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CHANGES IN NUCLEIC ACID CONTENT, PEROXIDASE CONTENT AND ITS ISOZYMIC FORMS IN *IN VITRO* PROPAGATED CALLI OF CHICK PEA[1]

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Abstract

Culture conditions were developed to facilitate callus inducation by seeds and from explants of shoot tip, root tip, node and leaf of chick pea, *Cicer arietinum* L. cv. CM 72. DNA RNA content and peroxidase activity of calli raised from seeds showed a linear increase upto third subculture and then a gradual decrease upto the fifth one. Maximum and minimum amount of DNA, RNA and peroxidase activity did not synchronise in calli obtained from different explants. The number of isozymic forms was 2 in the parent culture which increased to 4, 5 and 6 in the 1st, 2nd and 3rd subcultures, respectively. The number got reduced to 2 in the 4th and finally to 1 in the 5th subculture. Four bands were observed in calli raised from shoot tip, 3 in node and one band each in leaf and shoot tip derived calli. Changes in nucleic acids content, peroxidase activity and its isozymic forms are in correlation with the callus growth and are manifestation of metabolic activity associated with active and stationary growth phase of the calli.

Introduction

In *in vitro* growth studies with appropriate manipulation of growth media, callus and organ formation can be induced for any plant on *a priori* basis. The success in this regard has been remarkable as reports on numerous plants from diverse groups are available (Even *et al.*, 1983). However, there still are a number of plants, specially among seed legumes which pose a problem both for callogenesis and organogenesis. Chick pea (*Cicer arietinum*) is such an example, where callus formation has been surmounted (Gosal & Bajaj, 1979; Altaf & Ahmad, 1986), but the processes of organogenesis are enigmatic, as shoot regeneration could not be obtained beyond the second subculture (Altaf & Ahmad, 1986). From the morphogenetic view point, it is a serious problem, which necessitates looking deeply at the underlying causes responsible for blocking the regeneration process. The present work reports the changes in nucleic acids (DNA, RNA) levels, peroxidase activity and its isozymic forms in calli obtained from different subcultures of parent callus and of different explants of chick pea.

Material and Methods

(i) *Induction of callus formation*: Growth conditions for raising aseptic plants and for callogenesis were the same as reported by Altaf & Ahmad (1986). Seeds of chick pea cv.

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CM72 were surface sterilised for 30 min with freshly prepared commercial sodium hypochlorite (NaOC1), washed with sterilized distilled water and germinated aseptically on MS basal media without amino acids, vitamins, auxins and cytokinins. Five to seven days after germination, the seedlings were cut into 0.5 to 1 cm segments and shoot meristem, root meristem, node and leaf were cultured alongwith the seeds on Murashige & Skoog (MS) medium (1962) supplemented with 1 mg/1 2,4-D, 1 mg/1 BA and 0.5 mg/1NAA for induction of callogenesis. The pH of the medium was adjusted to 5.7 before adding agar (1-2%). Temperature of growth room was 27 ± 2°C, with light intensity of 2000 lux for 16 h.

Callus tissue obtained from seed (parent callus) was subcultured after every 5 weeks. Calli were subcultured upto 5 times and thus callus was maintained for about 6 months. Changes in nucleic acid content, peroxidase activity and its isozymic forms were studied in the parent callus, the subcultures and in calli obtained from the shoot, root tip, leaf and node explants.

(ii) *Estimation of DNA and RNA content*: For the extraction of nucleic acids Ogur & Rosen's(1950) method as modified by Shakoori & Ahmad (1973) was used. One g callus tissue was boiled in ethanol for 4-5 min, crushed in 10 ml of 10% PCA and incubated at 4°C for 18 h, centrifuged for 10 min at 3,000 rpm and supernatant used for RNA estimation spectrophotometrically at 260 nm. The residue was extracted in 10% PCA at 60°C for 25 min and DNA estimated by the improved diphenylamine method of Giles & Myers (1965).

