Am. Chem. Soc, 1988, **110**, 6204–6210.
29 Webb, T. H, Suh, H and Wilcox, C. S., *J. Am. Chem. Soc.*, 1991, **113**, 8554–8555
30. Adrian, J C. Jr and Wilcox, C. S, *J. Am Chem. Soc.*, 1989, **111**, 8055–8057. The design principle, synthesis and study of this receptor has been nicely described by C. S. Wilcox in ref. 1(c).
31. Wilcox *et al.* synthesized chiral host **18** starting from the chiral precursors. However, this strategy lacks the general applicability for the asymmetric synthesis of Tröger's base analogues.
32. Baker, J. F. and Blickenstaff, R. T., *J. Org. Chem.*, 1975, **40**, 1579–1586.
33. Yashima, E., Akashi, M. and Miyauchi, N., *Chem. Lett.*, 1991, 1017–1020.
34. For examples of binding nucleotide bases through both hydrogen bonding and aromatic–aromatic interaction see Zimmerman, S. C., Zeng, Z., Wu, W. and Reichert, D. E., *J. Am. Chem. Soc.*, 1991, **113**, 183–196; Zimmerman, S. C., Wu, W. and Zeng, Z., *J. Am. Chem. Soc.*, 1991, **113**, 196–201.

ACKNOWLEDGEMENTS. I thank Dr Uday Maitra for his encouragement for writing this particular article, for providing unpublished results, and for useful discussions while preparing this manuscript. Prof. P. Balaram, Dr S. Bhattacharya and Dr S. Balasubramanian are gratefully acknowledged for critically going through this manuscript. CSIR is acknowledged for providing a research fellowship.

Received 20 May 1994; accepted 1 July 1994

Lathyrus sativus: A future pulse crop free of neurotoxin

V. K. Yadav and S. L. Mehta*
Department of Biochemistry, Rajasthan Agricultural University, SKN College of Agriculture, Jobner 303 329, India
*Division of Biochemistry, Indian Agricultural Research Institute, New Delhi, 110 012, India

***Lathyrus sativus* is popular among farmers due to its ease of cultivation and high climatic adaptability. However, full potential of this crop has not been realized due to the presence of a toxin, β-*N*-oxalyl-L-α,β-diaminopropionic acid (ODAP) which causes a paralytic disorder known as neurolathyrism in humans. Conventional breeding and selection methods have failed to produce varieties free of the neurotoxin. Research utilizing recombinant DNA technology and tissue culture has been initiated in the recent years to produce *Lathyrus sativus* plants free of neurotoxin. The progress in this area of research includes isolation and characterization of ODAP-degrading gene from pure cultures of bacteria. It offers the scope for introducing this gene into *L. sativus* by *Agrobacterium*-mediated transformation. As part of the second approach, oxalyl-CoA (coenzyme A) synthetase, which is a key enzyme in the biosynthesis of ODAP, has been purified and monoclonal antibodies raised against it. This can be used to construct antisense gene of this enzyme for introducing into *L. sativus*. Somaclones having very low toxin contents have also been developed. All these results show the potential of producing neurotoxin-free *L. sativus* plants in the near future.**

LATHYRUS SATIVUS L., commonly called the chickling vetch, is an exceptionally hardy, protein-rich (28–40%) legume crop cultivated in many parts of the world. It is popular among the farmers due to its ease of cultivation and high climatic adaptability that permits growth even under such extreme conditions as drought or water logging. All these factors make *L. sativus* a potentially valuable food crop for arid regions ot the world. In India, it occupies nearly 5,000,000 acres under cultivation, which is 4% of the total area under pulse crops and constitutes 3% of the total pulse production. Madhya Pradesh produces more than 50% of the total produce in the country[1]. Besides, it is also cultivated in eastern Uttar Pradesh, Maharashtra, Bihar, West Bengal and Assam. However, the full potential of *L. sativus* has not been realized since prolonged or excessive consumption of this pulse leads to a paralytic disorder known as neurolathyrism or human lathyrism[2], caused by a neurotoxin. The disease has been documented in a number of countries in Europe, Africa and Asia. Human lathyrism continues to be a public health problem in parts of Bangladesh, China, Ethiopia and India.

