DETECTION OF FREE AMINO-ACIDS IN THE HOMOGENATE AND SECRETIONS OF PRATYLENCHUS COFFEAE (Zimm., 1898) Filip. & Stekh., 1941

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BY

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DEDICATED
TO THE SWEET MEMORY
OF
MY GRAND MOTHER

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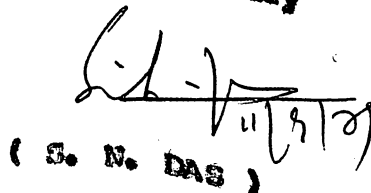
September 11, 1979.

CERTIFICATE

This is to certify that the thesis entitled " DETECTION OF FREE AMINO ACIDS IN THE HOMOGENATE AND SECRETIONS OF PRATYLENCHUS COFFEAE (Zimm., 1898) Filip. & Stekh., 1941 " submitted in partial fulfilment of the requirements for the award of the degree of MASTER OF SCIENCE (AGRICULTURE) of the Orissa University of Agriculture and Technology, Bhubaneswar, is a faithful record of bonafide research work carried out by Shri Madhusudan Pradhan under my guidance and supervision. No part of the thesis has been submitted for any other degree or diploma.

The help and information as have been availed of in course of this investigation have been duly acknowledged by him.



(S. N. DAS)

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CHAPTER-I
INTRODUCTION

INTRODUCTION

It is now well established that modern crop production technology envisages extensive but economic plant protection measures, where the nematologists come to play their useful roles. Although the science of Nematology is fairly young, it has made significant contributions to world-agriculture and has made sufficient impact on the society to earn its own recognition and place of importance as much as other disciplines in the field of crop protection.

The world-wide occurrence of the phytonematodes often cause serious problems, weakening our agricultural economy. One such pest of great concern happens to be the commonly known lesion nematode, the endoparasitic species of *Pratylenchus* Filipjev, 1936. These nematodes are undoubtedly the primary factors in the root destruction of many cultivated crops as well as forest trees.

The representatives of this genus are cosmopolitan in distribution, and have a wide host-range. More than 40 species of *Pratylenchus* are presently reported to occur abundantly throughout the temperate regions and the tropics. They have been reported from the Netherlands (De Man, 1880), England (De Man, 1884), Java (Zimmermann, 1898), West Indies (Cobb, 1919), Germany (Loof, 1957), Hawaii (Godfrey, 1929), California (Thorne, 1934), Nile delta of Egypt and U.A.R. (Oteifa, 1962), Bolivia (Lordello *et al.*, 1961), Japan (Yokoo, 1962), Ceylon (Hutchinson

and Vythilingam, 1963), Brazil (Lordello, 1967), Guatemala (Schieber, 1966), Spain (Tobarjimenez, 1966), New Zealand (Egunjobi, 1968), Poland (Wilski, 1968), U.S.S.R. (Romaniko, 1960), Ivory Coast (Guerout, 1968), Singapore (Chin, 1969), Ontario, Canada (Cornelisse _et al._, 1970), Iraq (Katcho and Allow, 1968), French West Indies (Scotto la massese, 1969), Cuba (Baranovskay and Krilov, 1968).

The host range of different species of Pratylenchs comprises of many crops like vegetables and fruits, such as, Potato, Rice, Tea, Barley, Corn, Banana, Tobaco, Citrus, Cowpea, Coffee and many other field crops including grasses. Due to their association with a broad spectrum of plants most of them are considered economically important.

In India, 9 species of _Pratylenchus_ have been reported from Delhi, Punjab, Rajsthan and Himachal Pradesh (Sethi and Swarup, 1972). They are _P. brachyurus_, _P. crenatus_, _P. coffeae_, _P. loosi_, _P. neglectus_, _P. penetrans_, _P. scribnery_, _P. thronei_ and _P. zeae_, out of which _P. crenatus_, _P. loosi_, _P. neglectus_ and _P. penetrans_ are new records for India. _P. zeae_ has also been reported from M.P. on maize (Pall and Chand, 1971). Recently, five new species, _P. ranjani_, _P. similis_, _P. impar_, _P. neocapitatus_ and _P. teres_ have been reported from northern India (Khan and Sing, 1974). _P. coffeae_ (Zimm., 1898), Filip. and Stekh., 1941 is another important species reported from south India from maize, sorghum, banana etc.

In spite of this reported occurrence of these nematodes on so many economic plants, their importance does not seem

to have been properly appreciated as not much work has been done to reveal their host-parasite relationship, nutrition, biochemistry, histochemistry, histo-pathology, bionomics and ecology.

A sound knowledge of the free amino-acids discharged or utilised by a plant parasitic nematode is essential to understand the biochemical basis of host-parasite relationship in its appropriate perspective of complexity with possible implication to disease resistance in plants, and to provide further insight into the physiopathology. Moreover, this knowledge could possibly be utilised in nematode taxonemy in future, as has been the case with Meloidogyne exigua, where females collected from different areas and of different pathogenicity to coffee, showed significant difference in their protein composition (Neto, Lordello and Lordello, 1975).

The addition of free amino acid may cause pathogenic reactions and produce symptoms at times. D. dipsaci caused alfalfa root galls by injection of tryptophan, as it was not found in healthy roots (Krusberg, 1961). Aphelenchoides ritzemabosi was also found to add free tyrosine to affected parts.

Animal parasites damage host tissue by ammonia liberated due to enzymatic action on free amino acids, the possibility of which may be suggested for plant parasites (Myuge, 1957) though it is yet to be demonstrated.

For physiological studies of the nematodes or for culturing in artificial media, it is very much essential to know the amino acid nutrition or their bio-synthesis in the phytonematodes.

Besides, transamination and metabolic pathways in nematodes leading to production of different amino acid may have evolutionary significance. Hence with proper knowledge of this protein-bound amino acid patterns of freeliving, plant parasitic and animal parasitic nematodes, we can better understand their physiological inter-relationship.

The antimetabolitic action of a few amino acids on nematodes will enhence the scope for control of nematodes, which do need to have a broad outlook of the free amino acids of the plant parasitic nematodes. As foliar spray, three amino acids DL-alanine, DL-serine and DL-threonine were tried against M. incognita on tomato. DL-serine and DL-alanine significantly decreased the development and reproduction of the nematode without slightest affect on the host (Krishna Prasad and Setty, 1974).

The contents of free amino acids in the nematode exeretion may lead us to posses a better knowledge regarding biosynthesis, catabolism, or utilisation of these compounds by the pathogen.

Pratylenchus coffeae is widely prevalent and its pathogenicity has been established with coffee, tea, banana, citrus and many other crops. Keeping its economic importance in view the nematode homogenate and exeretion have been analysed qualitively for free amino acids which may be pertinent to some of the above mentioned implications.

CHAPTER-II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The literature on biochemical studies relating to phyto-parasitic nematodes and their inter-relationship, in general, have received little attention, although in current decades many scientists have expressed wide appreciation for such investigations. However, some of the important literatures available concerning free and bound amino acids, their requirements, bio-synthesis and antimetabolism, which are indispensable to highlight any possible relationship to the present study, have been briefly cited below.

A. FREE AMINO ACIDS :

(i) Free amino acids in nematode excretion :-

While working on the discharged materials of *Ditylenchus dipsaci* and *Meloidogyne incognita*, Myers (1963) observed the presence of 15 to 20 amino acids in aseptic incubates of *D. triformis*, *D. dipasci* and *D. myceliophagous*.

Rothstein (1963) analysed the excretion products of a free living nematode *Caenorhabditis briggsae* after feeding them with radio active carbon isotope, ^{14}C. He reported that only radioactive alanine, aspartic acid, glutamic acid, glycine and serine were excreted by the nematode.

