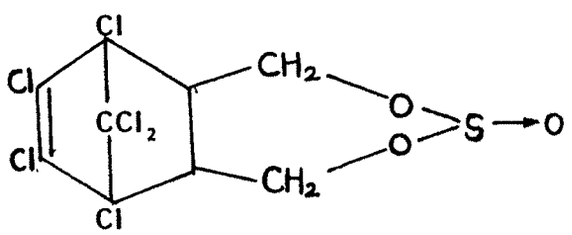
III MATERIALS AND METHODS

A. Pesticides used for the study

1. Endosulfan [Thiodan, Thiotox]

Chemical name : 6, 7, 8, 9, 10, 10 - hexachloro - 1, 5, 5a, 6, 9, 9a - hexahydro - 6, 9 - methano 2, 4, 3 - benzodioxanthiepin - 3 - oxide.



Endosulfan is the adduct of hexachlor cyclopentadine and 1, 4 - dihydroxy - 2 - butene subsequently chlorinated with SO_2Cl_2, to produce the Endosulfan. The technical material is brownish solid with M.P. 70 to 100°C. It is widely used as a broad spectrum insecticide and a caricide for stem borer, red hairy caterpillar, armyworms, earhead caterpillar, sorghumn midge, ear head bug, blister beetles, leaf roller, aphids etc. in cereals.

Dosage : 500 to 1200 ml of Endosulfan 35% EC per Acre depending upon pest, mixed with 250 to 450 litres of water.

Toxicity : Endosulfan acts as a stomach and contact poison of low toxicity to beneficial insects like honey beas, parasites and predator animals when used at recommended dosages. It is toxic to fish but is well tolerated by crops. Acute oral LD_{50}

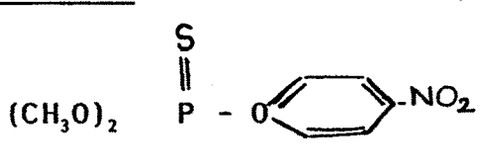
of active ingradient (Endosulfan)-30 mg/kg in alcohol suspension, 70 mg/kg in aqueous suspension, 110 mg/kg in oil. Acute Dermal LD_{50} (rat) of active ingr dient-1050 mg/kg.

Antidote : If swallowed, gastric lavage is given with half a print of water containing activated charcoal 2 parts, magnesium oxide 1 part and tannic acid 1 part to induce sedation and control convulsions. Phenobarbitone upto 0.7 gms daily may be given.

Compatibility : Endosulfan is compatible with most other pesticides except for Bordeaux mixture, calcium arsenate and other alkaline pesticides.

2. Methyl Parathion (Metaphos, dimethyl parathion, Metacid, Folidol - 80, Wafatox)

Chemical name : O, O - dimethyl O - 4 - nitrophenylthiosulphate.



Methylparathion is a white crystalline substance with a M.P. 35 to 36°C. It's solublity in water is about 55 mg/litre. It is slightly soluble in paraffinic hydrocarbons and most organic solvents.

It is usually marketed in the form of emulsion,

wettable powder and dust. It is widely used for controlling aphids, jassids, thrips, mites, white flies, caterpillars and grass hoppers in cereals. It is effective against sucking and chewing pests.

Dosage : 0.04 to 0.1% V|V for all these pests (Gangawane and Deshpande 1985).

Toxicity : Tolerable at recommended doses.

Antidote : 2 mg of atropine sulphate by intravenous route.

B. Procurement of seeds and pesticides :

The seeds of jowar (Sorghum bicolor L. var. Maldandi) were collected from Akluj (dist. Solapur) through local farmers.

Endosulfan (35% EC, United Fertilizer Industries, Bombay) and Methyl parathion (50% EC, Konkan pesticides, Bombay) were purchased from Shetkari Sahakari Sangh Pvt. Ltd. Kolhapur.

C. Pesticidal treatment :

Healthy seeds of uniform size were selected, surface sterilized with 0.1% $HgCl_2$ for 1 min. then washed repeatedly with Distilled water. These seeds were used for germination studies with various concentrations of Endosulfan and Methylparathion. Concentrations selected for both these pesticides are 0.05%, 0.1%, 0.2%, 0.4% and 0.6%

For Methyl parathion higher concentrations used are much above the recommended ones however for the sake of uniformity similar range of concentrations was choosen in the present study.

1. Germination : Sterilized seeds were placed in previously sterilized petridishes lined with Whatman No. 1 filter paper. The paper was moistened with 10 ml distilled water (control) or with respective concentration of pesticide solution, 50 seeds were used for germination in each petridish and the experiment was carried out in dark in an incubator at 25 ± 2°C. Germination percentage was recorded after every 24 h upto 96 h of germination. The pesticide solution was supplied uniformly during germination to all the seeds as and when required.