(iii) *Extraction and estimation of peroxidase activity*: One g sample of parent callus, its subcultures and explant calli was weighed and used for extraction of crude enzyme (Iqbal & Schraudolf, 1984). The activity of peroxidase was determined quantitatively by guaicol-H_2O_2 method of David & Murray (1965), spectrophotometrically at 470 nm and expressed as O.D./mg protein/30 seconds.

(iv) *Isozymic pattern of peroxidase*: Extracts were prepared in a manner as outlined above. Aliquots of the supernatant were taken for polyacrylamide slab gel disc electrophoresis according to Laemmli (1970). Peroxidase activity was detected on the gels by flooding with 0.005 M guaicol, 0.005 M hydrogen peroxide in a 0.2 M phosphate buffer adjusted to pH 5.8 (Siegel & Galston, 1967).

Results

(i) *Callogenesis from seeds*: For obtaining callus, seeds were grown in agar solidified MS + NAA (0.5 mg/1) + BA (1 mg/1) + 2, 4-D (1 mg/1). Initiation and proliferation of the calli was very rapid and vigorous from seed (parent callus) and in the first two sub-

cultures. Parent callus, i.e. seed callus appeared yellowish white and compact. Only the outer part of the callus was friable. The callus from the Ist subculture was pale yellow and proliferated extensively. Calli from 2nd and 3rd subcultures were greenish yellow in colour and proliferated vigorously. Proliferation slowed down in the 4th subculture, some signs of necrosis or partial browning of the external portion of the callus appeared. The growth of the 5th subculture was stationary and increased browning became apparent (Fig. 1; Table 1).

(ii) *Callogenesis from explants of seedlings:* For the callus induction from explants the same medium as used for callogenesis from seeds was used (Fig. 1). The callus inita-tion was observed in all cases. The most rapid and proliferating callus formation was ob-tained in leaf explant, while slowest in explants of shoot apices. Callogenesis in root and node was satisfactory. Callus obtained from the leaf was dark green colour while from node it was green and friable with compact portions. Shoot tip raised very friable callus of pale yellow colour while the callus from root tip was friable and greenish yellow (Table 2).

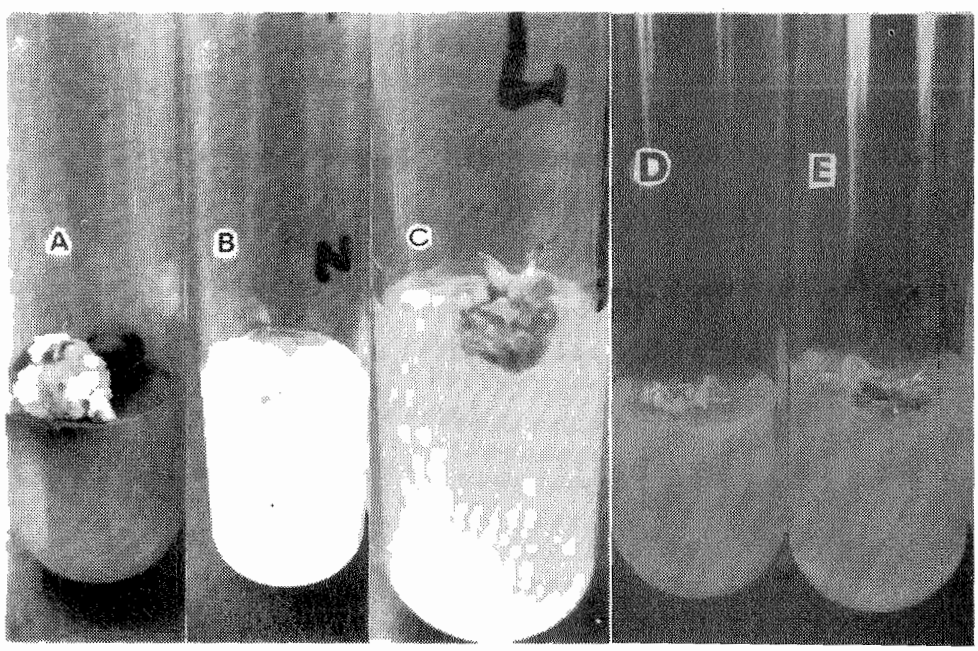

Fig. 1. Callogenesis response of seed and different seedling explants. A: Callus from seed. B: Seedling of chickpea. C: Callus fromleaf explant. D: Callus from shoot apex. E: Callus from root apex. F: Callus from node explant.

