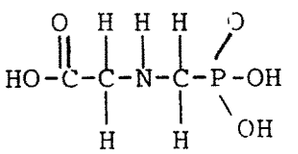
III Materials And Methods

A. GLYPHOSATE THE HERBICIDE

Common name : Glyphosate

Chemical name : N-(Phosphonomethyl) glycine

Chemical structure :



Trade name : Roundup

Brand name : Glycel 41% S.L.

Properties : It is white solid, soluble in water about 1.2% at 25°C, formulated as the isopropylamine salt for an aqueous spray.

Dosage : 2-3 litres Glycel 41% S.L. per hectare in 400 litres of water.

Applications :

Glyphosate is non-selective, broad-spectrum, post emergence, translocated herbicide, which is highly effective on annual, biennial and perennial grasses, sedges and broad leaf weeds. It is used to control trees and woody bush species in crop land, non-crop sites, industrial, recreational or public areas and turf grass establishments. Being non-selective in nature, it is applied with care in crops. Glyphosate is applied to the foliage and has little effect when applied to the soil Directed sprays and spot treatments are used to control serious weeds in crops. Soil active herbicides can be combined with glyphosate to control both emerged weeds and

later germinating weed seeds in the same treatment.

Toxicity : :

Glyphosate is relatively nontoxic to mammals. Acute oral LD50 is 5900 mg/kg for rats. It is primarily irritant to skin, eyes and respiratory track.

First aid : :

Wash contaminated skin with soap and water, flush contaminated eyes with fresh water for 15 minutes. If ingested in small amount (less than 10 mg/kg body wt.) the treatment of intubating the stomach and aspirating the content should be given within one hour.

Antidote : :

Gastric lavage, supportive and symptomatic treatment.

Mode of action:

Foliar chlorosis followed by necrosis is the common symptom of glyphosate injury. Malformed leaves, white spots and striations (Putnam 1976, Fernandez and Bayer, 1977, Marriage and Khan 1978) multiple shoots (Fernandez 1977) Yellowing and wrinkling of leaves (Votila et al. 1980) have been observed in regrowth, of perennial plants. Ultrastructural modifications such as disruption of chloroplast, swelling of endoplasmic reticulum (Campbell et al. 1975), deterioration of oil bodies, vacuolation of cytoplasm (Pihakaski and Pihakaski 1980) have been reported.

Glyphosate is readily absorbed by leaves and translocated via the symplastic system and afterwards through the apoplastic system. It is degraded slowly in higher plants (Gottrup et al. 1976). Glyphosate interferes with aromatic amino acid synthesis (Jaworski 1972). However, action of glyphosate is much more complex. Glyphosate interferes the metabolism of phenolic compounds (Hoagland et al. 1979, Duke et al. 1979, Hoagland and Duke 1982) and IAA metabolism (Lee and Dumas 1985). Specific enzymes of aromatic amino acid synthesis have been found inhibited by glyphosate (Steinrucken and Amrhein in 1980, Rubin et al. 1982). The reduction of aromatic amino acids especially phenylalanine and tyrosine was found due to the increased PAL activity (Duke and Hoagland 1979; Duke et al. 1979, Hoagland et al. 1978, 1979). At low rates of application the action of glyphosate is low and may not be visible up to 7 - 10 days.

B. PROCUREMENT OF SEEDS AND HERBICIDE

The seeds of wheat (Triticum aestivum L.) and soybean (Glycine max L.) were purchased from Shetkari Sahakari Sangh Pvt. Ltd., Kolhapur. The seeds of Celosia argentia L. and rhizomes of Cyperus rotundus L. were collected from the campus of Department of Botany, Shivaji University, Kolhapur.

Glycel 41% SL, Excel Industries Ltd., Bombay was purchased from Shetkari Sahakari Sangh Pvt. Ltd., Kolhapur.