Analysing the discharged organic materials by five plant parasitic nematodes, in one percent aqueous solution of glucose, Myers and Krusberg (1965) found a number of amino acids. *Ditylenchus triformis* discharged L-alanine, arginine, asparagine, aspartic acid, cysteine, cysteic acid, gamma-amino butyric acid, glutamic acid, glutamine, glycine,

histidine, hydroxy proline, iso-leucine, lysine, methionine sulfoxide, ornithine, phenyl alanine, proline, serine, threonine, tyrosine and valine. Ditylenchus dipsaci discharged L-alanine, arginine, asparagine, aspartic acid, cysteine, cysteic acid, glutamic acid, glutamine, glycine, iso-leucine, lysine, methionine, methionine sulfoxide, ornithine, proline, serine, threonine, tyrosine and valine but there was no trace of histidine, hydroxy proline; and perhaps phenyl alanine as well as gamma-amino butyric acid were present as indicated by faint ninhydrin positive spots. Discharged materials of D. myceliophagus contained L-alanine, arginine, asparagine, aspartic acid, glutamic acid, glycine, iso-leucine, lysine, methionine, methionine-sulfoxide, ornithine, serine, threonine, tyrosine, valine but no cysteine, cysteic acid, gamma-amino butyric acid, histidine, hydroxy-proline, proline and phenyl alanine. Pratylenchus penetrans excreted L-alanine, aspartic acid, glutamic acid, glycine, methionine sulfoxide, ornithine, serine, threonine but no arginine, asparagine, cysteine, cysteic acid, gamma-amino butyric acid, glutamine, histidine, hydroxy proline, iso-leucine, lysine, methionine, phenyl alanine, proline, tyrosine and valine. Meloidogyne icognita was found to discharge L-alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, histidine, iso-leucine, lysine, methionine sulfoxide, ornithine, phenyl alanine, proline, serine, threonine, but no cysteine, cysteic acid,

hydroxy proline and methionine. Cytrulline gave a very faint ninhydrin positive spot in the discharge chromatograms of all nematodes with the exception of P. penetrans. In M. incognita excreta chromatogram gamma-amino butyric acid, tyrosine and valine gave vary faint ninhydrin positive spots.

Asit and Riggs (1967) reported aspartic acid, glutamic acid, serine, glycine, alanine and two unidentified ninhydrin positive spots from the incubation solution of Heterodera glycines larvae. They also reported aspartic acid, glutamic acid, serine, glycine, alanine, threonine and glutamine and six other spots tentatively identified as lysine or ornithine, asparagine, tyrosine, methionine or valine, phenyl alanine, leucine or iso-leucine, as the free amino acids present in the homogenate of the same larvae.

Wright (1975) in his experiment with elimination of Panagrallus redivivus reported the presence of significant amount of urea and amino acids alongwith ammonia as major compounds. With increasing stress, elimination of amino acids increased while that of ammonia decreased.

Nordbring-hertz (1977) found that the excreted peptides and amino acids of living nematodes were partly responsible for nematode induced trap formation in Arthrobotrys oligospora. Additional effects on trap formation were due to volatile substances from nematodes.

(ii) Free amino acids in nematode homogenates :-

Krusberg (1961) had determined the free amino acid content in Ditylenchus dipsaci and Aphelenchoides ritzemabosi.

Extracts of *D. dipsaci* were found to contain aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, histidine, arginine, valine leucine, iso-leucine, gamma-amino butyric acid and cysteic acid; and that of *A. ritzemabosi* contained aspartic acid, glutamic acid, serine, threonine, glycine, alanine, arginine, valine, gamma-amino butyric acid, cysteic acid, lysine and methionine.

Smith and Ellenby (1967) while analysing the contents of *Heterodera rostochiensis* cysts during maturation found the free amino acids, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, iso-leucine and leucine. The quantity of total amino acids decreased with advancing maturation.

In 1969, Asit and Riggs compared the free amino acid content in adult and larvae of *Heterodera glycines* and found that on infection of soybean, proline increased.

Srivastava (1969) observed 8 free amino acids in gravid females of *Meloidogyne javanica* which were alanine, arginine, cysteine, lysine, glutamic acid, methionine, phenyl alanine and tyrosine.

Saxena (1972) analysed the free amino acids of mature *M. javanica* females and of the infected and uninfected roots of jute (*Corchorus capsularis* L.). He found 14, 13 and 9 amino acids in the nematode, root-knots and the healthy roots of jute respectively by 2-dimensional paper chromatography.

Rebois (1973) in studying the effect of *Rotylenchulus reniformis* on the amino acid content of seeds of *Glycine max*,

found lower leucine content of seeds from infected plants as compared to those from un-infected plants.

Doney, Fife and Whitney (1971) in their pot experiment with 11 varieties and selections of sugarbeet nematised with H. schachtii established the significant increase in the concentration of aspartic acid, glutamic acid, and glutamine in the fibrous roots of seedlings.

Epstein and Cohn (1973) analysed the galled root tips caused by Longidorous africanus which showed an increase in proteins, free aminoacids, phenols and sugars but decrease in cytokinin contents.

Bumbu, Derzhavina and Sobetskii (1967) reported that potato infected with D. destructor caused a decrease in free amino acids in the plant tissue, whilst those were increased significantly in cucumber infected with Meloidogyne incognita, particularly alanine, glutamic acid, glutamine, serine, histidine and arginine.

Lewis (1973) in his experiment with resistant and susceptible varieties of cotton (Gossipium hirsutum), nematosed with M. incognita, showed that the susceptible cultivar had a greater percentage increase of free-amino acids.

Midha and Swarup (1974) studied the wheat seed galls caused by Anguina tritici. The chemical analysis of the gall components showed the presence of amino acids and sugars.

Wang and Bergeson (1974) analysed the galled roots and xylem sap of infected tomato plants. The galled root exudate was found to contain 3 sugars, 12 amino acids and

three organic acids as compared to 4 sugars, 15 amino acids and 4 organic acids in healthy root exudates suggesting a reduction in amino acid content. But 18 and 17 amino acids were identified in healty and infected plant xylem saps respectively. The amino acids decreased moderately with an increase in inoculum.

Masood and Husain (1975) reported the role of amino acids and proteins in the resistance and susceptibility of tomato varieties, to infection of M. javanica. The concentration of amino acids increased with infection in resistant and moderately resistant varieties but decreased in highly susceptible ones. Protein content had an inverse relationship to the amino acids.

Giebel and Stobieka (1974) showed that in the potato roots infected with H. rostochiensis the ratio of proline to hydroxy proline increased significantly in susceptible varieties where as that decreased in resistant varieties. Possibly this decreased ratio was cause of resistance.

Lewis and McClure (1975) worked with the susceptible and resistant cultivars of cotton to root knot nematode M. incognita and found that the sum total of free amino acids was greater in the resistant cultivar. The concentration of glycine declined markedly over a 10-day period following inoculation, proline increased to a great extent and tyrosine and phenyl alanine varied as functions of infection, cultivar and time of harvest.

Bird and McClure (1976), in their chemical analysis

of the hydrolysis products of the egg shells of M. javanica, R. reniformis, T. semipenetrans and P. minyus have revealed a high proline content (upto 35%) which is apparently a characteristic of nematode egg shells examined so far.

B. BOUND AMINO ACIDS :

Bird (1958) while analysing the hydrolysis products of adult female cuticle and egg sac of Meloidogyne for bound amino acid content, found 15 to 14 amino acids to be present, respectively. The egg sac contained more histidine, threonine and aspartic acid than the cuticle and less proline and arginine.

Nicholas, Dougherty and Hansen (1960) examined the hydrolysate of Caenorhabditis briggsae by paper chromatography and radio-autography for determining the ^{14}C labelling ability of the amino acids present. The total bound amino acids detected were alanine, arginine, aspartic acid, cysteic acid, glutamic acid, glycine, histidine, lysine, serine, threonine, proline, tyrosine, valine, methionine, leucine, iso-leucine and phenyl alanine. The labelled amino acids observed were aspartic acid, glutamic acid, alanine, proline, glycine, serine, cysteine and cystine as cysteic acid.