Table 1. Physical condition of callus in calli of different subcultures raised from seeds of chick pea.

No. of Culture	Parent Culture	SUBCULTURES I	II	III	IV	V
*Time interval	4	8	12	16	20	24
Source of culture	Seeds (Parent callus)	Ist subculture	2nd subculture	3rd subculture	4th subculture	5th subculture
Physical condition of callus during 4 week duration	Yellowish white and compact	Pale Yellow and friable	Greenish yellow and friable	Greenish yellow, friable and proliferating	Cessation of excessive proliferation	Development of brown patches in few calli stationary phase

*Time Interval 'Weeks'.

(iii) *Changes in DNA and RNA content in different subcultures*: An increase in DNA content of calli was observed upto 4th subculture (4.28 mg/g callus) in relation to the parent callus (2.18 mg/g callus) obtained from seed (Fig. 2). The increase was linear upto the 3rd subculture. In the 4th subculture a slight decrease occurred as compared to 3rd one, but the amount was still significantly higher than the parent callus. In the 5th subculture a decrease of 8.84% as compared to parent callus was observed. Changes in RNA content were in conformity with the changes observed for DNA content. The RNA content gradually increased from the parent callus (6.85 mg/g callus) to the 4th subculture (7.79 mg/g callus). However, the maximum RNA content like the DNA were observed for the 3rd subculture (8.83 mg/g callus). A decline in RNA content was observed for the 5th subculture (6.52 mg/g callus), the decrease was insignificant.

Table 2. Comparative response to callus formation from different explants of chick pea (35 days after inoculation)

Explant	No. of Explants	Successful Callogenesis	Time for Callogenesis (days)	Callus Response	Physical character of Callus
Seed	10	9	12	++	Whitish yellow
Shoot tip	10	5	10	+	Pale yellow
Root tip	10	8	13	++	Greenish yellow
Node	10	7	8	++	Green friable
Leaf	10	8	7	+++	Dark green friable

+ = Slow growth; ++ = Medium growth; +++ = Fast growth

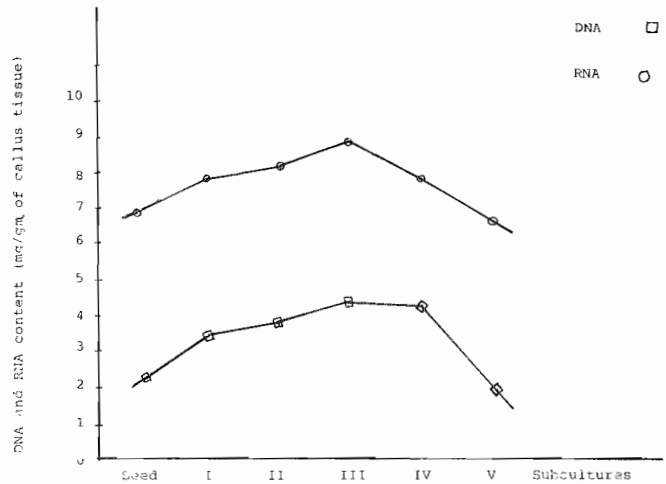

Fig. 2. Changes in DNA and RNA content (mg/g of callus tissue) in calli of different subcultures of chickpea. Time interval between successive subcultures (4 weeks).

(iv) *DNA and RNA content in calli of different explants*: The data on DNA and RNA content is also in conformity with the callogenesis responses of the explants (Fig. 3). The maximum DNA content was observed in leaf (2.51 mg/g callus) and minimum in node (0.73 mg/g callus). The gradient for DNA content in calli of explants is: leaf > root tip > shoot tip > seed > node. The pattern of RNA content was quite different from the DNA. Maximum RNA content was found in calli raised from seed (5.17 mg/g callus) and minimum in calli from leaf explant (2.17 mg/g callus). The gradient for RNA content is: seed > root tip > shoot tip > node > leaf.

