The Effect of Malathion on Amino Acid Incorporation into Plasma Membrane Proteins of *Vigna sinensis* (L): Effect of Plant Growth Hormone Supplementation

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Amino acid incorporation into plasma membrane proteins of *Vigna sinensis* (L) was inhibited by malathion, an organophosphorous insecticide, whereas some stimulatory effect was elicited by plant growth hormones, *viz.*, IAA, GA_3 and kinetin. The inhibitory effect of malathion towards amino acid incorporation into plasma membrane proteins was marked at concentrations above 50 ppm, and at 400 ppm the amino acid incorporation was much less. Up to 100 ppm of malathion treatment, the plant growth hormones could counteract the malathion-induced inhibition on amino acid incorporation into plasma membrane protein but above this concentration, the plant hormones gradually lost their effectiveness. Plasma membrane fraction was characterized by studying the activities of enzymes like IDPase, Oligomycin treated ATPase, Cytochrome *c*-oxidase and glucan synthetase, and also by a sterol/phospho lipid ratio study. From the above studies it appears that glucan synthetase may be the marker enzyme of the plasma membrane of *V. sinensis* (L), and some change at the membrane level on malathion exposure is suggested.

The trend towards large intensive live stock units and greater use of pesticides, herbicides, fertilizers and other environmental contaminants has clearly played a crucial role in increasing agricultural production. Although many aspects of the relationships between pollution and the environment have been studied extensively but studies on the effects of pollution on agriculture are scanty.[1~3]

Our previous observations revealed that malathion, a widely used organophosphorous insecticide, causes a reduction in the contents of protein and nucleic acids[4] and stimulates the activities of plasma membrane bound monovalent cation-stimulated ATPase[5] as well as some hydrolytic enzymes in the germinating seeds of *Vigna sinensis* (L) Chakraborti *et al.*, *Int. J. Env. Studies* (accepted)). Recently a few reports have been available on the isolation and characterization of plasma membrane from plant tissues[6,7] and also on the amino acid incorporation into different membranes proteins of animal cells,[8] but no such systematic studies have been made on amino acid incorporation into the plasma membrane proteins of plant cells.

This communication is concerned with the studies on the effects of malathion and plant growth hormones, alone and in combination, on the amino acid incorporation into the plasma membrane proteins of *Vigna sinensis* (L).

MATERIALS AND METHODS

Seed. Seeds of *Vigna sinensis* (L) were obtained from local sources.

Reagents. Tris-MES (morpholino ethane sulfinic acid), Indole-3-acetic acid (IAA), gibberellic acid (GA_3). kinetin, PPO, and POPOP were purchased from Sigma Chemical Co., U.S.A. L-(U-^{14}C)-phenylalanine and L-(U-^{14}C)-lysine were obtained from the Isotope Division, Bhabha Atomic Research Centre, Trombay, India. UDP-^{14}C-glucose was obtained from Radio Chemical Centre, Amarsham, England. Technical grades of Malathion (*O,O*-dimethyl *S*-(1,2-diethoxycarbonylethyl) phosphorodithioate) was obtained from Cyanamid India Ltd., Bombay, India. All other chemicals

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used were of analytical grade.

Germination of seed and in vivo incorporation of radio activity. Seeds of *Vigna sinensis* (L) were surface sterilized by 0.1 per cent mercuric chloride and after thorough washing with deionised water, the seeds were soaked overnight in water. The seedcoats were then removed and the dehulled seeds were allowed to germinate at 25°C in several petri dishes for 72 hours. After the specified germinating period 10 g of the seeds were incubated in 5 ml of buffer A (containing 0.01 M Tris-HCl, pH 7.4, 0.001 M potassium phosphate, 0.001 M NH_4Cl, 0.9% NaCl and 0.005 M $MgAc_2$) containing 5 μCi of L-(U-^{14}C)-phenyl alanine (specific activity 30 mci/mmol) or L-(U-^{14}C)-lysine (specific activity 25 mCi/mmol) in separate conical flasks with or without different concentration of malathion and/or plant growth hormones. The incubation was carried out for 2 hours at 37°C with constant shaking, air being the gas phase. At the end of the incubation period the seeds were washed three times with buffer A (ice-cold). The roots were excised, washed with the buffer A and were then ground with quartz powder in a morter. A 10% homogenate was prepared with 0.25 M sucrose from which mitochondria, microsomes and soluble supernatant were isolated by the method as described by Goswami *et al.*[9] and Dube *et al.*[10] respectively and were stored at −20°C.