In 1961, Krusberg determined the protein bound amino acids of Ditylenchus dipsaci and Aphelenchoides ritzemabosi on alfalfa tissue culture. D. dipsaci was found to posses aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, histidine, arginine, valine, leucine-

isoleucine, cysteic acid, proline, hydroxy proline, phenyl alanine and an unknown ninhydrin positive spot. Also in A. ritzemabosi aspartic acid, glutamic acid, serine, threonine, glycine, alanine, arginine, valine, cysteic acid, lysine, methionine, proline, hydroxy proline, phenyl alanine and two unknown ninhydrin positive spots were found.

Clarke and his associates (1967) investigating the chemical composition of the egg shells of Heterodera rostochiensis with Technicon Auto Analyser reported 18 amino acids. Those were proline, aspartic acid, glycine, serine, glutamic acid, hydroxy proline, lysine, tyrosine, alanine, cystine, arginine, leucine, threonine, phenyl alanine, histidine, valine, methionine and iso-leucine out of which proline was most abundant with aspartic acid, glycine and serine made up about 64% by weight of the total amino acids.

Clarke (1968) further made a chemical analysis of the cyst wall of Heterodera rostochiensis and found it to contain 72% protein which was collagen like. On acid hydrolysis of cyst wall of Heterodera schachtii he also found, in 1970, that glutamic acid, glycine, proline and hydroxy-proline constituted 54% of the total weight of the amino acids present.

In the year 1972, Viglierchio and Cortz determined the amino acid composition of whole cuticle, pseudocoelomic fluid and reproductive organs of Anisakis physeteris from

the sperm whale. Proline, glycine and arginine occurred abundantly in cuticle hydrolysate while that of reproductive organs, 62% of total nitrogen was given by lysine, glutamic acid, glycine, valine, leucine and histidine. Male cuticle contained more serine, more proline in the protein of female gonads and methionine in cuticular protein but absent in perienteric fluid.

Samoiloff (1973) using electron microscope auto radiography and adult male of *Panagrellus silusiae* labelled with ^{3}H leucine, observed that protein synthesised in hypodermal chords was inserted into the cuticle at the cuticle basement membrane invaginations.

Giebel (1973) found the increased activity of 2 deaminases, phenyl alanine ammonialyase (PAL) and tyrosine ammonia lyase (TAL) in the potato roots infected with *H. rostochiensis*, thereby increasing resistance.

Waterston, Epstein and Brenner (1974) detected paramyosin in the body wall and pharyngeal musculature of *Caenorhabditis elegans*. The body wall contained this protein in higher concentration than the pharynx.

Baron (1975) extracted proteins from *Meloidogyne* sp. on kenaf and tomato roots and by double gel diffusion technique showed that the protein extracts had strong antigenic relationship between the host and pathogen. He also detcted *Meloidogyne* protein in extracts of tomato roots.

Bumbu and Mel'nik (1975) showed that Ditylenchus destructor exuded protein which gave 8 fractions on electrophoresis. The hydrolysis products of each fraction contained cellobiose as detected by chromatography.

Lueshner and Pasternak (1975) investigated that incubation product of the whole worm of Panagrellus silusiae with ^{3}H proline contained collagen. The amount of collagen bound tritiated proline and hydroxy proline was measured by icorporation of tritium in to extracted materials, suggesting that the rate of collagen synthesis was discontinuous during post-embryonic development. Non-collagen proteins were also identified being labelled with ^{3}H tryptophan or ^{3}H leucine and the level of soluble radio active proline that accumulated in the organism, during incubation period remained constant.

Gus'keva and Makovskaya (1976) could differentiate the 6 populations of M. rostochiensis by electrophoretic mobility and by the number and density of protein groups by disc-electrophoresis.

Reversat in 1976, while studying the overall bio-chemical composition of M. javanica and M. oryzae juveniles put forth that they contained 40.9% and 40.7% protein by weight.

Greet and Firth (1977) reported that the electro-phoretic protein patterns of females of round cyst nematodes varies according to the host plant, they are cultured on.

Reznick and Gershon in 1977, purified fructose-1, 6-diphosphate aldolase from the free living nematode Turbotrix aceti which had proline at NH_2-terminus and tyrosine at COOH-terminus and resembled vertebrate aldolase A, but in amino acid composition this aldolase resembled vertebrate aldolase C.

C. BIOSYNTHESIS AND AMINO ACID REQUIREMENTS :

In Caenorhabditis briggsae, Rothstein and Tomlinson (1961) demonstrated the biosynthesis of 16 amino acids aspartic acid, glutamic acid, alanine, proline, glycine, cysteine, cystine (as cysteic acid), serine, arginine, threonine, tyrosine, valine, leucine, iso-leucine, histidine and lysine, by adding radio active amino acid precursor to the medium and subsequent determination of the labelled amino acids by chromatography and radio autography.

In 1962, the same authors again fed C. briggsae with ^{14}C labelled amino acids, and observed them to synthesize not only the non-essential amino acids like glutamic acid, aspartic acid, alanine, glycine, serine and arginine but also the essential amino acids namely threonine, tyrosine, valine, leucine, iso-leucine, histidine and lysine.

Rothstein and Mayoh (1964) showed the presence and utilisation of an enzyme " iso-cytrate lyase " by C. briggsae to synthesize glycine.

Miller and Roberts (1964) reported the synthesis of alanine in A. ritzemabosi from aspartic acid and glutamic

acid substrates.

Myers and Krusberg in the year 1965 demonstrated amino acid synthesis by Ditylenchus triformis and mixed populations of Meloidogyne incognita and M. arenaria larvae incubated in solutions containing glucose ^{14}C or acetate ^{14}C by chromatography and auto-radiography. D. triformis synthesized L-alanine, aspargine, aspartic acid, cysteic acid, cysteine, gamma-amino butyric acid, glutamine, glycine, serine and ornithine ; where as , Meloidogyne spp. synthesized L-alanine, asparagine, aspartic acid, glutamine, glycine, serine and tryptophan.

Rothstein (1965) reported the transfer of single carbon atom from formate to serine, glutamate, aspartate and alanine in C. briggsae.

In 1971, Balasubramaniam and Myers determined the amino acid requirements of Aphelenchoides sp. by lebelling and deletion methods. The absolute dietary requirements were histidine, methionine, phenyl alanine, tyrosine, threonine, lysine and leucine-isoleucine. Alanine, proline, serine, glycine and valine were of limited requirements. As aspartic acid and glutamic acid were found to be synthesized by the nematode, they were not required to be supplemented in the diet.

Vanfleteren (1973) worked on amino acid requirements of C. briggsae and reported that arginine, histidine, lysine, tryptophan, phenyl alanine, methionine, threonine, leucine,

iso-leucine and valine were not synthesized by the nematode, and hence, they were essential to be supplemented in the medium for growth and reproduction. On the other hand alanine, asparagine, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine were non-essential amino acids as they could be sufficiently synthesized by the nematode.

Myers and Balasubramaniam (1973) in axenic culturing of Aphelenchoides rutgersi demonstrated that iso-leucine, leucine, methionine, phenyl alanine, threonine, histidine, tryptophan and lysine were essential for the reproduction of this nematode.

Jackson (1973) cultured Neoaplectana glaseri on kidney slices and found that in addition to the 9 amino acids lysine, tryptophan, histidine, phenyl alanine, leucine, iso-leucine, threonine, methionine and valine, essential to mammals, arginine is essential to N. glaseri and tyrosine is marginally essential.

