both plant and plant cell culture levels have shown that some of these phytohormones may inhibit or activate the synthesis and/or accumulation of terpenes [18], phenols and alkaloids. The effects of phytohormones on secondary metabolites depend on the biochemical pathways involved and the level of tissue organization [6]. There are not many studies on tissue culture of *Trigonellafoenum-graecum*L.. By considering all these aspects, the following research work was intended to develop an efficient procedure for callus induction for Fenugreek. In this work, at first we studied the combinations of different plant hormones in callus production. Then hormones were tested on diosgenin production.

MATERIALS AND METHODS

Plant material

This experiment was carried out in the Tissue Culture Laboratory of the Department of Biology, Ferdowsi University of Mashhad, Iran. The seeds were sterilised by 20% hypochloride sodium for 10 min and then they were rinsed several times in sterile wate. The seeds were soaked in sterile wate for 24 h and placed in sterile lidded glass vessels on filter paper wetted with sterile water for germination. 7-day-old seedlings were transferred to sterile black polyethylene containers containing 3L of hoagland solution. Solution was changed every 5 days. Plants were put in growth chamber at 25 ˚C with a 16/8 h photoperiod (light/dark respectively) supplied by fluorescent tubes.

Callus culture

The leaves of four weeks old seedlings were used as explants and placed in different culture media for the corresponding calli to proliferate. For this purpose, The explants were washed with tape water up to 5 min to remove any pollution or dust particle and reduce the microbial load, then washed with distilled water and sterilized with 70% ethanol for 10 seconds. After sterilization with 70% ethanol, the explant were washed 3 times with autoclaved distilled water to remove the toxic effect of ethanol. Then, explants also were sterilized with 20% (v/v) hypochloride sodium for 2 min. Afterwards, explant were washed 3 times with autoclaved distilled water, again. Three different culture media were used to grow the explants. The first was composed of the basic Murashige and Skoog [12] medium; Type II contained the half basic Murashige and Skoog medium; Type III used Gamborg's B5 Basal Medium. MS basal media (pH 5.8) containing MS mineral and vitamins [12] supplemented with 30g/L sucrose as energy source was used.Different concentrations of 2, 4-D, NAA and Kin were added to basal media. Media was solidified with 7 g/l agar, added before autoclaving. All types of media were sterilized at 121˚C for 20 min. The calli were grown and maintained in a culture chamber at 25˚C with dark condition, at 25±2°C, relative humidity 40-60% was used throughout the experiment.

Measurement of growth

At different ages, 20 , 40 , 60 and 80 days after culture, the fresh and dry weight of the control calli and of those grown in the different concentrations of hormones were calculated in order to assess growth.

Spectrophotometric determination

At different times, 2 g of the calli were used for the isolation of diosgenin following the method of Uematsu (2000) and optimised for our work conditions. Diosgenin, (25R)-5-Spirosten-3β-ol, 3β-Hydroxy-5-spirostene, was purchased from Sigma (USA). Sulfuric acid and ethyl acetate were both of analytical grade. The diosgenin level was determined by measuring absorbance at 430 nm, based on the color reaction with anisaldehyde, sulfuric acid and ethyl acetate.The following color developing reagent solutions were prepared: (A) 0.5 mL p-anisaldehyde and 9.5 mL ethyl acetate, and (B) 50 mL concentrated sulfuric acid and 50 mL ethyl acetate. The ethyl acetate solution containing sapogenin was diluted with ethyl acetate to contain 2.5 to 10 mg/mL sapogenins. Two mL diluted sapogenin solution was placed in a 10 mL test tube. One mL each of reagent solutions (A) and (B) were added and the test tube sealed with a glass stopper. After stirring, the test tube was placed in a water bath maintained at 60˚C for 60 min to develop color, then allowed to cool for 10 min in a water bath maintained at room temperature. Because the boiling point of ethyl acetate is 76˚C, the water bath temperature should be controlled accurately. The absorbance of the color-developed solution was measured. Ethyl acetate was used as a control for the measurement of absorbance. As a reagent blank, 2mL ethyl acetate was placed in a test tube and assayed in a similar way as the sapogenin solution. Solutions containing 2–40 mg sapogenin in 2mL ethyl acetate were used to obtain a calibration curve [25].

