II MATERIALS AND METHODS

A About Insecticide and Fungicide used in Present Investigation:

1. MONOCROTOPHOS (Nuvacron) 36% SL.

Common name : Monocrotophos-

Chemical name : 3hydroxy-N-methyl-cis-crotonamide dimethyl phosphate

or

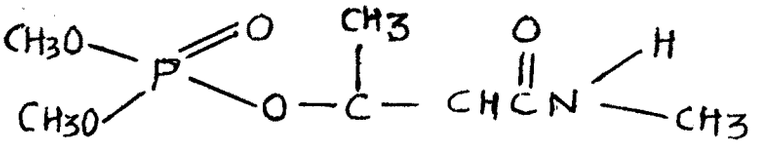
Dimethyl/phosphate of 3-hydroxy N-methyl-cis-crotonamide

or

Cis-(2-methyl carbomoyl-1–methyl/vinyl)-dimethyl phosphate

Empirical formula : $C_7H_{14}O_5NP$

Structural formula :



Solubility : Soluble in water at 25°C readily soluble in organic solvents.

Formultion : Emulsifiable concentrate.

Toxicity : Acute oral LD_{50}-6.9 to 11.3 mg/kg.

Acute dermal LD_{50}: 220 to 247 mg/kg body weight.

It is mild irritant to the mucous membrane. It is not skin irritant. Non-phytotoxic when used as recommended.

Antidote : Atropine sulphate.

Compatibility: Compatible with most of the commonly used pesticides and fungicides those of alkaline in nature.

It is water soluble organophosphorus concentrates containing 360g monocrotophos. [3-hydroxy-N-methyl-cis-crotonamide dimethyl phosphate] as an active ingradient in a Kg of product (m/m). This is equivalent to 400 g monocrotophos in a litre of product (w/v). It is a broad spectrum systemic and contact insecticide cum acaricide with long residual action effective against sucking, chewing and mining insects of paddy, sugarcane, cotton, pulses, vegetables and fruits and against mites on citrus and mango.

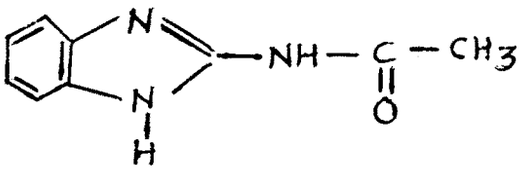
2. BAVISTIN (Carbendazim) 50 WP.

Common name : Bavistin

Chemical name : 2(methoxy-carbamoyl amino) benzimidazole

Empirical formula : $C_9H_9N_3O_2$

Structural formula :



Solubility : Highly soluble in water, actone, alcohol

Stability : Relatively stable in neutral and weak acids, aqueous solutions

Formulation : 50% w/w

Wettable powder containing 500 g/Kg of 2-methoxy carbamoyl)-benzimidazole (Carbendazim)

Toxicity : LD_{50} rats = 6400 mg/Kg.

Nonphytotoxic when used as recommended dose.

Antidote : There is no specific antidote but the universal antidote of 6-10 spoonfuls of medical charcoal per litre of water may be administrated for temporary relief before treatment.

Compatibility: Compatible with various fungicides, insecticides and acaricides should not be added to the spray mixture until before applications.

It is broad spectrum fungicide. It prevents as well as cures a large number of diseases like Scab, Blast, Blight, Loose smut, Anthracnose leaf spot of pulses, groundnut, cereals, grapes and vegetables. Two to three sprays are required at 2-3 weeks intervals starting approximately 2 weeks after emergence.

B Procurement of Seeds :

Seeds of Soybean [Glycine max (Linn.) Merrl.] Variety MACS-13 were obtained from M.A.C.S. Research Institute Pune-411004 (India). Monocrotophos (Nuvacron) 36% SL. (Emulsifiable concentrate CIBA GEIGY Ltd.) and Bavistin (Carbendazim) 50 WP. (B.A.S.F. India) were purchased from Shetkari Sahakari Sangh Pvt. Ltd., Kolhapur.

