Pak.J.Bot., 23(2): 227-235, 1991.

TOTAL PHENOLICS, PHENYL ALANINE AMMONIA LYASE AND POLYPHENOL OXIDASE IN *IN VITRO* CALLI OF CHICKPEA

JAVED IQBAL, SHAGUFTA NAZ, SARWAT NAZIR FAHEEM AFTAB AND M.S. AHMED**

Department of Botany,
Punjab University, Quaid-i-Azam Campus,
Lahore, Pakistan.

Abstract

Conditions for *in vitro* cultures of calli from root, cotyledon and hypocotyl explants of chickpea (*Cicer arietinum* L. cv. CM 72) in M.S. medium were established. Total phenolics, phenylalanine ammonia-lyase (PAL) and polyphenol oxidases (PPO) were estimated quantitatively from main callus to 6th sub-culture. The activity of PAL and PPO showed a linear increase upto third sub-culture and then a remarkable decrease from fourth to sixth sub-culture was observed. The data of phenolics was not in conformity with PAL and PPO activity. A gradual increase in total phenolics from main to sixth sub-culture was observed. The data obtained from PAL, PPO and total phenolics correlates with the callus growth.

Introduction

The brown colour that frequently develops in plant cell, tissue and protoplast cultures of diverse plants is thought to be brought about through the metabolism of phenolic compounds. The enzyme polyphenol oxidase (PPO) may oxidise these compounds to quinones which simultaneously polymerise into brown pigments (Siriphanich & Kader, 1985; Lee *et al.*, 1990). Quinones are well known to be toxic to microorganism and are inhibitory to plant cellular growth (Monaco *et al.*, 1977). Other inhibitory actions possibly result from bonding between phenols and proteins and subsequent oxidation of quinones resulting in the loss of enzyme activities (Loomis & Battailes, 1966). When culturing species prone to phenolic deposition, special precautions are necessary for preventing the accumulation of toxic products resulting from phenolic oxidation.

From an earlier work on nucleic acids, proteins and peroxidase content and its isozymic forms in calli of chickpea (Iqbal *et al.*, 1989), it was concluded that callus browning, necrosis and seizure of callus growth may be due to accumulation of phenolic substances to toxic levels. Accumulation of phenolics to a level which is detrimental for *in vitro* growth is common in some very important plants of economic significance such as tobacco (Anagnostasis, 1974); coffee (Monaco *et al.*, 1977); apple (Zimmerman, 1978); teak (Gupta *et al.*, 1980) sugarcane (Chen *et al.*, 1988); grapes (Lee & Wetzstein, 1988); mango (Dewald *et al.*, 1989) and chickpea (Iqbal *et al.*, 1989).

** Nuclear Institute for Agriculture and Biology, Jhang Road, Faisalabad.

Keeping the limiting effects of phenolics accumulation in *in vitro* cultures, it is considered worthwhile to systematically analyse:-
(i) The biosynthesis of phenolic compounds i.e., their production, accumulation, oxidation and role of PAL, PPO and peroxidase during callogenesis and organogenesis in *Cicer arietinum* L., and (ii) To develop methodology to keep the level of phenolics low in *in vitro* cultures by manipulating growth conditions and by use of growth regulators or specific inhibitors to control/regulate the accumulation of phenolic compounds. This should in turn improve the long term callogenesis and regeneration potential of the plant.

The present study reports the estimation of total phenolics and two important enzymes of phenolic metabolism i.e., Phenylalanine Ammonia-Lyase (PAL) and Polyphenol Oxidase (PPO) in main callus and sub-culture calli of chickpea. PAL is responsible for the first step reaction in the conversion of amino acid L-Phenylalanine and l-tyrosine to the whole range of phenolic compounds (Young *et al.*, 1966) while PPO is a group of enzymes associated with phenol oxidation.

