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Induction and Biochemical Parameters of Callus Growth from Three Peanut Cultivars[1]

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ABSTRACT

The phytohormones 2,4-dichlorophenoxyacetic acid, naphthalene acetic acid and kinetin have been employed to induce callus growth from the cotyledon tissue of three commercial peanut cultivars: 'Early Bunch' (EB), 'NC-Fla 14' (NC) and 'Florunner' (FR). Cultivar specific parameters have been examined for both cotyledon and callus tissue. The relative growth rates of callus tissues were always EB>NC> FR. SDS-polyacrylamide gel electrophoresis performed on the proteins from both types of tissue demonstrated that most cultivar specific differences seen in cotyledon tissue are maintained by callus tissue from the same plant. However, more high molecular weight protein fractions were observed in cotyledon tissue than in callus. Amino acid analysis of these tissues revealed a higher concentration of neutral and aromatic amino acids in cotyledon while callus tissue was higher in basic amino acids.

Key Words: Tissue Culture, Callus, Peanut, *Arachis hypogaea*, Cultivar.

The growth of plant cells and tissues in sterile culture opens broad new avenues of experimentation. The *in vitro* growth of plant tissue allows the researcher to grow large populations of plant cells in a shorter time, in less space and under a wider variety of growth conditions than would be possible with either whole plants or seedlings (Day, 1977). Recently, callus tissue generated in culture has been used as an experimental system to determine the metabolic response of plant cells to biochemical perturbation (Verma and van Huystee, 1970; van Huystee and Turcon, 1973; Kossatz and van Huystee, 1976; Polacco, 1976). The successful use of tissue culture, specifically callus culture, as an assay system relies in part on having a thorough familiarity of the physiological events involved in the transition from an organized tissue such as cotyledon or hypocotyl to callus, an unorganized, partially dedifferentiated tissue mass.

Several studies have been done which delineate some of the most important growth factors for generating maximum callus growth from peanut plant tissue (Kumar, 1974a, 1974b, 1974c). This work is an attempt to partially characterize some of the biochemical parameters of three individual peanut cultivars' responses to phytohormone stimulation. Three commercial cultivars of peanut: 'Early Bunch', 'Florunner' and 'NC-Fla 14' were tested for several parameters of cotyledon composition and for their ability to generate callus tissue under the influence of a variety of auxin and cytokinin concentrations. Comparisons are also made to determine compositional changes undergone by tissues after the induction of callus growth.

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Materials and Methods

The seeds of three commercial cultivars were employed in this study: 'Florunner', 'Early Bunch' and 'NC-Fla 14'. The seeds of these cultivars were soaked in water to remove the seed coats and the heart was removed to leave only cotyledon tissue. Cotyledons were surface sterilized by soaking 5 min each in 2.63% sodium hypochlorite, 0.01% Tween 80 and then 70% ethanol. New tissue surfaces were exposed by aseptic cutting during the last of three changes of sterile glass distilled water. Sterile cotyledon fragments were then placed on Murashige and Skoog medium solidified with 0.8% agar.

Commercially prepared Murashige and Skoog (MS) medium (Flow Labs) was supplemented with an additional 710 mg/l $CaCl_2 \cdot 2H_2o$, 1.0 g/l NZ-Amine (Humko-Sheffield Chem.) and varying concentrations of the phytohormones 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and 6-furfurylaminopurine (kinetin). Medium contents were added to glass distilled water and brought to a gentle boil with continuous stirring until the agar dissolved and the solution cleared. The pH of the medium was adjusted to 5.7±0.1 prior to autoclaving for 15 min at 121°C and 15 psi. Kinetin and NZ-Amine were filter sterilized and added after autoclaving due to their heat lability. Medium was distributed to 100 x 15 mm petri dishes and allowed to solidify.

Callus cultures were induced and maintained at 28°C on a 16/8 hr light-dark cycle under approximately 3500 lux. Routine maintenance of cultures was accomplished by transferring calluses at three week intervals. For growth tests, however, calluses were weighed and transferred to fresh medium every 15 days.

