III. MATERIALS AND METHODS

A. Collection of P. hygrophilus

About 250 g of rhizosphere soil in low-lying rice fields was processed by wet-sieving through 200 and 300 mesh sieves. The filtrates were reduced to a small amount (50 ml) and suspension was directly examined under 50 X magnification. P. hygrophilus were individually picked and colonised in water. The population was sexed and 50 adults of both sexes were together used to develop a nucleus culture.

B. Nucleus culture

The inoculum was surface sterilised with 0.07 per cent mercuric chloride and thoroughly washed in water for thrice. The basic medium for culture of Aphelenchoides besseyi, the white-tip nematode of rice (19 mg maltose, 5 g agar/wt., Voung Hu Haai, 1968) to which 200 ml aqueous extract of 2 gm potato tuber extract was added. To this medium in petri dishes the nematode inoculum was given. The nematodes developed and reproduced in this medium at 27 $\pm$ 1°C in an incubator.

C. Experimental procedures with nematodes

(1) Extraction procedure

The medium from each container (petri dish or Van Tieghem cell) was thoroughly mixed with water and the slurry was processed as in A above.

(2) Morphological and morphometric studies

(a) Adults and juveniles were heat relaxed at 60°C and mounted in a drop of water (Franklin, 1949) or in glycerin(2 per cent)(Seinhorst, 1962). These specimens were immediately used for measurements with calibrated occular and eye-piece micrometers fitted to BH Series Olympus Trinocular Microscope. All measurements were made at 100 to 350 X magnification from 20 specimens.

(b) Adults, juveniles and eggs were stained in-toto with acetic orcein 1% (Hirschmann, 1962) for study of reproductive system; in methyl red neutral and pH indicator (BDH) dyes for digestive system (Doncaster and Clark, 1964); or in silver nitrate 0.5% for body annulations, nerve ring and excretory system (Bedding, 1967).

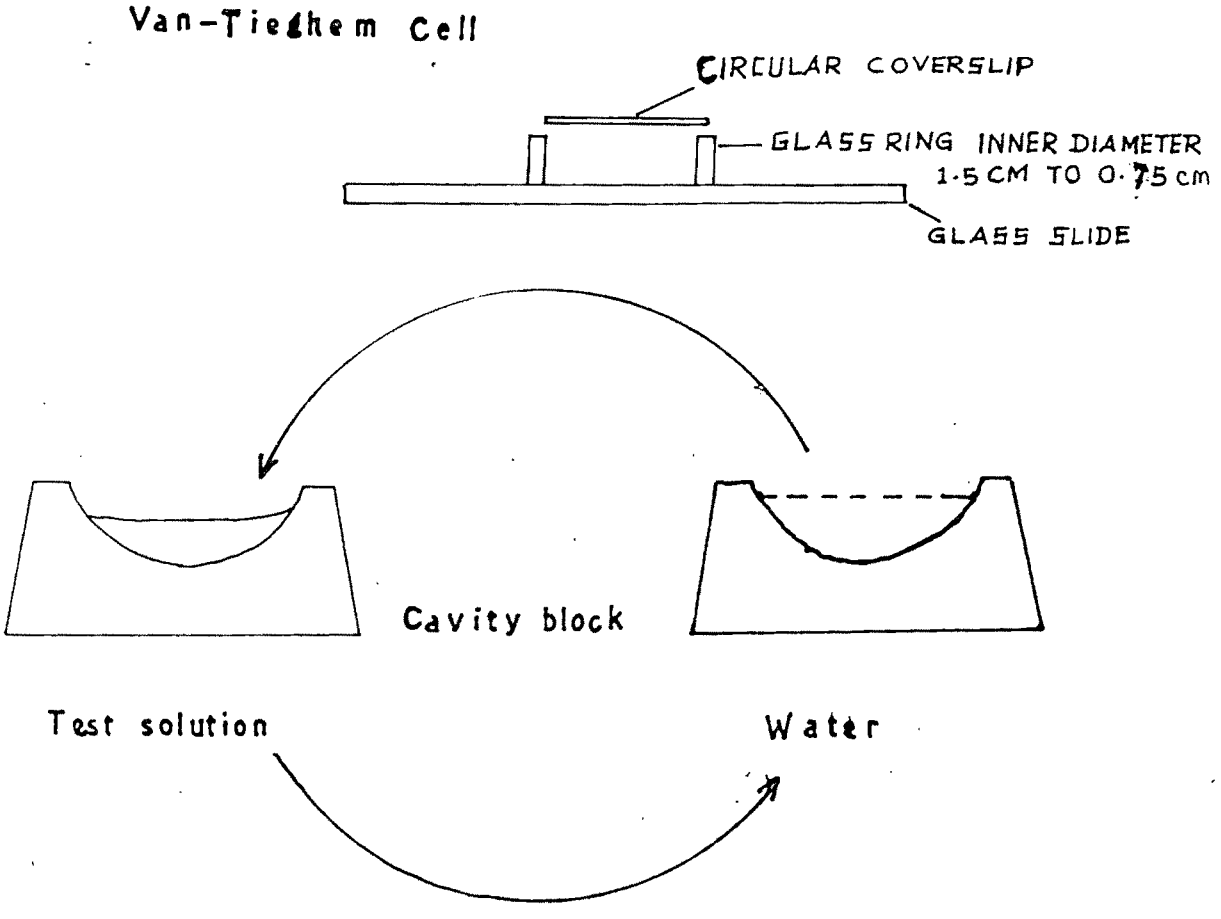
(c) The parameters included length of body and tail, maximum body width, distance of median bulb, posterior bulb, nerve ring, gonad, vulva from head end, the gonad length and deMann's indices a, b, b^1 and c.