Materials and Methods

Induction of callus formation: Methods of aseptic seed germination, seedling growth and callogenesis were the same as reported earlier (Iqbal *et al.*, 1989). Calli obtained from root, hypocotyl and cotyledon explants were sub-cultured after every four weeks. At the time of each sub-culturing the phenolic compounds (for both callus and upper 1 cm surface of growth medium), Phenylalanine Ammonia-Lyase (PAL) and Polyphenol oxidase (PPO) were estimated from the calli.
Estimation of phenolic compounds: For the estimation of total phenolics, the method of A.O.A.C (1965) was used. One gram callus of seed was crushed with 10 ml of methanol in a homogenizer for 5 minutes at top speed. The mixture was filtered and concentrated by evaporation in a rotary evaporator under reduced pressure. The aqueous extract was centrifuged at 6,000 rpm at 25^{o}C for 10 min. The supernatant was placed in a 100 ml flask, 5 ml of Folin-Dennis Reagent (A.O.A.C, 1965) and 10 ml of 20% Na_2CO_3 solution was added and volume was made upto 100 ml with distilled water. After shaking absorbance at 730-760 nm was taken and expressed as. O.D/mg of tannic acid, by comparison with a calibration curve prepared from a reference solution containing 100 mg of tannic acid per litre.
Changes in content of Phenylalanine Ammonia-Lyase: A procedure described by Zucker (1965) and modified by Pendharkar & Nair (1975) was used to assay PAL. According to this method, 0.1g of fresh callus was crushed with 0.4 ml of 0.1 M Borate buffer in an ice-chilled mortar and pestle. The slurry obtained was centrifuged at 10,000 rpm at 4^{o}C for 10 minutes. Of the crude enzyme extract 0.3 ml was mixed with 30 μ moles of phenylalanine and 200 μ moles of borate buffer. The total volume was made upto 3 ml with extraction buffer. This 3 ml was incubated for an hour at 40^{o}C. Reaction was terminated by adding 0.2 ml of 5 N HCl. Total volume was made upto 4 ml with distilled H_2O. The amount of transcinnamic acid formed in the reaction was calculated from change in O.D of the reaction mixture at 290 nm. A standard graph for trans-cinnamic acid under identical conditions was referred for

the calculation. The specific activity of the enzyme was expressed as moles of trans-cinnamic acid produced per hr/g of tissue. Suitable blanks were run simultaneously alongwith the samples in all assays.

Analysis of Polyphenol Oxidase Activity: The polyphenol oxidase activity was estimated using assay method outlined in Worthington Enzyme manual by Decker (1977). For every estimation 0.5 g callus was crushed with 0.5 M Phosphate buffer pH 6.5 in an ice-chilled pestle and mortar. The slurry was centrifuged at 10,000 rpm at 4°C for 10 min. According to this method to 0.1 ml of enzyme, 1.0 ml of phosphate buffer, 1.0 ml of 0.001 M tyrosine and 0.9 ml of H_2O was added. For blank instead of 0.1 ml of enzyme water was added. The activity of phenol oxidase was estimated at 280 nm for 10-15 minutes. Protein was determined by the method of Lowry *et al.*, (1951).

Results

Callogenesis: Results pertaining to callus induction, proliferation, behaviour and physical characteristics have been reported (Iqbal *et al.*, 1989). Calli were maintained upto 6th sub-culture. Afterwards because of excessive browning, further sub-culturing was not possible.

Total activity of polyphenol oxidase **(units/mg callus)**: As evident from Fig.1 and Table.1, a sharp increase in the total activity of polyphenol oxidase was observed in first sub-culture in comparison with the main callus. The enzyme activity although slightly dropped in 2nd and 3rd sub-culture, but was still 36.3 and 35.71% respectively, higher than the main callus. A sharp decline in the enzyme activity was observed in 4th sub-culture (-91.66%). More or less the same reduced activity was maintained at 5th and 6th sub-cultures (-92.85%).

Specific activity of polyphenol oxidase **(units/µg protein)**: Specific activity of enzyme showed a similar pattern of increase or decrease as for total activity (Fig.2, Table.1).