In the same year Jackson and Ratanarat-Brockelman determined the amino acid nutrition of two nematodes N. glaseri and Rhabditis maupasi. In both the cases lysine, tryptophan, histidine, phenyl alanine, leucine, iso-leucine, threonine, methionine, valine and arginine were essential for reproduction. Tyrosine increased the reproduction performance of N. glaseri and iso-leucine was essential for all stages of Rhabditis maupasi development.

Roy (1975) while culturing Chiloplacus lentus on nutrient broths supplemented with various aliphatic, aromatic and heterocyclic amino acids and many mono, di and oligo-saccharides. Amino acids were shown to promote high population levels whereas glucose, erythrose, fructose, raffinose, ribose, mannose and trehalose were suitable supplements for amino acids.

Pinnock and Stokstad in 1975 observed a 20 fold increase in the population of Caenorhabditis briggsae over control in leucyl-phenyl alanine supplemented medium with haemin. Also casaminoacids (Peptides + amino acids) stimulated growth to a population level but 1.5 times that of the control.

Lu, Hieb and Stokstad (1976) confirmed from experimental culture that methionine is an essential amino acid for C. briggsae and homocystein can substitute for methionine at optimal levels of vit. B_{12} and folic acid, thereby suggesting that C. briggsae possesses a mechanism for biosynthesis of methionine from homocystein as substrate.

Vanfleteren and Avau (1977) treated C. briggsae with aminopterin in growth promoting media which induced thymine deficiency and led to sterility. The omission of essential amino acids from the medium during thymine starvation prevented larval growth at every stage.

Rutherford, Webster and Barlow (1977) showed that uptake of glucose and amino acids by Mermis nigrescens

in vitro are trans-cuticular processes but are not energy requiring.

D. AMINO ACID ANTI - METABOLITES :

Prasad and Webster (1967) exhibited anti-metabolitic effects of some amino acids on *Aphelenchoides*, *Nacobbus* and *Heterodera*. They found that DL-amino-butyric acid significantly decreased the number of *Aphelenchoides* and *Nacobbus* galls by91% and 89% respectively. DL-alanine also significantly reduced the number of *Aphelenchoides* and *Heterodera* females over control. DL-valine and DL-methionine were reported to possess antimetabolitic activities.

Krishnamurthy Rao and Prasad (1969) demonstrated the soil application effects of 11 DL-forms of amino acids and 8 L-forms on *R. reniformis* infesting tomato seedlings, the DL-forms of tyrosine, serine, valine, threonine, proline and methionine were found effective in reducing the nematode population in roots as well as in soils, while their L-forms enhanced the population both in roots and soil. L-forms of serine, lysine and methionine showed lower population than control, but was more than that in DL-forms. However, treatments receiving DL-forms of amino acids had significantly lower population of nematodes both in soil and roots as compared to that in corresponding L-forms. Application of the total quantity of amino acids at one time was more effective than split application in two equal dosages.

In 1971, Evans and Trudgill investigated the effects of amino acids on the reproduction of H. rostochiensis. DL-methionine was found least toxic to the host plant potato and most toxic to the nematode.

Kisiel, Himmelhoch and Zuckerman (1973) studied the effect of aminopterin, the DNA synthesis inhibitor, on fine structure of Caenorhabditis briggsae and reported that this prevented reproduction of the nematode and reduced longevity, resistance to shock etc. which were symptomatics of accelerated aging.

Krishna Prasad and Setty (1974) investigated the effect of three amino acids DL-threonine, DL-serine and DL-alanine as anti-metabolites on root-knot nematode M. incognita on tomato. The amino acids as foliar spray did not have any adverse effect on the growth and vigour of tomato plant, but significantly affected the development and reproduction of nematode except DL-threonine.

Johnson and Shamiyeh (1975) while studying the effect of soil ammendments on hatching of M. incognita eggs found that addition of inorganic ammonium compounds, amino acids or protein hydrolysate to soil containing equivalent concentration of that in soil with 4% lucern inhibited hatching.

Dusenbery (1975) while testing the chemotactic responses of C. elegans to some amino acids indicated the avoidance of DL-tryptophan by the nematode. L-tryptophan had an inhibitory effect on the response, but no significant

reactions were found due to the D- and L-forms of alanine, leucine, methionine, phenyl alanine, serine and valine.

Andreeva, Ermolin and Tarakanov (1975) reported the presence of antigens in extracts of Aphelenchus avenae, N. glaseri and Dictyocaulus filaria by immuno-electrophoresis and double diffusion on gel. N. glaseri had 7 and A. avenae 9 antigenic components and D. filaria had 2 antigens in common with the other two.

Reddy, Govindu and Setty in 1976 studied the action of DL-methionine on M. incognita in tomato and found the amino acid to reduce root galling and egg mass production and also to delay life cycle of the nematode.

Naqvi and Saxena (1976) while testing the amino acids for mortality of R. reniformis in vitro revealed that highest mortality (100% after 48 hours in 10^{-1} M solutions) were obtained with glutamine and glycine. L-arginine and L-histidine did not have any lethal effect.

E. EFFECT OF ULTRASOUND ON NEMATODES :

Cairns (1961) exposed soil and plant nematodes to a 40 watt ultra-sonic generator operating at a frequency of 90,000 cycles/second. Nematodes suspended in water were killed quickly than those in moist sand. Small size nematodes were found to be more resistant to ultrasound than large ones.

In 1967, Clarke and his associates in course of their experiments on the amino acid composition of egg shell

protein of H. rostochiensis dis-integrated the egg shell by an MEL 60 watt ultrasonic disintegrator operating at a frequency of 20 kilo cycles/second for 10-15 minutes keeping the suspension constantly in an ice-bath with a view to restrict the temperature below 40°F.

CHAPTER-III
MATERIALS AND METHODS

MATERIALS AND METHODS

A. COLLECTION OF NEMATODES :

With a view to get a high population of the experimental nematode, *Pratylenchus* sp., a preliminary survey was made collecting soil samples from different locations, nearer to the Orissa University of Agriculture and Technology. Finally a highland area of the progeny orchard, in close proximity of the Khandagiri hills, and showing a very high population of the test organism, was selected.

The soil samples were collected mostly from the rhizosphere of banana (*Musa* sp.) plantation. Infected rhizomes of banana were also obtained which showed brown or dark brown conspicuous lesions, the characteristic symptoms of this nematode attack. The samples were brought to the laboratory for processing in polythene bags and stored, if necessary, in the lower most chamber of the refrigerator for not more than two days.

B. EXTRACTION OF NEMATODES :

(i) Extraction from plant samples (rhizomes) :-

The nematodes were extracted from the banana rhizomes, by Maceration-filtration technique (Stemerding, 1964). The infested rhizomes were first washed free of soil. The lesion portions were separated out and cut into small pieces of 1 to 1.5 cm in length. About 10 g of the chopped materials were placed in an electrically operated

warring blender along with about 10 ml of water. The blender was operated at maximum speed for 5 to 10 seconds in order to soften the tissue and liberate the nematodes from the plant parts. Then the suspension (the nematodes with macerated plant tissue) was poured over a piece of double layered tissue paper spread over a saucer shaped supporting wire gauge. The wiregauge-tissue paper assembly was then placed on an extraction dish (petridish containing enough water to touch the tissue paper. After two days the water suspension of the extraction dish was transferred to a beaker for examination as it contained the nematodes which had moved through the tissue paper into water down below.

(ii) Extraction from soil samples :-

The nematodes from the collected soil samples were wet-screened as per the Improved Baermann funnel technique (Schindler, 1961).

In a shallow aluminium pan about 250 g of soil was taken to which 3 litres of water was stirred in until the clods were broken. The soil suspension was allowed to stand for 10 seconds to let the sands and heavier particles settle down. The supernatant soil suspension was poured through phosphoro-bronze-wire-netted sieves of 60, 100 and 350 mesh sizes. The sediment at the bottom of the pan was flushed with water two more times, allowed to settle down for 10 seconds each time and screened through the same sieves.