The present article reviews the biochemical nature and the mode of action of *Lathyrus* neurotoxin, its occurrence and synthesis in different plant parts and the methods for removal of the toxin. Finally, the use of recombinant DNA technology and tissue culture methods in producing the neurotoxin-free *L. sativus* plants has been highlighted.

Nature of the neurotoxin

Various attempts have been made to identify the causative agent of human lathyrism. The presence of certain phenolic compounds[3], an excessive quantity of manganese in the seed[4], a water-soluble toxic amine[5], an excess amount of selenium, which interferes with methionine metabolism[6], have been attributed by different workers as causative factors for lathyrism. Subsequently, a toxic amino acid, β-*N*-oxalylamino-L-alanine (BOAA), was discovered in *L. sativus* seeds[7,8]. Later it was demonstrated that BOAA (β isomer) exists naturally in an isomeric form with the α isomer in 95 : 5 ratio[9]. The neurotoxic effect of BOAA has been demonstrated in mice[10], chicks[11] and rhesus monkeys[12].

Three different names have been suggested for the neurotoxin of *L. sativus*[13]. These are β-*N*-oxalylamino-L-alanine (BOAA), β-*N*-oxalyl-L-α,β-diaminopropionic acid (Ox-dapro or ODAP) and L-3-oxalylamino-2-aminopropionic acid (OAP).

Mode of action of the neurotoxin

Consumption of *L. sativus* for a period of 3–6 months afflicts the nervous system characterized by weakness, spasticity of leg muscles and subsequent development of lower limb paralysis (Figure 1). Continued ingestion of the seed meal can result in convulsions and finally death in extreme cases[2]. The onset of the disease is often sudden and the affected person feels difficulty in walking. The disease affects mostly young people between 20 and 29 years of age[14].

What are the mechanisms underlying the neurotoxicity of ODAP? It has been suggested that ODAP may mimic the action of putative excitatory neurotransmitters such as glutamic acid and aspartic acid either by directly overstimulating the sensitive nerve cells[15] or by interfering with reuptake mechanisms that normally terminate neurotransmitter action[16,21]. Extracellular ODAP may thus cause an influx of sodium and chloride into the nerve cells and a progressive retention of water that culminates in massive intracellular oedema and neuronal degeneration.

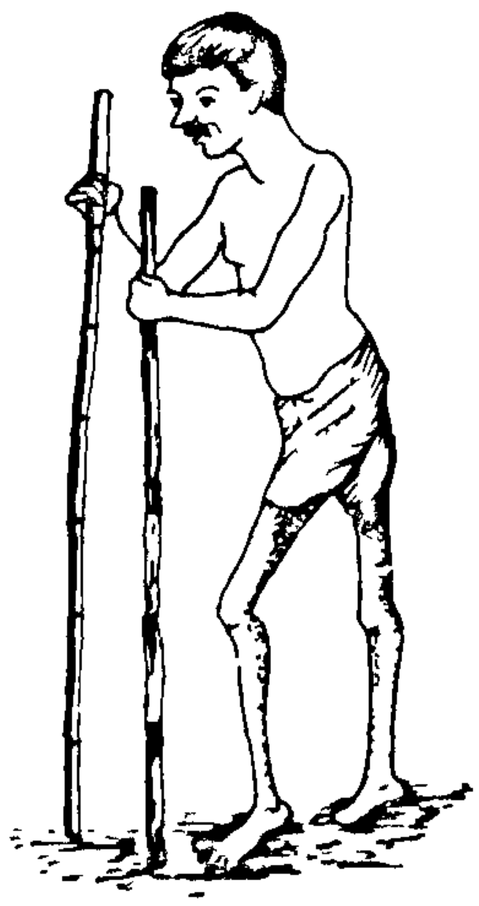

Figure 1. A man afflicted by neurolathyrism.

In another study, it was observed that ODAP undergoes transamination to produce a keto acid, β-*N*-oxalyl-L-keto-β-aminopyruvate, which inhibits the growth of several microorganisms[17]. Further, it was shown that ODAP interferes with ammonia metabolism and leads to chronic ammonia toxicity[18].

Appearance of neurological symptoms only in young animals and not in adults[14] has been explained by a hypothesis which suggests the existence of a blood-brain barrier to this toxin in adults[19] and its absence in young animals[20]. Studies on the effect of ODAP on glutamate metabolism revealed it as a stereospecific glutamate receptor agonist[21]. These investigations established that the amino group of ODAP is involved in causing neurotoxin symptoms.