C. HERBICIDAL TREATMENT

The seeds of wheat, soybean and Celosia were washed repeatedly with distilled water and sown in earthen pots containing three parts of soil and one part of farmyard manure. Similarly rhizomes of C. rotundus were washed with distilled water and raised in the pots. After stabilizing the seedlings upto 21 days the foliar application of 300 ppm glyphosate (50 ml) was given with the help of a hand sprayer. Simultaneously some plants were sprayed with an equal amount of distilled water and maintained as control. After second and tenth day of treatment the leaves of control and sprayed plants were used for analysis.

D. METHODS

1. STOMATAL BEHAVIOUR

Leaf diffusive resistance and transpiration rate were determined by using steady state porometer (LI-1600, LICOR U.S.A.) The readings were taken on II and X day of glyphosate treatment. Diffusive resistance for CO_2 was calculated using the formula suggested by Jarvis (1971).

$$\frac{1}{R} \text{ leaf} = \frac{1}{R} \text{ upper} + \frac{1}{R} \text{ lower}$$

$$R_L CO_2 = 1.6\ R_L H_2O$$

Where, R = Diffusive resistance.

Method suggested by Stoddard (1965) was followed to determine Stomatal Index. Epidermal peels were obtained

by applying skin coloured nail polish to the middle portion of upper as well as lower surfaces of leaves. After drying, the nail polish films were removed and observed under the precalibrated light microscope. The stomatal investigation were made at 12 noon in all the plants. The stomatal index was calculated using formula.

$$\text{Stomatal index (SI)} = \frac{S}{S + E} \times 100$$

Where, S = No. of stomata per mm^2

E = No. of Epidermal cells per mm^2

2. RELATIVE WATER CONTENT

One gram of leaf discs were taken from sprayed and control fresh leaves and floated in petriplates containing 20 ml of distilled water. After three hours the turgid leaf discs were taken out, blotted and weighed. Another set of leaf discs was kept in an oven at 60°C and allowed to dry completely. The dry weight of leaf discs was noted. The percent relative water content was calculated by using the formula :

$$\text{Relative Water Content [R.W.C. (\%)]} = \frac{\text{fresh weight - dry weight}}{\text{saturated weight-dry weight}} \times 100$$

3. OSMOTIC POTENTIAL OF CELL SAP

It was determined following the method of Janardhan et al. (1975).

One gram of fresh leaf material was thoroughly washed, blotted and crushed in a little amount of

distilled water. The extract was filtered through a four layered muslin cloth and volume was adjusted to 20 ml with distilled water. The electrical conductivity of this leaf extract was measured on the conductivity meter (ELICO Model PE-133). Simultaneously one gram of fresh leaf material was kept in an oven at 60^0 C for drying. From the dry weight of material moisture content was calculated Which was used to determine the dilution factor using the formula :

$$DF = \frac{\text{volume of extract X weight of plant material}}{\text{moisture content}}$$

From DF and EC readings osmotic potential was calculated

$$\text{Osmotic potential (O.P. in bars)} = \frac{0.36 \text{ X EC X DF}}{0.987}$$

Where, EC = Electrical conductivity

DF = Dilution Factor

0.35 = Constant

0.987 = Factor used for converting atmospheric pressure to bars

4. ORGANIC CONSTITUENTS

Fresh and mature leaves were harvested on II and X day of treatment and used for determination of various organic constituents.

i. Total Chlorophylls

Chlorophylls were estimated following the method suggested by Arnon (1949). 0.5 g fresh plant material was crushed in a mortar using 80% acetone. A pinch of $MgCO_3$

was added to enhance the extraction. 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 covered with a black paper to prevent photo-oxidation of chlorophylls. The absorbance was measured at 663 nm and 645 nm on Spectronic 20.

