# CHAPTER -III

# MATERIALS AND METHODS

## A. PROCUREMENT OF SEEDS AND PESTICIDES :

Seeds of *Glycine max* (L.) Merr. of variety Krishidhan JS - 335 were obtained from Shetakari Sahakari Sangh, Kolhapur. Fungicide Mandy M- 45 (Mancozeb 75 % W.P.) and insecticide Anth (Chlorpyriphos 50% and Cypermethrin 5 % EC) were purchased from Shetakari Sahakari Sangh, Kolhapur.

## B. ABOUT PESTICIDES :

### 1. Mandy M – 45 ( Mancozeb 75 % WP) :

Common name : Mancozeb

Trade names : Dithane M - 22, Dithane M - 45, Manzate, MEB, MnEBD, Manzeb, Mancofol,Manzin, Vondozeb, zimaneb.

Chemical name : Manganese ethylene bis (dithiocarbamate) (polymeric) complex with Zinc salt.

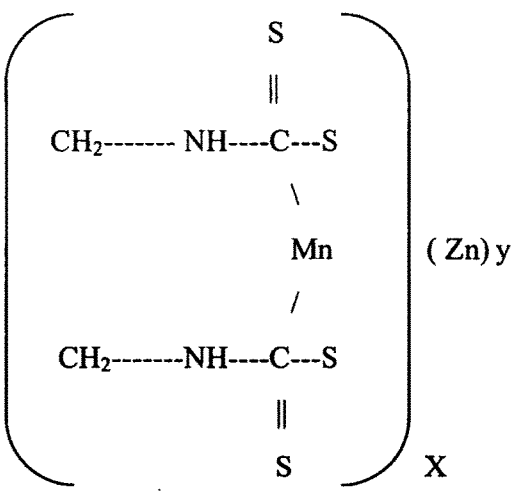
CAS No. : 8018 - 01 - 07

Empirical Formula : $(C_4H_6MnN_2S4)_x(Zn)_y$

Chemical class : ethylene (bis) dithiocarbamate

Molecular weight : 541.0

Structural formula :




Transformation product : Ethylene thiourea (ETU)

Synthesis : In 1950, by E. I. du Pont de Nemours and Company Rohm and Hoss Company and Bayer A. G. of Germany.

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|---|---|
| Physical state | : Solid, Amorphous |
| Colour | : Greyish yellow |
| Odour | : Odourless |
| Melting point | : Decomposes below melting point : ca. 150 $^{0}$ C |
| Solubility | : 6 - 20 mg / l in water, practically insoluble in most organic solvents. |
| Vapour pressure | : Pa at 20$^{O}$ C : negligible |
| Relative density (water = 1) : 1.92 | |
| Flash point | : 138 $^{0}$C o.c. |
| Corosive Properties | : Noncorosive |
| Explosive Properties | : Combustible. Liquid formulations containing organic solvents may be flammable. Finely dispersed particles form explosive mixtures in air. |
| Formulations | : Mancozeb is available as dusts, liquids, water –dispersible granules, wettable, powders |
| Application | : Mainly foliar spray and also soil drench. |
| Recommendations | : Mancozeb is used to protect many fruit, vegetables, nut and field crops against a wide spectrum of fungal diseases. It is used to control brown and black rust, blight of wheat, leaf blight and downy mildew of maize, blast of paddy, leaf spot and fungal diseases of guava. |
| Mode of action : | Mancozeb is classified as a contact fungicide with preventive activity. It inhibits enzyme activity in fungi by forming complex with metal containing enzymes including those involved in the production of adenosine triphosphate (ATP). |
| Toxicology | : Mancozeb is toxic to human beings when consumed orally, It causes irritation to nose and throat. |

**2. Anth (Chlorpyriphos 50% +Cypermethrin 5 % EC) :**

Classification : Moderatively hazardous

Acute oral $LD_{50}$ value : 135- 160 mg $kg^{-1}$

Mode of Action : It is a wide spectrum insecticide with three way action; stomach, contact and fumigant. It kill insects by direct contact or ingestion and by disrupting normal functioning of nervous system.

Application : Mainly foliar spray.

Recommendations : It is effective against a wide range of plant feeding, household and Soil insects. It is effectively used against most pests associated with crops like soybean, cotton, paddy and tobacco. It is very effective against foliar pests like leafworms, bollworms, white flies and soil dwelling pests like cutworms and wireworms.

