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CALLUS CULTURE OF *TRIGONELLA FOENUM GRAECUM* AS A PLANT TISSUE CULTURE ALTERNATIVE OF ANTIBACTERIAL ACTIVITY

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Abstract

In the present study, Fenugreek (*Trigonella foenum graecum*) seeds were surface sterilized and allowed to germinate on hormone free Murashige and Skoog's culture media for one week. Hypocotyls were cut into 0.5 mm length segments. Callus morphogenesis was achieved from the hypocotyls segments Using Murashige and Skoog's culture media supplemented with benzyl adenine and naphthalene acetic acid. The best callus biomass was obtained with 2 mg/l (BA) plus 1.5 mg/L (NAA) and the growth biomass was measured after 4 weeks. This concentration was then used to plot the growth curve of callus under both dark and light growth conditions. Calli obtained under light conditions exhibited a greenish yellow pigmentation, generally accumulated better biomass and their cells contained numerous and well formed discoid plastids. Calli obtained after 3, 6 and 9 weeks of growth were harvested, dried, and grinded into a fine powder and then extracted with ethanol 80%. Antibacterial activity of various calli extracts was checked by the disc-plate diffusion method and the results obtained have indicated that both hypocotyls and calli obtained from these explants exhibited activity against the tested bacteria and it was generally observed that calli were more potent than hypocotyls and that calli induced under light conditions were more potent in some cases than dark-induced calli in inhibiting the bacterial growth.

Key words: Callus, Antibacterial, Tissue culture, phytotherapeutics

Introduction

Approximately 60 – 80 % of the world's population still relies on traditional medicines as main phytotherapeutics for the treatment of common illness (Zhang, 2004). Gaines (2004) reported that the major disadvantage of traditional medicine is probably, over-harvesting of medicinal plants for extracting their valuable phytopharmaceutics and in turn making such valuable plants endangered, in addition to destroying the wild life associated with them.

Srivastava and Srivastava (2007) declared that production of biologically active metabolites *in vitro* via plant tissue culture technology is now considered as an attractive alternative that will save the environment and maximize the production of such ingredients. Bioreactors for callus and cell suspension cultures may be, in

future, the main source of bioactive compounds instead of field grown medicinal crops as well as collection of wild plants.

It has been proved that callus cultures were able to accumulate a lot of secondary metabolites with various biological activities. Examples of such metabolites are the sulfoxides (Vohoras *et al*. 1973), quinons (Duke, 1985), flavones (Kreis *et al*.1990), polysaccharides (Yao *et al*. 1992), flavonoid derivatives (Hunter and Hull 1993), polyacetylenes (Estevez-Braun *et al.*, 1994), tannins (Haslam 1996 and Stern *et al*., 1996), alkaloids (Omulokoli *et a*l.,1997), simple phenols (Peres *et al*., 1997), and diosgenin (Oncina *et al*., 2000). Many of such ingredients proved to exhibit antibacterial potential.

Fenugreek, which is adopted in this study, is probably a good target for plant tissue culturists who are interested in the production of valuable phytochemicals because it is a source of a lot of useful constituents that used in many industries (Rajagopalan, 1998) and exhibits various pharmacological activities like lowering blood sugar and cholesterol levels (Sharma, 1990), inhibition of bacterial growth (Shahidi Bonjar, 2004), prevention of ethanol-induced toxicity and apoptosis in chang liver cells (Kaviarasan *et al*., 2006) and potent antioxidant activity (Aqil *et al*., 2006).

Callus cultures were induced from fenugreek explants by Shekhawat and Galston (1983), Provorov *et al*., (1996) and Oncina *et al*., (2000) but for other purposes rather than studying their antibacterial properties. However, some more recent studies aimed at demonstrating the antimicrobial activities of callus extracts induced from other plants like *Bixa orellana* (Castello *et al.*, 2002), *Mimosa harmata* (Jain *et al.*, 2004), *Morus alba* (Pansuriya *et al*., 2006), *Rauvolfia tetraphylla* and *Physalis minima* (Shariff *et al.*, 2006), *Flaveria trinervea* (Hussein, 2007) and *Passiflora edulis* (Johnson *et al.*, 2008).

