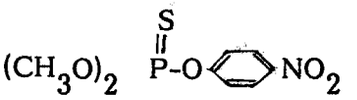
II MATERIALS AND METHODS

A. About the Pesticides used in the Present Investigation

1. Metacid-50 : (Methylparathion)

Chemical name : O, O-dimethyl O-4-nitrophenylthiophosphate



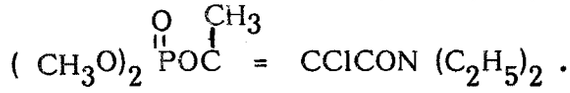
It is usually marketed in the form of emulsion, wettable powder and dust. It is thermally unstable, contact insecticide and is rapid in action. It is widely used for controlling aphids, jassids, thrips, mites, leaf rollers and beetles etc. in vegetables. The recommended dose for all these pests is in the range of 0.04 to 0.1% v/v (Gangawane and Deshpande, 1985).

Toxicity : Tolerable at recommended doses.

Antidote : Two mg of atropine sulphate by the intravenous route.

2. Dimecron (Phosphamidon)

Chemical name : O, O-dimethyl-O (2-chloro-N, N-diethylcarbamyl)-methylvinylphosphate



in vegetables. The recommended dose for all these pests is 0.03% v/v (Gangawane and Deshpande, 1985).

Toxicity : Acute oral LD_{50} for rats = 20 mg/kg body weight and acute dermal LD_{50} for rats = 312 mg/kg body weight.

Compatibility : It can be mixed with contact fungicides and insecticides of neutral reaction.

The above mentioned two organophosphorus insecticides have been used to study their effect on seed germination, seedling growth, hydrolytic enzymes, proline accumulation and cytotoxicity. Since their mode of application does not include seed treatments, such a study may not be of much agronomical significance. However, its importance for a complete understanding of the plant - pesticide relationship cannot be ignored. Further both these organophosphorus pesticides are widely used as an insecticide as a spray and the residual effect of these pesticides have been known to remain in the environment for a long time (Deshpande and Swamy, 1987). Above all, the recent studies on cyto and chromotoxic effects of environmental chemicals and their possible genotoxic effects have prompted several international agencies to recommend the screening of all the chemicals being used commercially for their biochemical and cytotoxic effects.

Hence the above mentioned insecticides have been used in studying their effect on some vegetable seeds in the present investigation. The pilot experiment using the recommended doses for spraying in seed treatment found to be lethal for seedling growth and hence the lower concentrations viz. 0.01, 0.02, 0.03 and 0.04 v/v for Metacid-50 and 0.005, 0.01, 0.015 and 0.02% v/v for Dimecron have been used.

B. Procurement of Seeds :

Seeds of Allium cepa L. (onion), Abelmoschus esculentus Moench, L. (okra), Phaseolus vulgaris L. (bean) and Cyamopsis tetragonolobus (guar) were obtained from Shetkari Sahakari Sangh, Kolhapur; Beej Bhandar, Kolhapur and Agriculture College, Kolhapur.

C. Pesticidal Treatment :

Seeds of uniform size were selected and surface sterilized with 0.1% $HgCl_2$ for 1 min and then were washed repeatedly with distilled water. The seeds were allowed to germinate on filter paper in dark in an incubator at $30^{o}C$ in corning glass petridishes each containing 10 ml solution of different concentrations of Metacid-50 (emulsifiable concentrate containing 50% w/w methylparathion : Bayer (India) Ltd.) viz. 0.01, 0.02, 0.03 and 0.04% and Dimecron (85% phosphamidon : Hindustan CIBA-GEIGY Ltd.) viz. 0.005, 0.01, 0.015 and 0.02% (all v/v). Simultaneously adequate controls were maintained under identical conditions by using distilled water. Seedling age was determined from the time of seed soaking in different concentrations. The solutions were supplied to the seedlings uniformly to all, as and when required. The germination percentage and root, shoot length were measured at the interval of 24 h while the enzyme activities were studied after 72 h germination. The germination percentage was recorded after 24, 48, 72, 96 and 120 h. The experiment has three replications and in each replication 100 seeds were used. Root and shoot length was measured by routine method after 48, 72, 96 and 120 h germination. From each treatment and control randomly sampled seedlings were used for experimental purpose. Abnormalities induced in seedlings by pesticides were recorded time to time and photographed.