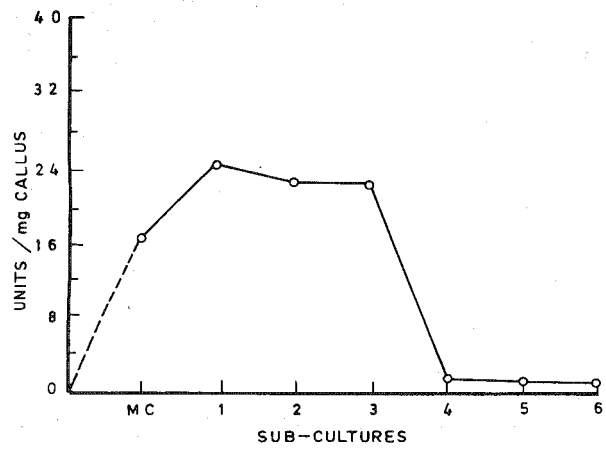

Fig.1. PPO-Total activity (units/mg callus) in seed callus from main to successive sub-cultures.

Table 1. Activity of polyphenol oxidase, Phenylalanine Ammonia-Lyase and phenolic compounds in different calli raised asceptically from main to successive sub-cultures.

No. of sub-culture	Poly phenol oxidase Total activity (units/mg callus)	Specific activity (units/µg protein)	Phenylalanine Ammonia -Lyase activity n moles of trans-cinnamic acid /hr/mg protein)	Phenolic compounds In Calli (O.D/mg tannic acid	In Calli medium (O.D/mg tannic acid	Physical appearance
Main culture	16.80[a]	3.24[a]	0.020[a]	0.110[a]	0.098[a]	Whitish yellow compact.
First sub-culture	24.40[b]	4.93[b]	0.078[b]	0.122[ab]	0.142[b]	Greenish yellow, gre
	(+45.23)	(+52.16)	(+290.0)	(+10.90)	(+44.89)	proliferating part.
Second sub-culture	22.90[c]	4.40[bc]	0.104[c]	0.170[c]	0.156[c]	Greenish yellow prol
	(+36.30)	(+35.80)	(+420.0)	(+54.54)	(+59.18)	ferating.
Third sub-culture	22.80[d]	4.38[bc]	0.041[d]	0.202[d]	0.160[c]	Green, friable.
	(+35.71)	(+35.18)	(+105.0)	(+83.63)	(+63.20)	
Fourth sub-culture	1.40[e]	1.89[d]	0.018[e]	0.218[de]	0.201[d]	Dark green, lesser
	(-91.66)	(-41.66)	(-10.0)	(+98.18)	(+105.10)	proliferation.
Fifth sub-culture	1.20[e]	1.66[d]	0.016[e]	0.224[e]	0.208[d]	Browning of calli
	(-92.85)	(-48.76)	(-20.0)	(103.6)	(+112.28)	starts. Dark brown patches on calli
Sixth sub-culture	1.20[e]	1.50[d]	0.012[e]	0.240[f]	0.230[e]	70% of the calli are
	(-92.85)	(-53.70)	(-40.0)	(+118.18)	(+134.69)	completely brown. proliferation ceases.

+/- percent increase or decrease. a.b.c. = Values with different alphabets are significantly different from each other at 5% level of significance. (Duncan's multiple range test of composite means).

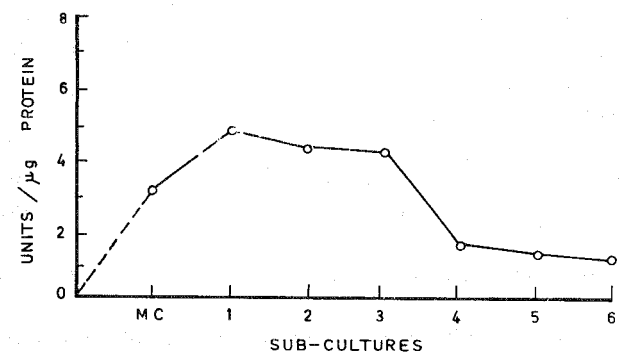

Fig.2. PPO-specific activity (units/µg protein) in seed callus from main to successive sub-cultures.

