II. MATERIALS AND METHODS

A. Selection of field

A huge fallow land having dense population of Parthenium hysterophorus L. was selected for the collection of plant material. From this field a small sector having uniform growth of P. hysterophorus was used for the present study.

B. Disease intensity

The incidance of phyllody disease was studied by laying the quadrats by the method of Misra (1968). The frequency, abundance and density of the diseased and healthy plants from this field was studied by using the formulae given below -

1. Frequency (%) = $\frac{\text{No.of occupied quadrats}}{\text{Total no. of quadrats}} \times 100$

2. Abundance (%) = $\frac{\text{Total no. of individuals}}{\text{No. of occupied quadrats}} \times 100$

3. Density (%) = $\frac{\text{Total No. of individuals}}{\text{Total No. of quadrats}} \times 100$

Morphological aspects of healthy and infected plants were studied by refering standard books.

C. Detection of MLOs under light microscope

A quick light microscopic method for MLO detection suggested by Deeley et al. (1979) was used. Free hand trans-sections of healthy and MLO infected plant parts (preferably stem) of P. hysterophorus were stained with 'phloroglucinol' to locate phloem tissue (Phloem tissue remains unstained). After locating the phloem tissue under light microscope, the sections were stained with Diene's stain for 10 min. The repeatedly washed sections in distilled water, were examined immediately under light microscope (JENAVAL). The MLOs in phloem of diseased plants stain blue. The greater details were observed under JENAVAL microscope than under ordinary light microscope.

1. Preparation of Diene's stain

Diene's stain was prepared by dissolving 2.5g methylene blue, 1.25 g Azure II, 10 g maltose and 00.25 g sodium carbonate in 100ml distiled water. The

stain was filtered through Whatman No.1 filter paper and stored in a freeze at 4°C.

(Hint : The stain should always be prepared fresh for detecting the MLOs by staining technique.)

D. Organic constituents

1. Chlorophylls

Chlorophyll content from healthy and MLO infected plant was estimated by the method suggested by Arnon (1949). 0.5 g randomly sampled fresh plant material was crushed in a mortar with pestle and extracted in 80% chilled acetone containing 4 ml liquor ammonia per litre in a dark and cold room. A pinch of magnessium carbonate ($MgCO_3$) was added during crushing. This extract was filtered through Buchner's funnel using Whatman No. 1 filter paper and the volume of filterate was measured. In order to avoid destruction of chlorophyll by light, the flasks containing chlorophyll extracts were covered with black paper and stored at low temperature. The absorbance was measured at 645 and 663 nm on double beam spectrophotometer (Shimadzu).

Chlorophylls (mg 100^{-1}g fresh weight) were calculated using the formulae given below -

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)'	=	8.02 x A 663 + 20.2 x A 645	= z

$$\text{Total chlorophylls mg } 100^{-1}\text{g fresh tissue} = \frac{x/y/z \times \text{Volume of extract} \times 100}{\text{Weight of plant material (g)} \times 1000}$$

Photooxidative degradation of chlorophyll was studied by exposing acetone extract of chlorophyll to diffused light (5 $\mu E\ m^{-2}s^{-1}$) at room temperature (30°C ± 2)

2. Starch test :

In order to test the Photosynthetic capability of MLO infected plant, the leafy part was studied to detect the starch. Starch detection was studied in the late afternoon assuring high level of starch in plant. Lafy material harvested from inected plant was placed

in 80% (v/v) boiling ethanol until the chlorophyll was fully extracted and then it was placed in 10% NaOH to attain clarity. The material was rinsed with distilled water and stained with I_2KI solution. The bluish black colour indicated presence of starch.

3. Polyphenols

Polyphenols from healthy and infected plant tissue were estimated following the method of Folin and Denis (1915). 1 g dried plant material was crushed in a mortar with pestle using 80% acetone. The 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 50ml using 80% acetone. 2ml of plant extract was taken in a Nessler's tube along with the series of standards (Std. tannic acid having concentration 0.1 mg ml^{-1}) to which 10 ml 20% Na_2CO_3 was added. The volume was adjusted to 35 ml with distilled water. Then 2 ml of Folin-Denis reagent was added in each test tube and the final volume was adjusted to 50ml with distilled water. After about 20-30 min, absorbance was measured at 660 mm using reaction blank. Polyphenols

were calculated from standard curve of tannic acid and the values are expressed in g 100^{-1}g dry tissue.

a) Preparation of Folin-Denis reagent :

100 g sodium tungstate and 20 g phosphomolybdic acid were dissolved in 800ml 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 1 litre and stored in an amber coloured bottle at low temperature.