Chlorophylls (mg/100 g of fresh weight) were calculated using formulae :

$$\text{Chlorophyll a, X} = 12.7 \times A_{663} - 2.69 \times A_{645}$$

$$\text{Chlorophyll b, Y} = 22.9 \times A_{645} - 4.68 \times A_{663}$$

$$\text{Chlorophyll (a+b), Z} = 8.02 \times A_{663} + 20.2 \times A_{645}$$

$$\underset{(mg)}{\text{Chla/Chlb/Chl(a+b)}} = \frac{\text{X or Y or Z X volume of extract X 100}}{\text{1000X weight of plant material (g)}}$$

ii. Carotenoids

The method described by Kirk and Allen (1965) was used to determine carotenoid content using the same acetone extract. Absorbance was read at 480 nm and carotenoids were calculated using the formula :

$$\underset{(g/100\ g)}{\text{Carotenoids}} = \frac{A_{480} \times \text{volume of extract X 10 X 100}}{\text{2500 X wt of plant material (g)}}$$

Where, 2500 = Average extinction

iii. Total Polyphenols

Polyphenols were estimated following the method suggested by Folin and Dennis (1915). 0.5 g fresh plant material was crushed using 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 final volume of extract was adjusted to 50 ml with 80% acetone.

2 ml extract was treated with 10 ml of 20% Na_2CO_3 in Nessler's tubes. The volume was adjusted to 35 ml with distilled water followed by 2 ml of Folin-Dennis reagent. The final volume of reaction was adjusted to 50 ml with distilled water. After 20-30 min. absorbance was read at 660 nm using a reaction blank. Polyphenols were calculated from a standard curve obtained by using various concentrations of standard tannic acid (0.1 mg/ml).

iv. Carbohydrates

Reducing sugars, total sugars and starch were estimated following the method suggested by Nelson (1944). Extract was prepared from .5 g fresh leaves in 80% alcohol. It was filtered through Buchner's funnel using Whatman No.1 filter paper. This filtrate was used to estimate soluble sugars and residue left over the filter paper was used to estimate the starch.

The filtrate was then condensed on a water bath to about 2-3 ml. To this were added about 2 g of mixture of lead acetate and potassium oxalate (1:1) with constant stirring . 15 ml distilled water were added and it was filtered through Buchner's funnel using Whatman No.1 filter paper. The volume of extract was adjusted to 50 ml

with distilled water. This filtrate served as 'A' and was used for estimation of reducing sugars.

In a 150 ml conical flask 20 ml of filtrate from 'A was taken and to it was added 2 ml of concentrated HCl. The flask was closed using a cotton plug and was then hydrolysed for 30 min under 15 lbs pressure in an autoclave. After cooling to room temperature the contents were neutralised with unhydrous Na_2CO_3 and filtered. The volume of the filtrate was noted down. This filtrate 'B' was used as the extract for estimation of total sugars.

The residue on filter paper during alcoholic extraction was transferred to a 150 ml conical flask alongwith the filter paper. To this, 50 ml of distilled water and 5 ml of concentrated HCl were added and it was hydrolysed at 15 lbs pressure for half an hour. After cooling to room temperature it was neutralised with unhydrous Na_2CO_3 and filtered. The volume of filtrate was measured and noted down. This filtrate 'C' was used as the extract for the estimation of starch.

The sugars were estimated colorimetrically by employing arsenomolybdate reagent to determine cuprous oxide formed in the oxidation of sugars by alkaline copper tartarate reagent. Two ml each of 'A' and 'B' and 1 ml of C were heated seperately with one ml alkaline copper tartarate on a boiling water both for 10 min. After cooling to room temperature, 1 ml of arsenomolybdate

reagent was added to each tube and the volume of each reaction mixture was adjusted to 10 ml with distilled water. Simultaneously a set of standard tubes containing various concentrations of glucose (.1 mg/ml) was prepared in the same manner alongwith a blank without any standard glucose. After 10 min. absorbance was read at 560 nm on a spectrophotometer.

Using calibration curve of standard glucose, the amount of reducing sugars in each fraction was calculated. The values were expressed in terms of g per 100 g of fresh weight.

v) **Soluble proteins**

Fresh plant material (0.5 g) of treated and control plants was homogenised in mortar using .14 M NaCl and filtered through a double layered muslin cloth. The filtrate was centrifuged at full speed for 15 minutes and the supernatant was used for estimation of proteins following method given by Lowry et al. (1951).

