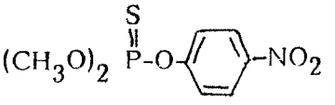
II MATERIALS AND METHODS

A. About the Pesticides used in the Present Investigation

1. METHYLPARATHION (Metacid-50)

Chemical name - 0, 0-dimethyl O-(P nitrophenyl) phosphorothioate

Structural formula - 

Empirical formula $C_8H_{10}NO_5PS$

Molecular wt. - 263.2

Solubility - 55 to 60 ppm in water at $25^{o}C$, readily soluble in most organic solvents, less soluble in petroleum ether and mineral oils.

Stability - Relatively stable at pH 1 to 7
Undergoes fast decomposition at pH 8-9.

Formulations - Metacid-50 (50% w/w Emulsifiable concentrate of Methyl parathion).

Biological Properties - Metacid is an insecticide with a very broad spectrum of activity. It kills nearly all sucking and biting pests. Metacid acts as a contact, stomach and breathing poison. It is notable for its fast killing action. As the active ingradient penetrates into the plant tissue, it controls both concealed and mining pests. Metacid displays good plant tolerance and therefore can be used in nearly all crops.

Uses and recommendations -

Metacid is widely used to control aphids, jassids, thrips, mites, leaf rollers, cut worms and cricket on crops like cotton, pome, grapes, citrus fruits and vegetables.

Toxicity

Oral toxicity - LD_{50} male rats - 14.0 mg/kg

Dermal toxicity - LD_{50} male and female rate 67.0 mg/kg (in Xylene) Active ingradient applied to dorsal skin, not removed.

Inhalation toxicity - LC_{50} male rats 200 mg/m^3 1 hour exposure.

Antidote - Two mg of Atropine sulphate by intravenous route.

2. PHOSPHAMIDON (Dimecron)

Chemical name - 0,0-dimethyl-O (2-chloro-N,N-diethyl-carbamyl)-methylvinyl phosphate.

Structural formula - $(CH_3O)_2 \overset{O}{\overset{\|}{P}}OC\overset{CH_3}{\overset{|}{}} = CClCON\ (C_2H_5)_2$

Solubility - Highly soluble in water, acetone, alcohol.

Stability - Relatively stable in neutral and weak acidic aqueous solution. Undergoes fast decomposition in alkaline medium.

Systemic Action - Active ingradient phosphamidon is absorbed by leaves within 1-3 hours after spray and is translocated more towards the top through plant sap. Being stomach poison it kills sucking insects on sap ingestion.

Properties -

(a) Effective against sucking, chewing and mining insects as well as sucking instars of spidermites.

(b) Has both insecticidal and acaricidal action.

(c) Being systemic it does not get washed away by rains.

(d) Had growth stimulating effect on certain crops.

(e) Regular applications do not give rise to secondary pest infestation.

It is used in the form of spray to control the pests like aphids, jassids, mites, fruitflies, etc. on vegetables and other field crops.

Toxicity -

(i) Oral toxicity - LD_{50} for rats 10.4 to 18 mg/kg.

(ii) Dermal toxicity - LD_{50} for rats 261 to 497 mg/kg.

Antidote -

2-4 mg Atropine sulphate should be administered intravenously in case of positive poisoning.

B. Procurement of seeds :

Seeds of Lycopersicon esculentum Mill. (tomato - variety Pusa Rubi), Abelmoschus esculentus Moench, L. (Okra - variety Pusa Savani) and Cyamopsis tetragonoloba (L.) Taub. (guar - variety Pusa Navbahar) were obtained from Shetakari Sahakari Sangh, Kolhapur; Beej Bhandar Kolhapur and College of Agriculture, 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. Seeds of okra and guar were sown in earthen pots filled with 3 parts of soil and one part farm yard manure and seedlings were allowed to establish for one month. Tomato seeds were sown on the soil bed and one month old seedlings were transplanted in earthen pots (30 cm in diameter) filled with 3 parts soil and 1 part farm yard manure. The seedlings were allowed to establish for 15 days under Photosynthetic Active Radiation (PAR) Ca 1900 $\mu E\ m^{-2}s^{-1}$ and temp. regime 30 $\pm$ 2 / 19 $\pm$ 2°C day/night.

