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EFFECT OF DIFFERENT CONCENTRATIONS OF AUXINS AND COMBINATION WITH KINETIN ON CALLUS INITIATION OF *TRIGONELLA FOENUM- GRAECUM*.L

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***Abstract*— MS medium supplemented with different concentrations of auxins 2,4-dichloro-phenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA) and combination with kinetin were studied to obtain a suitable protocol of callus initiation of *Trigonella foenum graecum*. Callus was induced from hypocotyls and cotyledons explants which were collected from the seedlings of the mentioned plant. The explants were cultured in MS medium supplemented with two auxins 2,4-D and NAA separately with different concentrations (0.0 as control, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l). Concentration of kinetin (0.5 mg/l) was used in combination with all concentrations of 2, 4-D and NAA hormones. The callus was successfully induced in all different concentrations of two mentioned auxins and combinations of different concentrations of two auxins separately with (0.5 mg/l) of kinetin. No callus formation was observed in the absence of plant growth regulators. Hypocotyl explants of *T.foenum- graecum* were much better in inducing callus than cotyledons explants. Combinations of NAA+Kin and 2,4-D+Kin were found to be more effective for inducing callus from hypocotyls explants compared to 2,4-D and NAA alone, at the same time callus induced by 2,4-D using cotyledons explants gave the best results compared to the other hormone. Among the different concentrations of auxin and combinations with kinetin ,the highest mean of callusing index from hypocotyls segments was (3.50±0.15) with 100% 0f callusing in sixth week by 4.0 mg/l NAA+ 0.5mg/l Kin. In the case of callus induction from cotyledons segments the highest mean of callusing index (2.41±0.18) with 100% of callusing in sixth week was observed by 1.0 mg/l 2, 4-D.**

Key words: *Trigonella foenum- graecum*. 2, 4-dichloro-phenoxyacetic (2, 4-D), α-naphthalene acetic acid (NAA), Kinetin, Callus initiation.

I. INTRODUCTION

Plant tissue culture is *in vitro* cultivation of plant cell or tissue under aseptic and controlled environmental conditions, in liquid or on semisolid well defined nutrient medium for the production of primary and secondary metabolites or to regenerate plant. This technique affords alternative solutions to problems arising due to current rate of extinction and decimation of flora and ecosystem. Thewhole process requires a well-equipped culture laboratory and nutrient medium [1]. Plant callus is a mass of undifferentiated cells derived from plant tissue (explants) for use in biological research and biotechnology. In plant biology, callus cells are those cells that cover a plant wound. As a first step in many tissue culture experiments it is necessary to induce callus formation from the primary explants. Callus formation is controlled by the level of plant growth regulators (auxin and cytokinin) in the culture medium. Concentrations of the plant growth regulators can vary depending on plant species. Culture conditions (temperature, light…etc) are also important in callus formation and development. Callus culture may be used for variety of experiments and studies like protoplast isolation, cell type, cellular selection, somatic embryogenesis, and secondary product production [2]. It has been reported that callus and regenerated plants have shown enhancement of secondary metabolites, when compared to parent plants [3]. *T. foenum-graecum* is an annual crop and dicotyledonous plant belonging to the family *Fabaceae* [4]. *T. foenum-graecum* is commonly used as a condiment and seasoning in food preparations, is assumed to possess nutritive and restorative properties [5]. It has been used in folk medicine for centuries for a wide range of diseases including diabetes, fever and abdominal colic as a poultice for abscesses, boils, and carbuncles [6]. *T. foenum-graecum* tissue and cell cultures have been used for either plant regeneration or for the production of secondary products of economic interest. The demand for *T. foenum-graecum* metabolites, mainly with a higher diosgenin and trigonelline content, prompted more directed tissue culturing efforts [7].

II. MATERIALS AND METHODS

A. Plant Materials:

The mature seeds of *T. foenum-graecum* were purchased from local market of Khartoum city.

B. Sterilization of equipment and glassware:

All dissection instruments, glassware and other accessories were sterilized by autoclaving at 121^0C with 15 1b/in^2 for 15 min. Dissection instrument like scalpel and forceps after autoclaving were further sterilized by dipping in 90% ethanol for at least 15 minutes and flaming before use. The laminar-air flow cabinet was sprayed with 70% (v/v) ethanol. Irradiation of instruments with Ultraviolet light in laminar-air flow cabinet for 30 minutes prior to inoculation was carried out.