Cotyledon and callus tissues were dried at 60°C to constant weight to determine moisture content. Dried cotyledon tissue was then defatted by perchloroethylene extraction and oil content determined by Foss-Let analysis (Heinis and Saunders, 1974). Callus tissue was defatted by acetone extraction during a Soxhlet determination of total oil (Ayers, 1968). Total nitrogen and protein were determined by micro-Kjeldahl analysis performed according to the methodology of the Association of Official Analytical Chemists except that a factor of 5.46 was used to convert Kjeldahl nitrogen to total protein (AOAC, 1970). Amino acid analysis of samples was performed using a JEOL JLC-6AH automated analyzer according to previously described methods (Savoy, *et al*, 1975). Sample preparation includes an acid hydrolysis of tissues which destroys tryptophan and it has therefore, not been included in the calculation of total amino acids.

Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by defatting both cotyledon and callus tissues with perchloroethylene and drying the meal to constant weight at 60°C. SDS-PAGE was performed on each tissue type no less than 12 times according to methods previously described (Weber and Osborn, 1969) using 10% gel columns. A 60 μl sample containing 60 μg of cotyledon protein or 70 μg of callus tissue protein was applied to gels. Protein quantities for electrophoretic comparison were determined by using the percent protein calculated by micro-Kjeladahl analysis of defatted callus or cotyledon meal. Two protein standards: highly purified chicken albumin (Sigma) and cytochrome c (Sigma) were used to ensure constant migration characteristics and to help estimate molecular weights. Standards were not run on the same gel as callus or cotyledon tissues but on different gel columns of identical composition and developed at the same time. A tracker dye, malachite green, was employed to monitor migration rates. Gels were developed for 1 hr at 1 mA/gel tube and then for 7.5 hr at 5 mA/gel tube. Gels were subsequently stained 2 hr in Coomasie Brilliant Blue and destained according to the methods of Weber and Osborn (1969). Destained gels were scanned at 597 nm in a silica cuvette using a Beckman 25 spectrophotometer equipped with a gel-scanning attachment.

Results and Discussion

Several concentrations of auxin and cytokinin added to Murashige and Skoog medium have been tested for their ability to induce the production of callus tissue from the surface sterilized cotyledons of three peanut cultivars. The addition of 2,4-D, NAA and kinetin at 2 mg/l each (designated as 2:2:2) was shown to induce the most vigorous callus growth (Table 1) in the shortest time. While the relative concentrations of 1:1:1, 4:1:1 and 4:0.05:0.05 also induced callus production, no evidence of cell division was seen prior to 30 days. Figure 1 compares the growth rate of callus tissue generated by all three cultivars when placed on 2:2:2. Little difference in the callus growth rates of the cultivars was observed before 30 days. At all times of measurement after 30 days, EB callus tissue grew at a substantially faster rate than did NC, which grew slightly faster than FR. It should be noted here that maximum callus growth was obtained by transferring callus cultures at 3 week intervals rather than every 15 days as was done in these growth tests.

Figure 2 shows a photographic series of typical callus growth from EB cotyledons. The other two cultivars had much the same appearance but callus growth was somewhat less robust. The ability of EB to develop callus tissue fastest does not appear to be a function of any one factor observed in this study, such as the cotyledon protein contents of the cultivars. A compositional analysis of the tissues (Table 2) shows that NC had the highest protein content in both cotyledon and callus tissues and the highest oil in its cotyledons. Callus growth rates, therefore, may reflect the relative ability of each cultivar to store essential nutrients other than protein or oil in the mature seed. It is interesting to note that the order of relative callus growth rates is the same as the order of maturation in the field for these 3 cultivars (EB>NC>FR).