(3) Life history of P. hygrophilus

(a) Embryology : Gravid females were colonised individually in hanging drops of water on 7.5 x 2.5 cm glass slides (Dasgupta and Raski, 1968) and kept in humid chamber at 27 ± 1°C. The nematodes were examined at intervals of 5 to 15 min. for oviposition and individual eggs were isolated on same slide chronologically, to follow the hatching and emergence of juveniles.

(b) Post-embryonic development : Van Tieghem cells were made by cementing glass rings (int. diam. 1.75 cm) to microslides (Southey, 1970). Each well was filled with the culture medium comprising of 4% oat meal agar (Fig.1). Eggs of P. hygrophilus were placed at the rate of 25 in each cell. At intervals, the hatching of juveniles and growth and development of adults were studied by extracting the population from four cells and recording the stages based on morphological differences until new eggs were oviposited by the females.

FIG.1. CELLS FOR CULTURE AND BIOASSAY OF P. HYGROPHILUS



(c) Developmental duration : The growth of nematodes per se was based on the increase in dimensions of successive developmental stages over the newly emerged juvenile. The first appearance of any advanced stage was considered as development and duration between the two such stages was expressed as the period for development of the stage.

(d) Growth of nematode populations : The ratio of initial population (pI-inoculum) given to the medium and final population (pF) of active adults and juveniles at 14 days was expressed as the build-up index. The efficiency of media or substrates for the culture of the nematode was also based on the build-up index and on the biomass production. The biomass was expressed by

$$G = \frac{a^2 b}{16 \times 10,00,00}$$

where G was the biomass of nematodes in micrograms, 'a' the maximum body diameter, 'b' the total body length and '16' was the emperical value (Andrassey, 1956).

(e) Feeding and excretion of nematodes : Van Tieghem cells with basic medium were used to colonise and provide substrate for juveniles and adults of *P. hygrophilus* as in 3(b) above. Methyl red indicator was

added to impart colour (Doncaster and Clark, 1964). The feeding, colouration of intestine and visual contents were examined at intervals by drawing six individuals from the medium, colonizing in a drop of water and examining at intervals under magnification.