**C. SEED TREATMENT :**

Healthy seeds of *Glycine max* (L.) Merr. were treated by wet treatment of Mancozeb (75 % WP) and Anth (Chlorpyriphos 50 % + Cypermethrin 5 % EC). For both the pesticide treatments, concentrations ranging from 0.025 % to 2 % (w / v) were used. Seeds were exposed for 6 and 12 hours to the respective concentrations of both the pesticides. Seeds of control set were soaked in distilled water for 6 and 12 hours. The treated seeds were thoroughly washed with distilled water after the completion of the treatment and then treated and untreated seeds were allowed to germinate in replicates of three in petriplates lined with moist filter paper. Fifty seeds were inoculated for each treatment in the petriplates incubated at 27 $^0$C -30 $^0$C in germinating chamber.

The percent germination of seeds was recorded after every 24 hours upto 72 hours of seeds. Growth analysis was carried out after 7 days growth of seedlings. Percent phytotoxicity was calculated using the formula given by Chiou and Muller (1972). From the germination study, the concentrations selected for both the pesticide treatments were 0.25, 0.5, 1 and 2 % with exposure period of 6 and 12 hours.

## D. SEED TREATMENT AND FOLIAR SPRAY :

Healthy seeds of *Glycine max* (L.) Merr. were subjected to wet treatment of Mancozeb and Anth (Chloropyriphos +Cypermethrin). Seeds were exposed for 6 and 12 h to the concentrations ranging from 0.25 to 2 % of both the pesticides. After the desired exposure period, the treated seeds were thoroughly washed with distilled water and sown in earthen pots containing a mixture of garden soil and manure (3 : 1) . For both the pesticides seeds treated with distilled water for 6 and 12 h and sown in earthen pots were used as control. Foliar sprays of Mancozeb and Anth (chlorpyriphos +Cypermethrin) of the same respective concentrations were applied on tenth day of growth. Growth analysis was carried out on fifteenth day of growth.

## E. PHOTOSYNTHETIC PIGMENTS :

### 1. Chlorophylls :

Chlorophyll pigments were estimated following the method of Arnon (1949). Randomly sampled leaves (0.5g) were homogenized and extracted in 80 % chilled acetone contained 4 ml ammonia per litre. A pinch of $MgCO_3$ was added to neutralize the acids released during extraction. The extract was filtered through Whatman No. 1 filter paper using Buckner's funnel under suction. Final volume of the extract was made to 50 ml with 80 % acetone. The filtrate was transferred into a conical flask and wrapped with black paper to prevent photooxidation of the pigments. Absorbance was read at 663 nm and 645 nm on a spectrophotometer.

Chlorophyll 'a', chlorophyll 'b' and total chlorophylls were calculated using the following equations of Arnon (1949).

Chl. 'a' (X) = $(12.7 \times A_{663}) - (2.69 \times A_{645})$

Chl. 'b' (Y) = $(22.9 \times A_{645}) - (4.68 \times A_{663})$

Total chlorophylls (Z) = $(8.02 \times A_{663}) - (20.20 \times A_{645})$.

$$\text{Chlorophyll a /b / chl (a+ b) (mg / 100 g fresh wt.)} = \frac{\text{X/Y/Z x volume of extract x100}}{\text{1000 x weight of plant material (g)}}$$

**2. Carotenoids :**

Carotenoids were estimated from the same acetone extract of chlorophylls as per the method described by Krik and Allen (1965). The absorbance was measured at 480 nm on a double beam spectrophotometer. The amount of carotenoids was calculated using the following formula.

$$\text{Total carotenoids (mg / 100 g fresh wt.)} = \frac{A_{480} \times \text{volume of extract} \times 10 \times 100}{2500 \times \text{weight of plant material (g)}}$$