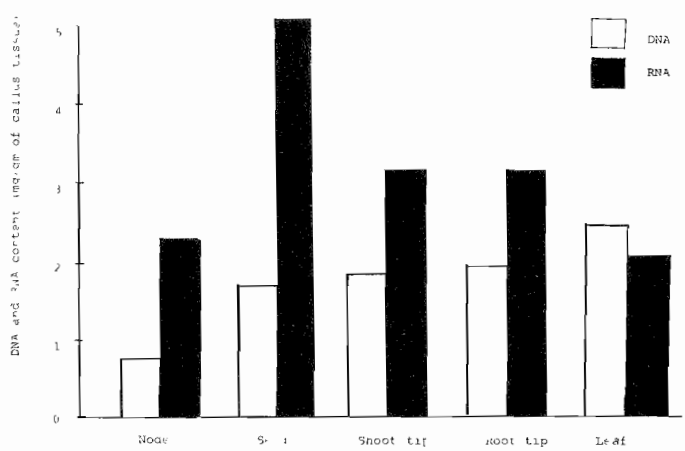

Fig. 3. Changes in DNA and RNA content (mg/g of callus tissue) calli raised from seed and different explants of chickpea.

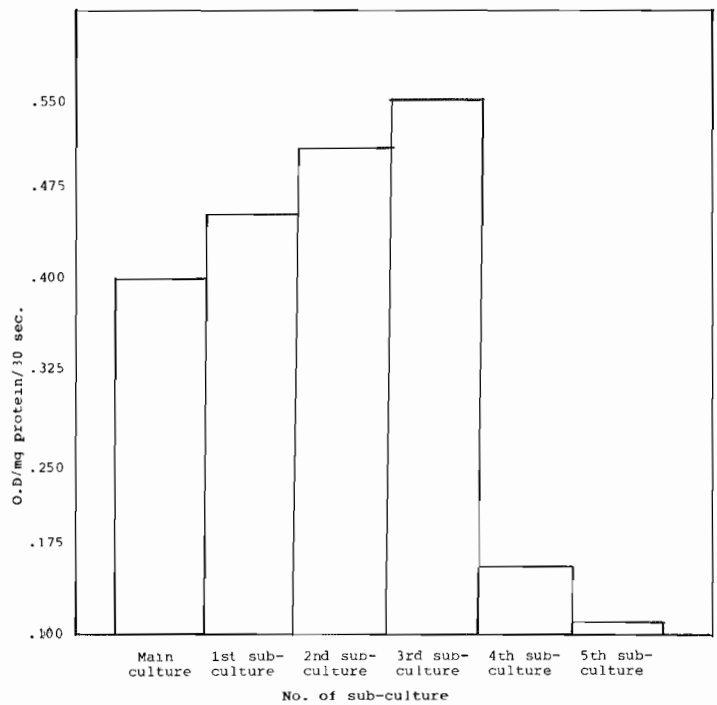

Fig. 4. Peroxidase activity in calli of different sub-cultures of chickpea (O.D = 470 nm).

(v) *Peroxidase activity of calli of the seed and its subcultures*: Starting from the parent seed callus the peroxidase activity increased upto 3rd subculture and declined afterwards, i.e. in the 4th and 5th sub culture (Fig. 4). The mean peroxidase activity of 0.395 was observed in parent callus, which increased to 0.452, 0.501 and 0.546 in Ist, 2nd and 3rd subcultures, respectively. The peroxidase activity decreased to 0.160 in the 4th and to 0.111 in the 5th subculture.

(vi) *Peroxidase activity of calli from explants*: Maximum peroxidase activity was observed in calli obtained from root and minimum from seed callus (Fig. 5). The gradient of peroxidase activity is: root (7.44) > Leaf (6.1) > node (5.42) > shoot tip (3.65) > and seed (3.42).