(4) Substrate suitability for nematode nutrition

(a) Preparation of media : Synthetic and non-synthetic media, with agar base were prepared in water and adjusted to pH 7.0 with 1N HCl.

PA	-	Plain agar 1% and 2%
OMA	-	Oat meal agar 4% and 2% (OM c.a., agar 2%, sugar 1%)
PDA	-	Potato dextrose agar 20% (boiled potato mash 20%, agar 1.5%)
MEA	-	Malt extract agar 2% (malt extract 2%, agar 2%)
SDA	-	Sarboudex agar (dextrose 4.0%, peptone 1%, agar 1.5%)
BEA	-	Beef extract agar (Beef extract 2%)

About 1.5 ml of above medium was dispensed into the well of Van Tieghem cell in 3 replicates. The media were exposed to ultra violet radiation for 1 hr before inoculations.

(b) Inoculation and recovery of nematodes : The medium in each cell was inoculated with ten surface sterilized gravid females of P. hygrophilus under UV light and the top of the well covered with cover slip (Fig.1). Inoculations were given in 3 replicates per substrate.

The inoculated assembly was kept in a glass chamber, inner walls of which were rinsed in rectified spirit and dried earlier. The cultures were maintained for 14 days after which the contents of each Van Tieghem cell were processed for extraction of nematodes.

(c) Testing the efficacy of substrate : For mass culture assay, the dimensions of 20 males and females drawn from each Van Tieghem cell were measured. The build up index and biomass production were compared. The data were subjected to statistical analysis of variance (Goulden, 1968).

(5) Effects of nutrients in substrate on P. hygrophilus

(a) Procedure : With a view to investigate the nutritional requirements for the growth and build up of the nematode populations and to study the adverse effects if any of synthetic chemicals, a series of experiments

were designed and conducted.

Unless otherwise stated, the basic substrate was OMA 4% which was previously sterilized and dispensed in the well of the Van Tieghem cell at the rate of 1.5 ml in each. Substrate was enriched with nutrients and chemicals (Table I) in cold condition before filling in the Van Tieghem cells and then 10 adult (5 male + 5 female) nematodes were colonised in each cell (under UV light) representing one treatment. After inoculation, the assembly was kept at 27 $\pm$ 1°C in an incubator.

On the 14-day after inoculation, the entire contents of the cell were collected for extraction of nematodes as in 4(b) above. The efficacy of treatments was compared on the basis of dimensions of 20 adults and on the build up of final population of nematodes as in 5(c) above.

(b) Effects of starvation : In sterilized agar 2% (without any nutrient) dispensed in Van Tieghem cell, 200 adults (male : female :: 1:1) of P. hygrophilus were colonized. About 20 colonies were prepared and daily two were drawn for study of morphometrics for 20 adults and for mortality rate.

(c) Effect of sugars : D-glucose, sucrose and maltose were enriched to OMA to give 0, 12, 25, 50 and

Table I. Quantity (gm) of chemicals required per litre of water to provide 100 millimolar concentration

Salts		
Potassium carbonate	...	13.82
Potassium nitrate	...	8.10
Potassium phosphate	...	13.61
Potassium chloride	...	7.46
Sodium chloride	...	5.85
Sodium sulphate	...	14.02
Sodium phosphate	...	15.60
Sodium carbonate	...	5.30
Amino acids		
Alanine	...	8.91
Threonine	...	14.61
Serine	...	10.51
Glutamic acid	...	14.71
Phenylalanine	...	16.52
Methionine	...	14.92
Glycine	...	7.51
Lysine	...	14.62
Hormones		
Insulin	...	600 units
Thiourea	...	7.61
SR 26	...	
Cholesterol	...	38.66
Fatty acids		
Formic acid	...	4.60
Butyric acid	...	15.82
Acetic acid	...	6.01
Sugars		
Sucrose	...	34.23
Glucose	...	18.06
Maltose	...	36.03
Vitamins		
Multi vitamin	...	*
Vitamin-C	...	17.61
Becozyme	...	17.61
Ravingon	...	17.61
Dexasol (cold)	...	*
Dexasol (hot)	...	
Adrenaline chloride	...	21.76

* See Appendix

100 µm concentration in 3 replicates.