The transition from an organized mass of tissue such as cotyledon to an unorganized cell mass as found in callus tissue produces a striking visual change (see Figure 2). However, electrophoretic analysis of the protein composition of callus tissue demonstrated a remarkably similar pattern to that of cotyledon (Figure 3). Each peak shown in the electropherograms represents one resolvable band of protein on a SDS-PAGE column. When the electropherogram patterns of cotyledon and callus tissues are compared, there appears to be a greater amount of high molecular weight protein species (Figure 3A; components 1, 2, 3 and 4) in cotyledon. Component 1 is the largest protein detected and does not appear in callus tissue. This observation is not surprizing since the bulk of the protein found in cotyledon tissues is probably the large storage proteins arachin and conarachin. The arrows in Figure 3A and 3B represent the major peaks of two standards of known molecular weight run on identical gels. The larger standard (right arrow) is purified chicken albumin having a molecular weight of approximately 45,000 and migrates coincidently with component 2. The smaller standard (left arrow) is highly purified cytochrome c having a molecular weight of approximately 12, 384 and is coincident with component 8. FR cotyledon and callus are both lacking component 4 and EB cotyledon apparently contains very little of component 5, enough to produce merely a "shoulder" on peak 6. The protein in component 6 appears to be present in the callus tissue from all three cultivars. In the cotyledon tissue, however, NC possesses what appears to be a smaller molecular weight component (6A) than either EB or FR.

The cultivar specific differences demonstrated in Figure 3 are not well supported by the amino acid analysis of callus and cotyledon tissues (Table 3). The amino acid composition of the three cultivars was very similar for all cotyledon tissues and for all callus tissues. Cotyledon composition was, however, quite different when compared to callus tissue amino acids. In general, cotyledon shows higher levels of aromatic amino acids, glutamic acid, glycine, alanine and valine while callus tissue appears to have higher concentrations of the basic amino acids.

This study is an attempt to provide some of the basic information necessary to understand the physiology

Table 1. Callus growth from cotyledon tissues.

Phytohormone Concentration (mg/l)			Cultivar								
			EB (days)			NC (days)			FR (days)		
2,4-D	NAA	Kinetin	15	30	45	15	30	45	15	30	45
1	1	1	0	+	+	0	+	0	0	+	+
2	2	2	+	+++	‡‡	+	++	+++	+	++	+++
4	1	1	0	+	+	0	0	0	0	0	0
1	4	1	0	0	0	0	0	0	0	0	0
1	1	4	0	0	0	0	0	0	0	0	0
4	0.05	0.05	0	+	++	0	+	+	0	+	++

0 = no growth, + = callus cells evident, ++ = moderate growth, +++ = normal growth, ‡‡ = robust growth.

Table 2. Composition of cotyledon and callus tissues.

Tissue	Cultivar	Moisture in tissue (%)	Protein in dry tissue (%)	Oil in dry tissue (%)
Cotyledon	EB	2.44	23.78	48.80
"	NC	2.22	29.19	49.20
"	FR	2.27	24.13	47.80
Callus	EB	83.07	24.25	6.56
"	NC	79.67	27.37	3.48
"	FR	85.41	19.55	8.83

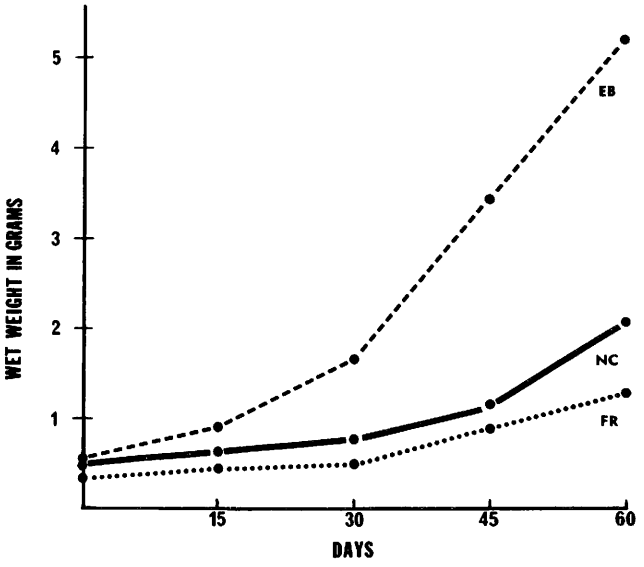

Fig. 1. Growth curves of callus tissue derived from the cotyledons of cultivars 'Early Bunch' (EB), 'NC-Fla 14' (NC) and 'Florunner' (FR) on supplemented MS medium containing the phytohormones 2,4-D, NAA and Kinetin (2 mg/l each).

of callus tissue growth. To date, however, the proteins which comprize the electropherograms in Figure 3 and the amino acid compositions in Table 3 remain unidentified. The identity of at least some of these proteins will be needed to determine the significance of the differences noted between cultivars and between organized plant and callus tissues. Experiments are in progress to establish identities for several of these specific proteins.