RESULTS

Time-course study of the callus growth

Three different culture media were used to growth explants. Leaf callus growth were examined in Murashige and Skoog (1962) (MS), 1/2 MS and Gamborg (1970) B5 media supplemented with different concentrations of regulators growth include NAA(1, 1.5 and 2 mg/l), 2,4-D (1,1.5 and 2 mg/l) and Kinetin(0.25 and 0.5 mg/l) for 80 days. The calli were harvested on days 20, 40, 60 and 80 after were cultured. Table 1 shows the rate of growth of the leaf calli in the first stage (callogenesis) and in the growing stage. As the data show, the callus growth was done as a sigmoid curve. The data show that the lowest and highest of growth occur on days 20 and 60, respectively. For leaf calli, Fig. 1 shows an exponential growth phase from 10 to 40 days, followed by a linear phase up to about 60 days, when the stationary phase started. Then from day 60 to 80 the growth remained relatively constant. So the best time to harvest was 60 days.

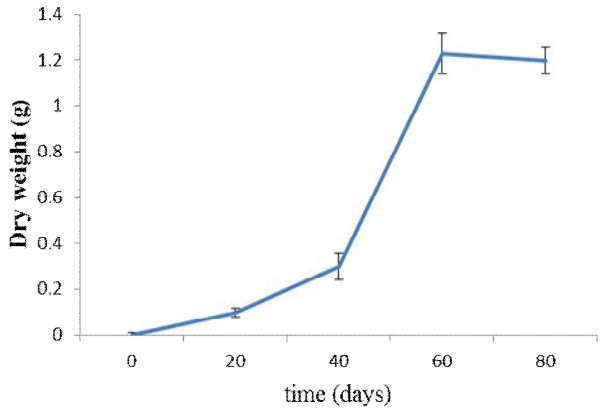

Fig. 1.Growth of *Trigonella foenum-graecum* leaf callus cultures. Experiments were replicated three times. Vertical bars denote ±S.E.

Table 1. Dry weight of leaf callus in three different culture media with different concentrations of hormones include: NAA(0, 1, 1.5 and 2 mg/l), 2,4-D(0, 1, 1.5 and 2 mg/l) and Kinetin(0, 0.25 and 0.5 mg/l). Data are expressed as the means±S.E. of three independent experiments