Occurrence and biosynthesis of the neurotoxin

The content of neurotoxin varies considerably among *L. sativus* genotypes, ranging from 0.1 to 2.5% by weight of tissues[22]. Neurotoxin levels also vary in different parts of the plant, with seeds being the greatest site of accumulation[2]. Fluctuations in ODAP concentration have been reported to occur throughout development, with maximum concentration in leaves during vegetative growth and in seed embryos during germination[23]. Foliar spray of micronutrients, especially cobalt and molybdenum has been shown to decrease the neurotoxin content of the plants[24].

A terminal two-step process has been shown to be involved in the synthesis of ODAP[25]. This involves oxalyl activation, followed by the condensation of oxalyl-CoA with L-α,β-diaminopropionic acid. The first reaction is catalysed by oxalyl-CoA synthetase and the second by oxalyl-DAP synthase as shown below:

(a) Oxalate + ATP + Coenzyme A = Oxalyl-CoA + AMP + PPi
(b) Oxalyl-CoA + L-α,β-diaminopropionic acid = ODAP + Coenzyme A

The first enzyme, oxalyl-CoA synthetase, has been purified from three-day-old seedlings of *L. sativus* using affinity chromatography and electroelution[26]. The enzyme has been shown to exist in three forms with molecular weights 63.1, 39.3 and 17.7 kDa. The second enzyme, ODAP synthase, is yet to be isolated to homogeneity and characterized. These two enzyme activities have

also been reported in other legume species but do not accumulate a significant amount of neurotoxin[27].

Removal of the neurotoxin

In spite of several efforts, human lathyrism continues to be a public health hazard. Attempts have been made to develop practical methods to detoxify *L. sativus* seeds for its safe use[28]. These include soaking the dehusked seeds overnight and then rejecting the extract. Seed roasting at 140°C for 15–20 min and parboiling have also been advocated. The utility of such domestic procedures could not be adopted on a large scale because of practical difficulties.

As an alternative approach, considerable emphasis has been placed on the identification of low ODAP content varieties over the last several decades[22,29]. Analysis of a large collection of *L. sativus* seeds revealed[30] wide variation in the amount of ODAP, ranging from 0.1 to 2.5%. Conventional breeding and selection methods have resulted in the production of a few varieties with low neurotoxin content. However, these varieties do not have either good yield or contain other compounds such as α,β-diaminobutyric acid[31], γ-cyanoalanine[32], which are lathyrogenic. In these varieties, a negative correlation has also been observed between the crude protein content and ODAP levels[22]. To date, no variety has been developed that lacks neurotoxin. The development of *L. sativus* cultivar devoid of neurotoxin is, therefore, still an important requirement for a fuller exploitation of the potential of this crop. To accomplish this goal, three well-established biotechnology approaches are being undertaken and significant achievements have already been attained. These approaches are: (i) insertion and expression of the enzyme that degrades the neurotoxin; (ii) antisense RNA technology; and (iii) somaclonal variations.

Insertion and expression of the neurotoxin-degrading enzyme

Many bacterial genes for xenobiotic degradation have originated from strains isolated from contaminated waste sites and are often found on plasmids[33]. Isolation of such strains is essential since laboratory selection can speed up this process and provide control unavailable under natural conditions. Further, biodegradation in the soil may be limited if a complex mixture of xenobiotics is present or the pathway is blocked by inhibitors[34]. Such difficulties can be overcome by cloning of genes for modified enzymes that have useful catabolic properties such as relaxed substrate specificities or enhanced induction capabilities. Alternatively, genetic tools can be used to develop specific catabolic pathways for degradation of toxic substances by bacteria that can function under a wide range of environmental conditions.

Exploring these possibilities for degradation of ODAP, pure cultures of bacteria have been isolated from soil–sludge filtrates[35] (Table 1). One of the isolates having highest competency for ODAP degradation has been identified as *Enterobacter cloacae*[35]. It has also been demonstrated that genetic information for ODAP degradation is contained on a plasmid (PBYA1) approximately 40–50 kb in size (Figure 2). Further, cloning of various restriction fragments by different enzymes showed localization of ODAP-degrading sequences on 3.0 kb Not1 fragment contained in a 9.7 kb EcoR1 fragment[35].