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

E. Oxidative enzymes

1. Polyphenol oxidase (E.C. 1.10.3.2)

The activity of an oxidative enzyme polyphenol oxidase was studied spectrophotometrically by using the extraction and assay procedure suggested by Mahadevan and Sridhar (1982) with slight modification to suit our laboratory conditions.

a) Extraction :

1 g randomly sampled and cleanly washed leaf material from healthy and MLO infected plant (leafy material) was cut into small pieces and extracted in 15 ml cold 0.1 M phosphate buffer (pH - 6.1) in prechilled mortar with pestle. The homogenate was filtered through 4 layers of muslin cloth and centrifuged for 10 min. at 5000 x g in refrigerated centrifuge at 4°C. The supernatant was used for assaying the enzyme activity.

b) Assay :

In order to score the activity of polyphenol oxidase the oxidation of catechol was measured from the reaction mixture containing 2 ml phosphate buffer (pH 6.1), 0.5ml enzyme extract and 1 ml 0.01 M catechol at 495 nm. The change in the absorbance between the first 30 sec. and 150 sec. of incubation at room temperature (27°C) was measured. The control reaction was maintained with heated enzyme.

1 ml enzyme extract + 8 ml pure acetone + 1 ml extraction medium was kept aside for enzyme chlorophyll estimation (Arnon 1949).

2. IAA oxidase

The activity of IAA oxidase was measured by using the extraction and assay procedure given by Mahadevan and Sridhar (1982) with slight modification.

a) Extraction :

2 g randomly sampled and cleanly washed fresh leaf material from healthy and MLO infected plant (leafy material) was prechilled at 4°C for 1 h and crushed in prechilled mortar with pestle in 15 ml cold McIlvaine's buffer (PH 4.8). The homogenate was filtered through 4 layers of muslim cloth and filterate was centrifuged at 5000 x g for 10 min. at 4°C. The supernatant was used for enzyme assay.

b) Assay :

The activity of IAA oxidase was determined by measuring the residual amount of IAA in the reaction mixture containing 2 ml McIlvaine's buffer (pH 4.8), 0.5 ml, 0.05 M MnCl2, 0.5 ml 0.01 M 2,4 - Dichlorophenol, 1 ml 0.01 M IAA and enzyme in a total volume of 5 ml. The reaction mixture was incubated at 30°C in a waterbath and at an interval of 15 min, 1.5 ml

Salper reagent was added and the intensity of stable pink colour developed was measured at 535 nm.

F. Chromatography of polyphenols

1. Preparation of extract

The polyphenols were extracted by the method suggested by Glass and Bohm (1969). 5 g dried powder of entire aboveground plant parts of healthy and MLO infected plant was crushed separately in 80% ethanol. The extract was refluxed for 2 h on water bath using water condenser and filtered hot through a sintered glass funnel. The solvent was removed under reduced pressure. The residue was triturated in 50 ml hot water. The combined extract was reduced to 3 ml by condensation, centrifuged at 5000 g for 5 min and supernatant was used for chromatography.

2. Unidimensional paper chromatography of polyphenols.

Unidimensional paper chromatography was performed using Whatman No. 1 filter paper (size 28x14 cm) with slight modification in the method used by Shetty (1971). Chromatograms were spotted with 20 µl healthy and infected plant extract using microsyringe with frequent drying. The solvent system used was

n-butanol, acetic acid and water in the proportion of 80 : 20 : 44 (v/v). The position of individual phenolic compounds on chromatogram was determined by marking flourescent area under UV light as well as under UV light in presence of ammonia fumes. Phenolic compounds and flavonoids that could not be located under UV and UV + NH_3 were detected by deeping the chromatogram in a mixture of 0.3% $FeCl_3$ and 0.3% $K_3Fe(CN)_6$ in equal proportion.

The probable identification of the compounds was made by calculating the Rf values, observing colour under UV and UV + NH_3 and by comparing with the Rf values of authentic standards obtained from Dr. P. Neuman, University of Texas, Austin.