Isolation of Plasma membrane. Plasma membrane of the roots of *Vigna sinensis* (L) from the control and experimental plates were isolated by the method as described by Hodges *et al.*[6]

Enzyme assays. Adenosine triphosphatase activity was measured at 38°C using 3.75 mM ATP (tris salt) in a final volume of 1 ml at pH 6.0. The reaction was initiated by addition of appropriate amount of membrane protein and the Pi released was estimated according to Fiske and SubbaRow.[11]

IDPase assay was performed by the method as described by Hodges *et al.*[12]

Cytochrome *c*-oxidase assays were performed by the method as described by Wharton *et al.*[13]

Glucan synthetase assays were done by the method as described by Vanderwoude *et al.*[14]

Proteins were determined by the method of Lowry *et al.*[15] using bovine serum albumin as the standard.

Phospholipid and sterol estimation. Lipidphosphorous was determined by the procedure of Rouser *et al.*[16] and total sterol was determined as described by Stadtman.[17]

Measurement of Radioactivity. Measurement of radioactivity in protein was done by the filter paper-disk method of Mans and Novelli[18] as described by Bollum.[19] The suspensions of different subcellular fractions stored at −20°C, were removed and thawed at 5°C, and 0.1 ml of each was placed on duplicate disks of Whatman 3 mm paper. Those were then dried in a stream of air and washed three times with ice cold 10% TCA containing 0.1 mM unlabelled amino acid (corresponding to the ^{14}C-amino acid used), the papers were then washed thrice with ice cold 5% TCA, twice with ether–ethanol (v/v) at 37°C, twice with diethyl ether, and finally air dried. In each experiment a blank disk was carried through the washing procedure together with the sample disk to provide for a background determination. Each disk was laid flat in a vial and the radio-activity was determined by liquid-scintillation counting in a Beckman LS-300 Scintillation Counter (efficiency 95% for ^{14}C and 40% for ^{3}H) using a mixture containing 0.01% POPOP and 0.4% PPO in toluene.

RESULTS

Effect of malathion on the seedling growth of Vigna sinensis (L)

Malathion at a concentration up to 50 ppm was found to have some stimulatory effects towards seedling growth of *Vigna sinensis* (L). Above this concentration the seedling growth of the seeds decreases. After treatment with 400 ppm of malathion, marked decrease in seedling height was observed with almost no visible growth (Fig. 1). No growth was recorded above this concentration.

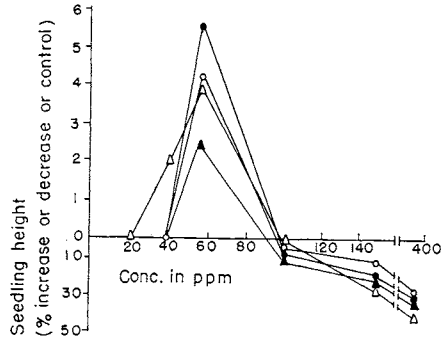

FIG. 1. Effect of Malathion Exposure on Seedling Height of *Vigna sinensis* (L) at Different Hours of Germination

○—○, 20 hr; ●—●, 44 hr; ▽—△, 64 hr; ▲—▲, 72 hr.

Characterization of plasma membrane fraction

The enrichment of the fraction obtained between 34% and 45% sucrose interface with plasma membrane was ascertained by assaying

TABLE I. SPECIFIC ACTIVITY OF IDP-ASE, CYT. *c*-OXIDASE AND GLUCAN SYNTHETASE IN VARIOUS FRACTIONS ISOLATED FRAM THE ROOT CELLS OF 72-HR GERMINATING SEEDS OF *Vigna sinensis* (L)

System	Specific activity (μmole/min/mg protein)		
	IDPase[a]	Cyt. *c*-oxidase[b]	Glucan synthetase[c] (1×10^3)
13,000 *g* fraction	0.028±0.004	0.924±0.012	1.66±0.019
13,000 *g* to 80,000 *g* fraction	0.046±0.005	0.078±0.004	5.75±0.131
Plasma membrane fraction	0.022±0.003	0.086±0.005	9.78±0.083

Results are expressed as means±SD of four sets of experiments.