One ml of extract was taken in a test tube. To this 5 ml of reagent 'A' was added, then it was mixed well and allowed to stand for 30 min. at room temperature. To this were added 5 ml of Folin-Phenol reagent with immediate mixing. It was allowed to stand for 30 minutes and colour intensity was measured at 660 nm on a spectrophotometer. A blank was prepared using distilled water in place of protein solution. A standard curve of proteins

was obtained with different concentrations of crystalline BSA (0.1 mg/ml) and used to calculate the amount of proteins.

vi) **Total nitrogen**

Nitrogen was estimated by the method of Hawk et al. (1948). .5 g of oven dried plant material was transferred to 300 ml Kjeldahl's flask containing 10 ml of 1:1 H_2SO_4; a pinch of microsalt and few glass bids (to avoid bumping). The flask was heated on a low flame till a colourless solution was obtained. Then it was cooled, diluted with distilled water and transferred quantitatively to a 100 ml volumetric flask. The volume was made with distilled water. It was then filtered through Whatman No.1 filter paper. The filtrate was used for nitrogen estimation.

Two ml of filtrate was taken in a 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 of Nessler's reagent (freshly prepared) were added to it. After 10-15 minutes the absorbance was recorded at 520 nm on spectronic 20. The blank contained all the ingradients except nitrogen source. Standard curve was obtained by using different concentrations of ammonium sulphate (0.05 mg/ml) in place of the filtrate.

vii. **Free proline**

Proline content was estimated by the method of Bates et al. (1973). .5 g oven dried plant material was homogenised in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was filtered through Whatman No. 1 filter paper. Two ml of filtrate was reacted with 2 ml of fresh acid ninhydrine and 2 ml of glacial acetic acid in a test tube and heated for one hour in a boiling water bath. The tubes were transferred to ice bath immediately. After 15 minutes the reaction mixture was extracted with 4 ml of toluene by shaking vigorously with a test tube stirrer for 20 seconds. Red coloured toluene phase was separated from the aqueous phase with the help of a small separating funnel and absorbance was measured at 520 nm on spectronic 20 using toluene as the blank. Amount of proline was calculated from the standard curve of proline using different concentrations of standard proline (0.05 mg/ml).

viii. **Detection of amino acids**

Preparation of extract

Fresh leaves (0.5 g) from control and sprayed plants were crushed in a mortar in 80% alcohol and the extract was condensed in an evaporating dish on water bath to reduce to 2-3 ml. By adding little distilled water it was centrifuged at full speed for 10 minutes. The supernatant was used for detection of amino acids using ascending paper chromatography.

Strips of Whatman No.1 paper were utilised for loading the samples with the help of micropipette. After loading, the strips were developed using a suitable solvent system (n-butanol : acetic acid : distilled water in 4:1:5 proportion). After sufficient development papers were removed, air dried and then sprayed with ninhydrine (0.5%). The separated compounds on chromatograms were marked and identified by comparing their R_f values with the standard amino acids.

5. INORGANIC CONSTITUENTS

Preparation of extract

Acid digest was prepared using the method of Toth *et al*. (1948). 0.5 g oven dried plant material was taken in 150 ml beaker to which 20 ml conc HNO_3 was added. The beaker was covered with a watch glass and kept aside till initial reaction was subsided. The beaker was heated slowly on a hot plate to dissolve the plant material. After cooling to room temperature, 10 ml of perchloric acid were added in the beaker and it was once again heated till mixture becomes clear and reduces to 2-3 ml. It was then cooled to room temperature, diluted to 100 ml with distilled water and kept over night. Next day the acid digest was filtered through Whatman No. 1 filter paper and this filtrate was used to analyse inorganic constituents such as phosphorus, potassium, calcium, magnesium and iron.

i) **Phosphorus**

It was estimated following the method suggested by Sekine (1965). Two ml of acid digest was reacted in a test tube with two ml of 2N HNO_3 followed by one ml molybdate vanadate reagent. The mixture was shaken thoroughly and the final volume was made to 10 ml with distilled water. It was allowed to stand for 20 minutes and absorbance readings were taken at 495 nm on spectronic 20. Phosphorus content was calculated by using a standard curve of phosphorus.

ii) **Potassium**

Potassium was estimated flame photometrically [Model ELICO-(L22A)]. Standards of known concentrations in parts per million of K^+ as KCl (10 to 40 ppm) were used for calibration curves of K^+. From the calibration curves concentration of K^+ in the acid digested samples was calculated.

iii) **Calcium, Magnesium and Iron**

Calcium (Ca^{++}), Magnesium (Mg^{++}) and Iron (Fe^{++}) were analysed using atomic absorption spectrophotometer (PERKIN ELMER 3030 ATOMIC ABSORPTION SPECTROPHOTOMETER). The readings were available in ppm concentrations which were further converted to g per 100 g of dry weight.

