

PHENOLIC CONTENT *IN VITRO* CULTURES OF CHICK PEA (*CICER ARIETINUM* L.) DURING CALLOGENESIS AND ORGANOGENESIS

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Abstract

The callogenic response of different explants i.e. cotyledon, leaf, node, shoot and root apices of *Cicer arietinum* was investigated on MS basal media supplemented with different concentrations of auxins and cytokinins. A combination of BAP and 2,4-D or NAA enhanced proliferation response. Of different combinations MS+1.0 2,4-D+0.5 NAA+0.5 BAP mg/l proved exceptionally good both for callus induction and proliferation. Callus masses initiated from different explants were maintained from main to 10th sub cultures. The morphogenic behaviour of different calli was also investigated. It was observed that the rate of browning and necrosis was increased with the increasing subcultures. To elucidate the biochemical basis of growth inhibition in non-morphogenic and morphogenic calli, total phenolics were estimated from main to 10th sub cultures both quantitatively and qualitatively. A gradual increase in total phenolic content was noticed from main to 10th sub cultures in both non-morphogenic and morphogenic calli. Non-morphogenic calli exhibited higher phenolic content as compared to morphogenic calli. The qualitative analysis of phenolic compound was also in conformity with quantitative data as the number of phenolic spots increased from main to 10th subcultures.

Introduction

In vitro callus maintenance and organogenesis from chickpea callus cultures poses great problems. Limited callus maintenance response normally results in restricted regeneration. In the present study, one major reason identified for poor callus maintenance was the initiation of browning and necrosis of callus tissues at various level of growth and differentiation.

It is well established fact that browning of the tissue is correlated with excessive accumulation of phenolics (Dubravina *et al.*, 2005). The plant phenolics can be defined as compounds having an aromatic ring with at least one hydroxyl functional group or derivatives of aromatic hydroxyl compounds. The biosynthesis of phenolic compound is complicated and not entirely understood. It begins with the biosynthesis of aromatic amino acids typtophan, phenylalanine and tyrosine. The cinnamic acid is believed to be the first step in the biosynthesis of phenolic compounds. It is synthesized from amino acid-phenylalanine ammonia lyase (PAL) (Sreenivasula, 1989). Two groups of enzymes, the phenol oxidases (PPO) and peroxidase (PEO) are involved in the oxidation of phenolic compounds (Cochrane, 1994). Phenolic compounds are most commonly distributed in plant kingdom. No tissue lacks phenolic compounds and high concentrations can be found in actively growing cells (Ozyigit *et al.*, 2007) Phenolic compounds play many roles in higher plants. They may combine with proteins either reversibly by hydrogen bonding or irreversibly by oxidation. When phenols become oxidized they form compound called quinones. Being an important group of secondary

metabolites, phenolics may act as modulators of plant development by regulating indole acetic acid catabolism (Arnaldos *et al.*, 2001). They are effective in plant growth regulation, cell differentiation and organogenesis (Ozyigit *et al.*, 2007).

Tissue browning and blackening are major impediments of *In vitro* culture of plants. Explants of high-tannin *Sorghum bicolor* cultivars produce very small, darkened callus. This callus browning, necrosis and seizure of callus growth in many *In vitro* grown plants may be due to accumulation of phenolic compounds (Lee *et al.*, 1990; Michael & John, 1986). The brown colour that develops in callus cultures of diverse plant cultures is due to the formation of quinones which are inhibitory to plant cellular growth. Accumulation of quinones to a level which is detrimental for *In vitro* growth is common in some very important plants of economic importance e.g. coffee, mango, chickpea, (Iqbal *et al.*, 1991), sugarcane (Chen *et al.*, 1990), guava, date palm, (Daayf *et al.*, 2003), cotton (Ozyigit *et al.*, 2007) and pistachio.

Keeping the limiting effects of phenolic accumulation *In vitro* cultures, it is considered worthwhile:

1. To develop optimum culture conditions for prolonged maintenance of calli.
2. To establish a correlation of phenolics, with the growth inhibition of callus and its impact on regeneration.
3. To estimate phenolic compounds both quantitatively and qualitatively from main to 10th sub cultures in morphogenic and non-morphogenic calli.

Materials and Methods

The seeds of *Cicer arietinum* L. CV, CM 72 were thoroughly washed with household detergent for about five minutes. This was followed by second washing with tap H₂O to make the material free from soap. These seeds were treated with 10% of commercial sodium hypochlorite for 20 minutes. The seeds were washed with distilled H₂O at least three times or more till the smell of Sodium hypochlorite was completely removed. These sterilized seeds were grown completely aseptically on MS basal media (Murashige & Skoog, 1962). These *In vitro* grown two weeks old seedlings were used for the preparation of node, leaf, shoot and root apices explants. The freshly prepared cultures were grown under carefully regulated temperature and light conditions. The temperature of culture room was 24±2°C and the light intensity varied from 2000-3000 lux with photoperiod of 16 hrs light and 8 hrs dark.

To standardize the media for induction of callus and regeneration, different concentrations of auxins and cytokinins were used on MS media. Calli from different explants were sub cultured after every four weeks. Phenolic compounds were estimated both quantitatively and qualitatively at the time of each sub culturing.

Quantitative estimation of phenolic compounds: For the estimation of total phenolics, from main to successive subcultures, method of Bray & Thorpe (1964) was used. One gram of callus was taken and boiled in methanol for 5 minutes. The boiled calli were homogenized in pestle mortar with methanol. The mixture was filtered and debris were washed with 80% acidified methanol. Two ml of H₂O was added. The extract was concentrated to aqueous volume by evaporation in a rotary evaporator under reduced pressure at 40°C. The aqueous extract was centrifuged at 6,000 rpm to remove chlorophyll and fine debris.