Metacid-50 (Emulsifiable concentrate containing 50% w/w methyl-parathion - Bayer (India) Ltd.) and Dimecron (85% phosphamidon : Hindustan CIBA GEIGY Ltd.), the organophosphorus insecticides were sprayed to run off on foliage by using air pneumatic manual pump at 10 to 11 a.m. Control plants were sprayed with equal volume of water. The concentrations used for spray were 0.1, 0.15 and 0.2% (all v/v) for methyl-parathion and 0.02, 0.04 and 0.06% (all v/v) for phosphamidon. These

concentrations were below, at and above recommended levels respectively for both the insecticides.

D. Relative Water Content (R.W.C.) :

One gram leaf discs (5 mm in diameter) were taken from sprayed and nonsdprayed fresh leaves. These leaf discs were kept in a conical flask containing water. After three to four hours, when the leaf discs attained full turgid level, weight was taken and then the leaf discs were kept in an oven at $60^{o}C$. The leaf discs were allowed to dry completely and then dry wt. of leaf discs was noted. The per cent relative water content was calculated by using the formula -

$$\underset{(\%)}{\text{R.W.C.}} = \frac{\text{Fresh wt. - Dry wt.}}{\text{Wt. at full turgid level - Dry wt.}} \times 100$$

E. Stomatal behaviour :

Leaf diffusive resistance and transpiration rate were determined by using steady state porometer, LI 1600 (LICOR, USA). The readings were taken continuously for 6 days in all the three crops and diffusive resistance for CO_2 was calculated using the formulae suggested by Jarvis (1971)

$$1/R \text{ Leaf} = 1/R \text{ upper} + 1/R \text{ Lower}$$

$$R_L \, CO_2 = 1.6 \, R_L \, H_2O$$

F. Organic Constituents :

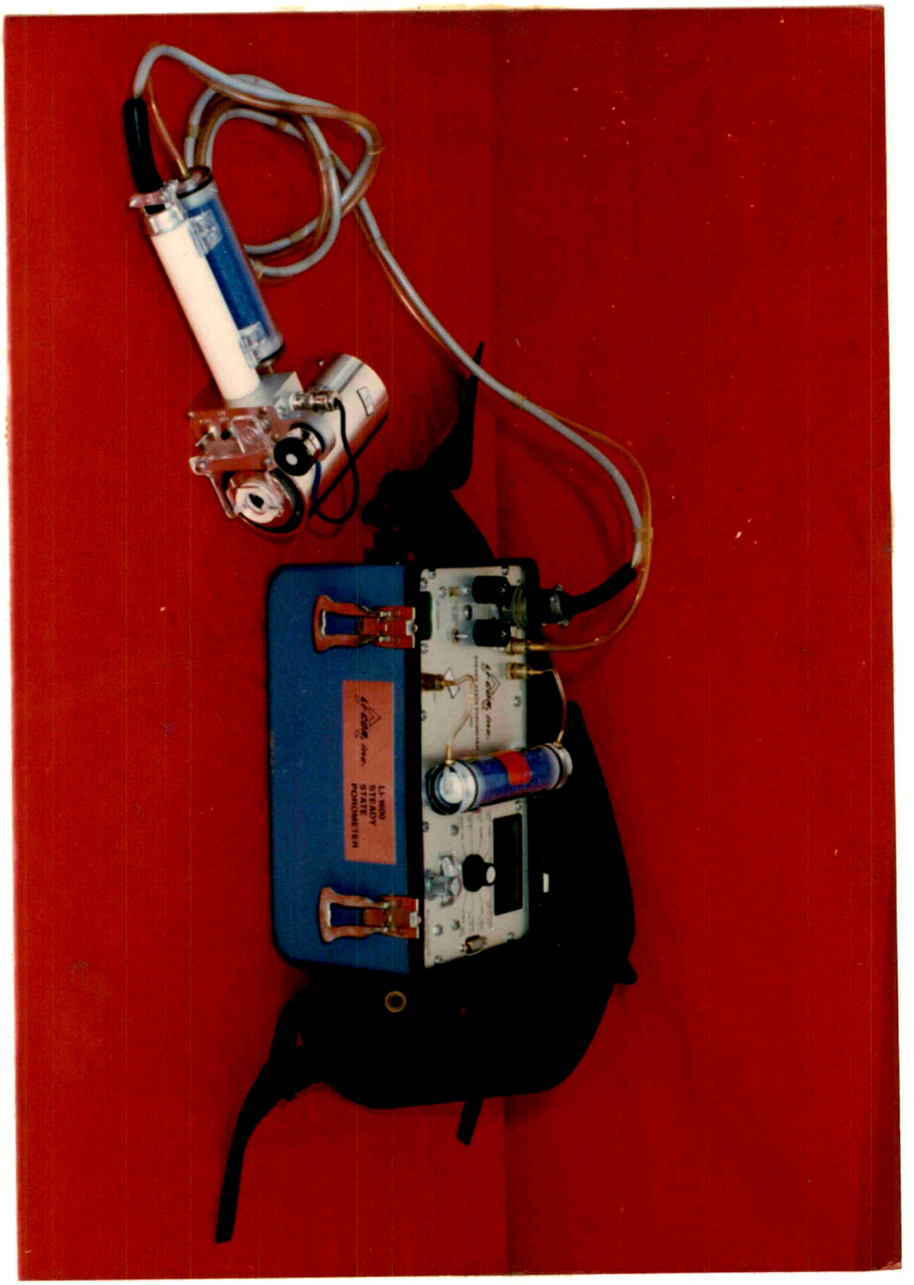
Plant samples were analysed for chlorophylls, polyphenols, and carbohydrates on 1st, 2nd, 4th and 7th day after spray.