(d) Effect of vitamins : Pharmaceutical preparations of vitamins viz., vitamin-A, vitamin A+E and vitamin-C were powdered and dissolved in water and enriched to the medium to provide 0, 2, 4, 6 and 8 ppm of each vitamin in 3 replicates.

(e) Effects of amino acids : Calcium pentothenate, DL-serine and L forms of glutamic acid, methionine, analynine, threonine, lysine, phenylalanine, were prepared as aqueous stock solutions and were enriched to the substrate to give 0, 12, 25, 50 and 100 µm concentration of each amino acid individually in 3 replicates.

(f) Effect of inorganic chemicals

(i) Effect of calcium and iron : Pharmaceutical preparations containing calcium and iron were dissolved in water and enriched to the substrate to provide 0 and 1 ppm of calcium or iron individually in 3 replicates.

(ii) Laboratory pure (ANALAR, BDH, Merk and Boots) sodium salts viz., carbonate, chloride, phosphate; potassium salts viz., bromide, phosphate, sulphate, ferrocyanide and magnesium carbonate were dissolved in water. These were individually enriched into the media to provide 0, 10, 50, 250 and 500 ppm of each salt to

substrate in 3 replicates.

(g) Effect of hormones

(i) Pharmaceutical products of insulin, dexasol and adrenaline chloride and cholesterol were dissolved in cold condition in water and enriched to the medium individually to provide levels of 0, 2, 4, 6 and 8 ppm in 3 replicates.

(ii) Juvenile hormones viz., SR and thiourea were dissolved in water and enriched to the medium individually to provide 0, 50, 100 and 200 ppm to substrate in 3 replications.

(6) Biochemical characteristics in P. hygrophilus

Both histochemical and biochemical investigations were conducted with P. hygrophilus with a view to find out the cuticular permeability, biochemical constituents and enzyme secretions of the nematode.

(a) Cuticular permeability : Response of nematode to hyper-hypotonic solutions of mineral salts, fatty acids, buffers was investigated.

(i) Laboratory pure (ANALAR, BDH, Merk and Boots) sodium hydroxide, copper sulphate, potassium nitrate, potassium carbonate of 0.1, 0.2, 0.3 and 0.4 M conc. were

prepared in water.

(ii) Formic acid, acetic acid, butyric acid and sucrose were dissolved in water to give test solutions of 0, 0.005, 0.1, 0.2, 0.3 and 0.4 M conc.

(iii) Potassium chloride was prepared in concentrations of 0, 1%, 2%, 3%, 4% and 5% and buffer (phosphate) was adjusted to pH 5.8, 6.0, 6.4, 6.8, 7.0, 7.4 and 8.0.

Each of the above test solutions was dispensed into a glass cavity block (Fig.1) at the rate of 0.5 ml in 3 replicates. Into each block was released 10 (5 male + 5 female) adults. After recording the immobilization time, the immobolized worms were transferred to cavity blocks containing water, for revival. Observations were continued at intervals of 5 sec (in the 1st hour) 10 min (2nd hour) and at interval of one hour thereafter. Dead nematodes between any two readings indicated immobolization time. All nematodes surviving after 24 hrs in any solution were transferred to test solution and the process repeated.

(b) Biochemical analysis : From the culture of P. hygrophilus maintained in 4% OMA, 25,000 active nematodes were harvested, washed and prepared for analyses. Batches were colonized in water to obtain material for studies on effects of starvation. Ethanol extracts

of the nematodes was made of the tissue homogenate of 5 ml (Mahadevan et al., 1965). These extracts were used for estimation of total sugars, reducing sugars and total phenolics by colorimetric methods using a Spectrophotometer (Elico Model CL-24).

(i) Reducing sugars : Reducing sugars were estimated by Nelson's method (Nelson, 1949). Glucose was used as the standard.