Table 1. ODAP utilization by sludge-derived cultures

Time after inoculation[a] (h)	A_{600}	ODAP[b] (μg/ml)	A_{600}	ODAP[b] (μg/ml)
0	0.00	200.0	0 00	400.00
6	0.01	177.8	0.06	356 00
8	0.02	75.7	—	—
12	0.08	2.5	0 17	Nil
16	0.08	Nil	0.18	Nil
24	0.09	Nil	0.18	Nil

[a]Observations were recorded from cultures during the ninth cycle of growth on MF media containing initial concentrations of neurotoxin ODAP indicated at 0 h.
[b]ODAP concentration remained in the media.
— MF medium contained per litre 0.05 g K_2HPO_4, 0.1 g $MgSO_4$, 0.01 g $FeSO_4$.
After ref. 35.

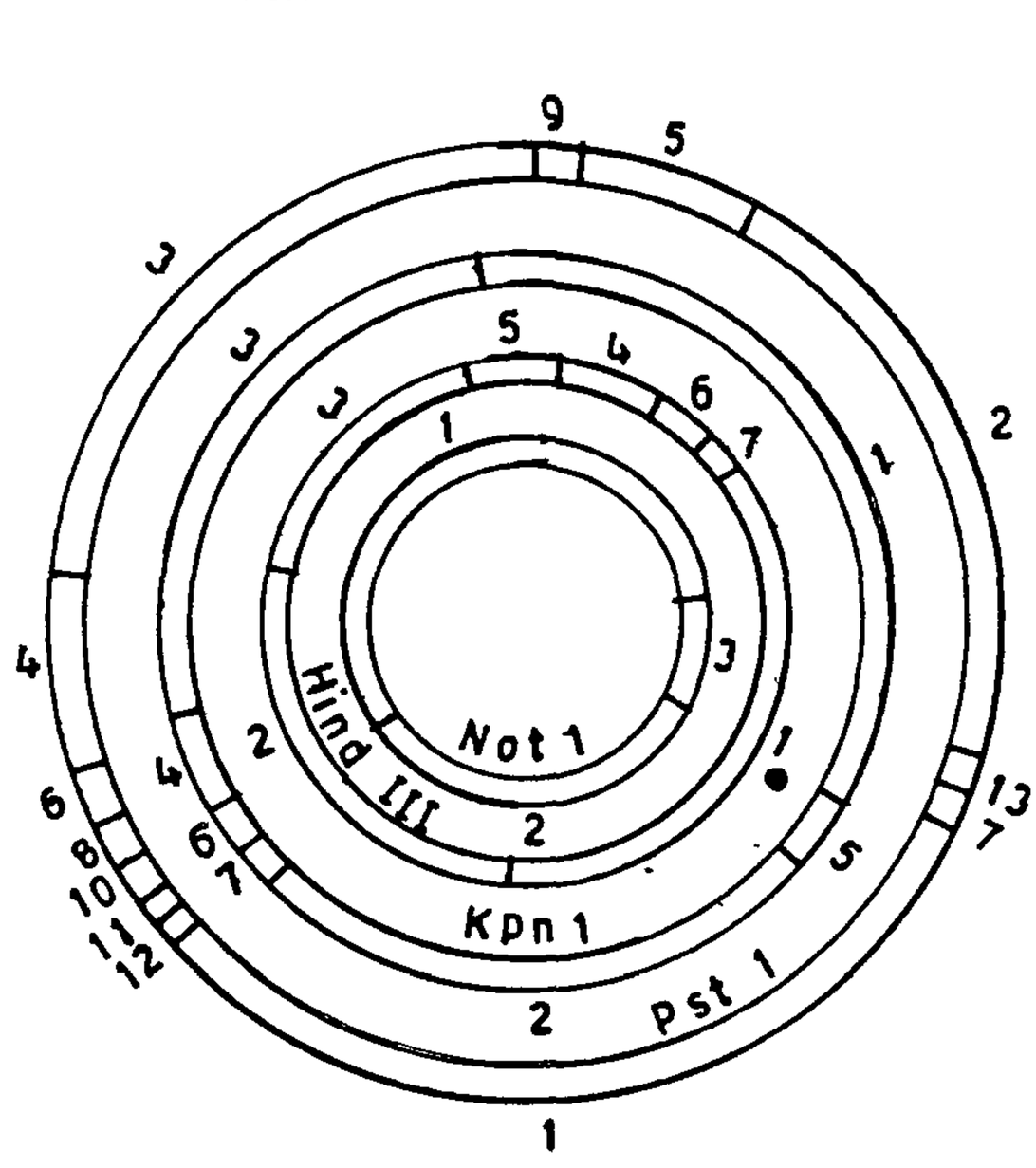

Figure 2. An overlapping restriction map of the ODAP-degrading plasmid pBYA1 with respect to the restriction enzymes Not1, Kpn1, Pst1 and HindIII.

Table 2. Purification of oxalyl-CoA synthetase (OCS) from *Lathyrus sativus* seedlings

Preparation	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units)	Purification fold
OCS-1	3.0	6.15	0.30	20.50	48.8
OCS-2	2.0	6.30	0.40	15.75	37.5
OCS-3	2.0	6.10	0.56	10.89	25.9
Crude extract	4.8	31.20	73.60	0.42	—

The enzyme was purified using affinity chromatography and electroelution.
1 Unit = change of 0.1 in absorbance at 540 nm.
After ref. 26.

A partial Sau3A library of the plasmid pBYA1 in the expression vector pUC18 and its screening has shown a 1.8 kb fragment responsible for ODAP degradation[36]. With these findings, it should be possible to introduce and express this gene in *L. sativus* so that the neurotoxin is degraded *in vivo*.

Antisense RNA technology

This technique is based on blocking the information flow from DNA via RNA to protein, by introducing an RNA strand complementary to (part of) the sequences of target mRNA. Subsequently, the duplex is either degraded or the mRNA is impaired in nuclear processing or it is blocked for translation[37]. The first report on artificial antisense regulation of gene expression in plants came from Ecker and Davis[38], who described the effective transient inhibition of CAT activity in carrot cell cultures.