In the present study, trials were conducted to induce callus formation from fenugreek hypocotyls explants under dark and light conditions using Murashige and Skoog's culture media supplemented with various banzyladenine and naphthalene acetic acid combinations, to plot callus growth curve and to demonstrate antibacterial properties of callus at different stages of growth.

Materials and Methods

Explant preparation

Seeds of fenugreek were surface sterilized according to Chawla (2003) by submerging the explants into a solution of 70% ethanol with continuous and gentile

stirring for one minute, transferring them to 100 ml conical flask containing 20 % solution of commercial sodium hypochlorite (1% active chlorine) with continuous gentle stirring for 7 minutes. The sterilant was decanted and the seeds were washed with 3 successive rinses of sterile distilled water under aseptic conditions. The seeds were then dried between two layers of sterile filter papers in a Petri-dish. Using sterile scalpel the seeds were transferred to Petri-dishes containing hormone free Murashige and Skoog's (1962) culture media and were allowed to germinate for one week at room temperature. Hypocotyls of the sprouts were then cut under aseptic conditions into 1cm length explants for callus initiation.

Callus initiation

For induction of callus from hypocotyls explants, Murashige and Skoog's (1962) culture media containing 3% sucrose supplemented with various combinations of benzyl adenine (BA) and naphthalene acetic acid (NAA) were used. The concentrations used of each growth substance were 0.0, 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l., accordingly, a total of 49 different hormonal combinations were prepared. pH value of the culture media was adjusted to 5.7 prior to autoclaving with 0.8% (w/v) agar-agar (Glaxo, bacteriological grade). Autoclave sterilization (15 minutes, 121 ^{o}C and a pressure equal to 1.5 atmosphere) was then carried out. Culture media were dispensed into sterile 9cm diameter Petri dishes. Each treatment was represented by 6 replicates and each replicate contained 3 hypocotyls explants. The cultures were divided into two groups, the first of which was incubated at room temperature in light while the second was incubated in dark. Morphogenic responses recorded after 4 weeks and the optimum hormonal combination for callus induction was determined.

For production of callus to study antibacterial activity, explants were cultured on the same culture media supplemented with 2 mg/l BA plus 1.5 mg/l NAA. Plates were divided into two parts, the first of which was incubated in dark while the other was subjected to normal day light. Calli were harvested after 3, 6 and 9 weeks of growth for studying the antibacterial activity.

Growth curve under light and dark conditions

For measuring the growth of the callus, hypocotyls explants were cultured on MS culture medium supplemented with BA (2.0 mg/l) and NAA (1.5 mg/l). Calli were collected after1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 weeks of incubation (in dark and light conditions) and a growth curve was plotted (dry weight of callus/explant versus time in weeks).

Preparation of callus extract for assay of antibacterial activity

Calli (3,6 and 9 weeks old) were harvested and their fresh weights were recorded. They were then dried in hot air oven at 50 º C. After constant weights were attained, calli were grinded into a fine powder**.** One gram of dry fine calli powder was soaked in 70% ethanol (150 ml) at room temperature for one week with stirring from time to time. After that, filtration was carried out and the residue was washed with 3 successive rinses (100 ml) of ethanol 80%. The filtrate and washings were combined and evaporated to dryness, weighed and then redissolved in 1 ml of 80% ethanol**.**

Test microorganisms and assay of antibacterial activity:

The bacterial strains used in this study were *Escherichia coli* (ATCC 11229), *Pseudomonas auruginosa* (CS.25*), Klebsiella pneumonia* (ATCC 13883), and *Staphylococcus aureus* (NCTC7447). Assay of antibacterial activity was carried out by the disk-diffusion method described by Salie *et al.*, (1996). Five mm (diameter) filter paper disks were allowed to imbibe different aliquots of ethanolic extract in order to have an exact amount of dry callus extract equal to 25 mg per disc. After the organic solvent was completely evaporated, the discs were put on the surface of nutrient agar seeded with test bacteria in 9 cm diameter Petri-dishes. All the plates were incubated at 37°C for 24 h. The experiment was performed 3 times under strict aseptic conditions. Antibacterial activity was determined by measuring the diameter of the inhibition zone and the mean values were calculated.