For estimation of total phenolics, 0.2 ml of phenolic extract was added in 4ml of 20% Na₂CO₃. In blank 0.2 ml of H₂O was used instead of phenolic extract. Tubes were shaken vigorously. After 2 minutes, 0.2 ml of 1:1 diluted Folin's Reagent was added with constant shaking. The colour was allowed to develop for half an hour and absorbance at 750 nm was taken and expressed as mg/g tissue, by comparison with calibration curve prepared from a reference solution containing chlorogenic acid.

Qualitative analysis: The search for a specific system for the separation of a mixture is best carried out by thin layer chromatography. Stahl-type of apparatus is used for preparation of thin uniform layers. Glass plates having sizes 20*20 cm and 5*20 cm were used. Plates were cleaned and oven dried at 160°C for one hour.

Plates were adjusted on the aligning tray. A weighed amount of fluorescent Silica gel which is best adsorbent for separation of phenolic compound was shaken to a homogenous suspension with distilled water. The quantity of H₂O to be added in gel was in ratio 1:2 (w/v). These suspensions must be shaken for 60-90 seconds. A 0.50 mm of layer thickness was selected by the spreading trough. Homogenous suspension was immediately poured in spreading trough, and trough was passed in one movement over a row of glass plates which held firmly on an aligning tray. The surface of the layer had initially a moist lustre. The freshly coated plates were left on tray, until the transparency of layer disappeared. After about 10 min, the plates were stacked in drying rack in vertical position for two hours at 120°C. The hot rack was taken out and placed in dry cabinet until used.

Spot application and development of TLC plates: The methanolic extract was used for spot application. Methanol extract 20 µl was applied on TLC plates by micropipette. The starting line was marked 1.5 cm from lower edge of plate. The spot were placed 1.5 cm apart from each neighbouring spot.

The choice of solvent was governed primarily by nature of the compound of different solvent used for TLC, Toluene: Ethyleformate: Formic acid, 50:40:10 showed better separation of phenolics. Fresh solvent was used for each experiment. Chamber was saturated before developing of TLC plates. A smooth sheet of filter paper was laid in glass trough and soaked in solvent. After this, moistened paper was than pressed against the sides of chromatographic chamber, before plate was introduced. Separation was carried out at room temperature generally. Ascending development was used in TLC. The plates were placed in chamber with solvent to a depth of about 0.5 cm. The plates were allowed until the solvent front reached near the upper end of the plate. Now the plates were removed from the chamber and were air-dried.

For identifying fluorescent compounds, UV lamps (emission maxima 254 and 365nm) were used. The chromatograms were viewed in both UV lights and all the compounds which were separated were marked. For identification authentic markers were used. The R_f values and fluorescent colours of standard phenolic compounds separated on TLC in the same solvent system have been recorded for comparison with the unknown phenolic compounds. For visualization of colourless substances, Folin-ciocalteus reagent was used. It gave dark blue spots on light background.

Results

In the present study, restricted callus maintenance and growth remained the major obstacle to trigger the desired differentiation response. One major reason identified for

poor callus maintenance was the initiation of browning and necrosis of callus tissues at various levels of growth and differentiation. Generally the browning and necrosis activities are correlated with the accumulation of excessive phenolics. Interference of phenolics with the process of growth and differentiation is a common phenomenon. So present study was undertaken to determine the precise role of phenolic compound and to develop a possible link between browning and accumulation of phenolics.

Quantitative analysis: The morphogenic and non-morphogenic calli derived from cotyledon, node, leaf, shoot and root apices were used as a source material for these investigations.

A comparison of total phenolic content of non-morphogenic and morphogenic calli obtained from different explants from main to 10th subcultures is given in Table 1. The non-morphogenic and morphogenic calli derived from every individual explants showed consistent increase in phenolic content from main to their successive subcultures. Of the main cultures of non-morphogenic callus, the highest phenolic contents were estimated from cotyledon (0.677 ± 0.015) followed by node (0.650 ± 0.026), shoot apex (0.602 ± 0.016), root apex (0.559 ± 0.020) and leaf (0.535 ± 0.018).

An increase in phenolic content from the initial to 10th subculture was from 0.677 to 1.65, 0.650 to 1.77, 0.602 to 1.55, 0.559 to 1.38 and 0.535 to 1.64 in cotyledon, node, shoot apex, root apex and leaf calli respectively. Maximum percent increase, was observed in 10th subcultures of every explant in relation to the main. The increases in decreasing order were from leaf callus (+206.54) to node (+172.30), to shoot apex (+157.47), to root apex (+146.86) and to cotyledon (+143.72). The increase in phenolic content of all above calli was statistically significant in relation to their respective callus (Table 1).

Like non-morphogenic callus, the morphogenic calli also showed a linear increase in the phenolic content in the successive subcultures. For example, the phenolic contents of node were 0.580 in main culture which increased to 1.21 in 10th subculture, significant increase being +108.62% from the main callus. In shoot apex callus, the increase was lowest i.e., +73.09% for 10th subculture as compared to main culture. A statistically significant increase (+87.07%) was also noticed in leaf callus.

Qualitative analysis: A comparative account of qualitative analysis of non-morphogenic and morphogenic calli derived from node, leaf, shoot-apex and root apex from main to successive subcultures is resolved by thin layer chromatography.