Harvest time (days)				Hormonal treatments	Culture media
80	60	40	20		
0	0	0	0	NAA0-Kin0	MS
0.99±0.111	1.07±0.235	0.33±0.055	0.04±0.007	NAA1-Kin0.25	MS
0.8±0.031	0.77±0.063	0.32±0.035	0.05±0.011	NAA1.5-Kin0.25	MS
0.61±0.058	0.59±0.031	0.163±0.016	0.01±0.002	NAA2-Kin0.25	MS
0.54±0.030	0.54±0.030	0.17±0.021	0.03±0.007	NAA1-Kin0.5	MS
0.82±0.014	0.81±0.013	0.3±0.006	0.03±0.008	NAA1.5-Kin0.5	MS
0.62±0.014	0.59±0.026	0.14±0.033	0.04±0.008	NAA2-Kin0.5	MS
0	0	0	0	2,4-D0-Kin0	MS
1.3±0.133	1.2±0.145	0.25±0.014	0.12±0.031	2,4-D1-Kin0.25	MS
1.08±0.030	1.06±0.088	0.29±0.041	0.12±0.012	2,4-D1.5-Kin0.25	MS
0.82±0.017	0.76±0.016	0.19±0.017	0.09±0.014	2,4-D2-Kin0.25	MS
0.67±0.017	0.63±0.020	0.17±0.20	0.48±0.247	2,4-D1-Kin0.5	MS
1.13±0.166	1.23±0.088	0.30±0.058	0.14±0.018	2,4-D1.5-Kin0.5	MS
1.03±0.120	1.06±0.088	0.23±0.030	0.05±0.005	2,4-D2Kin0.5	MS
0	0	0	0	NAA0-Kin0	MS/2
0.4±0.029	0.45±0.037	0.1±0.008	0.06±0.011	NAA1-Kin0.25	MS/2
0.4±0.040	0.4±0.031	0.09±0.006	0.04±0.003	NAA1.5-Kin0.25	MS/2
0.4±0.015	0.4±0.020	0.07±0.031	0.06±0.005	NAA2-Kin0.25	MS/2
0.34±0.031	0.35±0.032	0.1±0.020	0.05±0.003	NAA1-Kin0.5	MS/2
0.58±0.023	0.55±0.023	0.18±0.017	0.09±0.015	NAA1.5-Kin0.5	MS/2
0.47±0.025	0.42±0.012	0.11±0.017	0.05±0.012	NAA2-Kin0.5	MS/2
0	0	0	0	2,4-D0-Kin0	MS/2
0.5±0.012	0.49±0.013	0.16±0.020	0.05±0.010	2,4-D1-Kin0.25	MS/2
0.49±0.012	0.49±0.041	0.18±0.017	0.02±0.006	2,4-D1.5-Kin0.25	MS/2
0.40±0.025	0.41±0.054	0.14±0.010	0.04±0.009	2,4-D2-Kin0.25	MS/2
0.49±0.038	0.46±0.027	0.16±0.020	0.05±0.005	2,4-D1-Kin0.5	MS/2
0.41±0.017	0.39±0.030	0.1±0.011	0.04±0.010	2,4-D1.5-Kin0.5	MS/2
0.38±0.033	0.37±0.053	0.11±0.020	0.03±0.003	2,4-D2Kin0.5	MS/2
0	0	0	0	NAA0-Kin0	B5

0.4±0.049	0.4±0.029	0.13±0.011	0.05±0.009	NAA1-Kin0.25	B5
0.43±0.050	0.46±0.045	0.17±0.026	0.06±0.005	NAA1.5-Kin0.25	B5
0.36±0.032	0.33±0.046	0.06±0.012	0.04±0.004	NAA2-Kin0.25	B5
0.35±0.020	0.34±0.040	0.09±0.012	0.04±0.006	NAA1-Kin0.5	B5
0.4±0.055	0.38±0.037	0.09±0.008	0.04±0.003	NAA1.5-Kin0.5	B5
0.34±0.073	0.34±0.036	0.11±0.005	0.03±0.004	NAA2-Kin0.5	B5
0	0	0	0	2,4-D0-Kin0	B5
0.36±0.028	0.35±0.017	0.12±0.023	0.04±0.014	2,4-D1-Kin0.25	B5
0.42±0.039	0.42±0.037	0.12±0.026	0.03±0.007	2,4-D1.5-Kin0.25	B5
0.31±0.035	0.27±0.045	0.06±0.010	0.04±0.005	2,4-D2-Kin0.25	B5
0.38±0.056	0.35±0.049	0.09±0.012	0.04±0.008	2,4-D1-Kin0.5	B5
0.34±0.035	0.36±0.044	0.1±0.010	0.04±0.012	2,4-D1.5-Kin0.5	B5
0.34±0.023	0.32±0.040	0.09±0.011	0.03±0.008	2,4-D2Kin0.5	B5

Influence of culture media on callus growth

To evaluate the effect of culture medium on callogenesis and callus growth, callogenesis percentage and fresh and dry weight of calli were studied in three culture medium. According to Figure 2, callus induction in MS, MS/2 and B5 media is 67.7, 60.1 and 55 %, respectively. The data showed that the callogenesis percentage in B5 medium is significantly($P<0.05$) lower than MS medium. The study of fresh and dry weight of calli showed that the highest and lowest of weight attributed to MS and B5 media, respectively.Callus growth on medium MS/2 and B5 showed no significant difference, while callus weight significantly increased in the MS medium.