Results and discussion

Results of the present study, as shown in table (1), have clearly revealed that both qualitative and quantitative changes in the hormonal composition of the culture media was influenced by various qualitative and quantitative morphogenic responses like swelling of explants, callus formation, direct and indirect root morphogenesis. Dry weights of calli produced from explants varied considerably. The best callus dry weight was obtained with 2mg/l BA+1.5 mg/l NAA. Probably, this was the proper concentration that matches with endogenous phytohormones for inducing callus formation. It has been discussed elsewhere that phytohormones, perhaps, play the major role in the processes of callus formation, differentiation and biosynthesis of secondary metabolites by plant cell cultures *in vitro*. However callus formation from fenugreek explants was obtained in earlier studies like Shekhawat and Galston (1983), Provorov *et al.*, (1996) and Oncina *et al.*, (2000).

Results of table (2) and figure (1) indicate that callus growth, as measured by biomass dry weight, exhibited a sigmoid curve. Callus growth in the first four weeks was better in dark conditions than light condition but in the following weeks, growth under light conditions exceeded growth under dark condition. Probably the growth under light conditions induced plastid formation and the biosynthesis of essential intermediates which, in turn, encouraged callus growth. Under light conditions, callus was generally greenish, yellowish and soft. The cells loosely connected to each other, more or less parenchyma, containing numerous and well developed discoid plastids. Under dark conditions, the callus produced was yellowish white colored and its cells were thinner and much more elongated, branched, bone like, thread like, or banana shaped. Calli of different stages of growth and corresponding smears showing their cells are illustrated in plate (1).

Shahidi Bonjar (2004) reported that extracts of fenugreek showed antibacterial activity. The additional and fruitful part of the present study may be the evidence of the potential of fenugreek callus tissue to biosynthesize antibacterially active ingredients. Results of the present study (table 3 and plate 2) indicate that hypocotyls segments of fenugreek seeds germinated for one week exhibited some antibacterial properties against all the tested bacteria (fortunately, it is a common habit in Egypt to eat such sprouts fresh). After three weeks of growth callus extract under light conditions remained active against the tested bacteria and even exceeded the hypocotyls extracts in inhibiting bacterial growth while under dark conditions the extract was non active against *E. coli and K. pneumonia.* After 6 weeks of growth, extract of light induced callus continued to inhibit bacterial growth and exceeded both the control extracts and callus formed under dark conditions too. After nine weeks of growth both types of calli were active against all the tested bacteria and the effect generally exceeded the corresponding control and it was generally observed that the extract of light induced callus was more active than the dark induced one. It has been reported in earlier studies that callus cultures exhibited antibacterial properties against a variety of bacterial strains. Examples of such observations can be seen on calli induced from explants of *Bixa orellana* (Castello *et al.,* 2002), *Alternanthera maritima* (Marcos *et al.,* 2003) , *Satureja hortensis* (Gulluce *et al.,* 2003), *Mimosa harmata* (Jain *et al.,* 2004), *Morus alba* (Pansuriya *et al.,* 2006), *Rauvolfia tetraphylla* and *Physalis minima* (Shariff *et al.,* 2006), *Flaveria trinervea* (Hussein, 2007) and *Passiflora edulis* (Johnson *et al.,* 2008). Such promising biological activities of callus cultures may come back to the fact that calli induced *in vitro* can do synthesize a variety of active substances belonging to