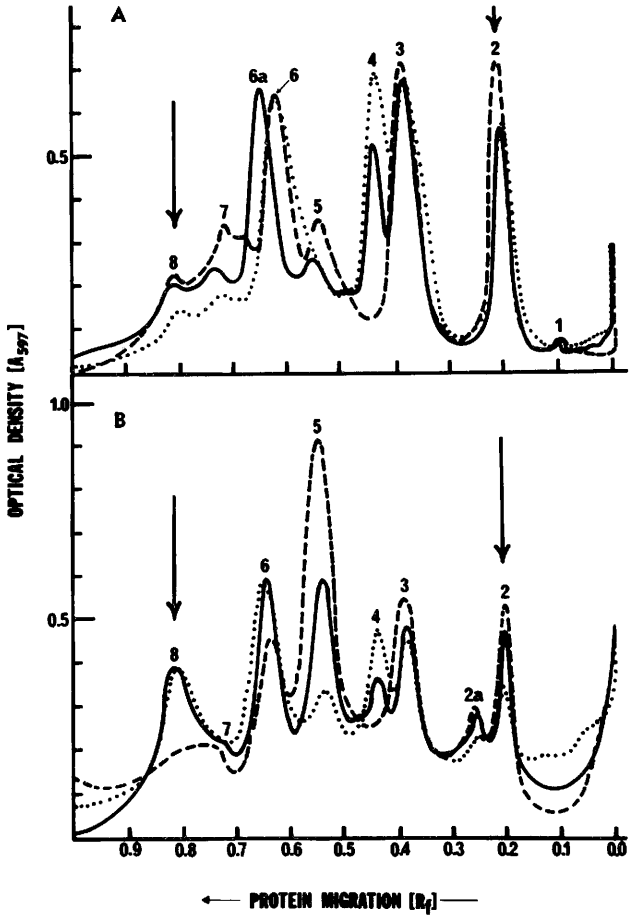

Fig. 3. Electropherograms of proteins of cotyledon (A) and callus (B) tissues from EB, NC and FR developed on 10% polyacrylamide gels. Migration is from right to left. Arrows correspond to the location in the gel to which standards of known molecular weight migrate: albumin (right arrow) and cytochrome c (left arrow).

..... EB, ———— NC, and - - - - - - FR.