Nodal callus: The description of phenolic spots and their respective Rf values of nodal explant are presented in Table 2; Figs. 1 and 2. In main culture of non-morphogenic callus, only 2 phenolic spots with Rf 0.83 and 0.90 were detected on chromatogram. The number of spots increased to 3 in 1st sub culture. All the spots were new with Rfs of 0.05, 0.21 and 0.81. A gradual increase in number of spots was observed in remaining sub cultures. The maximum number of spots i.e., 6 were visualized in the calli obtained from 7th sub culture. In the calli of 8th, 9th and 10th subcultures the number of spots was the same but the pattern was different. The morphogenic calli yielded one spot Rf 0.21 in main and 1st subculture. One more spot with Rf 0.05 was present in 2nd and 3rd subculture. The number of spots increased to 3 in 4th, 5th and 6th subcultures. Four spots were observed (Rfs 0.12, 0.21, 0.74, 0.76) were observed in calli of 7th, 8th, 9th and 10th subcultures.

Table 1. Total phenolic content (mg/g callus) in non-morphogenic and morphogenic callus from main to 10th subculture.

Explant cultures	Node			Leaf			Shoot apex			Cotyledon			Root apex		
	N.M. ^a	M. ^a	N.M. ^a	M. ^a	N.M. ^a	M. ^a	N.M. ^a	M. ^a	N.M. ^a	M. ^a	N.M. ^a	M. ^a	N.M. ^a	M. ^a	
Main culture	0.650 ^a ±0.026	0.580 ^a ±0.0027	0.535 ^a ±0.018	0.495 ^a ±0.0021	0.602 ^a ±0.016	0.550 ^a ±0.0031	0.667 ^a ±0.015	0.559 ^a ±0.020	-	-	-	-	0.559 ^a ±0.020	-	
1 st Subculture	0.743 ^b ±0.026	0.620 ^b ±0.0031	0.599 ^b ±0.013	0.507 ^b ±0.0028	0.670 ^b ±0.012	0.578 ^b ±0.0034	0.722 ^a ±0.058	0.620 ^b ±0.012	-	-	-	-	0.620 ^b ±0.012	-	
2 nd Subculture	(+14.30) 0.812 ^c	(+6.89) 0.650 ^c	(+12.38) 0.624 ^b	(+2.44) 0.520 ^b	(+11.29) 0.720 ^b	(+5.09) 0.581 ^b	(+6.64) 0.817 ^b	(+10.91) 0.680 ^c	-	-	-	-	(+10.91) 0.680 ^c	-	
3 rd Subculture	(+24.92) 0.833 ^d	(+12.06) 0.67 ^c	(+16.63) 0.744 ^c	(+2.44) 0.535 ^c	(+19.60) 0.811 ^c	(+5.63) 0.597 ^c	(+20.67) 0.896 ^{bc}	(+21.64) 0.744 ^c	-	-	-	-	(+21.64) 0.744 ^c	-	
4 th Subculture	(+35.84) 0.907 ^d	(+15.68) 0.680 ^c	(+39.06) 0.792 ^{cd}	(+5.05) 0.595 ^d	(+34.71) 0.915 ^d	(+8.54) 0.597 ^c	(+32.34) 0.896 ^{bc}	(+33.63) 0.744 ^c	-	-	-	-	(+33.63) 0.744 ^c	-	
5 th Subculture	(+39.53) 1.26 ^e	(+17.24) 0.783 ^d	(+48.22) 0.852 ^{de}	(+8.08) 0.622 ^e	(+51.99) 1.11 ^e	(+12.9) 0.713 ^e	(+36.64) 1.13 ^d	(+39.71) 0.835 ^e	-	-	-	-	(+39.71) 0.835 ^e	-	
6 th Subculture	(+93.84) 1.51 ^f	(+35.00) 0.815 ^d	(+59.25) 0.887 ^e	(+20.20) 0.649 ^e	(+84.38) 1.24 ^f	(+29.63) 0.751 ^f	(+66.91) 1.18 ^d	(+49.37) 0.909 ^f	-	-	-	-	(+49.37) 0.909 ^f	-	
7 th Subculture	(+132.0) 1.71 ^g	(+40.51) 0.941 ^e	(+65.79) 1.20 ^f	(+25.65) 0.780 ^g	(+105.98) 1.40 ^g	(+36.54) 0.823 ^g	(+74.29) 1.56 ^a	(+62.61) 1.17 ^g	-	-	-	-	(+62.61) 1.17 ^g	-	
8 th Subculture	(+167.79) 1.75 ^g	(+106.89) 1.21 ^f	(+124.29) 1.61 ^g	(+31.11) 0.921 ^h	(+132.55) 1.53 ^h	(+49.63) 0.949 ^h	(+130.24) 1.59 ^a	(109.30) 1.30 ^b	-	-	-	-	(109.30) 1.30 ^b	-	
9 th Subculture	(+169.29) 1.77 ^g	(+108.62) 1.21 ^f	(+204.46) 1.64 ^g	(+57.57) 0.926 ^h	(+154.15) 1.53 ^h	(+72.54) 0.951 ^h	(+134.85) 1.65 ^a	(+132.55) 1.35 ^f	-	-	-	-	(+132.55) 1.35 ^f	-	
10 th Subculture	(+172.30) 1.77 ^g	(+108.62) 1.21 ^f	(+206.54) 1.64 ^g	(+87.07) 0.926 ^h	(+154.15) 1.53 ^h	(+72.90) 0.952 ^h	(+143.72) 1.65 ^a	(+141.50) 1.38 ^f	-	-	-	-	(+141.50) 1.38 ^f	-	
				(+87.07) 0.926 ^h	(+157.47) 1.55 ^h	(+73.09) 0.952 ^h	(+143.72) 1.65 ^a	(+146.86) 1.38 ^f	-	-	-	-	(+146.86) 1.38 ^f	-	

*:N.M. =Non-morphogenic, M. = Morphogenic; **: Each value is a mean of three replicates with standard error (Mean±S.E.); (+) Present increase; a,b,c: Means with same superscript are not significantly different from each other at 5% level by Duncan's new multiple range test; ***: Cotyledons and Root Apex only exhibit non-morphogenic callus. So morphogenic callus was not presents

Table 2. Rf values on TLC and fluorescent colours of the spots of phenolic compound isolated from non-morphogenic and morphogenic nodal calli from main to 10th subculture.