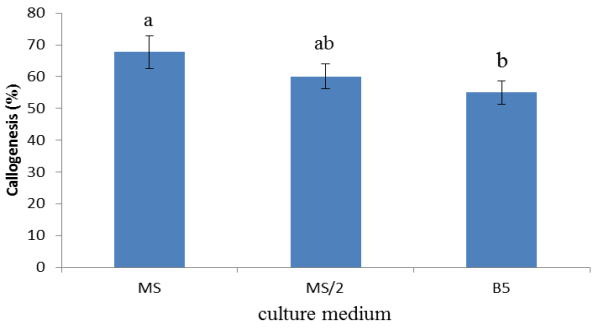

Figure 2.callogenesis percentage of *Trigonella foenum-graecum* leaf explant in three culture medium. Experiments were replicated three times. Vertical bars denote ±S.E. Non-identical letters indicate significant difference ($P<0.05$)

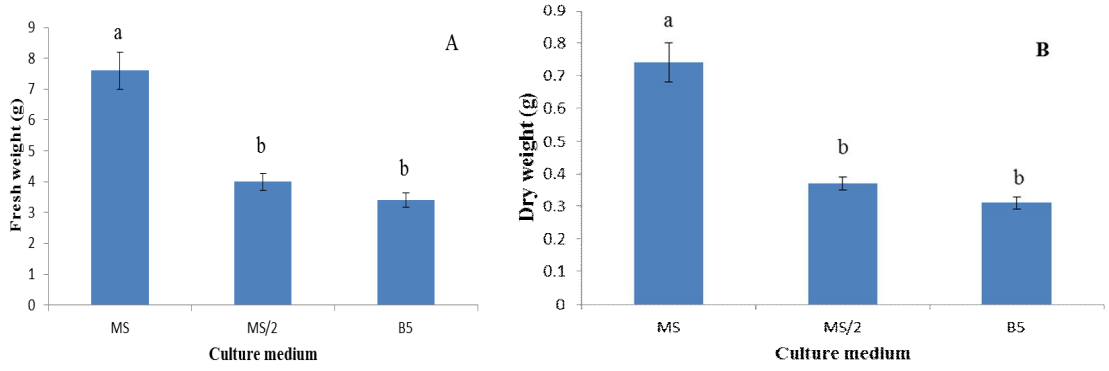

Figure 3.Growth of *Trigonella foenum-graecum* leaf callus cultures.A, Fresh weight (g) and B, Dry weight (g). Experiments were replicated three times. Vertical bars denote ±S.E. Non identical letters indicate significant difference ($P<0.05$)

Influence of hormones on callus growth

Comparison of fresh and dry weight of callus on MS medium containing different concentrations of the hormone NAA(1,1.5 and 2 mg/l), 2,4-D(1, 1.5 and 2 mg/l) and Kin(0.25 and 0.5 mg/l) showed that 1.5 mg/l of 2,4-D with 0.5 mg/l Kin had the greatest effect on callus weight. In Ms medium supplemented

with 0.5 mg/l Kin, increasing concentration of 2,4-D up to 1.5mg/l caused increased callus weight (7.1 g). The results showed that the lowest fresh weight was related to 1 mg/l NAA with 0.5 mg/l Kin(figure 4).