In pursuance of this, oxalyl-CoA synthetase (OCS), a key enzyme in the biosynthesis of ODAP[25], has been purified from *L. sativus* seedlings using affinity chromatography and electroelution (Table 2). The enzyme exists in three forms designated as OCS-1, OCS-2 and OCS-3 of molecular weights 63.1, 39.3 and 17.7 kDa, respectively. Monoclonal antibodies raised against this enzyme[26] can be used for screening of C-DNA library of the enzyme OCS in expression vectors. Use of antiserum for isolation of the desired genes has been reported by many workers[39]. Antisense OCS gene can be constructed from OCS C-DNA and introduced in *L. sativus*, which would inhibit the biosynthesis of the neurotoxin.

Somaclonal variation

Somaclonal variation has been exploited by various workers for crop improvement. Several varieties showing resistance to pathogens and herbicides have been developed in a number of crops such as sugarcane[40], potato[41] and tomato[42]. Somaclonal variation may, therefore, be exploited to reduce the toxin content in *Lathyrus*. Following this approach, protocols have been developed

Table 3. Variation in seed weight and ODAP content in leaf and seed of *in vitro* regenerated plants from internode explant

Pedigree	100-seed wt. (g)	ODAP (%) Leaf	ODAP (%) Seed
1-01	10.1	0.206	0.289
1-02	7.2	0.460	0.445
1-03	6.0	0.133	0.305
1-04	4.8	0.445	0.484
1-05	9.0	0.015	0.086
1-06	9.0	0.031	0.062
1-07	8.3	0.129	0.250
1-08	5.0	0.016	0.031
1-10	7.7	0.308	0.469
1-11	6.5	0.164	0.398
1-12	7.3	0.234	0.539
1-13	5.8	0.016	0.062
1-14	6.5	0.062	0.101
1-15	7.4	0.125	0.539
1-16	7.7	0.156	0.219
1-17	6.8	0.156	0.266
1-18	10.0	0.034	0.086
1-19	6.8	1.109	0.258
1-20	6.5	0.087	0.312
1-21	9.1	0.156	0.351
1-22	8.0	0.021	0.055
1-23	7.8	0.187	0.383
1-24	7.9	0.203	0.453
1-27	3.2	0.150	0.422
1-30	5.3	0.062	0.094
P24 (parent)	7.8	0.258	0.406