The residue at the bottom of the pan apparently free from nematodes, was discarded. The nematodes collected on the 100 and 350 mesh sieves were transferred to a aluminium pan with a gentle flow of water and then to 250 ml beakers in reduced volume of water. The nematode suspension thus obtained was poured over a piece of tissue paper spread on the supporting aluminium wire-gauge. The wire-gauge-tissue paper assembly was placed on a petridish containing sufficient amount of water so as to submerge slightly the bottom of the wire-gauge. The living and active nematodes wriggled through the tissue paper and were collected at the bottom of the petridish. They were recovered after 48 hours.

Thus the contents of several petridishes were pooled into one beaker and it was kept in the refrigerator at 4°C allowing the nematodes to settle down. Then, discarding the supernatant, a concentrated nematode suspension was obtained for isolation by picking.

C. IDENTIFICATION OF THE SPECIES :

(i) Killing and fixing of Nematodes :-

Some of the nematodes which were collected as above were killed, fixed and preserved for future identification. About 5 ml of the nematode suspension was taken in a small specimen tube. The tube was held over a flaming spirit lamp, rotating it for 8-10 seconds. During warming the nematode suspension, the nematodes were observed under stereoscopic microscope periodically.

The nematodes twisted first and then they straightened out suddenly. At this point heat rigor occurred, hence the specimen tube was removed from over the flame immediately. A fixative (F.A.) was prepared by mixing formaldehyde and glacial acetic acid in 4:1 (V/V) proportion. Subsequently, the dead nematodes were transferred into 5 ml of this fixative solution.

(ii) <u>Processing of Nematodes</u> :-

. Glycerol-ethanol Method (Seinhorst, 1959 and 1962)

The nematodes from the fixative solution were transferred to a small glass dish containing 0.5 ml of the following mixture :

Ethanol 96%	20 parts
Glycerol	1 part
Distilled Water	79 parts.

The small glass dish with nematodes in Seinhorst's solution was covered with a cover slip and was placed in a closed glass vessel (a desiccator) containing an excess (1/10th of its volume) of 96% ethanol and closed air tight. Then this was placed in an incubator at 36°C atleast for 12 hours. This procedure removed water from the solution and left the nematodes in a mixture of glycerol and ethanol. The container with the nematodes was refilled with a solution of 96% ethanol and glycerol (93:7, V/V) and placed in a closed petridish at 40°C till the ethanol evaporated. Thus the nematodes were

left in pure glycerol suitable to be mounted on the slides.

(iii) Preparation of permanent slides :-

Glycerine method was adopted for preparation of the permanent mounts. Glycerine was filtered through filter paper and preserved in a dropping bottle inside a desiccator containing fused calcium chloride ($CaCl_2$) for dehydration. A small drop of dehydrated glycerine from the dropping bottle was placed in the centre of a clean aluminium slide. Nematodes of approximately equal shape and size were transferred into the drop with the help of a clean sharp hand-pick. They were arranged properly with their heads pointing to the same direction at the centre of the glycerine drop. They were pressed gently with a bamboo splinter, so that they rested on the bottom surface of the slide. Three glass-rod pieces of almost equal diameter to that of the nematodes, were placed radially and peripherally. Then a clean round glass cover slip was warmed over a flame and placed carefully over the glycerine drop. The cover slip rested over the supporting glass rods quickly without disturbing the nematodes much.

The cover slip, then, was fixed at three points with droplets of diluted zut (Thorne's cement), applied with the help of a fine brush. These drops were allowed to dry and then the cover slip was ringed with a layer of thin zut first and later with a coating of thick zut. The permanent mounts thus prepared were labelled properly and

preserved for further identification of the nematode species. Subsequently they were identified as <u>Pratylenchus coffeae</u> (Zimm. 1898) Filip. and Stekh., 1941.

D. <u>SURFACE STERILIZATION OF NEMATODES</u> :

The collected nematodes were surface sterilized by holding them in aqueous mercurochrome solution (1%) for a period of one hour. First the nematodes were brought under the field of a binocular stereoscope and transferred to a petridish by sucking in individual nematodes through a capillary pipette. In the petridish the nematodes were washed 3 times with sterile distilled water. The nematode suspension was then measured and an equal volume of 2% mercurochrome was added to it. The nematodes were kept as such for one hour. They were then washed free of mercurochrome in 5 changes of sterile distilled water in a culture room.

E. <u>PRESERVATION AND COUNTING OF COLLECTED NEMATODE SAMPLE</u> :

After surface-sterilization, the nematode suspension was re-filtered by pouring it over a single layer tissue-paper-wire-gauge-assembly placed over an extraction dish containing sufficient sterile distilled water just to touch the tissue paper and kept for 24 hours. All the living and active nematodes (Adults & larvae) escaped to the dish thereby separating all other foreign materials. Now the pure and clear nematode suspension in the dish was put under a binocular stereoscopic microscope from which

the nematodes were picked up one by one with the help of a specially made and alcohol treated bamboo pick. They were collected in 100 ml sterile distilled water contained in a sterilized conical flask of 250 ml capacity. During picking the nematodes were counted to determine the total population. The conical flash was kept in deep freeze for subsequent use (Smith and Ellenby, 1967) till the required number of 75,000 nematodes was reached (Nicholas, Dougherty and Hansen, 1960).

F. COLLECTION OF NEMATODE EXCRETION :

With a view to get the excretory materials out of the nematode quickly it was necessary to apply some physical stress. The nematode excretions are generally water-soluble. Hence, the conical flash containing 75,000 of the living specimens in 100 ml of distilled water was put over an electrically operated reciprocating shaker operating at medium speed, shaking it over night. Next day the suspension was taken out and was centrifused at 2,000 rpm for 5-7 minutes using sterilized plastic centrifuse tubes. The supernatant liquid was collected in a sterilized specimen tube which contained the water soluble nemic excretions. The volume of this solution was reduced to a few ml by evaporating over an ice-bath by means of a hot air-blower. This was preserved in sterilized small glass vials in the refrigerator for subsequent use in chromatography.

The bottom portion of the centrifuse tubes retained

all the nematodes which were collected into sterilized snap-top plastic containers, with distilled water and were kept in freezer chamber.

G. PROCESSING OF NEMATODES :

Subsequently the contents of all the plastic containers were poured into a 500 ml sterilized measuring cylinder and the cylinder was kept in a refrigerator at 4°C. After every 4 hours, the supernatant liquid from the cylinder was removed by aspiration leaving the bottom 20 ml that contained almost all the nematodes (Nicholas, Dougherty and Hansen, 1960). In this way several washings were done to remove the particulate materials that might have inadvertently got into the suspension. The bottom suspension was then centrifused at 2,000 rpm for 10 minutes in order to concentrate the nematodes. The supernatant was poured down and the centrifused materials were put in a previously weighed clean watch glass. The watch glass with the nematodes was put in an oven at 105°C until it gave a constant weight (Nicholas, Dougherty and Hansen, 1960). The dry weight of the nematode mass was 7.6 mg.

The remnants of the watch glass were washed carefully into a 50 ml sterilized beaker with 80% ethanol. The amount of ethanol did not necessarily exceed 5 ml.

H. SONICATION AND HOMOGENISATION OF NEMATODES :

High frequency sound waves were used to disintegrate

the tissue of nematodes to release the free amino acids. They were sonicated with an ultrasonic disintegrator of 1,000 volts (80 millimpere) with a stainless steel probe of 5 mm diameter operating at 28 kilocycles per second for 15 minutes (Clarke, 1968). But this quantum energy failed to disintegrate the tissues completely but softened the tissue that facilitated easy homogenisation. After sonication the nematodes were homogenized by an electrically operated tissue homogeniser (mortar and pestle type) for 10 minutes. All these operations were carried on at very low temperature, keeping the material constantly in ice-bath (Clarke, 1968).