[a] Specific activity of IDPase is expressed as μmol inorganic phosphate/mg protein/min.

[b] Specific activity of Cyt. *c*-oxidase is expressed as μmole Cyt. *c* utilized/min/mg protein.

[c] Glucan synthetase assays were performed by W. J. VanDerwoude *et al.* (ref. No. 14 of the text), and represented as total glucose-^{14}C (1 μmol of ^{14}C UDPG per millilitre) incorporated into polysaccharide.

TABLE II. PHOSPHOLIPID AND STEROL CONTENT IN VARIOUS FRACTIONS FROM THE ROOTS OF 72-HR GERMINATING *Vigna sinensis* (L)[a]

System	mg of phospholipid / mg of protein	mg of sterol / mg of protein	Sterol / Phospholipid (molar basis)*
13,000 *g* pellet	0.229	0.042	0.355
13,000 *g* to 80,000 *g* pellet	0.423	0.097	0.444
Plasma membrane fraction	0.328	0.178	1.051

[a] Results are expressed as the average of three replications.

* Average molecular weights for phospholipids and sterols of 750 and 387, respectively, are assumed.

TABLE III. EFFECT OF OLIGOMYCIN ON THE SPECIFIC ACTIVITY OF PLASMA MEMBRANE BOUND Mg^{+2} ACTIVATED MONOVALENT ION STIMULATED ATPase OF THE ROOTS OF *V. sinensis* (L)

Results are expressed as mean±SD of four sets of experiments.

Concentration oligomycin in the reaction mixture (1 ml volume)	Specific activity of ATPase[a]
Nil	3.8878±0.358
0.005 M	3.4476±0.385
0.05 M	3.1089±0.613
0.5 M	2.8792±0.435

[a] Specific activity is expressed as μmol Pi/mg enzyme protein/hour.

the activity of the enzymes *viz.* glucan synthetase, IDP-ase, Cyt-*c* oxidase (Table 1). Plasma membrane fraction has high glucan synthetase activity compared to the other fractions. Whereas the activity of Cyt. *c*-oxidase, IDPase in the isolated plasma membrane fraction is low compared to that of other fractions (Table I). The types of sterols and phospholipids in plant membranes are unknown, but if we assume that the average molecular weights for phospholipids and sterols are 750 and 387, respectively, molar ratios of sterols: phospholipid of the plasma membrane was determined to be 1.051 (Table II). The plasma membrane bound ATPase of *V. sinensis* (L) was found to be insensitive to oligomycin whereas ATPases of other fractions were significantly inhibited by oligomycin (Table III).

Distribution of radioactivity in different fractions

After incubation of the seeds of *V. sinensis* (L) in the incubation medium containing L-(U-^{14}C)-phenylalanine or L-(U-^{14}C)-lysine for 2 hours, it was found that the amount of incorporation of ^{14}C-amino acids was maximum in the supernatant fraction isolated from the roots of the seeds followed by mitochondria, microsomes and plasma membrane fractions in the decreasing order (Table IV).

TABLE IV. *In vivo* INCORPORATION OF L-(U-^{14}C)-PHENYLALANINE AND L-(U-^{14}C)-LYSINE BY THE ROOTS OF 72-HR GERMINATING SEEDS OF *Vigna sinensis* (L) AND DISTRIBUTION OF RADIOACTIVITY IN PROTEINS OF DIFFERENT SUBCELLULAR FRACTIONS

Results are means of 10 experiments with ±SD and given as cpm/mg protein.

Cell fraction	L-(U-^{14}C)-Phenylalanine	L-(U-^{14}C)-Lysine
Mitochondria	1010±22	1150±20
Microsome	714±17	850±16
Soluble supernatant	1215±24	1290±28
Plasma membrane	560± 8	690±15

Effect of malathion on the amino acid incorporation into plasma membrane proteins

Incorporation of ^{14}C phenylalanine into plasma membrane protein is sensitive to malathion. Malathion at a concentration of 50 ppm has got some stimulatory effect on amino acid incorporation into plasma membrane protein (Table V). Above 50 ppm there is observed a significant inhibition of amino acid incorporation into plasma membrane proteins. The amino acid incorporation into plasma membrane protein of *V. sinensis* (L) was found to be significantly inhibited when exposed to 400 ppm malathion.