various classes of plant secondary metabolites. Examples of such observations were mentioned by Vohoras *et al.,* 1973; Duke, 1985; Kreis *et al.*,1990; *Yao et al.,* 1992; Hunter and Hull 1993; Estevez-Braun 1994; Haslam 1996; Stern *et al.,* 1996; Omulokoli *et al.*,1997; Peres *et al.,* 1997; Oncina *et al.,* 2000. It is to be added that callus cultures (especially if it is composed of undifferentiated cells) do not always succeed in the synthesis of active metabolites because biosynthesis sometimes take place in only highly organized structures (Sevo'n and Oksman-Caldenty, 2002), but fortunately, in the present study, it was possible for calli of fenugreek to carry on secondary metabolism. The reason that light induced callus was more potent than dark induced callus may be due to the development of plastids under light conditions and the synthesis of special intermediate metabolites that encouraged secondary metabolism. It was reported by Jacop and Malpathak (2004) green hairy root cultures of *Solanum khasianum* were more potent than dark grown hairy roots in solasodine production.

Table (1): Effect of different hormonal treatments on morphogenic responses of fenugreek hypocotyls explants and callus fresh weight determination (mg/explant). Each value is a mean of 10 determinations ± standard error (after 4 weeks of growth).

BA / NAA	0.0	0.1	0.5	1.0	1.5	2.0	2.5
0.0	NR	NR	163±12	158±17	RM	118±15	NR
0.1	C+RM	NR	127±16	NR	C+RM	265±32	NR
0.5	216±26	222±19	238±21	198±18	207±24	289±27	212±17
1.0	365±32	C+RM	NR	349±25	C+RM	227±17	SW
1.5	SW	189±37	C+RM	304±41	171±19	**410±20**	111±23
2.0	RM	398±26	383±43	189±29	350±34	217±27	SW
2.5	89±52	98±46	112±66	159±22	396±31	118±21	143±26

NR = No response, SW = Swelling, C+RM = Callus and root morphogenesis, RM = Root morphogenesis

Table (2): Growth of callus under light and dark conditions as measured by calli dry weight in milligrams versus time in weeks using MS culture media plus 2 mg /l of BA and 1.5 mg/l of NAA.

Time (weeks)		0	1	2	3	4	5	6	7	8	9
Dry wt. mg/explant	Light conditions	0	2	5	12	18	30	35	38	41	42
	Dark conditions	0	4	9	17	22	27	32	35	36	37

Table (3): Antibacterial activity of alcoholic extracts of calli obtained after 3, 6 and 9 weeks of growth under light and dark conditions. Diameter of filter paper discs=5mm, diameter of inhibition zones is measured in mm. Each value is mean of 3 determinations.

Tested bacteria			*E. coli*	*S. aureus*	*P. aeruginosa*	*K. pneumonia*
Control (hypocotyls tissue)			8	7	8	8
Callus tissue	3 weeks	Dark	0	9	9	0
		Light	10	9	12	9
	6 weeks	Dark	9	13	10	0
		Light	14	15	13	13
	9 weeks	Dark	10	12	11	12
		Light	14	14	13	15

Figure (1): Growth curve of callus under light and dark conditions, callus dry weight in milligrams versus time in weeks.