However, the relative percent decreases at 4th to 6th sub-culture were less in comparison with the total activity. Maximum specific activity was observed at 1st sub-culture (+52.16%) with decline at 2nd (+35.80%) and 3rd sub-culture (+35.18%). The decreases at 4th to 6th sub-culture were -41.66, -48.76 and -53.70%, respectively.

***Total activity of Phenylalanine Ammonia-Lyase* (n moles of trans-cinnamic acid/mg protein):** The phenylalanine ammonia-lyase activity increased upto third sub-culture in relation to the main callus and declined afterwards i.e., in fourth, fifth and sixth sub-cultures (Fig.3, Table 1). In main culture, the PAL activity was 0.020 which

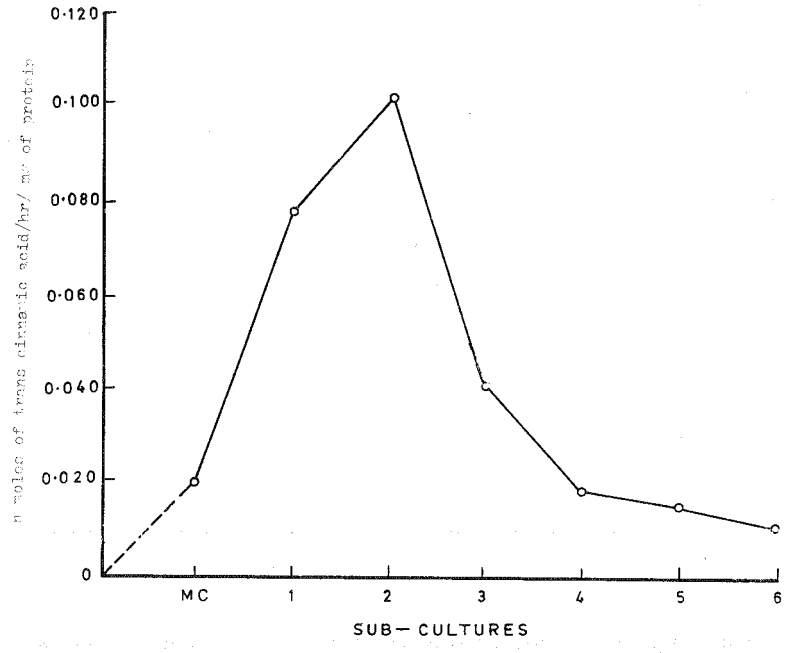

Fig.3. Phenylalanine Ammonia-Lyase activity in seed callus from main to successive sub-cultures. (n moles of trans cinnamic acid per hour per mg of protein).

increased to 0.078 and 0.104 in 1st and 2nd sub-cultures. In 3rd sub-culture a decrease occured as compared to 2nd sub-culture but the activity was still higher (+105%) as compared to parent callus. In fourth, fifth and sixth sub-cultures a decrease was observed. The activity was -10.00, -20.00 and -40.00%, respectively in relation to the main callus.

***Total Phenolics in Calli* (O.D/mg of tannic acid):** An increase in total phenolics was observed from main culture to sixth sub-culture as is evident from the Fig.4 and Table 1. The increase was linear. In main culture, the amount of total phenolics was 0.110 which increased to 0.240 in sixth sub-culture, an increase of 118.186% from the main callus.

***Total phenolics in culture medium* (O.D/mg of tannic acid):** The data of phenolics assayed from upper 1 cm growth medium is in conformity with the data on calli (Fig.4, Table 1). An increase in phenolics was observed in growth media of all sub-cultures (except for 1st sub-culture) in relation to their respective calli.