After ref. 45.

for *in vitro* regeneration of *Lathyrus* from leaf[43] and root explants[44] of this species. Subsequently, somaclones with low toxin contents have been developed[45] from internode explants of *L. sativus* cv. P24. The toxin contents varied from 0.015% to 0.46% in leaf and 0.030% to 0.539% in seed in R_1 generation of these somaclones, as compared to 0.258% in leaf and 0.406% in seed for the parent P24 (Table 3). Mean seed toxin content in R_2 generation of some of the somaclones varied from 0.039 to 0.057%.

Conclusion

Use of recombinant DNA technology and tissue culture

methods offer an opportunity to produce neurotoxin-free *Lathyrus sativus* plants. Three approaches are being pursued to accomplish this goal: (i) transformation of *L. sativus* plants with ODAP-degrading gene; (ii) introduction of antisense gene of oxalyl-CoA synthetase (a key enzyme in the biosynthesis of ODAP) in *L. sativus*; (iii) production of *L. sativus* somaclones containing very little or no neurotoxin. Considerable success has been achieved through each of these approaches. These include isolation and characterization of ODAP-degrading plasmid-borne gene from a pure culture of bacteria isolated from soil–sludge filtrate. The enzyme oxalyl-CoA synthetase has also been purified and monoclonal antibodies raised against it. This can be utilized to construct antisense gene of this enzyme. Therefore, now it should be possible to introduce either ODAP-degrading gene, antisense gene of oxalyl-CoA synthetase, or both, into *L. sativus* by *Agarobacterium*-mediated transformation. Such transgenic plants would be totally free of the neurotoxin. At the same time, somaclones of *L. sativus* having very little neurotoxin have also been developed. But it is yet to be seen whether these plants maintain this low level of neurotoxin in their successive generations. A caution is, however, added that removal of the toxin may end up in having a crop which may not have the advantage of being as sturdy as it is now. Nevertheless, this apprehension should not come in the way of the ongoing efforts to make this crop neurotoxin-free.