**F. ORGANIC CONSTITUENTS :**

1. **Carbohydrates :**

Total soluble sugars and starch content were estimated according to the method given by Nelson (1944). 1 g germinating seeds were extracted in 80 % alcohol and filtered through Buckner's funnel using Whatman No. 1 filter paper. The filtrate was condensed on a water bath to about 2-3 ml. Add 2 g of mixture of lead acetate and potassium oxalate (1:1) with constant stirring and then the contents were mixed with 20 ml of distilled water. After passing through Buckner's funnel, using Whatman No. 1 filter paper, volume of filtrate was measured and recorded. 20 ml of extract was transferred into a conical flask containing 2 ml concentrated HCl. The flask was plugged with cotton and autoclaved for 30 min under 15 lbs pressure. After cooling to room temperature the contents were neutralised by adding anhydrous $Na_2CO_3$ and filtered again. The volume of filtrate was recorded and it was used for the estimation of total sugars. The residue left on filter paper during the alcoholic extraction was transferred along with the filter paper into conical flask, containing 5 ml concentrated HCl and 15 ml distilled water. It was hydrolysed at 15 lbs pressure for 30 min and then cooled to room temperature. The contents were neutralised with anhydrous $Na_2CO_3$ and filtered. The volume of the filtrate was recorded. This filtrate was used for the estimation of starch. Estimation of sugar was carried out colorimetrically by using Arsenomolybdate reagent to determine cuprous oxide formed in the oxidation of reducing sugar by alkaline copper tartarate reagent. One ml extract was heated with 1ml copper tartarate on water bath for 10 minutes. One ml of Arsenomolybdate reagent was added after cooling and the volume was adjusted to 10 ml with distilled water. Absorbance was measured at 560 nm after 10 min on spectrophotometer against blank.

**2. Total Soluble Proteins :**

Soluble proteins were estimated from germinating seeds of *Glycine max* L. Merr. according to the method described by Lowry et al. (1951). Germinated seeds (0.5 g) of *Glycine max* L. Merr. were homogenized in 10 ml 0.1 M phosphate buffer (pH 7), filtered through moist muslin cloth and centrifuged for 10 min at 5000 rpm. 0.5 ml supernatant was taken into a test tube to prepare an assay, followed by 5 ml alkaline copper tartarate solution [ prepared by mixing 50 ml of reagent A (2% $Na_2CO_3$ in 0.1N aqueous NaOH ) with 1 ml of reagent B ( 0.5 % $CuSO_4$, 5 $H_2O$ in 1 % sodium tartarate)] . After 15 minutes 0.5 ml Folin - Phenol reagent was mixed and it was kept for 30 min at room temperature. Absorbance was read at 660 nm against a blank prepared with distilled water. Amount of soluble protein was calculated with the help of a standard curve obtained using different concentrations of bovine serum albumin, by a similar procedure as employed for the plant extract.

**3. Total polyphenols :**

Polyphenols were estimated following the method of Folin and Denis (1915).0.5 g germinating seeds were crushed in 80 % acetone and filtered through Buckner's funnel using Whatman No.1. filter paper. The residue on the filter paper was washed several times with 80 % acetone and added to final volume made to 50 ml. Two ml extract was treated with 10 ml of $Na_2CO_3$ in a Nesseler's tube. The volume was made to 35 ml with distilled water. After adding 2 ml of Folin Denis reagent, the final volume of the reaction mixture was adjusted to 50 ml with distilled water. After half an hour absorbance of blue colour was measured at 660 nm using a blank reaction mixture.

**G. MINERAL ANALYSIS :**

**1. Preparation of Acid Digest :**

For the determination of mineral elements extract was prepared by following the method of Toth et al.(1948). 0.5 g oven dried plant material was treated with 20 ml concentrated $HNO_3$ in a beaker covered with a watch glass and kept till the primary reaction is subsided. It was then subjected to slow heating on a hot plate to dissolve the plant material. After cooling to room temperature 10 ml of perchloric acid (70 %) were added into the

beaker and it was heated again until reduced to 2 -3 ml clear solution. This extract was then cooled to room temperature, diluted to 100 ml with distilled water and kept overnight. Next day it was filtered through Whatman No.1 filter paper and stored at room temperature. This acid digest was used to estimate different inorganic elements.

**Calcium, Magnesium, Iron, Manganese, Zinc, Copper, Cobalt**

These elements were analysed from the acid digest on an atomic absorption spectrophotometer (Perkin-Elmer 3030 model). The readings were recorded in ppm and then converted into g / 100 g of dry weight.

## H. ENZYME STUDIES :

### 1. Protease [ EC 3.4.3.2]

Protease activity was assayed according to the method of Penner and Ashton (1967) as described by Chinoy et al. (1969). 0.5 g fresh germinating seeds were crushed in a prechilled mortar in 10 ml cold phosphate buffer (0.1 M pH 7.0 ). The homogenate was filtered through a four layered muslin cloth and filtrate was centrifuged at 6000 rpm for 15 minutes. Supernatant served as the enzyme source.