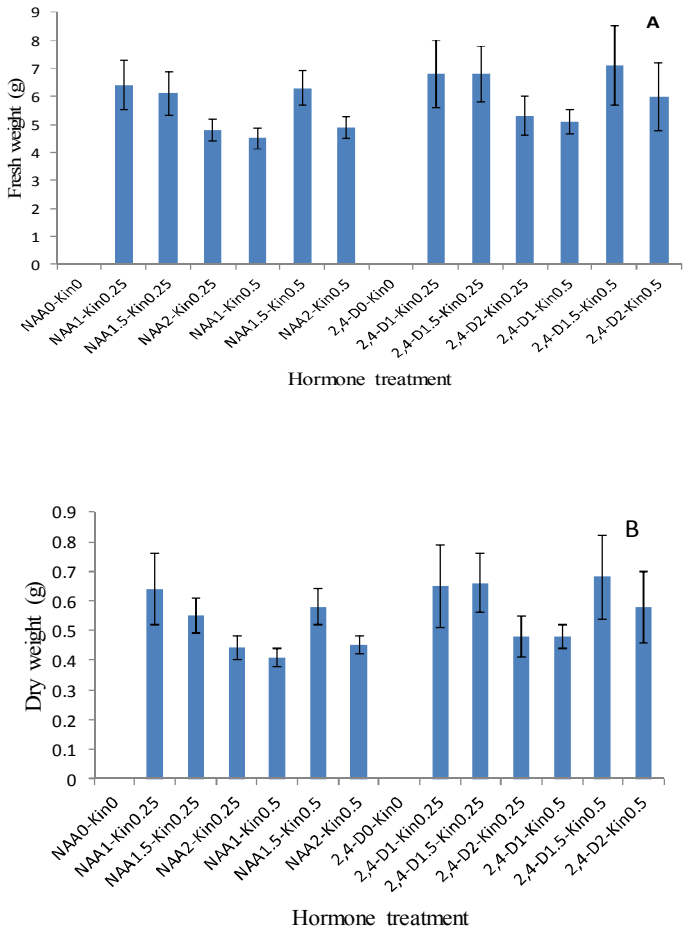

Figure 4: Growth of *Trigonella foenum-graecum* leaf callus cultures in media supplemented with different concentration of NAA(1, 1.5 ,2 mg/l), 2,4-D (1, 1.5 and 2 mg/l) and Kin (0.25 and 0.5 mg/l). A, Fresh weight (g) and B, Dry weight (g). Experiments were replicated three times. Vertical bars denote ±S.E.

In accordance with the results obtained, in hormone-free medium wasn't any kind of induction and growth of callus(figure 4). According to Figure 4, the highest and lowest dry weight of callus is related to 1.5 mg/l 2,4-D with 0.5 mg/l Kin and 1 mg/l NAA with 0.5 mg/l Kin, respectively. The data showed that calli were grown better in medium containing 2,4-D than NAA.

Diosgenin content in callus cultures

The results show that the diosgenin accumulated at all the ages analysed (20, 40, 60 and 80 days after culture). These results suggest that maximum and minimum production of diosgenin occurs at 60 and 20 days(figure 5). In accordance with the results obtained, accumulated diosgenin from day 20 to day 60 increased while after that, it decreased.

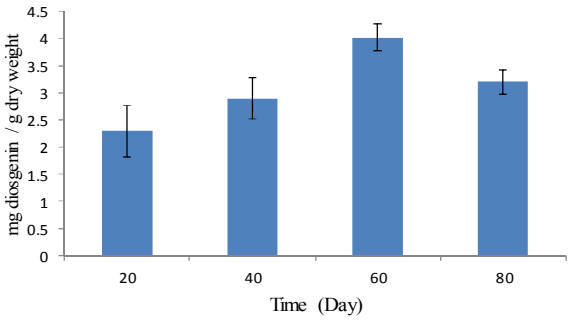

Figure 5: Time course of diosgenin levels in calli of leave obtained from explants of *Trigonellafoenum-graecum*. Experiments were replicated three times. Vertical bars denote ±S.E

Influence of hormones on diosgenin content

Comparison of callus to investigate the stored diosgenin in MS medium containing various concentrations of hormones shows that accumulated diosgenin in MS medium containing 2,4-D is 32% more than NAA. Although the diosgenin content in MS medium supplemented with various concentrations of 2,4-D has not significant difference, the MS medium supplemented with 1.5 mg/l 2,4-D has a greater impact on increasing diosgenin. The highest and lowest diosgenin was in the 1.5 mg/l 2,4-D with 0.5 mg/l Kin(4 mg/g dry weight) and 2 mg/l NAA with 0.5 mg/l Kin(1.9 mg/g dry weight), respectively.