The homogenised mass was centrifused at 3,000 rpm for 15 minutes. The supernatant liquid was poured into a clean watch glass where it was evaporated to free it from alcohol. To reduce the volume of extract, the water was allowed to evaporate by the help of hot-air blower. The viscous liquid was taken, dissolved in 1 ml of 10% iso-propanol and transferred into small, clean glass vials for subsequent use in paper chromotography. This was preserved in the freeze compartment of a refrigerator until further use.

I. <u>TECHNIQUE FOR PAPER CHROMATOGRAPHY</u> :

Whatman No.1 chromatographic paper of size 27.5 cm x 27.5 cm was used for paper chromatography. It was appropriately marked and spotted with the test sample which had been prepared earlier. For spotting of the

test sample on the paper very fine capillary glass tube was used. The size of the spot was restricted to smallest possible area on the origin, which was facilitated by means of a hot air-blower during sample application on the paper.

Two dimensional ascending paper chromatography technique was used. The two solvents used were; n-butanol-acetic acid-water and phenol-water-ammonia in the proporation of 160:40:40 and 180:20:1, respectively (Smith, 1960).

The n-butanol-acetic acid-water was used as first solvent, whereas, phenol-water-ammonia as the second solvent.

After spotting with the test sample, the paper was folded so as to form the shape of a cylinder; its edges were fastened together carefully avoiding overlapping. This paper cylinder was placed in an upright position in a petridish (15 cm diameter) containing 50 ml of n-butanol-acetic acid-water, the first solvent. The petriplate was placed on a glass sheet and these were covered with a glass bell jar (32.5 cm x 30 cm), which served the purpose of a chromatographic chamber. The bell jar was made air tight by sealing its rim with vaseline in contact with the glass sheet. This ensured a saturated atmosphere inside the bell jar so as to provide saturation of the stationary phase of the paper.

The test sample spot was at the lower end of the paper just above the solvent, but not in direct contact. The bell jar chamber was made airtight as before. The paper cylinder was left as such for about 10 hours when the first solvent reached the close proximity of the upper edge of the paper. After the flow of the first solvent was completed, the paper was carefully taken out of the chamber and dried for about 2 hours in room temperature to allow the evaporation of n-butanol-acetic acid-water solvent. Subsequently, the paper was run in 50 ml of phenol-water-ammonia, the second solvent. The dried chromatogram was again folded in the form of a cylinder in perpendicularly opposite direction. It was placed in 50 ml of the second solvent in the petriplate (15 cm diameter) and run in the same manner for about 10 hours as for the first solvent inside the air tight chamber. By the end of this period the second solvent reached very close to the upper edge of the paper. Again the chromatogram was carefully taken out of the bell jar, unfastened and dried in air.

After the chromatograms were completely air-dried by hanging with wooden clips inside the laboratory at room temperature (30°C) for 12 hours (over night), and when phenol-water-ammonia solvent got evaporated, they were developed with 0.25% ninhydrin solution dissolved in acetone. The chromotograms were developed by dipping

them in an enamel tray containing the reagent. Sufficient amount of freshly prepared ninhydrin color reagent was poured into the tray and the chromatograms were individually immersed into it. To provide uniform flow of the colour reagent, one edge of the paper was first immersed in it and from the other edge the chromatogram was briskly pulled out, after allowing it to remain in contact with the ninhydrin solution for few seconds. Acetone was allowed to evaporate from the chromatogram again by hanging them at room-temperature (30°C). After it was air-dried, the chromatogram was put in an oven, maintained at 75°C, for 15 minutes when the coloured spots of various amino acids became distinctly visible on the chromatograms. Immediately after detecting the coloured spots on chromatograms, they were outlined with a pencil so as to retain their original location. The central points, the drakest points of the spots were also marked simultaneously for calculating Rf values later on.

The identification of free amino acids was done by preparing standard chromatograms with pure amino acids. Twenty one amino acids (including two amide forms) were used for the purpose of preparing standard maps. Those were aspartic acid, glutamic acid, L-serine, DL-threonine, L-(-)tyrosine, histidine, L-methionine, L-leucine, L-proline, glycine, L-arginine, L-(+)cysteine, L-cystine, L-phenyl alanine, L-tryptophan, L-alanine, valine, lysine, iso-leucine,

L-asparagine and L-glutamine. The standard solution of each amino acid was made by dissolving 10 mg of each into 20 ml of 10% iso-propanol. Four amino acids namely, cystine, glutamic acid, phenyl alanine and tryptophan could not be dissolved very eassily requiring addition of few drops of 100% hydrochloric acid to dissolve them. With a view to concentrate the solutions, the volume of each was reduced from 20 ml to 10 ml by evaporating the iso-propanol. To prepare a mixture of the twenty one amino acids 0.2 ml from each stock solution was pipetted out and mixed in a small glass vial. Each of these twenty one amino acids was individually spotted on Whatman No.1 chromatographic paper by the procedure prescribed above. On a second sheet of paper the mixture of all the 21 amino acids was spotted. The former paper was run in single dimension by n-butanol-acetic acid-water (first solvent) and the latter paper (spotted with mixture) was also similarly run two dimensionally, as described earlier, with n-butanol-acetic acid-water first and then with phenol water-ammonia solvent. These were also developed with 0.25% ninhydrin colour reagent solution in acetone after proper drying. Differently coloured spots of amino acids on the chromatograms were outlined with a pencil and their central points marked. These standard maps were used to identify the free amino acids developed on the chromatograms with unknown nematode homogenate and excreted samples.

Throughout the chromatographic technique, same proportion and grade of solvent chemicals and the colour reagents were used for each treatment with a view to maintain maximum possible uniformity in the experimental procedures.

CHAPTER-IV
EXPERIMENTAL FINDINGS

EXPERIMENTAL FINDINGS

The free amino acids of the nematode homogenate and excretion which, on oxidative deamination with ninhydrin, gave ninhydrin positive (coloured) spots on the chromatograms were identified mainly on the basis of their positions on the papers, the colour reactions with the spray reagents and the Rf values in comparision with the standard chromatograms drawn using known aminoacids.

Rf VALUES :

When the solvent front is not allowed to travel off the paper, the Rf value is defined as :

$$Rf = \frac{\text{distance the substance has travelled from origin}}{\text{distance the solvent front has travelled from origin}}$$

As the Rf value, thus obtained, is a fraction, it was conveniently expressed as a percentage. Further, the Rf value is an arbitrary value, hence for identification the basic configuration and colour of the amino acid spots on chromatograms were also considered giving due emphasis.

A. Rf VALUE OF STANDARD AMINO ACIDS :

All the twentyone amino acids of known strength were spotted individually and run in n-butanol-acetic acid-water solvent one-dimensionally. After developing them with ninhydrin solution in acetone the central points of the ninhydrin positive spots were marked and measured from the base line (x-axis), which gave the distance the substance has travelled from the origin. It was divided by the distance solvent front has travelled and the Rf values were

calculated in percentage, as presented in Table 1.

Table 1: The Rf values of standard amino acids
(1-D, chromatography).