Rf values	Flourescent colours (uv 366nm)	No. of Subcultures																					
		Main culture		1st sub-culture		2nd sub-culture		3rd sub-culture		4th sub-culture		5th sub-culture		6th sub-culture		7th sub-culture		8th sub-culture		9th sub-culture		10th sub-culture	
		N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M
0.05 ¹	Green	-	-	+	-	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
0.12 ²	Deep violet	-	-	-	-	+	-	+	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+
0.21	Light green	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	+	-	+
0.57 ³	Light pink	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-
0.62 ⁴	Blue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-
0.71	Light violet	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
0.74 ⁵	Light blue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
0.76 ⁶	Blue purple	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+	-	+
0.81 ⁷	Blue	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	-	+	-
0.83 ⁸	Green	+	-	-	-	+	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-
0.89 ⁹	Light blue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-
0.90	Light green	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.92 ¹⁰	Red	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
	Total:	2	1	3	1	4	2	4	2	5	3	5	3	5	3	6	4	6	4	6	4	6	4

1: Chlorogenic acid; 2: Sinapic acid; 3: Syringic acid; 4: Ferulic acid; 5: Iso-rhamnetin; 6: Salicylic acid; 7: Daphnetin; 8: Scopoletin; 9: Umbelliferone; 10: Coumarins. Solvent system: Toluene : Ethyle Formate : Formic acid (50 : 40 : 10)

Of the total phenolics detected from main to 10th subcultures, 5 of these (Rfs 0.05, 0.12, 0.57, 0.62, 0.76) were identified as chlorogenic acid, sinapic acid, syringic acid, ferulic acid and salicylic acid respectively by chromatography with commercially available authentic markers. The five more spots (Rfs 0.74, 0.81, 0.83, 0.89 and 0.92) were identified as iso-rhamnetin, daphnetin, scopoletin, umbelliferone and coumarins respectively by comparing Rfs and colour with literature.

Leaf callus: The qualitative analysis for total phenolics of non-morphogenic and morphogenic calli (main to 10th subcultures) derived from leaf is given in Table 3; Figs 3 and 4. The non-morphogenic callus yielded 3 spots (Rfs 0.12, 0.15, 0.67) in main cultures. An increase of one spot with Rf 0.73 was observed in 1st, 2nd and 3rd subcultures. In calli of 4th and 5th subcultures, although the number of phenolic spots was the same 4 but the pattern was different. Five spots were visualized in calli of 6th and 7th subcultures. In this case only one spot was new, while the rest were common with 5th subculture. The maximum number of spots i.e., 8 was detected in calli of 8th, 9th and 10th subcultures. Like non-morphogenic callus, the morphogenic callus also exhibited an increase in the number of phenolic spots from main to 10th subcultures. In main culture, only one spot with Rf 0.15 was observed. Two phenolic spots (Rfs 0.15, 0.76) were detected in calli of 1st to 4th subcultures which increased to 3 in 5th and 6th subcultures. In 8th, 9th and 10th subcultures the number of spots increased to 4. Among the various spots detected, three of these were identified as sinapic acid (0.12), vanilic acid (0.67) and salicylic acid (0.76) by comparing with authentic markers. The other three were identified as tricetin (0.73), Scopoletin (0.83) and coumarins (0.92) by comparing the colours and Rf values with literature.

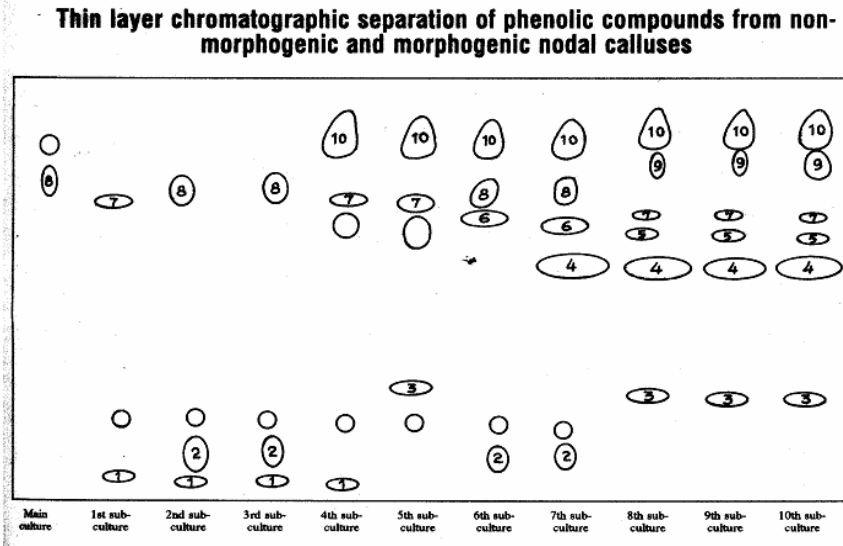


Fig. 1. Non-morphogenic calli.