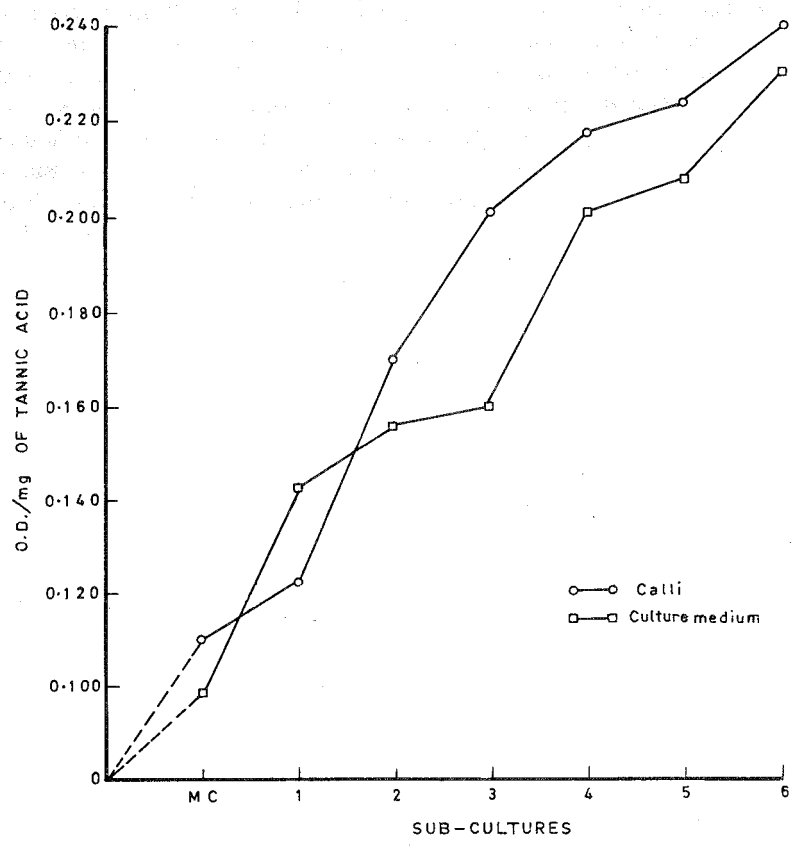

Fig.4. Concentration of phenolic compounds (O.D/mg of tannic acid) from main to successive sub-cultures in calli and culture medium.

Discussion

In the tissue culture studies callus has great potential particularly in creating genetic variability. Callus has been successfully induced from numerous explants belonging to diverse groups of plants. However, regeneration potential of callusing in many plants is very low. This is particularly true of chickpea. The callus of chickpea can be initiated from number of explants, yet plant regeneration is limited (Iqbal *et al.*, 1989; Rao & Chopra, 1987; Altaf & Ahmed, 1986; Anil *et al.*, 1982; Kartha *et al.*, 1981). Physical appearance of calli after 5th sub-culture (Iqbal *et al.*, 1989) suggests on *a priori* basis that phenolics may somehow be involved in suppression of callus proliferation and regeneration.

Phenolic compounds are widely distributed among wide variety of plants. With certain exceptions, the function of most of the phenolics are obscure (Clarri & Philipe, 1988; Wall *et al.*, 1988; Jacques *et al.*, 1988; Sheen, 1969; Galston, 1969). Most of the work regarding phenolic compounds and the related enzymes such as phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO), in general is found with respect to disease resistance (Chander & Chandravedana, 1988; Kalia & Sharma 1988; Fehrmann & Diamond, 1967; Bard Ya, 1984). The inhibitory role of phenolics in callogenesis has not been investigated properly.