~~Sl.~~ No. of spots	Name of the amino acid	Rf value in 100 %
1.	Aspartic acid	4.8/24.0 = 0.20 x 100 = 20
2.	Glutamic acid	6.0/24.0 = 0.25 x 100 = 25
3.	L-serine	4.5/24.0 = 0.19 x 100 = 19
4.	Glycine	5.1/24.0 = 0.21 x 100 = 21
5.	DL-threonine	6.4/24.0 = 0.27 x 100 = 27
6.	Valine	12.9/24.0= 0.54 x 100 = 54
7.	Iso-leucine	15.6/24.0 = 0.65 x 100 = 65
8.	L-leucine	16.9/24.0 = 0.70 x 100 = 70
9.	L-histidine	2.6/24.0 = 0.11 x 100 = 11
10.	Lysine	2.8/24.0 = 0.12 x 100 = 12
11.	L-arginine	3.8/24.0 = 0.16 x 100 = 16
12.	L-phenyl alanine	14.4/24.0 = 0.60 x 100 = 60
13.	L-(-)tyrosine	10.1/24.0 = 0.42 x 100 = 42
14.	L-tryptophan	12.7/24.0 = 0.53 x 100 = 53
15.	L-proline	8.6/24.0 = 0.36 x 100 = 36
16.	L-(+) cysteine	1.7/24.0 = 0.07 x 100 = 7
17.	L-cystine	1.2/24.0 = 0.05 x 100 = 5
18.	L-methionine	12.0/24.0 = 0.50 x 100 = 50
19.	L-alanine	7.5/24.0 = 0.31 x 100 = 31
20.	L-asparagine	3.6/24.0 = 0.15 x 100 = 15
21.	L-glutamine	5.0/24.0 = 0.21 x 100 = 21

B. <u>Rf VALUE OF STANDARD AMINO ACIDS IN MIXTURE</u> :

A mixture of the 21 amino acids was spotted and separated by two-dimensional chromotography. The Rf values of single amino acid spots were calculated against n-butanol acetic acid-water front. Subsequently the amino acids were

identified by comparing their colour reaction and Rf values with the colour and Rf values of individual amino acid. The Rf values of the separated ninhydrin positive spots from the mixture (Fig. 1) are tabulated below.

Table 2 : The Rf values of spots separated from standard amino acid mixture (2-D chromatography).

Serial No. of spots	Rf values in 100 %
1.	3.6/22.2 = 0.16 x 100 = 16
2.	5.3/22.2 = 0.23 x 100 = 23
3.	6.1/22.2 = 0.27 x 100 = 27
4.	5.6/22.2 = 0.25 x 100 = 25
5.	4.6/22.2 = 0.20 x 100 = 20
6.	5.0/22.2 = 0.22 x 100 = 22
7.	6.3/22.2 = 0.28 x 100 = 28
8.	6.7/22.2 = 0.30 x 100 = 30
9.	1.5/22.2 = 0.06 x 100 = 6
10.	2.0/22.2 = 0.09 x 100 = 9
11.	3.2/22.2 = 0.14 x 100 = 14
12.	2.5/22.2 = 0.11 x 100 = 11
13.	2.6/22.2 = 0.11 x 100 = 11
14.	8.2/22.2 = 0.36 x 100 = 36
15.	10.0/22.2 = 0.45 x 100 = 45
16.	11.1/22.2 = 0.50 x 100 = 50
17.	12.1/22.2 = 0.54 x 100 = 54
18.	12.7/22.2 = 0.57 x 100 = 57
19.	15.0/22.2 = 0.67 x 100 = 67
20.	16.0/22.2 = 0.72 x 100 = 72
21	14.2/22.2 = 0.63 x 100 = 63

Comparing the Rf values of the above ninhydrin positive spots on the chromatogram of the mixture with that of the Rf values and colour of single amino acid

chromatograms, the relative positions of the amino acids on mixture chromatogram were found out. They were identified as follows corresponding to their respective serial numbers :

1. L-asparagine
2. L-glutamine
3. DL-threonine
4. Glycine
5. L-serine
6. Aspartic acid
7. Glutamic acid
8. L-alanine
9. L-cystine
10. L-(+) cysteine
11. L-arginine
12. L-histidine
13. Lysine
14. L-proline
15. L-(-)tyrosine
16. L-methionine
17. L-tryptophan
18. Valine
19. Iso-leucine
20. L-leucine
21. L-phenyl alanine

IDENTIFICATION OF AMINO ACIDS IN HOMOGENATE CHROMATOGRAM :

The calculated Rf values of ninhydrin positive spots on the chromatogram of the homogenate (Fig.2) have been indicated in Table 3 .

Table 3 : Rf values (in percentage) of ninhydrin positive spots on homogenate chromatogram (2-D chromatography).

Serial No. of spots	Rf values
1.	3.5/20.1 = 0.17 x 100 = 17
2.	5.1/20.1 = 0.25 x 100 = 25
3.	3.7/20.1 = 0.18 x 100 = 18
4.	4.3/20.1 = 0.21 x 100 = 21
5.	5.3/20.1 = 0.26 x 100 = 26
6.	6.8/20.1 = 0.33 x 100 = 33
7.	3.1/20.1 = 0.15 x 100 = 15
8.	1.6/20.1 = 0.07 x 100 = 7
9.	6.9/20.1 = 0.34 x 100 = 34

Comparing the Rf values, colour offered by the reagent and configuration of ninhydrin positive spots on sample chromatogram with that of the mixture chromatogram, the following free amino acids, arranged according to the serial number of the spots, were identified to be present in the homogenate of Pratylenchus coffeae :

1. L-serine
2. DL-threonine
3. Aspartic acid
4. Glycine
5. Glutamic acid
6. L-alanine
7. L-arginine
8. L-(+)cysteine
9. L-proline

FIGURES

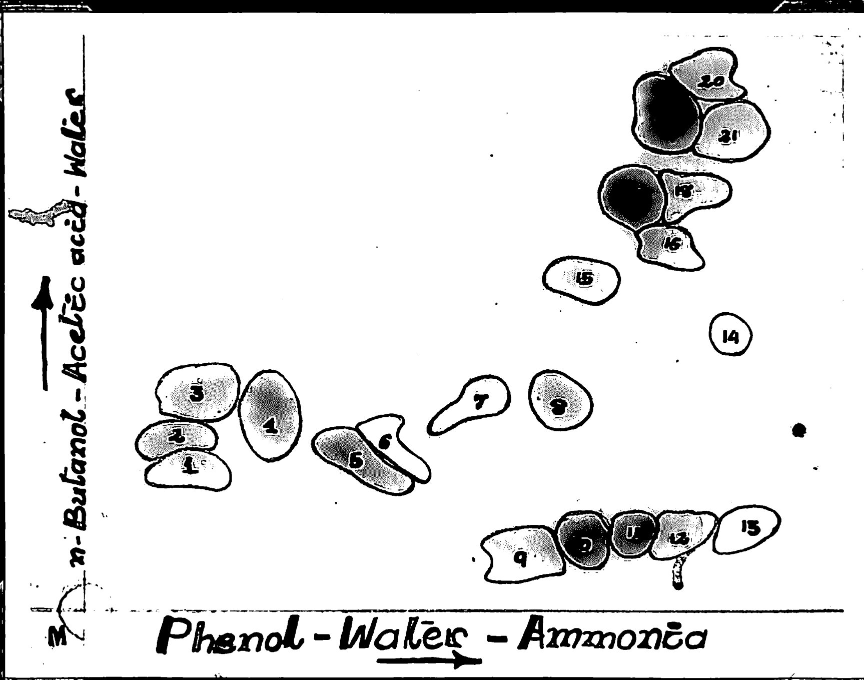


Fig. 1.