6.Enzymes

i) ∝- amylase (E.C. 3.2.1.1)

A modified method of Katsumi and Fukuharu (1969) was used to study the ∝-amylase activity. 0.5 g of fresh leaves from treated and control plants were washed with distilled water and homogenised in pre-chilled mortor in ten ml. cold acetate buffer (0.1 M, pH 5.00). The extract was filtered through four layered muslin cloth and centrifuged at 6000 rpm for 10 minutes. The supernatant was stored on an ice bath and used for enzyme assay. All the operations were carried out at 0-4°C.

The activity of ∝-amylase was assayed by incubating one ml enzyme, one ml amylose (0.2%) and acetate buffer (0.1 M, pH 5.00). The reaction was killed after '0' and '30' minutes with 2 ml acetic acid (0.5 N). The aliquote(one ml) from killed reaction mixture was then treated with ten ml dilute I_2KI solution (0.25% I_2 prepared in 0.1% aqueous KI solution). The absorbance was measured on spectronic 20 at 700 nm using I_2KI solution as a blank. Enzyme activity was calculated using the following formula :

$$DB = 2 \times \frac{d - D}{d} \times \frac{100}{10}$$

Where, d = O.D at '0' minute

D = O.D at '30' minutes

D.B is the unit enzyme activity expressed as mg of hydrolysed amylose under the conditions in which the

O.D. of amylose Iodine complex at 700 nm was decreased 10% with one ml of enzyme in 30 minutes reaction at 40^0 C.

ii) **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). 0.5 g fresh leaves of treated and plants were crushed in a pre-chilled mortar in ten ml. cold phosphate buffer (0.1 M, pH 7.0). The homogenate was filtered through four layered muslin cloth and filtrate was centrifuged at 6000 rpm for 15 minutes. Supernatant served as the enzyme source.

Assay of protease consisted of one ml casein (0.5% pH 7.0), three ml. phosphate buffer (0.2 M pH 7.0) and one ml enzyme. The reaction was incubated for one hour at 37°C. Then it was terminated by adding two ml. of 5% perchloric acid. Untreated protein was precipitated on an ice bath followed by centrifugation for 20 minutes at full speed. 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 the reaction was measured at 660 nm on a spectrophotometer. For blank, distilled water was used instead of enzyme source and the same procedure was followed.

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

Enzyme activity = μg of tyrosine liberated h^{-1}

= (142.4 X A) - 0.13

Where, A = change in optical density

iii) **Acid Phosphatase** (E.C. 3.1.3.2)

The method adopted by McLachlan (1980) was used to study enzyme acid phosphatase. The extraction procedure for acid phosphatase was exactly same as that of α-amylase described earlier.

The assay mixture contained three ml. of p-nitrophenyl phosphate (0.1 mg per ml of acetate buffer pH 5.0) two ml. acetate buffer (1.0 M pH 5.0) and one ml enzyme. The reaction was terminated at 0 minute or 30 minutes (as per the requirement) by the addition of 1.5 ml. of 1.68 N NaOH. Blank contained the same ingradients as the assay mixture except for the enzyme source. The optical density of yellow colour was measured at 420 nm on spectronic 20. The enzyme activity was expressed as OD. per hour per gram of fresh weight.

iv) **Polyphenol Oxidase** (E.C. 1.10.3.2)

The enzyme polyphenol oxidase was assayed according to method given by Mahadevan and Sridhar (1982). 0.5 g fresh leaves of treated and control plants were homogenised in pre-chilled mortar in ten ml. of cold phosphate buffer (0.1 M pH 6.0). The homogenate was filtered through four layered muslin cloth and centrifusgd

at full speed for 15 minutes. Supernatant served as the enzyme source.