1. Hartman, C. P., Diwakar, N. G. and Nagaraja Rao, U. N, *Indian J. Nutr. Dietet.*, 1974, **11**, 178–191.
2. Rao, S. L. N, Adiga, P. R. and Sarma, P. S., *Biochemistry*, 1964, **3**, 432–436.
3. Nagarajan, V., Mohan, V. S. and Gopalan, C., *Indian J. Biochem.*, 1966, **3**, 130–131.
4. Berry, J. N, *Text Book of Medicine*, (ed. Vakil, R. J.), 2nd edn. Association of Physicians of India, 1973, p 300.
5. Dwivedi, M. P, *Indian Med. Gaz.*, 1965–66, **4**, 41.
6. Rudra, M. N., Choudhury, L. M. and Sinha, S. P., *Indian Med Gaz.*, 1952, **87**, 89–91.
7. Selye, H., *Rev. Can. Biol.*, 1957, **16**, 1–82.
8. Murti, V. V. S., Seshadri, T. R. and Venkitasubramanian, T. A., *Phytochemistry*, 1964, **3**, 73–78.
9. Roy, D. N. and Narasinga Rao, B. S., *Curr Sci.*, 1968, **37**, 395–396.
10. Mehta, T., Zarghami, N. S., Parker, A. J., Cusick, P. K. and Hasell, B. E., *Toxicol. Appl. Pharmacol.*, 1979, **48**, 1–9.
11. Roy, D. N., *Environ. Physiol. Chem.*, 1973, **3**, 192–195.
12. Srinivasa Rao, P. and Roy, D. N., *Baroda J Nutr.*, 1981, **8**, 36–38
13. Roy, D. N., *Nutr. Abs. and Rev. Ser.*, 1981, **51**, 691–707.
14. Nagarajan, V., Mohan, V. S. and Gopalan, C., *Indian J. Med. Res.*, 1965, **53**, 269–272.
15. Olney, J. W., in *Experimental and Clinical Neurotoxicology* (eds Spencer, P. S. and Schaumburg, H. H), Williams and Wilkins, Baltimore, 1980, pp 272–294.
16. Ross, S. M, Roy, D. N. and Spencer, P. S, *J. Neurochem*, 1985, **44**, 886–892.
17. Cheema, P. S, Padmanabhan, G and Sarma, P S, *Indian J Biochem*, 1971, **8**, 16–19.
18. Cheema, P. S, Padmanabhan, G. and Sarma, P. S, *J Neurochem.*, 1971, **18**, 2137–2144
19. Curtis, D R. and Watkins, J C., *Pharmacol. Rev*, 1965, **17**, 347–391.
20 Sperry, W. M, in *Neurochemistry* (eds Elliot, K A. C., Page, I H and Quastel, J H), Thomas Springfield, Illinois, 1962, pp. 55–84
21. Laxman, J and Padmanabhan, G, *Nature*, 1974, **249**, 469–471.
22. Misra, B. K., Panda, P. C. and Barat, G. K, *Plant Biochem J*, 1979, **6**, 1246.
23. Prakash, S, Misra, B K., Adsule, R N. and Barat, G K., *Biochem Physiol. Pflanzen*, 1977, **171**, 369
24 Misra, B K. and Barat, G. K, *J Plant Nutr*, 1981, **3**, 997.
25. Malathi, K., Padmanabhan, G. and Sarma, P S, *Phytochemistry*, 1970, **9**, 1603–1610.
26 Sehgal, D, Santha, I M and Mehta, S L., *J. Plant Biochem. Biotech*, 1992, **1**, 97–100.
27 Malathi, K., Padmanabhan, G and Sarma, P. S, *Biochem Biophys. Acta*, 1967, **141**, 71–78
28 Mohan, V. S., Nagarajan, V. and Gopalan, C., *Indian J Med Res*, 1966, **54**, 410–414.
29. Swaminathan, M S, Naik, M. S, Kaul, A K. and Austin, A., in *Improving Plant Proteins by Nuclear Techniques*, IAEA, Vienna, 1970, p. 16.
30. Nagarajan, V and Gopalan, C., *Indian J. Med Res*, 1968, **56**, 95–99.
31. Bell, E. and O'Donovan, J. P, *Phytochemistry*, 1966, **5**, 1211–1219.
32. Ressler, C., *J. Biol. Chem.*, 1962, **237**, 733–735
33. Focht, D. D, in *Environmental Biotechnology Reducing Risks from Environmental Chemicals* (ed Omenn, G. S), Plenum Press, New York, 1988, pp. 15–29
34. Timmis, K. N., Roja, F. and Ramos, J L, in *Environmental Biotechnology Reducing Risks from Environmental Chemicals* (ed Omenn, G. S), Plenum Press, New York, 1988, pp 61–79
35. Yadav, V. K., Santha, I M., Timko, M. P. and Mehta, S. L., *J Plant Biochem. Biotech*, 1992, **1**, 87–92.
36. Sukanya, R., Santha, I. M. and Mehta, S L., *J Plant Biochem Biotech*, 1993, **2**, 77–82.
37. Alexander, R, Vander, K., Joseph, N. M M and Anteine, R. S, *Science*, 1988, **72**, 45–50.
38. Ecker, J. R and Davis, R. W., *Proc. Natl Acad Sci USA*, 1986, **83**, 5372–5376.
39. Roesler, K. R. and Organ, W. L., *Plant Physiol*, 1990, **93**, 188.
40. Liu, M C., in *Plant Tissue Culture* (ed. Thorpe, T. A), Academic Press, New York, 1981, p. 299
41. Shepard, J. E., Bindey, D. and Shahi, E., *Plant Cell Tiss. Org. Cult.*, 1983, **62**, 67.
42. Evans, D. A. and Sharp, W R., *Science*, 1983, **221**, 949.
43. Roy, P. K., Singh, B, Mehta, S. L., Barat, G K., Gupta, N, Kirti, P. B and Chopra, V. L., *Indian J. Exp Biol*, 1991, **29**, 327
44. Roy, P. K, Barat, G. K. and Mehta, S. L., *Plant Cell Tiss. Org Cult.*, 1992, **29**, 135.
45. Roy, P. K., Ali, K., Gupta, A., Barat, G. K. and Mehta, S L., *J. Plant Biochem. Biotech*, 1993, **2**, 9–13.

Received 3 May 1994; revised accepted 1 October 1994