Effect of plant hormones on the amino acid incorporation into plasma membrane proteins

The effects of different concentrations of plant hormones *viz.*, IAA, GA_3 and Kinetin on the amino acid incorporation into plasma membrane protein of *V. sinensis* (L) are shown in Fig. 2. It is evident that the optimum concentrations of IAA, GA_3 and kinetin needed to promote amino acid incorporation into plasma membrane proteins of *V. sinensis* (L) were 10^{-4} M, 10^{-3} M and 10^{-4} M respectively.

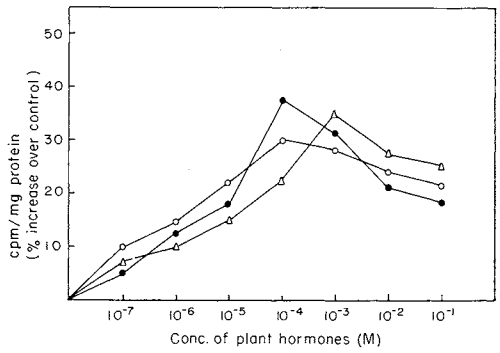

FIG. 2. Effect of Plant Hormones on the Amino Acid Incorporation into Plasma Membrane Protein of *V. sinensis* (L).

○—○, IAA induced amino acid incorporation; △—△, GA_3 induced amino acid incorporation; ●—●, kinetin induced amino acid incorporation.

Effect of plant hormones in reversing malathion inhibition

On simultaneous application of malathion and the plant hormones *viz.*, IAA, GA_3 and kinetin in the incubation mixture, it was found

TABLE V. EFFECT OF MALATHION AND PLANT GROWTH HORMONES ON *in vivo* INCORPORATION OF AMINO ACID INTO THE PLASMA MEMBRANE PROTEINS OF *Vigna sinensis* (L)*

Results are means of 10 experiments with ±SD and given as cpm/mg protein.

Condition	Additions			
	None	IAA	GA_3	Kinetin
Control	715±36	1055±35[a]	1268±63[a]	1378±74[a]
+ 50 ppm Malathion	740±28[b]	995±34[a]	1170±42[a]	1298±45[a]
+100 ppm Malathion	638±25[a]	728±38[b]	785±26[a]	826±29[a]
+200 ppm Malathion	517±27[a]	644±28[a]	656±32[c]	684±27[d]
+300 ppm Malathion	435±24[a]	468±26[a]	518±23[a]	535±26[a]
+400 ppm Malathon	217+12[a]	280±15[a]	257±18[a]	275±12[a]

* The determination of radioactivity and other details are given in the text. Mean values are significantly different from control.

[a] $p<0.001$. [b] p values are not significant. [c] $p<0.05$. [d] $p<0.10$.

that the amino acid incorporation into plasma membrane proteins of *Vigna sinensis* (L) was not inhibited up to 100 ppm of malathion. Table V also shows that the incorporation of amino acid into plasma membrane proteins was even stimulated under the above condition. But the plant growth hormones were ineffective to overcome the malathion induced inhibition of amino acid incorporation into plasma membrane proteins of *V. sinensis* (L) when the malathion concentration exceeds 100 ppm.

DISCUSSION

The present study indicates that the incorporation of ^{14}C-phenylalanine into plasma membrane proteins of *Vigna sinensis* (L) is sensitive to malathion (Table V). In the event of malathion treatment at concentrations up to 50 ppm, some stimulation was found in the amino acid incorporation into plasma membrane proteins of *V. sinensis* (L). Above 50 ppm, malathion has got some inhibitory effect on the amino acid incorporation, the inhibition being marked when the seeds were treated with 400 ppm malathion. The plasma membrane bound monovalent ion-stimulated ATPase activity was found to be significantly altered at 400 ppm malathion exposure.[5] Sterol and phospholipid status of the plasma membrane of *V. sinensis* (L) was also found to be altered under the above condition (Table II). Taken together, these observations provide a strong support that there may be some alteration of membrane permeability, composition and/or level of different membrane bound macromocules by malathion treatment.