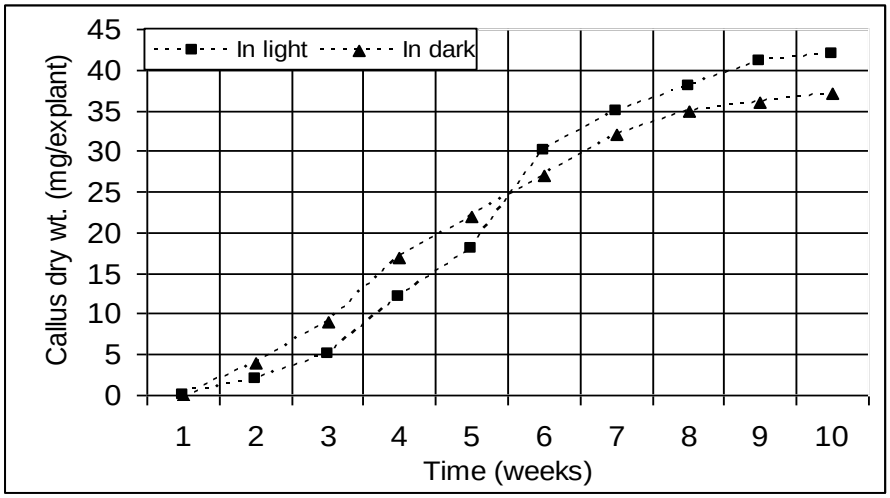


Plate (1): Photos showing callus and callus cells at different stages of growth under both light and dark conditions.

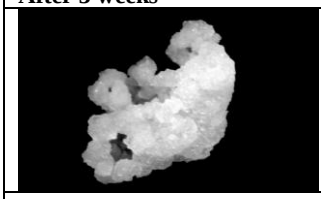
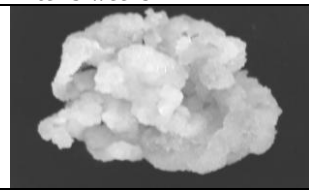
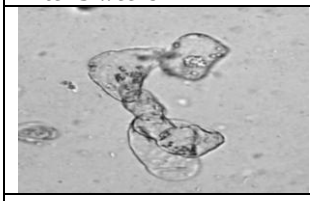
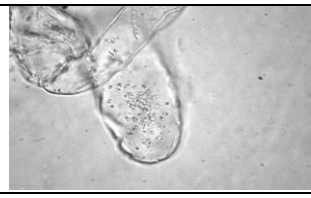
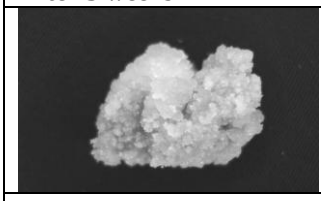
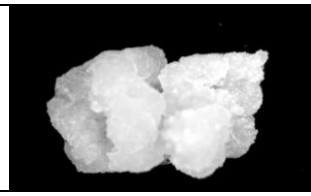
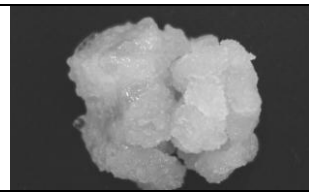
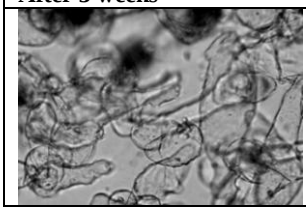
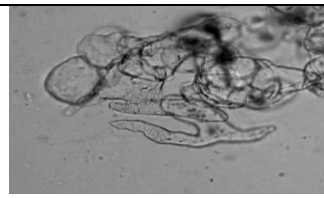
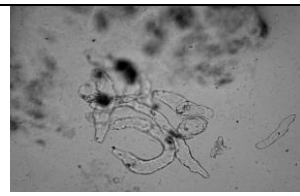
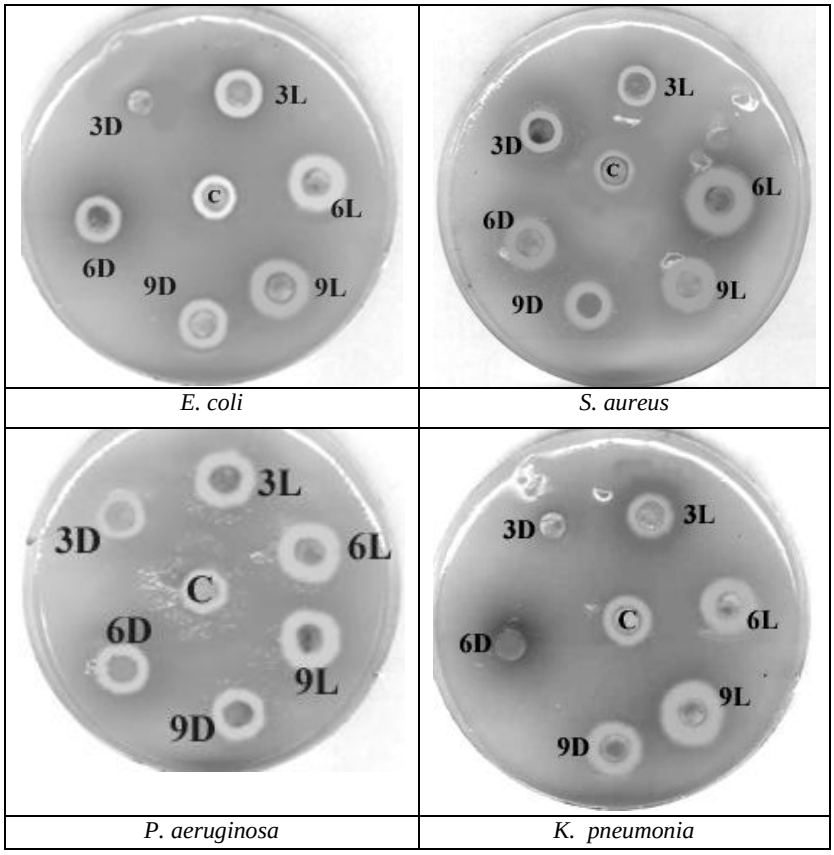
Callus formation under light conditions		
After 3 weeks	**After 6 weeks**	**After 9 weeks**
		