C. Seeds surface sterilization and germination:

Seeds of *T. foenum-graecum* were surface sterilized in 70% ethanol for 30 sec with hand shaking and rinsed 3 times in sterile distilled water to remove trace of alcohol, then seeds were soaked in 20% Clorox (0.5% free chlorine) with 2 drops

of Tween-20 for 15 minute, and rinsed 3-5 times in sterile distilled water. After surface sterilization, the seeds were directly cultured in the germination basal medium MS [8] at 25±2°C and photoperiod of 16 hrs light and 8 hrs dark for 10 days.

D. Preparation of explants and growth regulators:

The hypocotyls and cotyledons were used as explants for *T. foenum-graecum* in this study for callus induction, MS medium was used. Two types of auxin (2,4-D and NAA) were used separately at different concentrations (0.0 as control, 0.1, 0.5, 1.0, 2.0, 3.0, 4. and 5.0 mg/l) to assess their effects on callus induction from explants. To compare the effect of the presence of cytokinin in callus induction medium, (0.5 concentration mg/l) Kinetin was used in combination with the above concentrations of 2,4-D and NAA separately. Each of the sterilized explants were cut into 2-3 mm pieces using sterile scalpel. Four pieces were inoculated in each vial containing sterile culture medium MS, with different concentrations and combination of growth regulators. Cultures were incubated for 6 weeks in 16 hrs light and 8 hrs dark at 25±2°C and data were recorded every two weeks.

E. Statistical Analysis

Data were analyzed by SPSS statistical package software [9].The results are presented as mean ± standard error of three replicates, and analyzed with Duncan LSD.

III. RESULTS AND DISCUSSION

In vitro callus was induced from fenugreek (*T. foenum-graecum*). Hypocotyls and cotyledons explants were collected from the seedlings of the mentioned plant. The explants were cultured in MS medium supplemented with two auxins 2,4-D and NAA separately with different concentrations (0.0 as control, 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l). Concentration of kinetin (0.5 mg/l) was used in combination with all concentrations of 2, 4-D and NAA hormones. The callus was successfully induced in all different concentrations and hormones combinations. No callus formation was observed in the absence of plant growth regulators. These results agree with Ahmed *et al* [10], whom investigated optimum conditions for callus proliferation from leaf, stem and root explants of *T.foenum- graecum* variety (Giza-3). They found that the presence of 2, 4-D is necessary for callus induction and no callus formation was observed in the absence of 2, 4-D. Hypocotyls explants of *T.foenum- graecum* were much better in inducing callus than cotyledons explants. Aasim *et al*. [11] found that hypocotyls explants were very recalcitrant when compared to cotyledonary node explant in inducing callus in MS medium. The highest callus formation was observed at the sixth week of cultivation in most phytohormones (Fig.1).

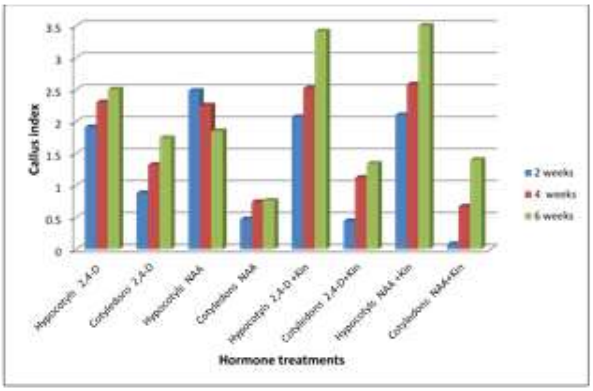


Fig (1). Effect of different hormones on callus induction of *Trigonella foenum- graecum* explants during six weeks.