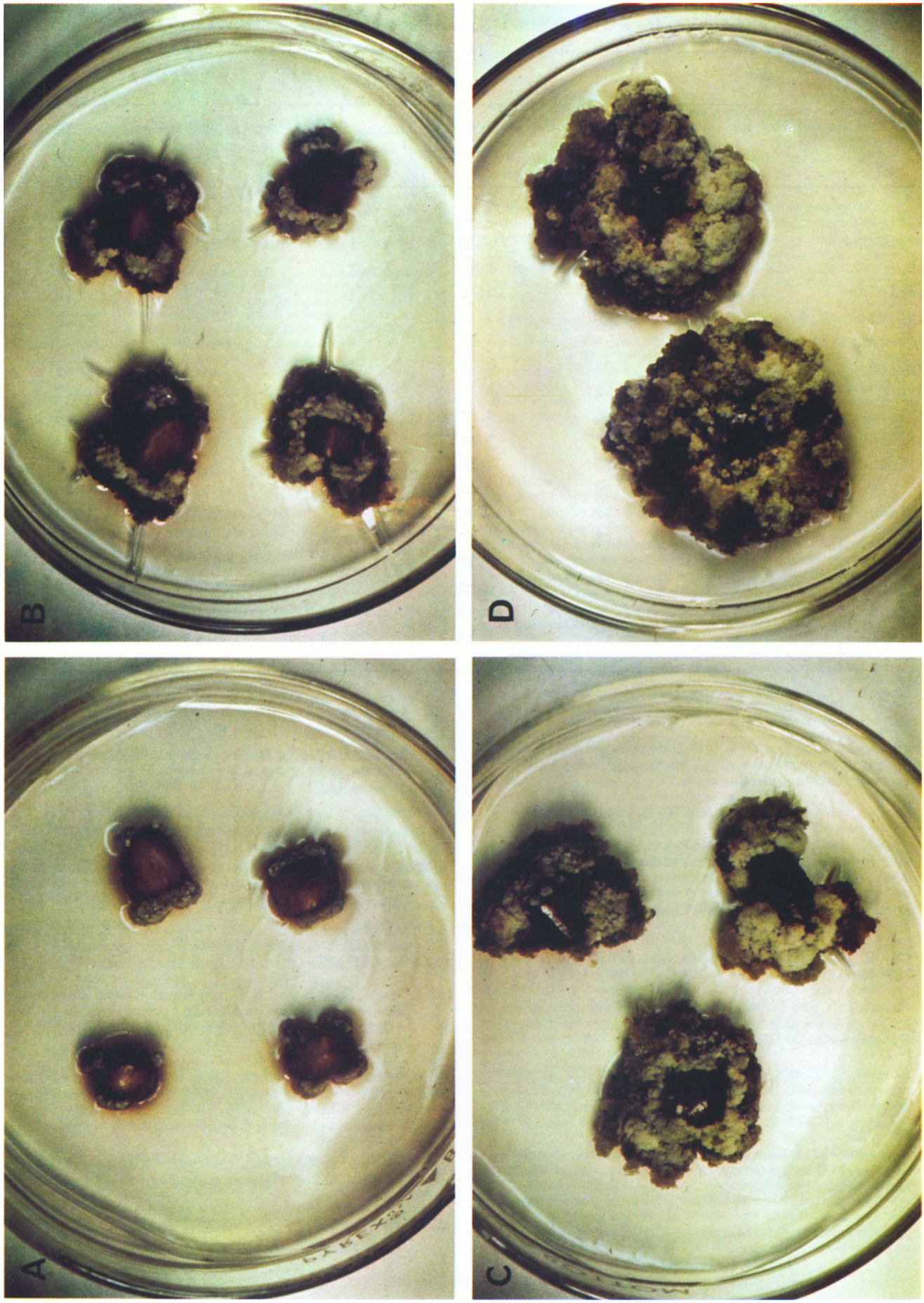

Fig. 2. Photographic series corresponding to the callus growth curve of 'Early Bunch' as seen in Figure 1. A. 15 days growth, B. 30 days growth, C. 45 days growth, D. 60 days growth.

Table 3. Amino acid composition of cotyledon and callus tissues (% of protein)

	Tissue					
	EB cotyledon	NC cotyledon	FR cotyledon	EB callus	NC callus	FR callus
lysine	3.49	3.58	3.78	4.68	4.12	4.59
histidine	2.46	2.48	2.37	3.19	3.71	3.81
NH_3	2.37	2.34	2.29	3.75	3.75	3.91
arginine	12.39	12.03	12.63	17.46	14.71	15.74
aspartic acid	11.98	12.15	12.22	19.89	20.60	22.65
threonine	4.33	4.38	4.19	4.43	4.49	4.68
serine	4.81	4.85	4.89	4.22	4.24	4.35
glutamic acid	18.79	19.09	19.26	12.05	12.90	11.01
proline	5.16	4.58	4.72	4.98	5.27	6.86
glycine	6.00	5.46	6.62	4.86	4.16	4.10
alanine	4.62	4.53	3.58	3.36	3.50	3.14
½ cysteine	trace	trace	trace	trace	trace	trace
valine	4.21	4.63	4.02	3.53	3.75	3.24
methionine	0.74	0.85	0.67	trace	trace	trace
isoleucine	3.18	3.29	3.19	3.07	3.21	2.66
leucine	6.38	6.41	6.42	4.77	5.15	4.01
tyrosine	3.90	3.92	3.90	2.43	2.51	2.32
phenylalanine	5.19	5.43	4.98	3.28	3.91	2.90

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