C Insectcidal and Fungicidal Treatment :

Monocrotophos 36% SL. the organophosphorus insecticide and mono-crotophos in combination with bavistin 50 WP. as broad spectrum systemic fungicide were selected as pesticides. The concentrations of monocrotophos for treatment were 0.075% (M_1), 0.15% (M_2) and 0.3% (M_3) (all v/v). While bavistin concentrations as 0.034% (B_1), 0.068% (B_2) and

0.136% (B_3) (w/v) were made in distilled water and then mixed with respective concentrations of monocrotophos. These concentrations were below, at and above recommended levels for monocrotophos and bavistin respectively.

D Germination :

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.

Sterilized seeds were placed in previously sterilized petri dishes lined with Whatman No.1 filter paper. The paper was moistened with 10 ml distilled water (control) and with respective concentrations of monocrotophos and monocrotophos in combination with bavistin. 10 seeds were used for germination in each petridish and germination was carried out in dark in an incubator at 27 ± 3°C. Germination percentage was recorded after every 24 h upto 96 h of germination. The known quantity of insecticide solution was supplied uniformly during germination to all seeds as and when required.

E Seedling Growth :

Root length and shoot length of each seedling was measured after 120 h of germination. Root to shoot ratio was determined by the routine method while percent phytotoxicity was obtained by the formula suggested by Chou and Muller (1972) was used.

$$\text{Percent Phytotoxicity} = \frac{\text{Radicle length of control - Radicle length of test}}{\text{Radicle length of control}} \times 100$$

For further studies surface sterilized seeds were sown in an earthen pots filled with three part of soil and one part Farm Yard Manure (FYM) and seedlings were allowed to establish for one month. After one month, first spray of different concentrations of monocrotophos (on one set) and monocrotophos in combination with bavistin (on another set) was given to run off point on foliage by using air pneumatic manual pump at 10.30 to 11.00 a.m. Simultaneously control plants were sprayed with equal volume of distilled water. The second spray was given after 15 days of first spray. From each treatment and control randomly sampled material was used for experimental purpose after 5 days of second spray.

F Stomatal Behaviour :

Leaf diffusive resistance and transpiration rate were determined by using Steady State Porometer, L 1600 (LICOR, U.S.A.). The readings were taken continuously for seven days after 1st spray and diffusive resistance for CO_2 was calculated using formulae suggested by Jarvis (1971).

1/R Leaf = 1/R Upper + 1/R Lower.

RL CO_2 = 1.6 RL H_2O

G Leaf area :

Leaf area was determined by tracing the outline on graph paper and the area covered by the leaf was measured in terms of cm^2.

H Organic constituents :

Organic constituents such as Chlorophylls, Polyphenols, Nitrogen, Proteins and Amino acids were analysed after 50 days of plant growth both from monocrotophos and morocrotophos in combination with bavistin sprayed plants.

1. Chlorophylls

Chlorophylls were estimated following the method of Arnon (1949).

0.5 g fresh material was crushed in mortor with pestle and extracted in 80% chilled acetone containing 4 ml liquor NH_3 per litre in dark. A pinch of $MgCO_3$ was added during crushing. The 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. The flask was covered with black paper to retain the activity of chlorophylls. The absorbance was measured at 663 and 645 nm on double beam spectrophotometer (Schimadzu) for chlorophylls a and b respectively. Chlorophylls (mg/100 fresh weight) were calculated using the following formulae :

Chlorophyll a = (12.7 x A 663) - (2.69 x A 645) ... X

Chlorophyll b = (22.9 x A 645) - (4.68 x A 663) ... Y

Chlorophyll a+b = (18.02 x A 663) + (20.2 x A 645) .... Z

$$\text{Total chlorophyll : } a/b/a+b = \frac{X/Y/Z \times \text{Volume of extract} \times 100}{1000 \times \text{Weight on plant material (g)}}$$

(mg 100^{-1}g fresh wt.)