The results in table (1) showed that callus index increased significantly($P<0.05$) through the increasing of 2,4-D hormone concentrations to 1.0mg/l, then started decreasing, when the concentration of hormone was increasing up to 2.0 mg/l. One mg/l of 2, 4-D proved to give maximum mean of callusing index (3.00±0.17) and (2.41±0.18) with %100 of callus induction from hypocotyls and cotyledons respectively (Fig.2&3). These results agree with that obtained by Rezaeian [12],who revealed that, the concentration of 2,4-D at 1.0 mg/l proved to be the best concentration for callus induction and proliferation in all kinds of explants of *T.foenum- graecum*, also he found that callus generation increased through increasing the 2,4-D hormone concentration to 1.0 mg/l, while it's increasing up to 1.5 mg/l decreased fresh and dry weight of callus significantly.

Table(1) Effects of different concentrations of 2,4-D on callus induction of hypocotyls and cotyledons explants of *T. Foenum – graecum* during 2, 4 and 6 weeks using MS medium

Explants	Parameter	Time (weeks)	2,4-D Concentration (mg/l)						
			0.1	0.5	1.00	2.00	3.00	4.00	5.00
Hypocotyls	Callus index (Mean ±SE)	2 week	0.58±0.14	1.33±0.14	1.41±0.14	1.50±0.15	1.25±0.13	1.16±0.11	1.00±0.08
		4 week	1.50±0.26	2.33±0.18	3.00±0.17	2.58±0.1	2.33±0.14	2.25±0.13	2.25±0.13
		6week	1.41±0.22	2.41±0.19	2.70±0.15	2.66±0.14	2.33±0.18	2.25±0.13	2.16±0.11
	% of callusing	2 week	58	100	100	100	100	100	100
		4 week	83	100	100	100	100	100	100
		6 week	83	100	100	100	100	100	100
	Callus colour		Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy
	Texture of callus		Variable	Variable	Variable	Variable	Variable	Variable	Variable
Cotyledons	Callus index (Mean ±SE	2 week	00.00±00	1.16±0.20	1.08±0.28	1.08±0.22	1.00±0.30	0.91±0.19	0.92±0.25
		4 week	0.25±0.12	1.83±0.20	1.83±0.26	1.50±0.35	1.42±0.37	1.33±0.33	1.08±0.33
		6week	0.50±0.19	2.08±0.19	2.41±0.18	1.91±0.22	1.75±0.40	1.75±0.40	1.67±0.39
	% of callusing	2 week	00	83	58	91	58	75	58
		4 week	25	91	83	91	58	66	58
		6 week	41	100	100	91	75	66	66
	Callus colour		Creamy	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy
	Texture of callus		compact	compact	compact	compact	compact	compact	compact

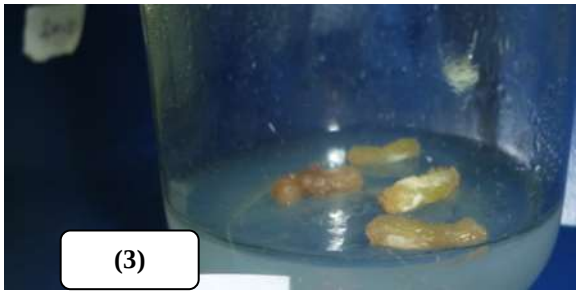
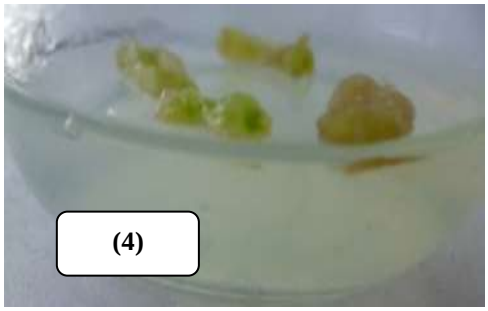


Fig. (2, 3). Callus induced from fenugreek hypocotyls and cotyledons explants by 1mg/l of 2, 4-D in MS medium

The other auxin used in this study is NAA, the effect of this hormone showed in table (2). The callus generation increased through increasing of the NAA hormone concentrations in MS medium. The maximum mean of callusing index and proliferation from hypocotyls was (3.10 ±0.16) with %100 of callus induction in second week by 2.0mg/l of NAA (Fig.4). There is no significant difference between the mean of callusing index at concentration of 2.0, 3.0 and 4.0 mg/l of NAA (Table 2). The maximum mean of callusing index from cotyledons by NAA was (1.25±0.24) with %80 of callus induction in fourth week by concentration 5.0mg/l of NAA (Fig. 5). These results similar to that obtained by Bahram et al. [12], whom used the leaves as explants instead of embryogenic leaves (cotyledons).