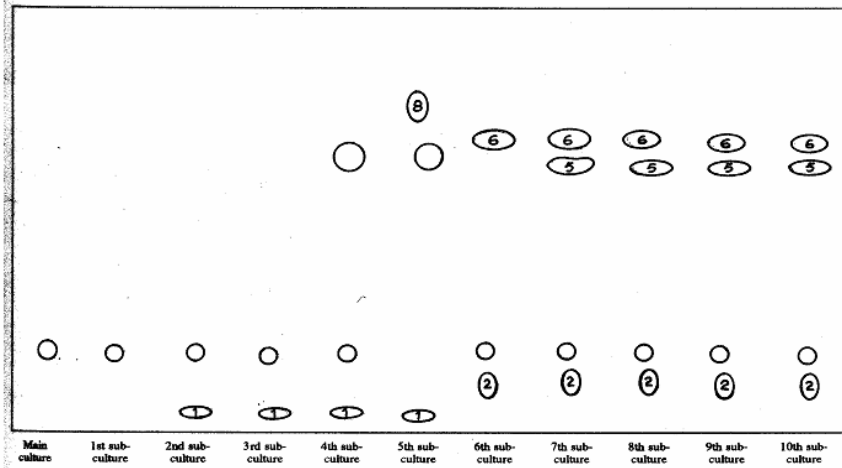


Fig. 2. Morphogenic calli.

1= Chlorogenic acid; 2= Sinapic acid; 3= Syringic acid; 4= Ferulic acid; 5= Iso-rhamnetin; 6= Salicylic acid; 7= Daphnetin; 8= Scopoleti; 9= Umbelliferone; 10= Coumarins
O: Unidentified.

Shoot-apex callus: Separation profiles of total phenolics on TLC from non-morphogenic and morphogenic calli of shoot-apices from main to successive subcultures is illustrated in Table 4; Figs. 5 & 6. The methanolic extract of non-morphogenic callus exhibited only one phenolic spot with Rf 0.67 in main culture. The number of spots increased to 2 from 1st to 3rd subcultures. Three spots were visualized in calli of 4th to 7th subcultures. In calli of 8th -10th subculture, the number of spots increased to maximum i.e., 5. In morphogenic

Table 3. Rf values on TLC and fluorescent colours of the spots of phenolic compound isolated from non-morphogenic and morphogenic leaf calli from main to 10th subculture.

Rf values	Flourescent colours (uv 366nm)	No. of Subcultures																					
		Main culture		1st sub-culture		2nd sub-culture		3rd sub-culture		4th sub-culture		5th sub-culture		6th sub-culture		7th sub-culture		8th sub-culture		9th sub-culture		10th sub-culture	
		N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M
0.12	Deep violet	+	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.15	Light violet	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	+	-	+	-	+	-
0.19	Pink	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.67 ²	Violet	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
0.73 ³	Light green	-	-	+	-	+	-	+	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-
0.76 ⁴	Blue purple	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	+
0.83 ⁵	Light green	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+
0.88	Light violet	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-
0.92 ⁶	Light blue	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
0.96	Pinkish red	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	-	-	-	-	-	-
	Total:	3	1	4	2	4	2	4	2	4	2	4	3	5	3	5	3	8	4	8	4	8	4

1: Sinapic acid; 2: Vanillic acid; 3: Tricin; 4: Salicylic acid; 5: Scopoletin; 6: Coumarins.
Solvent system: Toluene : Ethyl Formate : Formic acid (50 : 40 : 10).

calli, one phenolic spot (Rf 0.54) was present in main and 1st subculture. The addition of one spot (Rf 0.60) was noticed in calli of 2nd to 4th subcultures. In calli of 5th and 6th subcultures, the number of spots remained same; however the difference in Rf of one spot 0.67 was observed. The spots increased to 3 in 7th to 10th subcultures. Among the total phenolic spots detected from main to 10th subcultures, three phenolic spots were identified as Ferulic acid (0.62) Vanilic acid (0.67) and salicylic acids (0.76) by comparing with commercially available markers. Other spots were identified as quercetin (0.41) iso-rhamnetin (0.74) and daphnetin (0.81) by comparing the Rf values and colours with literature.

Cotyledon callus: Table 5; Fig. 7 shows the phenolic spots of cotyledon from main to successive subcultures. In main culture, the methanolic extract of cotyledon yielded one spot with Rf 0.54. In 1st, 2nd and 3rd subcultures, the number remained one but with different Rf value 0.67. In calli of 4th and 5th subcultures, the number of spots rose to 2. An increase of one more spot was noticed in 6th and 7th subcultures.

In calli of 8th, 9th and 10th subcultures, 5 spots were detected out of these, 3 spots with Rf 0.24, 0.34 and 0.54 were new while the rest of 2 (0.67, 0.74) were common with 4th to 7th subcultures. Two of these were identified as vanilic acid (0.67) and caffiec acid (0.50) by comparing with authentic markers. Identification of one more spot as iso-rhamnetin (0.74) was observed by comparing with Rf and colour of spot with literature.

Root-apex callus: A qualitative analysis of phenolics of non-morphogenic callus is presented in Table 6; Fig. 8. In main, 1st and 2nd subcultures, only one spot with Rf 0.67 was detected. The number of spots increased to 2 from 3rd to 7th subcultures. The calli derived from 8th to 10th subcultures exhibited 3 phenolic spots. Among the total phenolics detected, two of these were identified as vanilic acid (0.67) and salicylic acid (0.76) by comparing with authentic markers, the remaining two spots were identified as daphnetin (0.81) and scopoletin (0.83) by comparing the colour and Rf values with literature.

Table 4. R_f values on TLC and fluorescent colours of the spots of phenolic compound isolated from non-morphogenic and morphogenic shoot apex calli from main to 10th sub-culture.