PLATE - 2

Steady state porometer (LI-1600) LICOR. Inc. USA

used to study stomatal regulation

34



1. Total Chlorophylls :

Chlorophylls were estimated following the method suggested by Arnon (1948).

0.5 g fresh plant material was crushed in a mortor with pestle and extracted in 80% chilled acetone containing 4 ml liquid NH_3 per litre in a dark and cold room. A pinch of $MgCO_3$ was added during crushing. This extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The volume of filtrate was adjusted to 50 ml with 80% acetone. The extract was then transferred to a conical flask. This flask was covered with black paper to retain the activity of chlorophylls. The absorbance was read at 663 and 645 nm.

Chlorophylls (mg/100 g fresh weight) were calculated using the formula :

$$\text{Total chlorophylls (a+b)} = z = 8.2 \times A663 + 22.2 \times A645$$

$$\text{Total chlorophylls / 100 g fresh tissue} = \frac{z \times \text{volume of extract} \times 100}{1000 \times \text{wt. of plant material (g)}}$$

2. Polyphenols :

Polyphenols were estimated following the method suggested by Folin and Dennis (1915).

0.5 g fresh plant material was crushed in a mortor with pestle and was extracted in 80% acetone. This extract was filtered through Buchner's funnel using Whatman No.1 filter paper. The residue on the filter paper was washed several times with 80% acetone and the final volume of extract was adjusted to 50 ml with 80% acetone.

2 ml of plant extract was taken in Nessler's tube along with the series of standards (Standard tannic acid 0.1 mg/ml) to which 10 ml of 20% Na_2CO_3 was added. The volume was adjusted to 35 ml with distilled water. Then 2 ml of Folin Dennis reagent (Dissolve 100 g sodium tungstate and 20 g phosphomolybdic acid in 800 ml distilled water. Add 50 ml 80% phosphoric acid. Reflux for 2-1/2 hours using water condenser. Adjust the final volume to 1 lit. with distilled water) was added to each test tube and the final volume was adjusted to 50 ml with distilled water. After about 20-30 min absorbance was read at 660 nm using reaction blank. Polyphenols were calculated from standard curve of tannic acid and were expressed in g/100 fresh tissue.

Carbohydrates :

The sugars were estimated following the method suggested by Nelson (1944). Carbohydrates were extracted from known quantity of oven dried plant material with 80% ethanol. The extract was filtered through Buchner's funnel using Whatman Noi.1 filter paper. The filtrate thus obtained was used to estimate soluble sugars and the residue over the filter paper was used to estimate the starch.