Table(2) Effects of different concentrations of NAA on callus induction of hypocotyls and cotyledons explants of *T. foenum – graecum* during 2,4 and 6 weeks using MS medium.

Explants	Parameter	Time (weeks)	NAA Concentration mg/l						
			0.1	0.5	1.00	2.00	3.00	4.00	5.00
Hypocotyls	Callus index Mean±SE	2 week	1.00±0.17	2.41±0.14	3.00±0.08	3.10±0.16	2.91±0.19	2.58±0.14	2.25±0.13
		4 week	1.00±0.12	2.00±0.12	2.58±0.19	2.83±0. 20	2.50±0.19	2.58±0.14	2.25±0.13
		6week	0.75±0.17	1.75±0.17	1.91±0.19	2.33±0.28	2.16±0.16	2.33±0.25	1.75±0.13
	% of callusing	2 week	83	100	100	100	100	100	100
		4 week	91	100	100	100	100	100	100
		6 week	66	100	100	100	100	100	100
	Callus colour		Brown	Brown	Creamy	Creamy	Creamy	Creamy	Creamy
	Texture of callus		Variable	Variable	Variable	Variable	Variable	Variable	Variable
Cotyledons	Callus index Mean±SE	2 week	00.00±00	0.34±0.14	0.50±0.14	0.58±0.14	0.67±0.22	0.75±0.21	0.83±0.23
		4 week	00.00±00	0.58±0.19	0.75±0.17	1.00±0.17	1.08±0.19	1.16±0.26	1.25±0.24
		6week	00.00±00	0.50±0.14	0.58±0.14	0.58±0.19	1.08±0.19	0.83±0.20	0.92±0.28
	% of callusing	2 week	00	33	50	58	50	58	58
		4 week	00	50	66	80	83	75	83
		6 week	00	50	58	50	83	66	58
	Callus colour		-	Brownish	Brownish	Brownish	Creamy	Creamy	Creamy
	Texture of callus		-	compact	compact	compact	compact	compact	compact



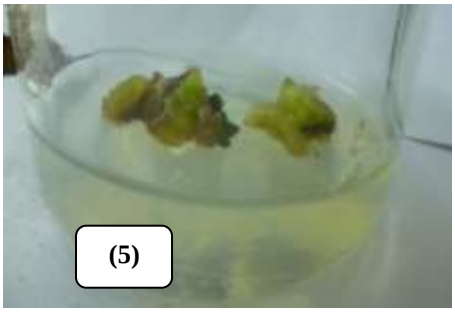


Fig.(4). Callus induced from fenugreek hypocotyls explants by 2 mg/l of NAA in MS medium.
Fig. (5). Callus induced from fenugreek cotyledons explants by 5mg/l of NAA in MS medium.

Combination of 2.0 mg/l of 2,4-D +0.5 mg/l Kinetin was more effective to induce callus from hypocotyls segments with maximum mean of callusing index (3.41±0.25) and 100% of callus induction in sixth week (Table 3& Fig.6). The present results similar to those of Afsharie et al. [14] results whom studied callus induction, somatic embryogenesis and plant regeneration of *T.foenum- graecum.* Their results revealed that medium containing 2.0 mg/l of 2, 4-D in combination with 0.5mg/l Kinetin gave the best treatment for callus induction in both MS and B5 media. Cotyledons segments induced callus by 0.5 mg/l of 2,4-D+ 0.5 mg/l Kin, the maximum mean of callusing index was (1.91±0.22) with %91 of callus induction in sixth week as shown in(Table3 &Fig.7).

Table(3) Effects of different combinatios of 2,4-D+0.5 mg/l KIN on callus induction of hypocotyls and cotyledons explants *of T. foenum – graecum* during 2,4 and 6 weeks using MS medium.