D. Hydrolytic Enzymes

1. α- Amylase (E.C. 3.2.1.1)

A modified method of Katsumi and Fukuharu (1969) was used to score the α-amylase activity.

a) Extraction :

2 g of seedlings from each treatments of Metacid and Dimecron were washed with distilled water and homogenised in pre-chilled mortar and pestle with 15 ml cold 0.1 M acetate buffer (pH 5.0). The extract was filtered through four layers of muslin cloth. The filtrate was centrifuged twice at 6000 rpm for 5 min and the supernatant was used for enzyme assay. All the operations were carried out at 0 - 4^{o}C.

b) Assay :

The activity of α-amylase was assayed by incubating 1 ml enzyme with 1 ml 0.1 M acetate buffer (pH 7.0) and then the reaction was killed after 0 and 30 min with 10 ml 0.5 N acetic acid. The aliquat (1 ml.) from killed reaction mixture was then treated with 10 ml dil. I_2KI solution (0.25% I_2 prepared in 0.1% aqueous KI solution). The absorbance was measured on double beam Shimadzu spectrophotometer at 700 nm using I_2KI solution as a blank.

2. Peroxidase (E.C. 1.11.1.7)

For study of enzyme peroxidase the method of Maehly (1954) was used.

a) Extraction :

The enzyme was extracted from seedlings subjected to different pesticidal treatments by using cold distilled water as an extraction medium. The remaining procedure was same to the one described for α-amylase.

X

b) Assay :

The assay consists of 2 ml 0.1 M phosphate buffer (pH 7.0), 1 ml 20 mM guiacol and 1 ml enzyme. The reaction was started by adding 0.04 ml 10 mM H_2O_2 and oxidation of guiacol was studied by measuring the change in optical density at 470 nm on double beam Shimadzu spectrophotometer.

3. Catalase (E.C. 1.11.1.6)

To score the activity of enzyme catalase a modified method of Herbert (1955) was followed.

a) Extraction :

The enzyme was extracted from seedlings treated with different concentration of pesticides in a manner similar to that of peroxidase.

b) Assay :

The assay mixture was prepared by mixing 1 ml of 0.045 M H_2O_2 in phosphate buffer (pH 6.8) and 1 ml aliquot of enzyme. Assay mixture was incubated at room temperature for 1 min and then the reaction was terminated by the addition of 5 ml of 5 N H_2SO_4. For zero min the reaction was stopped before addition of enzyme by 5 N H_2SO_4. To this 1 ml 10% aqueous KI solution and a drop of 2% ammonium molybdate were added. The amount of H_2O_2 utilized by liberation of iodine was determined by titrating the reaction mixture with 0.01 M sodium thiosulphate using starch indicator. The difference between 0 min and 1 min reaction was used as enzyme activity. Activity of enzyme is expressed as mg H_2O_2 broken down $min^{-1}g^{-1}$ fresh wt.

4. Acid Phosphatase (E.C. 3.1.3.2)

The method adopted by McLachlan (1980) was used for study of enzyme acid phosphatase.

a) Extraction :

The extraction procedure for acid phosphatase was exactly the same as that of α-amylase discribed earlier.

b) Assay :

The assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg-p-nitrophenyl phosphate/ml of 0.1 M acetate buffer, pH 5.0). 2 ml 0.1 M acetate buffer (pH 5.0) and 1 ml enzyme. The reaction was allowed to continue for 30 min and then was terminated by addition of 1.5 ml of 1.68 N NaOH. The reaction terminated immediately (0 min) served as control. The optical density of the yellow colour was measured at 420 nm using double beam Shimadzu spectrophotometer. The enzyme activity was expressed as ΔOD h^{-1} g^{-1} fresh wt.