(vii) *Isozyme pattern of peroxidase of calli of seed and subsequent subcultures* : Fig. 6 shows two bands for peroxidase in the seed callus (Rf values of 0.73 and 0.79). The number of bands increased to 4 in Ist subculture (Rf values of 0.13, 0.18, 0.26 and 0.29, respectively). These Rf values were quite different from those of the seed callus, indicating that all these four bands were new in their origin. In the calli of 2nd subculture, the number of bands rose to 5 (Rf values 0.18, 0.24, 0.29, 0.31 and 0.33, respectively). There were two bands (Rf 0.18 and 0.29) which were common in both Ist and 2nd subcultures.

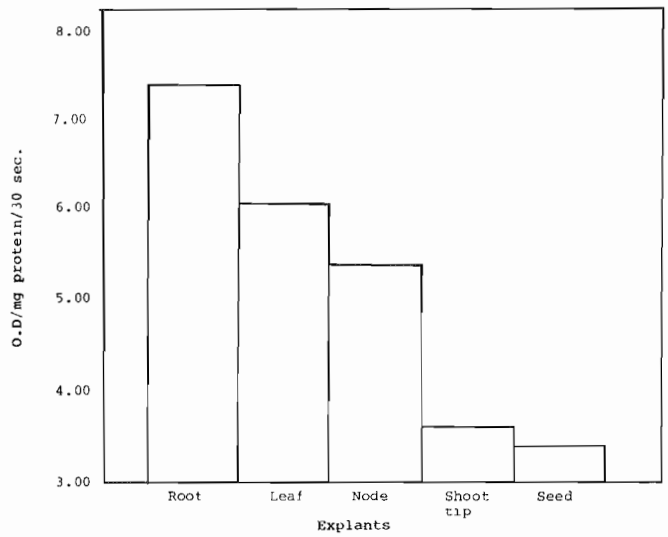

Fig. 5. Peroxidase activity in calli raised from different explants of chickpea (O.D. = 470 nm).

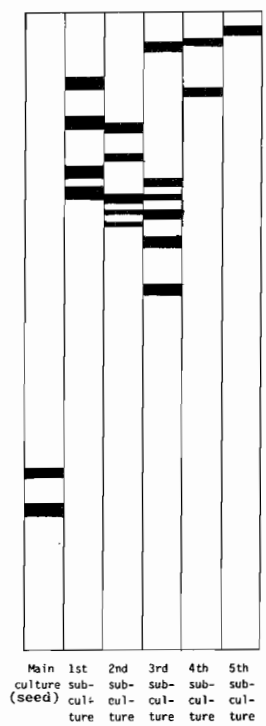

Fig. 6. Peroxidase isozymic pattern for main and different sub-cultures of chickpea.

Six bands were observed in the 3rd subculture (Rf values of 0.06, 0.27, 0.29, 0.32, 0.39 and 0.43). In this case also 4 bands were new, while two were common in both 2nd and 3rd subcultures. The number of bands got reduced to 2 in the 4th (Rf 0.05 and 0.13) and finally to one in the 5th subculture (Rf 0.04).

(viii) *Isozymic pattern of peroxidase of calli raised from different explants*: Maximum number of peroxidase isozymic forms (Fig.7) were observed in the calli raised from shoot tip (4 bands), followed by the node (3 bands), seed callus (2 bands), leaf and root (one band each). A comparison of Rf values of the isozymic forms reveal that only one isozymic form is common between shoot tip and node callus (Rf 0.92). All other bands from these two calli and from the calli of leaf and root exhibited a characteristic pattern specific to themselves.

Discussion

The present study shows that callus induction can be conveniently generated in chickpea from seeds and other seedling explants under appropriate growth conditions, as also reported by Gosal & Bajaj (1979) and Altaf & Ahmad (1986).The calli can be subpropagatged for a considerable length of time. However, growth potential gradually decreases as is indicated by physical characteristics of the calli and the present biochemical investigations.