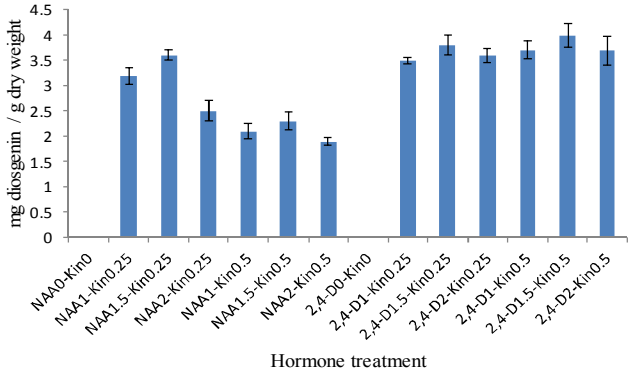

Figure 6: Diosgenin levels in callus of leaf in MS medium containing different concentrations of NAA(1, 1.5 and 2 mg/l), 2,4-D(1, 1.5 and 2 mg/l) and Kin(0.25 and 0.5 mg/l) hormones. Experiments were replicated three times. Vertical bars denote ±S.E

DISCUSSION

The results obtained in this study showed that in between MS, MS/2 and B5 media, MS medium was the best for induction and growth of callus. Data showed that the callus induction in MS/2 and B5 media was 8% and 13% lower than MS medium. These data reflect the results obtained by Oncinaet. al. (2000) that showed Murashige and Skoog's medium was given 100% callogenesis, while the White and Gamborg media led to callogenesis of around 50% [18]. The results also showed that 2,4-D hormone is better than NAA hormone since the callus grown in MS medium that supplemented with 2,4-D hormone grew approximately 12% more than in MS medium that supplemented with NAA hormone. The results suggested that the lowest dry weight was related to the calli was grown in MS medium that added 1 mg/l NAA with 0.5 mg/l Kin. While highest callus dry weight in MS medium containing 1.5 mg/l 2,4-D and 0.5 mg/l Kin With 40% increase reached to 0.68 g. Previous researchers have demonstrated that the callus grown in MS medium containing NAA (3×10^{-6} M) that supplemented with coconut milk was approximately 20% more than in MS medium containing NAA(3×10^{-6} M) that supplemented with malt extract [18]. Accordance with Table 1, the maximum callus growth in MS medium supplemented with 1.5 mg/l 2,4-D and 0.5 mg/l Kin was approximately 1.2 g whereas, Oncina*et al.* (2000) reported that dry weight of leaf callus in optimum medium (MS medium containing 3×10^{-6} M NAA that supplemented with coconut milk) was about 0.8 g, that suggest 1.5 fold increasing. We observed that diosgenin content was about 4 mg/g dry weight at 60 day. These results are in accordance with those obtained by Gomez et al. (2004) in cell suspensions culture of *Trigonella foenum-graecum*, which determined that diosgenin bioproduction was higher at the beginning of the stationary phase, when it reached about 3.4 mg/g dry weight(6). Previous studies showed that the diosgenin levels accumulated at all the ages analyzed (15, 27, 37, 45 and 60 days) were higher in leaf calli than in stem calli and root calli. In these calli, maximum levels were attained after 45 days, which coincided with the onset of the stationary phase of growth for leaf cultures [18]. In three media evaluation, MS medium was the best for diosgenin bioproduction, while the diosgenin levels in MS/2 and B5 media was associated with 17% and 30% decreases, respectively. Also, the data showed that 2,4-D hormone compared with NAA hormone caused 32% increase in diosgenin content.

CONCLUSION

The above results revealed that the best medium and hormonal treatment for callus induction, callus growth and diosgenin bioproduction, is MS medium supplemented with 1.5 mg/l 2,4-D and 0.5 mg/l Kin.

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