5. Protease (E.C. 3.4.3.2)

The enzyme protease was assayed according to the method of Penner and Ashton (1967) as described by Chinoy et al. (1969).

a) Extraction :

The known quantity of seedlings from 72 h germination stage of different treatments of pesticides were crushed in pre-chilled mortar and pestle in 15 ml cold 0.1 M phosphate buffer, pH 7.0 at 4^{o}C. The homogenate was filtered

through four layers of muslin cloth and filtrate was centrifuged twice at 6000 rpm for 5 min . Supernatant served as enzyme source.

b) Assay :

Assay of protease consists of 1 ml 0.5% Casein (pH 7.0), 3 ml 0.2 M phosphate buffer (pH 7.0) and 1 ml enzyme. The reaction was incubated for 1 h at 37°C then it was terminated by adding 2 ml. 5% Trichloro acetic acid. Unreacted protein was precipitated on an ice bath followed by centrifugation for 20 min. The aliquots (1 ml) of supernatant were used for measurement of protein as a free tyrosine. To this, 4 ml 0.5 N NaOH and 1.2 ml Folin-phenol reagent was added. The blue colour developed after shaking the reaction was measured at 660 nm on spectrophotometer. For blank distilled water was used instead of enzyme source and the same procedure was followed.

The enzyme activity was calculated by using the formula described by Chinoy et al. (1969).

$$\mu g \text{ of tyrosine liberated } h^{-1} = (142.4 \times A) - 0.13$$

where A = Change in optical density.

E. Proline

1. Material

In order to study proline content, the seeds of bean, okra, guar and onion were subjected to pesticidal treatment. All the seeds were soaked separately in 0.03% (v/v) Metacid and 0.015% (v/v) Dimecron for 1 h and then washed repeatedly with distilled water. Then they were allowed to germinate at room temperature (30 $\pm$ 1.5°C) on moist filter paper (soaked in distilled

water) kept in petridishes. The another set of seeds was directly allowed to germinate in petridish containing moist filter paper soaked with 0.03% (v/v) Metacid in one and with 0.015% (v/v) Dimecron in the other. In this case solutions of respective concentrations were supplied uniformly as and when required. The controls were maintained by using distilled water. The experiment was performed in replication. The proline content was determined from 1 h pesticide pre-treatment and from continuous pesticide treatment after 24 and 72 h germination.

2. Estimation of Proline

Proline content was estimated by the method of Bates et al. (1973). 0.5 g seedlings were homogenised in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate filtered through Whatman No.1 filter paper. 2 ml of filtrate was reacted with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C in water bath, and the reaction was terminated by transferring the tubes to ice bath immediately. After 15 min the reaction mixture was extracted with 4 ml toluene by shaking vigorously with a test tube stirrer for 20 sec . The red coloured toluene phase was separated from the aqueous phase with the help of small separating funnel and warmed to room temperature and the absorbance was measured at 520 nm using toluene as a blank on Shimadzu spectrophotometer. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

a) Preparation of acid ninhydrin

This reagent was prepared by warming 1.25 g ninhydrin (Unichem) in 30 ml glacial acetic acid (Merck) and 20 ml 6 M phosphoric acid (Glaxo) with agitation, until dissolved. The reagent remain stable for 24 h at 4°C.

F. Mitotic Aberrations

1. Material

To study mitotic aberrations the root tips of bean, okra, guar and onion seedlings grown in different concentrations of Metacid (0.01, 0.02, 0.03 and 0.04% v/v) and Dimecron (0.005, 0.01, 0.015 and 0.02% v/v) were excised and fixed in 1:3 acetic alchol by ensuring maximum cell division while fixing the material.

2. Squash preparation

A routine method was employed for squash preparation. The root tips were squashed in freshly prepared 2% acetoorcein. For each treatment and control a minimum of 10 root tip preparations were observed for cytotoxicity and about 200 cells were counted from each slide, under light microscope. The photographs were taken with the help of microphoto camera (Olympus).

a) Preparation of acetoorcein :

Acetoorcein stain was prepared by adding 45 ml of boiling glacial acetic acid and 55 ml of boiling distilled water to 2 gms of certified orcein powder in a beaker. It was stirred thoroughly and kept overnight in a cool and dark place. Then it was filtered thriough Whatman filter paper No.1 and stored in tightly stoppered bottle until use.