3. Quantification of individual phenolic compounds

Aliquot of healthy and inferted plant extract (20 µl each) was spotted on two sets of chromatographic paper. One set was used for detecting the spots under UV, UV + NH_3 and in a 1 : 1 mixture of 0.3% $FeCl_3$ + 0.3% $K_3Fe(CN)_6$. The other set was used to mark the area of separated phenolic compounds by overlapping it on the first set. The marked area of separated phenolic compounds on the second set of chromatogram was cut with

a sharp razor blade and the individual cut portions were transferred in a Nessler's tube containing 6 ml 80% ethanol and eluted by boiling it for 5 min. till the smell of ethanol get vanished. After removal of the paper and cooling the tubes at room temperature, 1 ml. 20% Na_2CO_3 and 0.2 ml Folin-Denis reagent were added. The volume was made to 10 ml with distilled water. The absorbance of the colour intensity was measured at 660 nm on double beam spectrophotometer (Shimadzu). Paper blanks of comparable size were taken as background colour. The calibration curve was prepared with tannic acid. The values of compounds were recorded as $\mu g\ spot^{-1}$.

G. Chromatography of amino acids

Amino acid composition of healthy and MLO infected plant of P. hysterophorus was studied by using the technique of ascending unidirectional paper chromatography (Block et al. 1955)

1. Preparation of Extract

The leaves of healthy and MLO infected plant (5 g) were crushed repeatedly in 80% ethanol and filtered through Buchner's funnel using Whatman No. 1

filter paper. The filterate was condensed under reduced pressure upto 2 ml and transferred into a centrifuge tube. The extract was then centrifuged at 5000 x g for 5 min. and the supernatant was collected in a small glass vial and stored at 4°C until use.

2. Unidimensional paper chromatography

The amino acids were separated by loading the aliquot of extract on chromatographic paper and 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 the chromatography of authentic standards and comparing their colours and Rf values. The colour intensity of spots were compared and according to their concentrations they were graded as least (+), less (++), moderate (+++) and high (++++).

H. Isolation of sesquiterpene lactone (SL)

Air- dried powder (20 g) of entire above ground plant parts of healthy and MLO infected Parthenium hysterophorus was repeatedly extracted with acetone

(analar grade) and filtered through 4 layers of muslin cloth. The filterate was collected in an evapourating dish and allowed to evapourate at room temperature. The green residue obtained was then redissolved in acetone in the proportion 1 part residue : 50 part acetone and the solution was passed through a Silica Gel - G (300 mesh) column (1 x 6 cm) preequilibrated with acetone. The flow rate through the column was adjusted to 4 drops per min. The yellowish brown mass thus obtained was redissolved in acetone and passed through a sintered glass filter. The filterate was then passed through 1x8 cm column packed with 1 cm layer of Silica Gel - G (300 mesh) followed by a 6 cm layer of activated charcoal (E. Merck, Germany) and 1 cm layer of Siloca Gel - G again. The sandwitched charcoal ensured removal of colouring matter. The effluent was collected in an evapourating dish by maintaining a flow rate 4 drops per min. and allowed to evapourate at room temperature (28±2°C) till a white crystalline residue was obtained.

1. Solubility of the SL

Solubility of sesquiterpene lactone in various solvents viz. Acetone, Methanol, Petroleum ether, Hexane, Benzene, Toluene, Xylene, Ethanol, Chloroform and Water was tested.

2. Determination of λ max

The λ max of sesquiterpene lactone was studied by dissolving known amount of compound in acetone and acetonitrile and the absorbance was measured on double beam spectrophotometer (Shimadzu) at 200 to 230 nm.

3. Allelopathic effects of SL

The allelopathic effects of sesquiterpene lactone were studied by preparing various concentrations of sesquiterpene lactone viz. 250, 500, 1000 ppm. The surface sterilized wheat seeds (0.1% $HgCl_2$ for 1 min) were kept in a petriplate containing blotting paper and treated with different concentrations of sesquiterpene lactone prepared as above. The germination percentage and root, shoot length were recorded upto 72 h of germination.

I. Nuclear Magnetic Resonance (NMR) spectra of SL

The compound was identified by obtaining NMR spectra on EM 390 90 MHz NMR spectrophotometer by the courtesy of Prof. E. Rodriguez, Irvine, U.S.A. and Dr. Manual Aregullin, California, U.S.A.

J. High performance liquid chromatographic (HPLC) analysis of S.L.

The S.L. was analysed on HPLC (Beckman gradient liquid chromatograph series 334) having analytical C_{18} RP column 25 : 75 water - acetonitrile isocratic system by the courtesy of Dr. Manuel Aregullin, California, U.S.A.

1. Detection :

The UV detector was set at 215 nm. This wavelength was found to be reasonable average of λ max for sesquiterepene lactone.

2. Sample preparation :

The pure crystalline sesquiterpene lactone isolated from healthy and MLO infected plants of P. hysterophorus was dissolved in acetonitrile and filtered through Millipore before injection.