R _f values	Flourescent colours (uv 366nm)	No. of Subcultures																						
		Main culture	1st sub-culture		2nd sub-culture		3rd sub-culture		4th sub-culture		5th sub-culture		6th sub-culture		7th sub-culture		8th sub-culture		9th sub-culture		10th sub-culture			
		N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	N.M	M	
0.41 ¹	Light blue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.54	Light green	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
0.59	Light blue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.60	Dark purple	-	-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
0.62 ²	Blue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.67 ³	Violet	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
0.74 ⁴	Green	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
0.76 ⁵	Blue purple	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.81 ⁶	Light blue	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.87	Pink	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total:	1	1	2	1	2	2	2	2	3	2	3	2	3	2	3	3	3	5	3	5	3	5	3

1: Quercetin; 2: Ferulic acid; 3: Vanillic acid; 4: Iso-rhamnetin; 5: Salicylic acid; 6: Daphnetin
 Solvent system: Toluene : Ethyle Formate : Formic acid (50 : 40 : 10).

Thin layer chromatographic separation of phenolic compound of non-morphogenic and morphogenic calluses of leaf explant

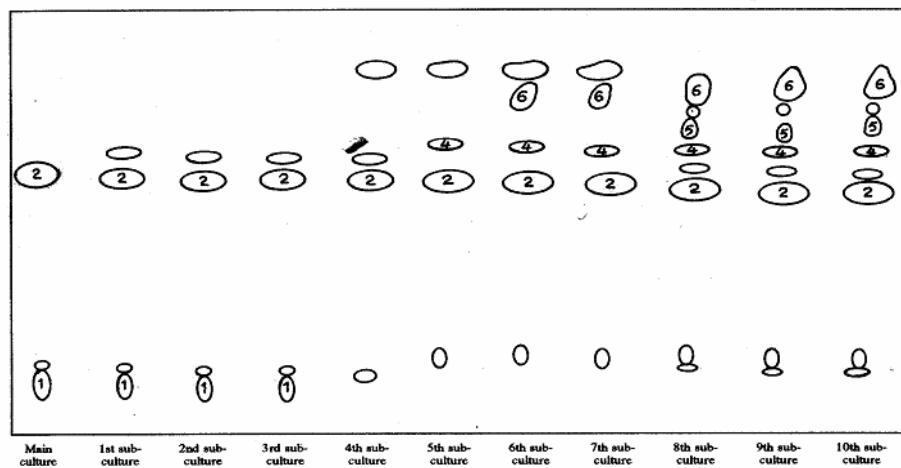


Fig. 3. Non-morphogenic calli.

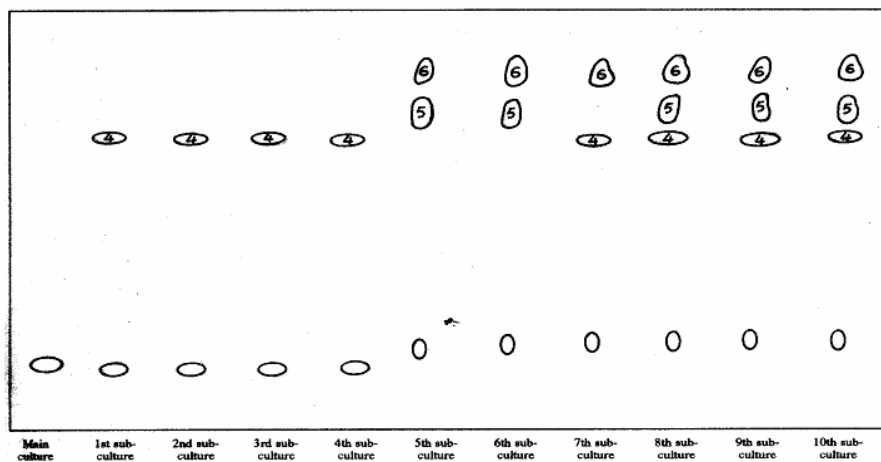


Fig. 4. Morphogenic calli.

1= Sinapic acid; 2= Vanillic acid; 3= Tricin; 4= Salicylic acid; 5= Scopoletin; 6= Coumarins.
O: Unidentified.

Thin layer chromatographic separation of phenolic compounds of non-morphogenic and morphogenic calluses of shoot-apex

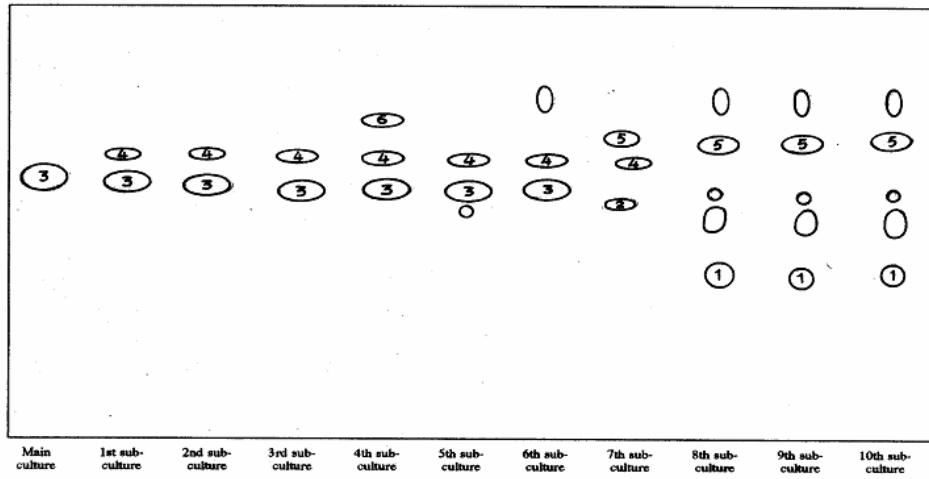


Fig. 5. Non-morphogenic calli.