On simultaneous application of malathion and either of the plant growth hormones *viz.*, IAA, GA_3 or kinetin in the incubation mixture it was found that the plant hormones can not only counteract the inhibition induced by 100ppm malathion but also augment the amino acid incorporation into the plasma membrane proteins of *Vigna sinensis* (L). But above 100 ppm of malathion, the plant hormones were found to be ineffective in reversing the inhibition.

A possible way of identifying the plasma membrane of the roots of *V. sinensis* (L) is by its chemical composition. In animal cells, the plasma membrane is unique in that it has a high cholesterol: phospholipid ratio.[20] We have determined the total sterol and the phospholipid content of plasma membrane fractions as well as other fractions of the roots of *V. sinensis* (L). Fractions obtained at the interface between 34% and 45% sucrose had a high sterol: phospholipid ratio compared to that of other fractions. The molar ratio of sterol: phospholipid for the plasma membrane was determined to be 1.1 (Table II). Typical molar ratios of cholesterol: phospholipid for purified animal plasma membrane range from 0.7 to 1.2.[20] These results lend further support to the conviction that the membrane collected between 34% and 45% sucrose interface was indeed the plasma membrane. This was also proved by glucan synthetase assays. Enrichment of this enzyme in the 34% and 45% sucrose interface is particularly interesting because this enzyme has been reported to be associated with Golgi apparatus membrane[21] and/or plasma membranes[22] of plant cells. It is unlikely that the fractions obtained between 34% and 45% sucrose contain Golgi membranes since IDPase is a marker enzyme for Golgi membranes in plant cells[21,28] and this enzyme is not enriched in the plasma membrane fraction as was evident from Table I. Thus the enrichment of glucan synthetase in the plasma membrane fractions of the root cells of *V. sinensis* (L) indicates that it is a marker enzyme of the plasma membrane of the root cells of *V. sinensis* (L).

Auxin has pleiotropic effects in the plant cells and these effects may be categorized into two types, (i) early effect and (ii) late effect. The early action of auxin is recorded at the membrane level. For sustained growth, RNA and protein synthesis are involved. This aspect of auxin action has already been elucidated by Biswas *et al.*[24] Receptor proteins for indole acetic acid (IAA) has been implicated for the differential RNA synthesis in the

plant cell both *in vivo* and *in vitro*.[25] The stimulatory effect of IAA, GA_3 and kinetin to nullify the inhibitory effects of malathion upto 100 ppm and promote the amino acid incorporation into plasma membrane protein of the root cells of *V. sinensis* (L) may be not only due to the fact that the plant hormones are effective growth stimulants but also in that they participate in a variety of developmental processes.[26] Fox and Erion[27] recently isolated a cytokinin binding protein from higher plant ribosomes, which is thought to mediate the effect of cytokinin on protein synthesis.

Evidence of membrane abnormalities by pesticide or other toxin treatment on plant cells was also reported by Charnetski *et al.*[28] and Samaddar *et al.*[29] Initial leakiness of membrane which is repaired by plant hormone treatment has also been reported.[30] Recent reports on the effect of plant hormones on membrane metabolism accompanied by membrane protein synthesis are also available.[31]

Apparently the plant growth hormones stimulate the synthesis of new molecules of mRNA[32] which acts as template for increased protein synthesis. This in turn got a specific recognition site(s) into the plasma membrane periphery. The macromolecules *viz.*, lipids and proteins at the cell interface are, to a great extent, responsible for the specificity of interaction of the cells with their environment, and maintenance of the level and integrity of structure of these macromolecules is an important aspect of physiological regulations to be maintained through the mediation of plasma membrane. A possible explanation of the plant hormones effect in reversing the malathion inhibition (up to 100 ppm) on the amino acid incorporation into the plasma membrane proteins of 72 hour germinating *V. sinensis* (L) may be due to the fact that the plant hormones interact in some way with malathion. It may not necessary to imply a direct reaction between malathion and the plant growth hormones. There are several possibilities which may include (a) Interaction with the availability of the added hormones, (b) Competition with the hormones for a binding site (s) or (c) Interaction with a subsequent hormones product or complex. Reports have been made that the hormones appears to counteract the effects of some growth inhibitors and pesticide.[33,34] While each of these is suggestive, a definite conclusion is yet to be established.

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