Two ml. of enzyme and five ml. phosphate buffer (0.1 M pH 6.0) were mixed well in a cuvette and it was inserted in the sample holder of spectrophotometer set at 495 nm. The absorbance was adjusted to zero. One ml of 0.01 M catechol was added into the cuvette to start the reaction. The reaction mixture was stirred quickly with a rod and change in absorbance was recorded from 0 to 180 seconds. Enzyme activity was expressed as OD. per hour per gram of fresh weight.

v) **IAA Oxidase**

The enzyme IAA oxidase was assayed according to the method of Tang and Bonner (1947). Enzyme was extracted in ten ml. cold phosphate buffer (0.1 M pH 6.2) using 0.5 g fresh leaves of treated and control plants. The homogenate was filtered through four layered muslin cloth and filtrate was centrifuged at full speed for 10 minutes. Supernatant was stored stored on an ice bath served as enzyme source.

Assay of IAA oxidase consisted of 4 ml. IAA. (0.05 mg per ml) two ml. enzyme, 0.5 ml each of 2.4-dichlorophenol (0.0001 M) and $MnCl_2$ (0.002 M) and three ml. phosphate buffer (0.1 M pH 6.2) mixed thoroughly in a test tube. Separate reaction mixture were prepared for 0 and 30 min. Then two ml. reaction mixture was taken and eight ml. of

Tang and Bonner's reagent after 0 and 30 minutes. The pink colour developed after shaking the reaction was measured at 520 nm on spectrophotometer. For blank, distilled water was used instead of enzyme source. Calibration curve for IAA was obtained using various concentrations of std IAA (0.1 mg per ml) and the amount of IAA broken down by enzyme per hour per gram of fresh weight was calculated.

vi) **Nitrate Reductase** (E.C. 1.6.6.1)

The method employed in assaying nitrate reductase activity was of Jaworski (1971). 0.5 g leaf discs of control and treated plants were suspended in conical flasks containing ten ml. incubation mixture i.e. one ml KNO_3 (1 M) two ml. n-propanol (5%), two ml. triton-x-100 (.5%), 5 ml phosphate buffer (.2 M pH 7.5). The conical flasks were sealed with cork and kept in dark for an hour. Simultaneously two flasks containing ten ml. incubation mixture and .5 g leaf discs were kept for a zero minute reaction. A control flask without leaf discs was also maintained during the experiment serves as the blank.

After the completion of reaction (0 min/60 min.) the contents of flasks were mixed well. One ml from each flask was taken in a test tube and two ml. sulfanyl amide (1%) and two ml. (N-1-Naphthylenthylene diamine di-hydrochloride) were added. Absorbance of the colour developed was measured at 540 nm on spectronic 20. 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 per hour per gram of fresh tissue.

vii) **Nitrite Reductase** (E.C. 1.6.6.4)

For this enzyme same procedure as above was followed except that in place of KNO_3, KNO_2 (0.03 M) was used. Activity of the enzyme was expressed as µg of NO_2 utilised per hour per gram of fresh tissue.

7. RESIDUAL ANALYSIS OF GLYPHOSATE

Qualitative Analysis

Residual content in the leaf samples after foliar spray of glyphosate was analysed qualitatively using the technique of thin layer chromatography.

i) Extraction :

The plant material was homogenised on II and X day after the treatment of glyphosate. The extraction was carried out by the method suggested by Sherma (1980) using oven dried plant material. 5 g leaf material of control and sprayed plants was extracted seperately in 25 ml acetonitrile in a mortar. Then the extract was filtered through sintered glass funnel having a plug of cotton, containing unhydrous sodium sulphate placed over it. The filtrate and washings were collected in an evaporating dish and then evaporated to dryness at room temperature.

ii) **Partitioning** :

The residue left in the evaporating dish was transferred to a 25 ml. glass stoppered separating funnel by using ten ml. of pre-equilibrated mixture of hexane and acetonitrile. The mixture was shaken for one minute. The hexane layer was discarded and acetonitrile layer was reserved for analysis by TLC.