2. Seedling growth : Root length & shoot length of each seedling was measured every day after 48 h of germination upto 96 h of germination. Root to shoot ratio and percent phytotoxicity was calculated from these observations. For percent phytotoxicity the formula suggested by Chou and Muller (1972) was used.

$$\text{Percent phytotoxicity} = \frac{\text{Radicle length of control} - \text{Radicle length of Test}}{\text{Radicle length of control}} \times 100$$

Development of secondary roots was recorded as the no. of lateral roots observed for each and every seedling. Fresh weight of seedlings was also recorded at the end of experiment.

3. Starch content : Amount of total starch content was estimated every day from the seeds germinated in presence of pesticide as well as from untreated seeds.

4. Enzyme studies : Activities of hydrolytic enzymes α-amylase, protease, acid and alkaline phosphatases and a few respiratory enzymes (peroxidase and catalase) were determined after every 24 h upto 120 h of germination.

D. Methods followed :-

1. Starch : The method described by Nelson (1944) was followed for estimation of starch.

0.5 g plant material was homogenised in 80% ethyl alchol. The extract was filtered through Buchner funnel using whatman No. 1 filter paper. The insoluble residue, left after filtering the alcholic extract, was washed 2-3 times with alchol and then transferred to a 100 ml conical flask, containing 50 ml distilled water and 5 ml concentrated HCL. It was stoppered with cotton plugue and hydrolysed at 15 lbs atm. pressure for half an hour, in an autoclave. After cooling to room temperature the extract was neutralised by addition of an-

hydrous Na_2CO_3 and filtered. The volume of filtrate was measured. This filtrate contained reducing sugars (mostly glucose) formed as a result of hydrolysis of starch in the plant residue. The sugar content was determined colorimetrically using arsenomolybdate reagent. When sugar solution is heated cuprous oxide is produced in the oxidation of sugars, by alkaline copper tartarate, which reacts with arsenomolybdate to give molybdenum blue. The intensity of blue colour developed is measured on spectrophotometer. For estimation, requisite quantity of extract was taken in test tubes along with different conc· of standard glucose solution (0.1 mg/ml) in other test tubes. To this required amount of distilled water was added to make final volume one ml. 1 ml Somogyi's alkaline copper tartarate reagent [4 g $CuSO_4$ $5H_2O$, 24 g anhydrous Na_2CO_3, 16 g Na-K tartarate (Rochelle salt) 180 g anhydrous Na_2SO_4 dissolved in 1 litre distilled water] was added in each test tube. A blank was maintained in similar way withougut sugar solution. All the tubes were transferred to boiling waterbath for 10 minutes. After cooling to room temprature, 1 ml Arsenomolybdate reagent (25 g ammonium molybdate in 450 ml distilled water to which 21 ml conc. H_2SO_4 added, followed by 3 g sodium arsenate [$Na_2HA_3O_4$, $7H_2O$] dissolved in 25 ml distilled water. These ingradients were mixed well and incubated for 48 h at 37°C before use) was added to each reaction mixture. The volume in each tube was adjusted to 10 ml with d.w. After 10 minutes absorbance was read at 560 nm on Shimadzu spectrophotometer.

Using calibration curve of standard glucose, the sugar percentage was calculated. Starch content is expressed as gram per gram of fresh weight.

2. Hydrolytic Enzymes :

i) α- Amylase (E. C. 3.2 1.1)

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

a) Extraction : 1 g of seedlings were washed with distilled water and homogenised in a mortar using 10 ml cold acetate buffer (0.1 M, PH 5.00). The extract was filtered through four layers of muslin cloth and the filtrate was centrifuged at 6000 rpm for 15 minutes. The supernatent was stored on an ice bath and used for enzyme assay.

b) Assay : The activity of α-amylase was assayed by incubating 1 ml enzyme with 1 ml of 0.1 M acetate buffer (PH.5). The reaction was killed after 0 and 30 min. with 10 ml of 0.5 N acetic acid. The aliquat (1 ml) from killed reaction mixture was then treated with 10 ml of dilute KI solution (0.25% I_2 prepared in 0.1% aqueous KI solution). The absorbance was measured on a double beam Shimadzu spectrophotometer at 700 nm using I_2KI solution as a blank. Enzyme activity is expressed in terms of ΔOD. $h^{-1}g^{-1}$ of fresh weight.

ii) Protease (E.C. 3.4.3.2)

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

a. Extraction : One gram of seedlings in each germinating stage (24 to 120 h) and different pesticidal treatments was washed with distilled water and crushed in mortar using 10 ml of phosphate buffer (0.1 M, PH 7) The homogenate was filtered through four layers of muslin cloth and filtrate was centrifuged at 6000 rpm for 10 min. Supernatent served as the enzyme source.