2. Carotenoids :

Total carotenoids were estimated by using the method and formula suggested by Jensen (1978)

$$\text{Total carotenoids mg } 100^{-1}\text{g fresh wt.} = \frac{D \times V \times F \times 10}{2500}$$

where D = Absorbance at 450 nm
V = Volume of extract
F = Dilution factor and
2500 = Average ext nction.

$$\text{Dilution factor} = \frac{\text{Total vol. of extract}}{\text{Moisture content in plant material used.}}$$

3. Polyphenols :

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

0.5 g fresh plant material was crushed in mortor with pestle and was extracted in 80% actone. 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 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^{-1}) to which 10ml 20% Na_2CO_3 was added. The volume was adjusted to 35 ml with distilled water and 2 ml of Folin Dennis reagent 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^{-1} g fresh tissue.

i) Preparation of Folin - Dennis Reagent :

100 g sodium tungstate and 20 g phosphomolybdic acid were dissolved in 800 ml distilled water. To that 50 ml 80% phosphoric acid was added. The entire mixture was refluxed for 2 h on water bath using water condenser. After cooling to room temperature the final volume of the mixture was made to one litre and stored in an amber coloured bottle at low temperature.

ii) Standard tannic acid was prepared by dissolving 250 mg tannic acid in 250 ml distilled water so as tc get the final concentration in the range of 0.1 mg ml^{-1}.

4 Nitrogen :

Nitrogen was estimated by the method of Hawk et al (1948). 0.5 g dried plant material was taken in Kjeldehl's flask containing 10 ml 1:1 H_2SO_4 and water. A pinch microsalt and few glass beads were added to the flask. This was digested on low flame till colourless solution was obtained. Then it was cooled and transferred quantitatively to volumetric flask and volume was made 100 ml with distilled water and filtered through Whatman No.1 filter paper. From the filtrate 2 ml of extract was taken in Nessler's tube to which a drop of 8% $KHSO_4$ was added and volume was made to 35 ml with distilled water. Then 15 ml Ness er's reagent (freshly prepared) was added to it.

After 10-15 min. the absorbance was recorded at 520 nm on double beam spectrophotometer (Schimadzu). The blank contained all the ingradients except nitrogen source.

Standard curve was obtained by using different concentrations of ammonium sulphate (0.1, 0.2, 0.3, 0.4 ml) by using same procedure of Nitrogen estimation. The values are expressed as g 100^{-1} g dry wt.

i) Preparation of microsalt : Microsalt was prepared by grinding anhydrous copper sulphate and potassium sulphate in proportion of 1:40 i.e. 0.1 g $CuSO_4$ + 4.0 g K_2SO_4.

ii) Preparation of Nessler's reagent :

a) 7 gm of KI + 1 gm of HgI_2 dissolved in 40 ml of distilled water.

b) 10 g of NaOH dissolved in 50 ml distilled water. Both a and b were mixed immediately before use.

iii) Preparation of Standard Ammonium Sulphate Solution :

$(NH_4)_2SO_4$ was kept in an oven for 10 h and 0.266 g of it was dissolved in water. A few drops of conc. H_2SO_4 were added to it and the volume was made to 1 litre. this contains 0.05 mg of nitrogen/ml.

5. Protein :

Protein content was obtained by multiplying the total nitrogen content by the factor 5.71 recommended for soybean (Sadasivan and Manikam 1992).

6. Amino acids :

Amino acid composition of control and sprayed plants of soybean (Glycine max (Linn.) Merrl) was studied by the technique of ascending unidirectional paper chromatography (Block et al.. 1955).

i. Preparation of Extract :

The leaves of control plant and sprayed plants (5 gm) were crushed repeatedly in 80% ethanol and filtered through Buchner's funnel using Whatman No.1 filter paper. The filtrate was condensed under reduced pressure upto 2 ml and transferred into centrifuge tube. The extract was then centrifuged at 5000 x g for 5 min. and the supernatant was collected in small glass vial and stored at 4°C until use.

ii. Ascending Paper Chromatography :

The amino acids were separated by loading the aliquot of extract on chromatographic paper by using the solvent system n-Butanol : Acetic acid : water (80:20:100 v/v). 0.5% Ninhydrin prepared in 95% Acetone was used as detecting reagent for amino acids. Identification of each amino acid was made by using chromatography of authentic standards and comparing their colours and Rf values. The colour intensity of spots were compared according to their concentrations they were graded as, not detectable (-), detectable (+), less (++), moderate (+++), optimum (++++) and maximum (+++++).