Explants	Parameter	Time (weeks)	2,4-D+0.5 KIN Concentration mg/l						
			0.1	0.5	1.00	2.00	3.00	4.00	5.00
Hypocotyls	Callus index (Mean±SE)	2 week	1.50±0.22	1.91±0.14	2.00±0.08	2.41±0.14	2.33±0.14	2.16±0.11	2.08±0.08
		4 week	1.83±0.29	2.83±0.20	2.91±0.19	3.00±0.17	2.66±0.18	2.25±0.17	2.16±0.11
		6week	2.25±0.24	3.08±0.19	3.33±0.18	3.41±0.25	3.16±0.16	2.75±0.21	2.41±0.19
	% of callusing	2 week	83	100	100	100	100	100	100
		4 week	83	100	100	100	100	100	100
		6 week	92	100	100	100	100	100	100
	Callus colour		Pale green	Pale green	Pale green	Pale green	Pale green	Pale green	Pale green
	Texture of callus		Variable	Variable	Variable	Variable	Variable	Variable	Variable
Cotyledons	Callus index (Mean±SE	2 week	0.09±0.08	0.67±0.14	0.75±0.17	0.67±0.22	0.42±0.14	0.34±0.18	0.17±0.11
		4 week	0.83±0.20	1.75±0.21	1.50±0.19	1.17±0.36	1.00±0.21	0.91±0.19	0.67±0.18
		6week	0.91±0.19	1.91±0.22	1.83±0.11	1.50±0.22	1.33±0.18	1.16±0.16	0.83±0.20
	% of callusing	2 week	0.08	66	66	58	41	25	16
		4 week	66	91	91	58	75	75	58
		6 week	75	91	100	91	91	91	75
	Callus colour		-	Creamy	Creamy	Creamy	Creamy	Creamy	Creamy
	Texture of callus		-	compact	compact	compact	compact	compact	compact

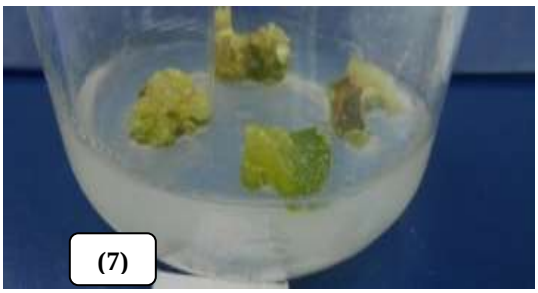


Fig.(6). Callus induced from fenugreek hypocotyls explants by 2 mg/l of 2, 4-D + (0.5mg/l) Kin in MS medium.
Fig.(7). Callus induced from fenugreek cotyledons explants by 0.5 mg/l of 2, 4- D + (0.5mg/l) Kin in MS medium.

The highest mean of callusing index(3.50±0.15)in sixth week which was recorded by 4mg/l NAA+ 0.5mg/l Kin from hypocotyls segments (Table 4& Fig.8), while 2.0 mg/l of NAA+0.5 mg/l of KIN gave maximum mean of callusing index (1.83±0.11) with 100% of callus induction in sixth week from cotyledons segments (Table4&figure9)

Table(4) Effects of different combination of NAA+0.5mg/l KIN on callus induction of hypocotyls and cotyledons explants of *T. foenum – graecum* during 2,4 and 6 weeks using MS medium.

Explants	Parameter	Time (weeks)	NAA+0.5mg/l KIN Concentration mg/l						
			0.1	0.5	1.00	2.00	3.00	4.00	5.00
Hypocotyls	Callus index (Mean±SE)	2 week	1.83±0.1	2.08±0.083	2.16±0.11	2.16±0.16	2.25±0.13	2.33±0.14	1.91±0.083
		4 week	2.00±0.17	2.50±0.15	2.58±0.19	2.75±0.13	2.83±0.20	2.91±0.22	2.50±0.19
		6week	2.16±0.16	2.58±0.22	2.66±0.14	3.08±0.22	3.33±0.14	3.50±0.15	2.19±0.28
	% of callusing	2 week	100	100	100	100	100	100	100
		4 week	100	100	100	100	100	100	100
		6 week	100	100	100	100	100	100	100
	Callus colour		Pale green	Pale green	Pale green	Pale green	Pale green	Pale green	Pale green
	Texture of callus		Variable	Variable	Variable	Variable	Variable	Nodular	Variable
Cotyledons	Callus index (Mean±SE)	2 week	00.06±00	0.09±0.08	0.09±0.08	0.17±0.11	0.09±0.08	0.09±0.08	00.00±00
		4 week	0.67+0.18	0.75±0.21	0.83±0.20	1.08±0.14	0.50±0.15	0.42±0.14	0.42±0.14
		6week	1.00±0.24	1.25±0.21	1.33±0.22	1.83±0.11	1.33±0.18	1.58±0.14	1.50±0.15
	% of callusing	2 week	00	0.08	0.08	16	0.08	0.08	00
		4 week	58	58	66	91	50	41	41
		6 week	66	83	83	100	91	100	100
	Callus colour		Pale green	Pale green	Pale green	Pale green	Pale green	Pale green	Pale green
	Texture of callus		compact	compact	compact	compact	compact	compact	compact