b. Assay : Assay mixture consisted of 1 ml casein (0.5%, PH 7.0), 3 ml phosphate buffer (0.2 M, PH 7.0) and 1 ml enzyme. The reaction was incubated for 1 h at 37°C and was then terminated by adding 2 ml of Trichloroacetic acid (5%). Untreated protein was precipitated on an ice bath followed by centrifugation for 20 minutes. Then the supernatent was taken for estimation of free tyrosine. Four ml NaOH (0.5 N) and 1.2 ml Folin - phenol reagent were added to 1 ml of supernatent and mixed thoroughly. Absorbance of developed blue colour was read at 660 nm on a spectrophotometer. Blank was prepared by using distilled water instead of enzyme source. Further procedure was the same as described above.

Enzyme activity was calculated using the formula given by Chinoy et al. (1969).

Enzyme activity = (142.4 x A) - 0.13 = µg of tyrosine liberated h^{-1}

Where A = change in optical density.

iii) Acid Phosphatase (E.C. 3.1.3.2)

For the study of Enzyme acid phosphatase method described by McLachlan (1980) was adopted.

a. Extraction : Accurately weighed (0.5g) seedlings from each germination stage (24 to 120 h) were homogenised separately in 10 ml cold acetate buffer (0.1 M, PH 5) in a chilled mortar with pestle. The resultant extract was filtered through four layers of muslin cloth and filtrate was centrifuged at 6000 rpm for 10 min. The supernatent was used as the enzyme source.

b. Assay : The assay mixture contained 3 ml of p-nitrophenyl phosphate (0.1 mg per ml of acetate buffer, PH 5.0), 2 ml of acetate buffer (0.1 M, PH 5.0) and 1 ml enzyme. The reaction was allowed to proceed for 30 minutes and then terminated by adding 1.5 ml of NaOH (1.68 N). The reaction was terminated immediatly to detain enzyme activity at 0 min. Optical density of the yellow colour developed was read at 420 nm using a substrate blank. The enzyme activity is expressed as Δ OD h^{-1} g^{-1} fresh wt.

iv) Alkaline Phosphatase (E.C. 3.1. 3.1)

Enzyme alkaline phosphatase was studied by the method described by Weimberg (1970).

a. Extraction : 0.5 g seedlings from each germination stages were homogenised separately in 10 ml of tris-HCl buffer (0.1 M, PH 5.0). The resultant homogenate was filtered through four layered muslin coth and filtrate was centrifuged at 6000 rpm for 10 min. The supernatent was used for enzyme assay.

b. Assay : Assay mixture consisted of 1 ml Tris-HCl buffer (0.1 M, PH 5), 0.1 ml 0.05 M $MgCl_2$, 0.1 ml 0.02 M þ- nitrophenyl phosphate and 1.7 ml distilled water. Absorbance was measured at 410 nm immediatly after addition of 0.1 ml crude enzyme and after 30 minutes of reaction. The difference in the two readings corresponded for the ΔOD for 30 min reaction. enzyme activity was then computed as ΔOD h^{-1} g^{-1} fresh weight.

3. Oxido - Reductases (Respiratory enzymes)

i) Peroxidase (E.C. 1.11. 1.7)

The method of Maehly and Chance (1954) was followed to study the activity of enzyme peroxidase.

a. Extraction : The enzyme was extracted separately using one g of seedlings subjected to different pesticidal treatments

by using 10 ml cold distilled water. The extract was filtered through four layered muslin cloth and centrifuged at 6000 rpm for 10 min. The supernatent was used as enzymes source as usual.

b. Assay : Enzyme assay mixture was prepared by adding 2 ml of phosphate buffer (0.1 M, PH .7), 1 ml 20 mM guiacol and 1 ml enzyme. The reaction was initiated by the addition of 0.04 ml of 10mM H_2O_2. The change in optical density due to oxidation of guiacol was recorded at every 30 seconds for 2 minutes, at 470 nm with frequent stirring of the reaction mixture. Enzyme activity is expressed as ΔOD min^{-1} g^{-1} fresh wt.

ii) Catalase (E.C. 1.11. 1.6)

For study of catalase a modified method of Herbert (1955) was adopted.

a. Extraction : The enzyme was extracted from seedlings treated with different concentrations 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 temp. for 1 min. and then reaction was terminated by the addition of 5 ml of 5N H_2SO_4. For zero min. the reaction was stopped by adding 5 N H_2SO_4 before the addition of enzyme. 1ml of 10%

aqueous KI solution and a drop of 2% ammonium molybdate were added to the resultant reaction mixture. 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 (1% in d.w.). The difference between 0 min. and 1 min. reaction was taken as the measure of enzyme activity.

Activity of enzyme is expressed as mg H_2O_2 broken down min^{-1}, g^{-1} fresh wt.