Assay of enzyme consist of one ml casein (0.5 % pH 7.0 ), three ml phosphate buffer (0.1 M pH 7.0) and one ml enzyme. The reaction was incubated for one hour at $37^{O}$ C and then terminated by adding 2 ml of 5 % perchloric acid. Untreated protein was precipitated by keeping on an icebath, followed by centrifugation for 20 minutes at 10,000 rpm. One ml supernatant was reacted with 4 ml of 0.5 N NaOH and 1.2 ml Folin- Phenol reagent. The blue colour developed after shaking was measured at 660 nm on a spectrophotometer. For blank assay, distilled water was used instead of enzyme source.

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

Enzyme activity = (142.4 x A) - 0.13

(µg Tyrosine liberated / hr)

where, A = change in optical density.

### 2. Nitrate Reductase [EC 1.6.6.1 ] :

The enzyme Nitrate Reductase was assayed according to the method of Jaworsky

(1971). 0.5 g fresh leaf discs were suspended in a conical flask containing 10 ml incubation mixture [ 1ml $KNO_3$ (0.1 M ), 2 ml n- propanol (5 %), 2ml triton - X (0.5 %) and 5 ml phosphate buffer (0.1 M pH 7.5 )]. The flask was sealed with a cork and kept in dark for an hour. Simultaneously another flask containing 10 ml incubation mixture and 0.5 g leaf discs was kept for a zero minute reaction. A control flask without leaf discs was maintained during the experiment served as blank.

After the completion of reaction (0 min / 60 min ) the contents of flasks were mixed well and used to determine the amount of nitrite. One ml from each flask was taken in a test tube and 2 ml sulfanilamide (1%) and 2 ml fresh N-1-(naphthyl)-Ethylenediamine Di-HCl (0.02 %) were added. Absorbance of pink colour developed was measured at 540 nm on a spectrophotometer.

Standard curve was obtained using various concentrations of $KNO_2$ (0.03 M). Enzyme activity was expressed in terms of µg of $NO_2$ produced / h /g of fresh tissue.

**3. Acid Phosphatase [EC 3.1.3.2] :**

Acid phosphatase activity was assayed according to the method of McLachlam (1980). Five hundred milligrams of plant material was homogenized in a prechilled mortar in 10 ml cold acetate buffer (0.1 M pH 5.0 ). The extract was filtered through a four layered muslin cloth and then centrifuged at 6000 rpm for 10 minutes. The supernatant was stored on an icebath and used for enzyme assay.

The assay mixture consisted of three ml p- nitrophenyl phosphate (0.1 mg / ml of acetate buffer pH 5.0), two ml acetate buffer (0.1 M pH 5.0 ) and one ml enzyme. The reaction was terminated at 0 and 30 minutes by adding 1.5 ml of 1.68 N NaOH. Blank reaction mixture was prepared by adding all the ingradients except the enzyme. The optical density of yellow colour was measured at 420 nm on a spectrophotometer. The enzyme activity was expressed as Δ OD / h / g of fresh tissue.

**4. Catalase [ EC 1.11.1.6 ] :**

For catalase a modified method of Hebert (1955) was adopted. The enzyme was extracted from 1 g fresh leaves in 10 ml cold distilled water. The homogenate was filtered through a four layered muslin cloth and centrifuged at 6000 rpm for 10 minutes. The

supernatant was used as the enzyme source.

The assay mixture was prepared by mixing 1 ml 0.045 M $H_2O_2$ (in phosphate buffer 0.1 M pH 6.8) and 1 ml enzyme. Assay mixture was incubated at room temperature for 1 minute and then reaction was terminated by the addition of 5 ml of 5 N $H_2SO_4$. For 0 minute assay, the reaction was stopped by adding 5 N $H_2SO_4$ before the addition of enzyme.1 ml 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 liberated iodine was determined by titrating the reaction mixture with 0.01 N sodium thiosulphate using starch indicator (1 % in d.w.). The difference between 0 min and 1 min reaction was taken as the measure of the enzyme activity.

Activity of enzyme was expressed as mg $H_2O_2$ broken down /min/g fresh weight.