In the present study the content of phenolics in particular and activity of PAL and PPO in general shows a relationship with general physical characteristics of calli i.e., callus colour, friability and growth rate. The callus growth is very active (+ + + +) at 1st sub-culture, is stable and fairly vigorous (+ + +) at 2nd, 3rd and 4th sub-cultures. There after it slows down in 5th sub-culture (+ +) and finally is static (+) showing signs of necrosis and extensive browning in the 6th sub-culture. As regard the total phenolics, activity shows a gradual continuous increase from main to 6th sub-culture. Contrary to this, the activity of PAL and PPO is quite high upto 3rd sub-culture, then after it shows a sudden fall in PPO and gradual decline for PAL, upto 6th sub-cultures. Apparently these are contradictory trends as with increase in phenolics, increase in the precursor enzyme PAL and oxidizing enzyme PPO should also exhibit parallel increase. Although the anamolous behaviour of enzymes is not clear, yet on *a priori* basis, it is suggested that upto the 3rd sub-culture both the enzymes keep pace with the synthesis and breakdown of phenolics. From 4th sub-culture the fall in the content and final cessation of synthesis of both enzymes be due to metabolic disorders caused by the phenolics, once the safe threshold level of cell tolerance to phenolics has been crossed. At this stage phenolics and other toxic substances may have been deposited to a level where cells cease to divide and the process of slow metabolic death initiates. This is substantiated by our earlier report where decrease in nucleic acids and protein content were observed from 4th sub-culture onward, an indication of disruption of cell anabolic activity (Iqbal *et al.*, 1989).

Acknowledgements

The first author is highly grateful to the Director, NIAB, Faisalabad and Pakistan Atomic Energy Commission for provision of funds for the Project 11(4)86 under "Collaboration with Universities" Programme.

References

Altaf, N. and M.S. Ahmed. 1986. Plant regeneration and propagation of chickpea (*Cicer arietinum*) through tissue culture technique. Symp. Nuclear techniques and *in vitro* cultures for plant improvement. August, 1985. IAEA-SM-282/31, PP. 408-417.

Anagnostalis, S.L. 1974. Haploid plants from anthers of tobacco enhancement with charcoal. *Planta*, 115: 281-283.

Anil, N., C.S. Redy. and G.M. Reddy. 1986. Plantlet regeneration from callus cultures of *Cicer arietinum* L. *Int. Chickpea News Letter*, 14: 12-13.

A.O.A.C. 1965. Methods of analysis of the Association of Official Agricultural Chemists, 10th Ed. P. 139. Washington. D.C.

Bard Ya, T. 1984. The effect of abscisic acid and kinetin on the resistance of asters to powdery mildew and on the polyphenol peroxidase system. *EEste NSv Tead Akad Ioim* Biol, 33: 131-136.

Chander, M.S. and M.V. Chandravadana. 1988. Enzymic properties associated with resistance to powdery mildew in peas. *J. Hortic. Sci.*, 63: 641-644.

Chen, W.H., M.R. Devy, J.B. Power. and E.C. Cocking. 1988. Sugarcane protoplasts: factors affecting division and plant regeneration. *Plant Cell Reports*, 7: 344-347.

Clarri, L.P. and L. Philipe. 1980. Flavonoid variability within *Pinus sylvestris Phytochemistry*, 27: 2663-66.

De wald, S.G., R.E. Litz and G.A. Moore. 1989. Optimizing somatic embryo production in mango. *J. Amer. Soc. Hort Sci.*, 114: 712-716.

Decker, A. 1977. Enzymes, enzyme reagents and related enzyme biochemicals. Worthington Enzyme manual. Worthington Biochemical Corporations Freehold; New Jersey, U.S.A. pp. 74-76.

Fehrmann, H. and A.E. Diamond. 1967. Peroxidase activity and *Phytophthora* resistance in different organs of potato plant. *Phytopathology*, 57: 68-72.

Galston, A.W. 1969. In: *Perspectives in phytochemistry*, (Eds.). J.B. Harborne and T.S. Swain pp. 193-204. Acad. Press. London.

Gupta, P.K., A.L. Nadhir, A.F. Mescarenhas. and V. Jagannathan. 1980. Tissue culture of forest trees: Clonal multiplication of *Tectona grandis* L. (Teak) by tissue culture. *Plant Sci. Lett.*, 17: 259-268.