iii) **Preparation of TLC Plates** :

20 g silica gel-G (BDH, 200 mesh with 13% $CaSO_4$ as a binder) was mixed with 100 ml distilled water. The slurry formed was quickly transferred to an applicator whose 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°C for an hour. They were cooled and used for loading the sample.

iv) **Application of Sample** :

Samples (100 λ each) were loaded with the help of a micropipette. Alongwith the sample, standard glyphosate (300 ppm) was also loaded on the plates. The spots were air dried and then plates were developed in a suitable solvent.

v) **Development of Chromatograms** :

The chromatographic chambers were prepared 20 min. before inserting the plates. The chamber was first lined with Whatman No. 1 on inner three sides which were

made moist with the developing solvent. The solvent system used was a mixture of hexane and acetone mixed in proportion of 3:1 (v/v). The plates were run to reach the solvent upto 15 cm from the original line of samples. The plates were then removed and dried at room temperature and sprayed by $AgNO_3$ reagent prepared in ammonia.

The brown coloured spots appearing on the plates were identified by comparing their R_f values with the standard herbicide.

E. PREPARATION OF REAGENTS

i. FOLIN-DENNIS REAGENT : (Estimation of total polyphenols)

100 g sodium tungstate and 20 g phosphomolybdic acid were dissolved in 800 ml. distilled water. 50 ml. 80% phosphoric acid were added and it was refluxed for 2-3 hours using water condensor. The volume was adjusted to 1 litre with distilled water.

ii. SOMOGYI'S ALKALINE COPPER TARTARATE REAGENT : (Estimation of carbohydrates)

4 g $CuSO_4$ $5H_2O$, 24 g unhydrous Na_2CO_3, 16 g Na-K tartarate and 180 g unhydrous Na_2SO_4 were dissolved in 1 litre distilled water.

iii. ARSENOMOLYBDATE REAGENT: (Estimation of carbohydrates)

25 g ammonium molybdate were dissolved in 450 ml distilled water to which 21 ml. concentrated.. H_2SO_4 were

added. 3 g of sodium arsenate ($Na_2HASO_4, 7H_2O$) were dissolved in 25 ml. distilled water. The solutions were mixed well and kept in an incubator at 37°C for 48 hours before use.

iv. REAGENT 'A' : (Estimation of soluble proteins)

50 ml of 2% sodium carbonate in 0.1 N aqueous NaOH and 1 ml of 0.5% copper sulphate in 1% sodium tartarate were mixed together.

v. FOLIN-PHENOL REAGENT : (Estimation of soluble proteins)

100 g of sodium tungstate, 25 g of sodium molybdate, 700 ml. of distilled water 50 ml. of 50% phosphoric acid and 100 ml. of concentratied HCl were mixed in one litre volumetric flask. The solution was refluxed gently for 10 hours. To this 50 g of lithium sulphate. 50 ml of distilled water and a few drops of bromine water were added. This mixture was boiled for 15 minutes without condensor to remove excess of bromine. It was then cooled and adjusted to 1 N acidity by titrating against 1 N NaOH to phenolphthalein end point and filtered. Care was taken that reagent did not have greenish tinge.

vi. NESSLER'S REAGENT : (Estimation of total nitrogen)

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

Solution b) 10 g of NaOH dissolved in 50 ml of distilled water.

The reagent was prepared fresh by mixing a and b (4:5) at the time of estimation.

vii. ACID NIN HYDRINE REAGENT : (Estimation of free proline)

This reagent was prepared by warming 1.25 g nin hydrine in 30 ml glacial acetic acid and 20 ml 6M phosphoric acid with agitation until dissolved. The reagent remains stable for 24 hours at 4°C.

viii. MOLYBDATE-VANADATE REAGENT : (Estimation of phosphorus)

Solution a) 1.25 g of ammonium vanadate dissolved in 500 ml of 1N HNO_3.

Solution b) 25 g of ammonium molybdate dissolved in 500 ml of distilled water.

The reagent was prepared fresh by mixing a and b in equal proportions at the time of estimation.