The filtrate was then condensed on water bath to about 2-3 ml. To this lead acetate and potassium oxalate (1:1) were added with constant stirring. To that, about 30 ml distilled water was added and was once again filtered through Buchner's funnel using Whatman No.1 filter paper and the volume of extract was adjusted to 50 ml with distilled water. From this extract reducing sugars were determined.

The residue on filter paper was transferred to 150 ml conical flask along with filter paper and to this 50 ml distilled water and 5 ml concentrated HCl were added. The same was hydrolysed at 15 lbs pressure for half an hour and cooled to room temperature, neutralized with anhydrous Na_2CO_3 and filtered. The volume of the filtrate was measured and was noted down. This filtrate contains reducing sugars (mostly glucose) formed as a result of hydrolysis of starch.

The sugars from both the filtrates were estimated by determining the reducing power by employing arsenomolybdate reagent introduced by Nelson (1944) for the colorimetric determination of cuprous oxide formed in the oxidation of sugars by alkaline copper tartarate reagent.

2 ml extract of reducing sugars and 0.5 ml extract of starch was taken in a test tube along with the stnadard series of glucose (standard glucose 0.1 mg/ml) in other test tubes. In case of blank, standard glucose or extract was not added, but distilled water was taken in a test tube. One ml of Somogyi's alkaline copper tartarate reagent (4 g $CuSO_4.5H_2O$, 24 g anhydrous Na_2CO_3, 16 g Na-K tartarate and 180 g of anhydrous Na_2SO_4 dissolved in 1 litre distilled water) was added in each test tube and all these test tubes were kept in boiling water bath for 10 min. After cooling to room temperature 1 ml of arsenomolybdate reagent (25 g ammonium molybdate dissolved in 450 ml distilled water to which 21 ml concentrated H_2SO_4 were added. 3 g of sodium arsenate ($Na_2HASO_4.7H_2O$) dissolved in 25 ml water. These solutions were mixed well and kept in an incubator for 37°C for 48 hours before use) was added to each test tube and then the volume of each reaction mixture was

adjusted to 10 ml with distilled water. After 10 minutes absorbance was read at 560 nm on spectrophotometer.

Using caliberation curve of standard glucose, the sugar percentage in each fraction was calculated. The values were expressed in terms of g/100 g dry tissue.

G. Inorganic Constituents :

Estimation of inorganic constituents was carried out from treated and control plants on 1st, 2nd, 4th and 7th day after spray. The plant material was cut into small pieces and was weighed acurately and kept in an oven for drying at $60^{o}C$. After 4-5 days when the dried plant material showed constant weight, known amount was taken for analysis. From this plant material inorganic constituents such as Sodium (Na^{+}), Potassium (K^{+}), Calcium (Ca^{2+}), Iron (Fe^{2+}), Magnesium (Mg^{2+}), Manganese (Mn^{2+}), Zinc (Zn^{2+}) and Copper (Cu^{2+}) were estimated.

1. Preparation of extract :

For the estimation of inorganic constituents method suggested by Toth et al (1948) was followed.

Known quantity of oven dried plant material was taken in 150 ml beaker to which 20 ml of conc. HNO_3 was added. The beaker was covered with a watchglass and kept aside till the initial reactions subside The beaker was then heated slowly till the plant material was dissolved. After cooling to room temperature 10 ml of perchlorice acid (about 60%) was added. The beaker was once again heated till the mixture becomes

clear and volume of the mixture was reduced to about 2-3 ml. Mixture was not allowed to dry. It was then cooled to room temperature and diluted to 100 ml with distilled water and kept overnight. Next day the acid digest was filtered through Whatman No.1 filter paper and this filtrate was used to analyse inorganic constituents.

Sodium (Na^+) and Potassium (K^+) :

Sodium and Potassium were estimated flame photometrically (Model ELICO-CL 22A). Standards of known concentrations in parts per million of K^+ as KCL (10 to 40 ppm) and Na^+ as NaCl (5 to 25 ppm) were used for calibration curves. From the calibration curves concentrations of Na^+ and K^+ in the acid digested samples were calculated.

Calcium (Ca^{2+}), Iron (Fe^{2+}), Magnesium (Mg^{2+}), Mangenese (Mn^{2+}) Zink (Zn^{2+}), and Copper (Cu^{2+}) were analysed using atomic absorption spectrophotometer (PERKIN ELMER - 3030 Atomic Absorption Spectrophotometer).