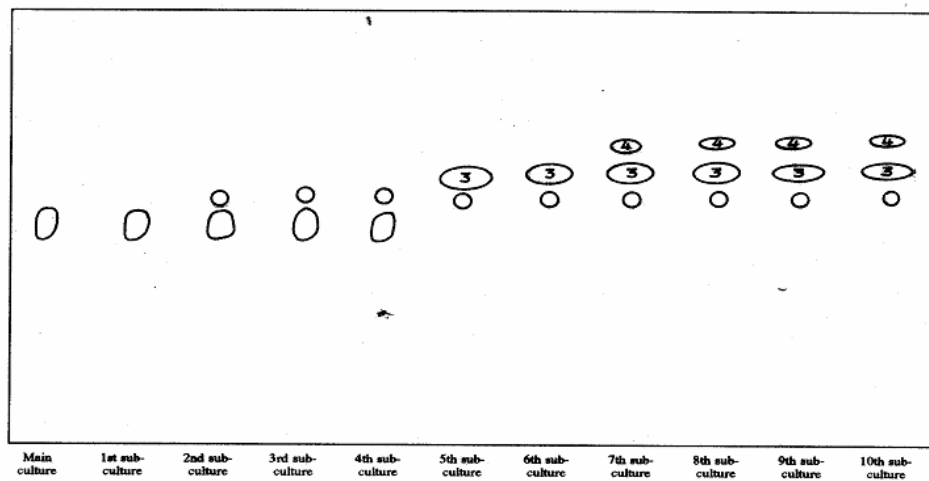


Fig. 6. Morphogenic calli.

1= Quercetin; 2= Ferulic acid; 3= Vanillic acid; 4= Iso-rhamnetin; 5= Salicylic acid; 6= Daphnetin.
 O: Unidentified.

Discussion

The present study was undertaken to view the correlation of phenolics with the browning of callus and eventual inhibition of growth and morphogenesis from callus. The data on above parameters revealed a gradual increase in total phenolic content from main to 10th subcultures both in non-morphogenic and morphogenic calli. However non-morphogenic calli exhibited greater phenolic content as compared to morphogenic calli. The results from qualitative analysis showed a general correlation with quantitative data of phenolics. Higher content of phenolics in non-morphogenic calli of *Juglans regia* L., were also reported by Rodriguez (1982). Accumulation of phenolic content in alfalfa callus in relation to their embryogenic ability was studied by Cvikrova *et al.*, (1991) and higher level of phenolic content was noticed in non-embryogenic callus as compared with embryogenic callus. Dubravina *et al.*, (2005) also reported the enhanced synthesis of phenolic compounds and flavans in cell dedifferentiation and growth of European and Canadian Yew *In vitro*. In tissue cultures studies normally, phenolic substances, especially oxidized phenolics generally effect *In vitro* proliferation negatively. Arnaldos *et al.*, (2001) and Thomas & Ravindra (1999) studied with four age groups of shoots ranging from one week to one year old and they observed maximum phenol oxidation, medium discoloration and rapid explants browning with one-week-old shoots in mango.

The result of the present study further revealed that phenolic content increased both quantitatively and qualitatively with the increase in age of calli i.e., from main to 10th subcultures. The percentage of browning and necrosis is also in conformity with increase in phenolic content in each subculture. In 1st to 4th subculture where the phenolic content were relatively low, the growth of calli is very active and vigorous, whereas after 5th subculture, a gradual increase in phenolic contents as well as in browning and necrosis with concomitant cessation of growth was observed up to 10th subculture.

This finding strongly suggests on a priori basis the inhibition of growth of calli with accumulation of phenolics. The relationship of phenolic content with the rate of browning is also reported by many workers. Hyser & Moft (1980) observed that *Pinus taeda* L., declined and generally turned brown as phenolic compounds were produced. The same phenomenon of phenolic accumulation in senescing calli was also reported by Nash & Davis (1972) in cell suspension of Paul's Scarlet rose. Hrubcove *et al.*, (1988) reported the 90% increase in non-extractable phenolics with the age of alfalfa callus. Cvikrova *et al.*, (1990) also reported that the content of total phenolics increased with the age of callus and reached 90% in the 4th subculture of *Medicago sativa* callus. Iqbal *et al.*, (1991) observed a gradual increase of total phenolics from main to 6th subculture with the increasing rate of browning. Effect of phenolics on growth and morphogenesis both *In vivo* and *In vitro* is investigated by various authors (Coseting & Lee, 1987; Lee, 1991; Lee *et al.*, 1990; Onyencho & Hettiaracheny, 1993; Provan *et al.*, 1994).

Increase in phenolic content is normally associated with increase in the enzymes that regulate the synthesis of phenolic compound, while the intensity of browning is related with the hyperactivity of oxidative enzymes (Ting, 1982; Barz & koster, 1981; Cochrane (1994) Laukkanen *et al.*, (1999) cultured calli from shoot tips of mature Scot pine and obtained brownish green calli in 14 days old, greenish brown in 28 days old and totally brown in 42 days old calli and examined phenol oxidase POD and polyphenol oxidase PPO activity in those explants. They found that PPO activity increased rapidly during culture but the increase slowed down after 28 days of culture. An increase in production of phenolic compounds has been associated with a decrease in growth, a decline in protein synthesis.