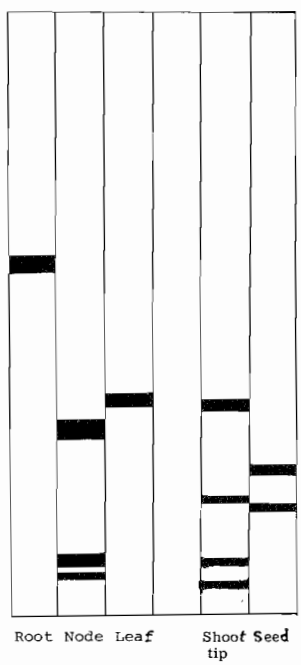

Fig. 7. Peroxidase isozymic pattern for calli raised from different explants of chickpea.

The changes observed in DNA, RNA, peroxidase activity and its isozymic forms from parent callus upto 5th subculture are in general conformity with the physical characteristics of these calli. The gradual increase in all these biochemical parameters upto the 3rd subculture correlates with active proliferation of the callus from parent to the 3rd subculture. This increase in nucleic acids and peroxidase activity is in turn correlated with the increased soluble protein content during these phases (parent callus 11.63 mg/g callus to 21.44 mg/g callus in the 3rd subculture). This all manifest that the observed quantitative (DNA, RNA, peroxidase activity and soluble protein content) and qualitative (isozymic forms of peroxidase) increase is anabolic, reflecting intense metabolic activity linked with active cell division associated with extensive proliferation of the calli from parent to 3rd subculture. The decrease in peroxidase activity, DNA and RNA content concomitant with soluble protein content in the 4th and 5th subculture (14.62 and 8.80 mg/g callus protein, respectively) and consequently reduction in isozymic forms of peroxidase can conveniently be correlated with the physical characteristics of the calli in the 4th and 5th subculture. In 4th subculture active proliferation subsided alongwith the initiation of browning of the callus. In the 5th subculture the growth of the callus became stationary, browning increased and necrotic spots appeared, which lead to the final death of the callus.

Changes both quantitative and qualitative in different enzymes and their isozymic forms during cell or callus cultures as observed in the present study in chickpea have also been reported in bean (Bassiri & Carlson, 1978); bush bean (Arnison & Boll, 1975) wild carrot (Lee & Dougal, 1973) and in tobacco (Ritzert & Turin, 1970). Peroxidase is an iron-porphyrin enzyme whose precise role in plant metabolism is rather obscure, although it has been implicated in numerous plant functions. In plant tissues, naturally occuring phenols and amines may serve as hydrogen donors and a possible metabolic role for peroxidase would be to oxidise these toxic compounds to less harmful products (Sullivan, 1946). Peroxidase may also serve to eliminate H_2O_2. Peroxidase enzymes may also be important for the destruction of the plant growth hormone IAA (McCune, 1961). Peroxidase is also implicated with high rates of cell division during cell differentiation (De Jong, 1967; Vandern, 1963; Van Fleet, 1959).

The present authors on *a priori* basis, view the increase in nucleic acids, soluble protein content upto 3rd subculture as linked with active cell divisions occurring in proliferating callus, and a drop in these parameters in 4th and 5th subcultures with reduction in cell division activity leading to the cessation of callus growth. The increase in the peroxidase activity can both be linked with cell division activity (De Jong, 1967) and for the elimination of increasing amounts of naturally occurring phenols (Sullivan, 1946). The later viewpoint is substantiated by the fact that in chickpea plant, isoflavones occur as 7-o-glycosides and flavonoles as the 3-glycosides (Hösel *et al*, 1972). Flavonol aglycones and flavonol 7-glycosides are readily degraded by peroxidases *in vitro*, as well as, in plant

suspension cultures (Hösel *et al*, 1975). In 4th and 5th subcultures the amount of phenols and other toxic substances may reach a level where sustaining of callus growth becomes an arduous task for the component cells. Consequently the seizure, browning and necrosis of the callus occurs, and number of normal metabolising cells fall. This brings the decrease in nucleic acid content, protein content, peroxidase activity and of its isozymic forms.

The present study also reveals the tissue specificity of different explants. The calli raised from different explants vary in their DNA, RNA content, peroxidase activity and its isozymic forms. The precise biological mechanism underlying the observed variations in these biochemical parameters is not known. These, however, could be a reflection of differences in genetic expression or gene functioning in different cell types.

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