Iqbal, J., N. Butt., F. Saeed. and M.S. Ahmed. 1989. Changes in nucleic acid content, peroxidase content and its isozymic forms in *in vitro* propagated calli of chickpea. *Pak. J. Bot.*, 21: 13-23.

Jacques, U., H. Kessmann. and W. Barz. 1988. Accumulation of phenolic compounds and phytoalexins in sliced and elicitor treated cotyledons of *Cicer arietinum* L. *Z. naturforsch.*, 42: 117-78.

Kallia, D. and S.K. Sharma. 1988. Biochemical genetics of powdery mildew. *Theor. Appl. Genet.*, 76: 795-799.

Kartha, K.K., N.L. Ceung and L.A. Mroginsoki. 1981. Plant regeneration from meristem of grain legumes, soyabean, cowpea, peanut and bean. *Can. J. Bot.*, 59: 167-79.

Lee, C.Y., V. Kagan, A.W. Jaworski and S.K. Brown. 1990. Enzymatic browning in relation to phenolic compounds and phenol oxidase activity among various peach cultivars. *J. Agric. Food. Chem.*, 30: 99-101.

Lee, N. and H.Y. Wetzstein. 1988. Protoplast isolation and callus production from leaves of tissue cultured *Vitis* spp. *Plant Cell Reports*, 7: 531-534.

Loomis, W.D. and J. Battailes. 1966. Plant phenolic compounds and isolation of plant enzymes. *Phytochemistry*, 5: 423-438.

Lowry, D.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.*, 193: 265-275.

Monaco, L.C., M.R. Sondhal, A. Cavalho, O.J. Crocomo. and W.R. Sharp. 1977. Application of tissue culture in the improvement of coffee. In; Applied and fundamental aspects of plant cell tissue and organ culture. (Ed.) J. Reinert and Y.P.S. Bajaj, Springer Verlag, Berlin, pp. 109-129.

Pendharkar, M.B. and P.M. Nair. 1975. Induction of Phenylalanine ammonia Lyase (PAL) in gamma irradiated potatoes. *Radiation Botany*, 15: 191-197.

Rao, B.G. and V.L. Chopra. 1987. The influence of media on callusing and organogenesis in chickpea. *Int. Chickpea News Letter*, 17: 7-10.

Sharma, R., B. Sharma. and G. Singh. 1984. Phenols as regulators of Nitrate reductase activity of *Cicer arietinum*. *Phyton. Rev. Int. Bot. Exp.*, 44: 185-88.

Sheen, S.J. 1969. The distribution of polyphenol, chlorogenic acid oxidase and peroxidase in different plant parts of tobacco *Nicotiana tabacum* L. *Phytochem*, 8: 1839-47.

Singh, R.P., H.K. Singh., H.K. Jaiswal and R.M. Singh. 1982. Organogenesis in callus culture of chickpea. *Indian Jour. Agric. Science*, 52: 86-90.

Siriphanich, J. and A.A. Kader. 1985. Effect of CO_2 on total phenolics, phenylalanine ammonia lyase and polyphenol oxidase in lettuce tissue. *J. Amer. Soc. Hort. Sci.*, 110: 249-253.

Wall, M.E., M.C. Wani, G. Manikumar, P. Abraham, H. Taylor, G. Warner and R. Mcgiveny. 1988. Plant antimutagenic agents 2-Flavonoids. *J. Nat. Prod.*, 51: 1084-91.

Young, M.R., G.H.N. Towers and A.C. Nesh. 1966. Taxonomic distribution of Ammonia Lyases for L. phenylalanine, *Can. J. Bot.*, 44: 341-49.

Zimmerman, R.H. 1978. Tissue culture of fruit trees and other plants. *Proc. Int. Plant Crop. Soc.*, 28: 539-545.

Zucker, M. 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.*, 40: 779-784.

(Received for Publication 24 February 1991)