To one ml of alcohol extract placed in a boiling tube, one ml of a mixture of 25 parts of reagent 'A' (25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartarate, 20 g of sodium bicarbonate and 200 g of anhydrous sodium sulphate dissolved in 800 ml of glass distilled water and diluted to one l) and 'B' (15 g of copper sulphate and one or two drops of conc sulphuric acid in 100 ml of glass distilled water) and one part of reagent 'B' was added. The mixture was heated on a boiling water bath for 20 min and cooled. One ml of arseno-molybdate colour reagent (25 g ammonium molybdate, 21 ml of conc sulphuric acid, 3 g of sodium arsenate dissolved in 25 ml of glass distilled water and mixed with 450 ml of glass distilled water and the mixture was incubated at 37°C for 48 hr) was added to the solution. The contents in the tubes were thoroughly mixed and diluted to 25 ml with glass distilled water. The absorbance of the

resulting blue coloured solution was read at 520 µm. Standards prepared with glucose were used to calculate the amount of reducing sugars present in the extract.

(ii) Total sugars : Non-reducing sugars present in alcohol extract were hydrolysed to reducing sugars and the concentration of total reducing sugars was estimated. Exactly one millilitre of the alcohol extract was placed in a boiling tube and evaporated to dryness in a boiling water bath. One ml of glass distilled water and one ml of 1N sulphuric acid were added to the tubes. The tubes containing the mixture were heated on a hot water bath at 49°C for 30 minutes to hydrolyze the non-reducing sugars to reducing sugars (Mahadevan and Sridhar, 1982).

The tubes were cooled in a running tap and a drop of methyl red indicator was added to each tube. The contents were neutralized by the addition of 1N NaOH. The concentration of total sugars (present in the form of reducing sugars) was estimated employing Nelson's modified Somogy's method (1944) as described earlier.

(iii) Total proteins : To one ml of tissue homogenate, 4 ml of absolute alcohol was added and boiled for 5 min at 80°C in water bath and then centrifuged and to the residue collected, 4 ml of 10% T.C.A.

solution was added and kept over night in refrigerator. This mixture was centrifuged and supernatant was collected (which was used also for total sugar and amino acid estimation) and the residue was again dissolved in 4 ml of T.C.A. 10% solution and centrifuged. Residue was washed in absolute alcohol, dried boiled in 2 ml 1N NaOH for 15 minutes and centrifuged. Supernatant was collected and made upto 20 ml in the 1N NaOH (protein extract).

(iv) Estimation of total soluble amino acids

The amount of total soluble amino acids present in the extract was determined by the ninhydrin method of Moore and Stein (1948).

One ml of the alcohol extract was placed in a boiling tube. One drop of methyl red indicator was added to it, and the extract was neutralized with 0.1 N sodium hydroxide. To this solution, 1 ml of freshly prepared ninhydrin reagent (800 mg of hydrated stannous chloride was dissolved in 500 ml of citrate buffer at pH 5.0. This solution was mixed thoroughly with 500 ml of methyl cellosolve containing 20 g of recrystalized ninhydrin) was added, mixed thoroughly and heated on a water bath for 20 min. The mouth of the tube was covered with glass marbles during heating. Five ml of the diluent

solution (n-propanol diluted with equal volume of glass distilled water) was added to the mixture while it was still on the water bath.

The tubes were removed and cooled under running tap water and the contents were thoroughly mixed. Suitable reagent blank was maintained with one ml of glass distilled water in place of the extract. The absorbance of the purple coloured solution was read in a Spectrophotometer (Elico Model, CL-24) at 570 nm. Standards prepared with glutamic acid were used to calculate the concentration of amino acids present in the extract.

(v) Enzyme assays : Several samples of 25,000 full-fed nematodes were treated to water as substrate so that they remain starved for at least 3 to 4 days prior to assays. Full-fed and starved batches of nematodes were separately homogenized with 10 ml phosphate buffer pH 6.8 (0.1 M) and cold centrifused at $2^{o}C$ for 15 min at 1700 g. The clear supernatant was used as the source for enzyme assays.