Thin layer chromatographic separation of phenolic compounds of non-morphogenic calluses derived from cotyledon

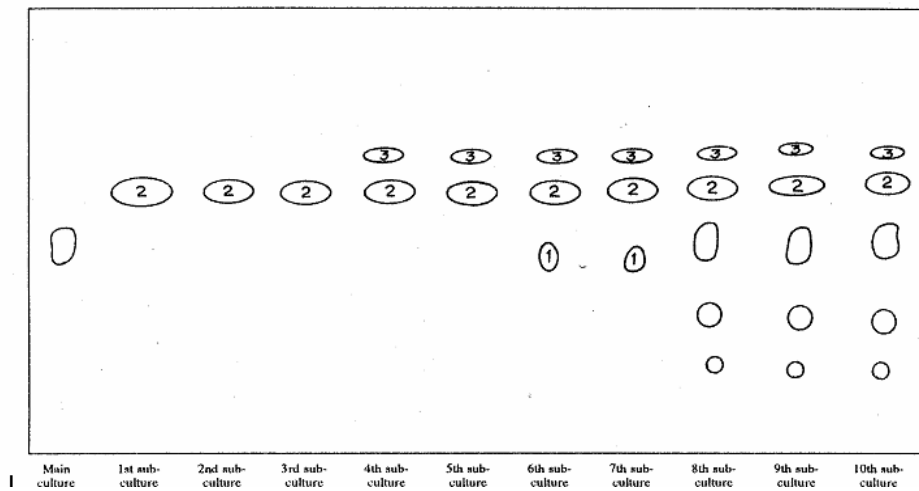


Fig. 7. Non-morphogenic calli
1= Caffeic acid; 2= Vanillic acid; 3=Iso-rhamnetin.

Thin layer chromatographic separation of phenolic compounds of non-morphogenic calluses of root-apex

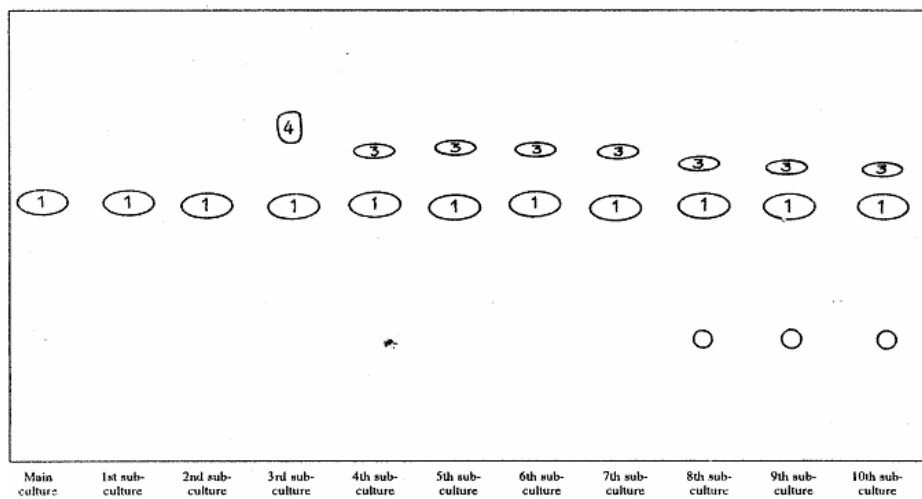


Fig. 8. Non-morphogenic calli.

Table 5. Rf values on TLC and fluorescent colours of the spots of phenolic compound isolated from non-morphogenic and morphogenic cotyledon calli from main to 10th subculture.

Rf values	Flourescent colour (uv 366nm)	No. of Subcultures										
		Main culture	1st sub-culture	2nd sub-culture	3rd sub-culture	4th sub-culture	5th sub-culture	6th sub-culture	7th sub-culture	8th sub-culture	9th sub-culture	10th sub-culture
0.24	Greenish blue	-	-	-	-	-	-	-	-	+	+	+
0.34	Light blue	-	-	-	-	-	-	-	-	+	+	+
0.50 ¹	Flourescent blue	‡	-	-	-	-	-	+	+	-	-	-
0.54	Light green	+	-	-	-	-	-	-	-	+	+	+
0.67 ²	Violet	-	+	+	+	+	+	+	+	+	+	+
0.74 ³	Green	-	-	-	-	+	+	+	+	+	+	+
	Total:	1	1	1	1	2	2	3	3	5	5	5

1: Caffeic acid; 2: Vanillic acid; 3: Iso-rhamnetin.
Solvent system: Toluene : Ethyle Formate : Formic acid (50 : 40 : 10)

Table 6. Rf values on TLC and fluorescent colours of the spots of phenolic compound isolated from non-morphogenic and morphogenic root apex calli from main to 10th subculture.

Rf values	Flourescent colour (uv 366nm)	No. of Subcultures										
		Main culture	1st sub-culture	2nd sub-culture	3rd sub-culture	4th sub-culture	5th sub-culture	6th sub-culture	7th sub-culture	8th sub-culture	9th sub-culture	10th sub-culture
0.31	Light blue	-	-	-	-	-	-	-	-	+	+	+
0.67 ¹	Violet	+	+	+	+	+	+	+	+	+	+	+
0.76 ²	Blue purple	‡	-	-	-	-	-	-	-	+	+	+
0.81 ³	Light blue	-	-	-	-	+	+	+	+	-	-	-
0.83 ⁴	Light green	-	-	-	+	-	-	-	-	-	-	-
	Total:	1	1	1	2	2	2	2	2	3	3	3

1: Vanillic acid; 2: Salicylic acid; 3: Daphnetin; 4: Scopoletin.
Solvent system: Toluene : Ethyle Formate : Formic acid (50 : 40 : 10)

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