Smear of callus induced under light conditions		
After 3 weeks	**After 6 weeks**	**After 9 weeks**
		
Callus formation under Dark conditions		
After 3 weeks	**After 6 weeks**	**After 9 weeks**
		
Smear of callus induced under dark conditions		
After 3 weeks	**After 6 weeks**	**After 9 weeks**
		

Plate (2): Antibacterial activity of alcoholic extracts of calli produced from fenugreek after 3, 6 and 9 weeks of growth under light and dark conditions.



E. coli | *S. aureus*

P. aeruginosa | *K. pneumonia*

L: (growth under light conditions), D: (growth under dark conditions)

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ملخص عربى

كالس نبات الحلبة : بديل بزراعة الأنسجة النباتية لإنتاج النشاط الضد بكتيرى

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لقد تم فى الدراسة الحالية إنبات بذور الحلبة بعد تعقيمها على وسط موراشيج و سكوج الخالى من الهرمونات لمدة أسبوع و قطعت بعد ذلك السويقات تحت الفلقية التى نتجت عن إنبات البذور إلى قطع طول كل منها 1 سم. زرعت تلك القطع على نفس الوسط ولكن مزودا بتراكيزات مختلفة من بنزيل أدنين و نفثالين حامض الخليك تحت ظروف كاملة التعقيم. لقد كان تركيز 2 مج للتر من بنزيل أدنين + 1.5 من نفثالين حامض الخليك هو الأفضل فى استحثاث تكوين الكالس مقدراً بالوزن الجاف للكتلة الحيوية المتكونه على كل منفصل وعلى أساسه تم عمل منحنى نمو الكالس على امتداد فترة زمنية مقدارها 10 أسابيع و أظهر المنحنى شكل سيجما و كان النمو فى حالة التحضين فى الضوء أفضل منه فى حالة التحضين فى الظلام. لقد تم فى هذه التجربة أيضا دراسة النشاط الضد بكتيرى للمستخلص الإيثانولى80% لكل من السويقات تحت الفلقية و الكالس المتكون منها فى ظروف التحضين فى الضوء و الظلام على ثلاث مراحل من نمو الكالس و هى بعد 3 و 6 و 9 أسابيع من النمو. أشارت النتائج المتحصل عليها إلى أن الكالس تفوق على السويقات تحت الفلقية فى تثبيط النمو البكتيرى و أن الضوء زاد من كفاءة الكالس فى ذلك و بدا أن التأثير زاد بزيادة عمر الكالس.