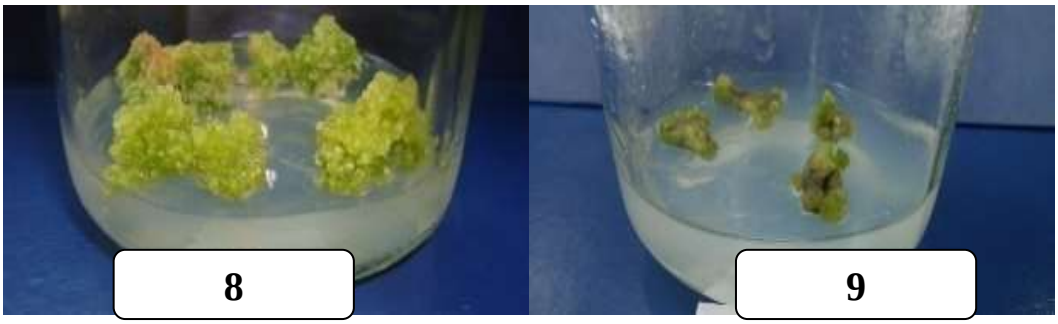


Fig. (8). Callus induced from fenugreek hypocotyls explants by 4 mg/l of NAA + (0.5mg/l) Kin in MS medium.
Fig. (9). Callus induced from fenugreek cotyledons explants by 2 mg/l of NAA + (0.5mg/l) Kin in MS medium.

Results showed that hormone types and combination with kinetin in the culture medium significantly effective ($P<0.05$) in inducing callus from hypocotyls and cotyledons explants. Combinations of NAA+Kin and 2,4-D+Kin were found to be more effective for inducing callus from hypocotyls explants compared to 2,4-D and NAA alone. At the same time callus induced by 2,4-D using cotyledons explants gave the best results compared to the other hormone. Among the different concentrations and combinations of NAA, 2,4-D, NAA+Kin and 2,4-D+Kin for callus induction from hypocotyls segments, the highest mean of callusing index was recorded (3.50±0.15)in sixth week by 4.0 mg/l NAA+ 0.5 Kin(Table4,Fig.8). In the case of callus induction from cotyledons segments the highest mean of callusing index (2.41±0.18) in sixth week was observed by 1.0 mg/l 2, 4-D (Table 1& Fig.3). Callus of *T.foenum- graecum* was compact in cotyledons segments and variable in hypocotyls segments, and showed creamy colour by 2, 4-D and NAA hormone. Sometimes callus was brown or brownish especially that induced by 0.1, 0.5, 1.0 and 2.0 mg/l of NAA. These results agree with results obtained by ELNour *et al*. [15], whom found that callus of *T.foenum- graecum* was compact in cotyledons segments, variable in hypocotyls segments and showed creamy colour. Callus from concentration 2.0 mg/l of NAA showed brown colour, this may be due to accumulation of secondary metabolite in the callus. The callus induced by combination of hormones was pale green colour (Table 3,4) this due to the presence of chlorophyll pigments in callus, as revealed by Prabakaran and Ravimycin [16],whom assessed chlorophyll pigment content in the callus of *T.foenum-graecum* and they noted that maximum peroxidase activity in green friable callus was obtained from a combination of hormones.

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