1) Catalase : Catalase was assayed following the method of Chance and Maehly (1955) with the following modifications (Kar and Mishra, 1976).

Five ml of the assay mixture contained 300 µmoles of phosphate buffer (pH 6.8), 100 µmoles of H_2O_2 and 1 ml

of the twice diluted enzyme extract.. After incubation of the reaction mixture at 25°C for 1 min, the reaction was stopped by the addition of 10 ml of 2% (v/v) H_2SO_4 and the residual H_2O_2 was titrated against 0.01 N $KMnO_4$ until a faint purple colour persisted for at least 15 sec. The control was run at the same time in which the enzyme activity was stopped at the start of the reaction itself. One unit of catalase activity was defined as that amount of enzyme which broke down 1 µmole of H_2O_2/ min.

2) Peroxidase : Peroxidase activity was assayed as described by Chance and Maehly (1955) with the following modifications (Kar and Mishra, 1976).

Five ml of the assay mixture contained 125 umoles of phosphate buffer (pH 6.8), 50 umoles of pyrogallol, 50 umoles of H_2O_2 and 1 ml of the 20 times diluted enzyme extract. The assay mixture was incubated for 5 min at 25°C, after which the reaction was stopped by the addition of 0.5 ml of 5% (v/v) H_2SO_4. The amount of purpurogallin formed was determined by recording the absorbancy at 420 nm in a Spectrophotometer.

3) Polyphenol oxidase : Five ml of assay mixture for polyphenol oxidase activity consisted of the same assay mixture as that of peroxidase without H_2O_2. The

absorbancy of purpurogallin formed was measured at 420 nm in a Spectrophotometer. Peroxidase and polyphenol oxidase activities were expressed in absorbancy units.

4) Acid phosphatase : The enzyme activity was determined by the method employed by Roberts (1956) and Mishra and Mohanty (1967).

5) Cellulase : One ml of extract was taken in a test tube to which 3 ml buffer and 1 ml substrate were added (CMC - carboxy methyl cellulase) and incubated at room temperature for 1 min. Then 5 ml inhibitor (0.3 M TRIS) was added to this solution. From this prepared mixture 0.2 ml was taken for both '0' time and full time, to which was added 0.8 ml water and 1 ml of copper reagent. To this solution 1 ml of arsenic molybdate and 7 ml of water was added in order to make the solution a total of 10 ml. Absorbance was read at 660 nm.

6) Urease : One ml of enzyme extract was taken in a test tube, to which 3 ml buffer 6.8 and 1 ml urea solution was added. It was left for 1 min, then to this solution, was added 10 ml KCl.

To one ml of above extract, 3 ml of colour reagent was added and incubated at 120^{o}C for 20-30 min after which

it was cooled for 15 min in a running water. One ml of water was added and the absorbancy was read at 527 nm.

7) Amylase : One ml of enzyme extract was taken in a test tube, 1 ml of citrate buffer pH 5.0 and 1.0 ml of starch solution (1%) were added. The mixture was incubated at room temperature for 5 min after which was added 2 ml of 3-5 dinitrosalycylic acid and heated in water bath for 5 min. Then the mixture was centrifuged. To 1.0 ml of aliquot, was added 5 ml of water and absorbancy was read at 540 nm.

8) Total phenolics : Total phenols were estimated by using Folin-Ciocaltaeu reagent (Bray and Thrope, 1954) using catechol as standard.

One ml of the alcohol extract was placed in a boiling tube. One ml of Folin-Ciocaltaeu reagent and two ml of 20 per cent sodium carbonate were added to it and the mixture was heated on a boiling water bath for exactly one min. The resulting mixture (blue in colour) was diluted to 25 ml with glass distilled water. Reagent blank was maintained with one ml of distilled water. The absorbance of the solution was read at 650 nm. The concentration of total phenols present in the extract was calculated from a standard curve prepared with ferrulic acid.