H. Residual Analysis :

1. Qualitative determination of pesticidal residue

The qualitative analysis of residual content of pesticides from the leaves and fruits was made by employing paper chromatographic technique for phosphamidon and thin layer chromatographic technique for methylaparathion.

(a) Paper Chromatography

The procedure followed for paper chromatography was suggested by Prasad and Ramasubbaiah (1982).

(i) Preparation of extract

Samples of fruits and leaves sprayed with 0.06% phosphamidon were harvested from tomato plants at 1 hour after spray and on 7th day after spray. From this 20 g leaf material and 100 g fruits were crushed separately in a mortor with pestle by using 40 ml distilled methanol (Anala-R grade). The extract thus obtained was filtered under vacuume through Buchner's funnel over Whatman filter paper No.1, a plug of cotton and anhydrous sodium sulphate placed in a sequence. The filtrate was evaporated at room temperature and the residue dissolved in methanol was centrifuged at 5000 x g for 5 min and the supernatent was used for spotting.

(ii) Preparation of standard solution

A solution of phosphamidon in methanol was prepared by mixing 0.03 ml phosphamidon in 0.1 ml methanol from which 20 was loaded on chromatogram.

(iii) Spotting and development of chromatogram

An aliquot of the sample was taken with 10 ml microsyringe and was spotted on chromatogram paper (Whatman No.1) strips of 30 cm length and 4 cm width. The standard solution was also spotted in the same manner. The paper strips were developed by ascending technique with the upper phase of sdolvent system (5 parts petroleum ether : 5 parts toluene : 7 parts methanol : 3 parts water). After allowing the solvent to reach 17 cm height, the strips were removed, dried in shade and developed by a solution of one volume of 0.1% tetrazolium blue

solution and 9 volumes of 2 N NaOH solution. Phosphamidon appeared as a blue smear and was confirmed by R_f value of the standard spot with those of the sample. The coloured band developed on a strip was not viable for long time. Hence an outline figure is given in the text.

b. Thin Layer Chromatography :

(i) Preparation of TLC plates

TLC plates were prepared by the method of Patil (1980).

20 g Silica Gel-G (BDH, 200 mesh with 13% $CaSO_4$ as a binder) was slurried with 40 ml distilled water. The slurry was quickly transferred to applicator, thickness was adjusted to 0.25 mm and applied to the glass plates (size 20 x 20 cm). The plates were activated in an oven at $110^{o}C$ for one hour. They were cooled and used further for loading.

(ii) Extraction

The extraction of plant material was carried out essentially by the method suggested by Sharma (1980).

20 g leaf material and 100 g fruits sprayed with 0.2% methyl-parathion were crushed in 100 ml acetonitrile in a mortor with pestle separately. Then the extract was filtered through scintered glass funnel having a plug of cotton and anhydrous sodium sulphate placed in a sequence. The filtrate and washings were then evaporated to dryness at room temperature for appropriate partitioning step.

(iii) Partitioning

The residue was transferred to a 25 ml glass stoppered separating funnel by using 10 ml of pre-equilibriated mixture of hexane and aceto-

nitrile. This mixture was shaken for 1 min. The acetonitrile layer was withdrawn and the hexane layer was discarded. Acetonitrate layer was reserved for direct application on TLC plates.

(iv) Application of sample

Alongwith the extract of sample, standard methylparathion was also loaded with the help of microsyringe and were run parallely.

(v) Development of Chromatograms

The chromatographic chambers were prepared 20 min. before insertion of plates. The chamber was first ligned with Whatman filter paper No.1 on three inner sides, which was wetted with developing solvent. The solvent system used was a mixture of Hexane and acetone mixed in the proportion of 3:1 (v/v). The solvent was run to a length of about 17 cm from the bottom of plates. The plates were then removed and dried at room temperature and spots were developed by spraying $AgNO_3$ reagent prepared in ammonia (1% solution of silver nitrate, prepared by dissolving 1.0 g of reagent grade $AgNO_3$ in redistilled ethanol containing 5.0 ml of concentrated ammonia solution and made upto 100 ml with ethanol).

The yellow coloured spots, appeared on TLC plates were identified by comparing with standards and their R_f values.