7. Phosphorus :

The phosphorus content was estimated from the acid digested extracts by using molybdate vanadate reagent (Sekine et al. 1965). 2 ml of acid digested extract was taken in a test tube in which 2 N HNO_3 and 1 ml molybdo- vanadate reagent was added. The volume was adjusted to 10 ml with distilled water. The test tubes were kept aside for 20 min to complete the reaction and to develop the colour. The absorbance was measured at

420 nm using blank. Blank contains all ingradients except plant extract and phosphorus source. The amount of phosphorus (g 100^{-1} g dry tissue) was calculated by obtaining standard curve using standard phosphorus solution (0.25 mg/ml).

8. Enzymes of Nitrogen Metabolism :

i. In vivo assay of nitrite reductase (E.C. 1.6.6.4):

The method used for this assay was that of Guerrero (1982). 0.5 g fresh plant material (cut pieces of plant parts of 5 mm^2) was placed in a testube containing 4.5 ml 100 mM phosphate buffer (PH 7.7) and 0.1 N $NaNO_2$. The tubes were evacuated with an oil pump for 2 min. They were then wrapped in aluminium foil and incubated at 30°C with gentle shaking on the rotatory shaker. After 30 min the tubes were transferred to a boiling water bath for 2 min to stop the reaction and then cooled. To determine initial nitrite concentration one set of test tubes was maintained as blank with no plant material. The final nitrite concentration was determined in 1 ml aliquots.

ii. In vivo assay of Nitrate reductase (E.C.1.6.6.1) :

The method employed in assaying in vivo nitrate reductase activity was of Guerrero (1982). 0.5 g cut pieces of fresh plant material (about 5 mm^2) were added to 5 ml incubation mixture containing 0.1 M potassium phosphate buffer (PH 7.7). 0.1 M KNO_3 and 1% V/V isopropanol. After flushing the mixture for about 5 min with argon the entire set was incubated in dark at 30°C. Aliquots (0.2 ml) were then removed for nitrite determination at zero time and after 60 min. Estimation of Nitrites : 1 ml of

1% sulphanyl amide in 1N Hcl and 1 ml of 0.02% aqueous solution of N-(1 Napthyl) ethylene diamide dihydrochloride were added to 1 ml aliquots. The colour was allowed to develop for 20 min. and total volume was made upto 10 ml with distilled water. The absorbance was read at 540 nm and the nitrite concentration was calculated from standard curve.

9) Inorganic Constituents :

Estimation of inorganic constituents was carried out from control and sprayed plants. The plant material was cut into small pieces and was weighed accurately and kept in oven for drying at 60°C. After 4-5 days when dried material showed constant weight known amount (1 g) was taken for analysis. From this plant material inorganic constituents such as Sodium (Na^+), Potassium (K^+), Calcium (Ca^{++}), Iron (Fe^{++}), Magnesium (Mg^{++}), Manganese (Mn^{++}), Zinc (Zn^{++}) and Cobalt (Co^{++}) were estimated.

i) Preparation of extract :

For the estimation of inorganic constituents methods suggested by Toth et al.. (1948) was followed . Known quantity (1 g) of oven dried plant material was taken in 150 ml beaker to which 20 ml of concentration HNO_3 was added. The beaker covered with a watch glass 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 perchloric acid (about 60%) was added. The beaker was again heated till the mixture becomes clear and volume of the mixture was reduced to about 2-3 ml. Mixture was not allowed to char. It was then cooled to room temperature and diluted to 100 ml with distilled water and kept over night.

Next day, acid digest extract was filtered throughout Whatman No.1 filter paper and this filtrate was used to analyse inorganic constituents.

Inorganic constituents from the filterate were analysed using atomic absorption spectrophotometer (PERKEN ELMER - 3030) by using standards of known concentrations in part per million.