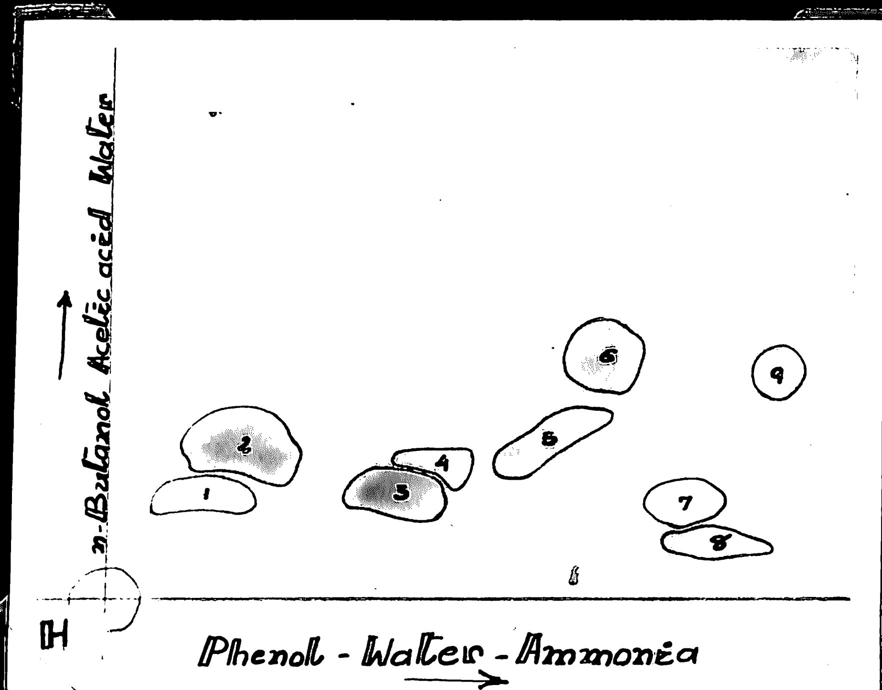


Fig. 2.

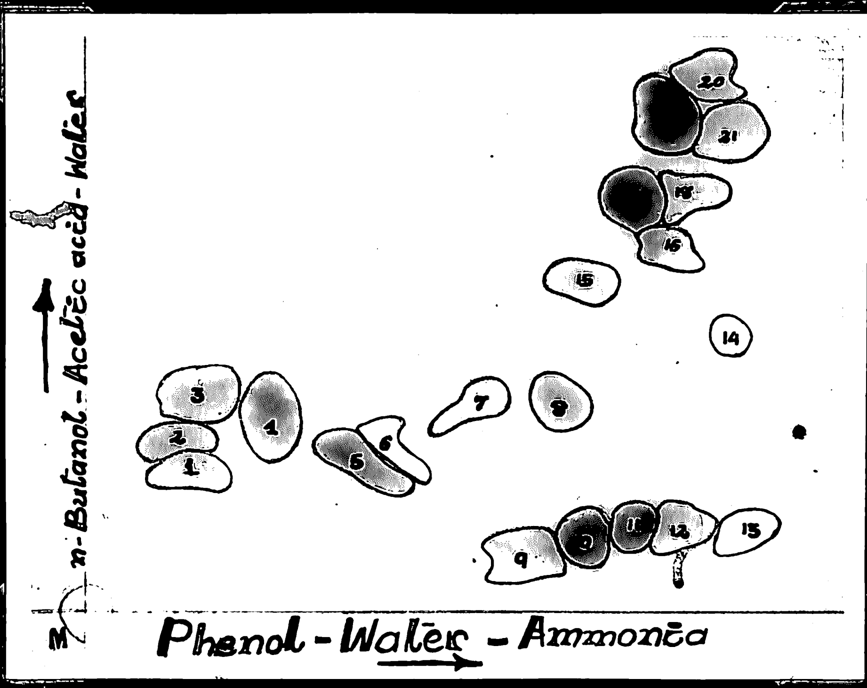


Fig. 1.

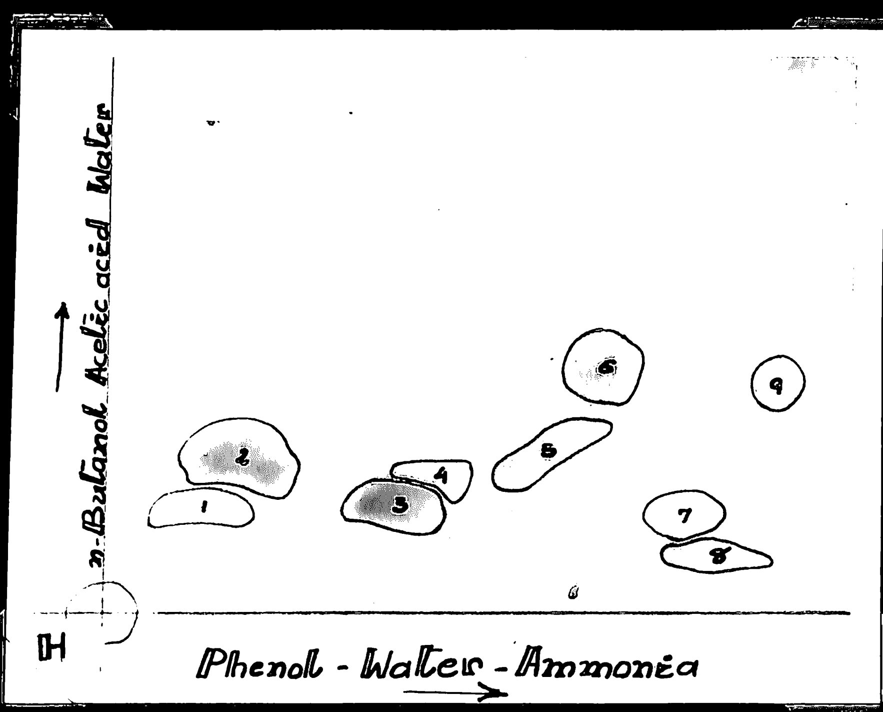
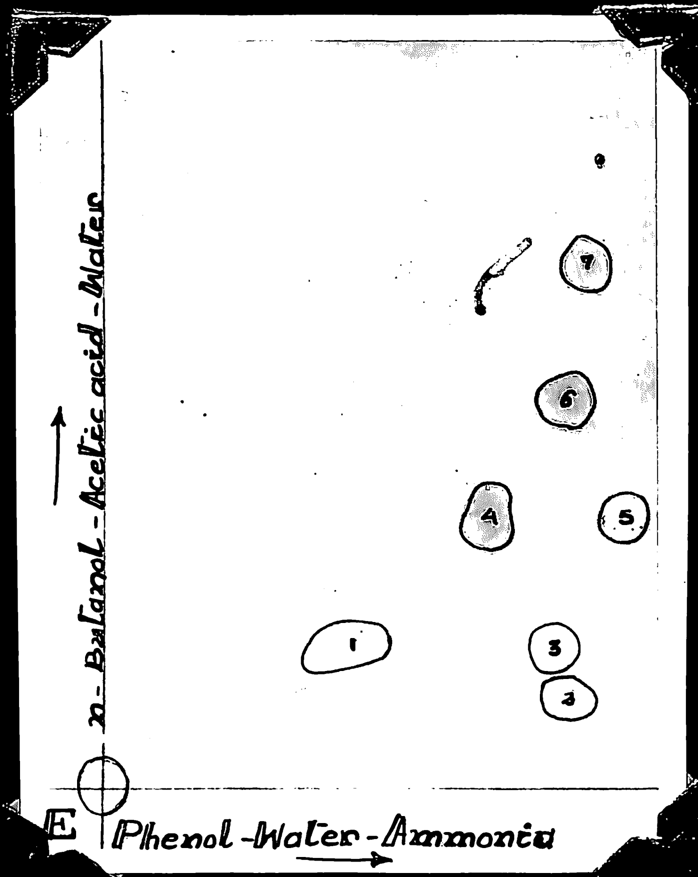


Fig. 2.

Fig. 3.



D. IDENTIFICATION OF AMINO ACIDS IN EXCRETION CHROMATOGRAM :

The Rf values of the ninhydrin positive spots on the chromatogram prepared with nematode excretion (Fig. 3) have been indicated in Table 4.

Table 4 : The Rf values (in percentage) of ninhydrin positive spots on excretion chromatogram (2-D chromatography).

Serial No. of spots	Rf values %
1.	4.6/24.0 = 0.19 x 100 = 19
2.	2.8/24.0 = 0.11 x 100 = 11
3.	4.4/24.0 = 0.18 x 100 = 18
4.	8.3/24.0 = 0.34 x 100 = 34
5.	8.9/24.0 = 0.37 x 100 = 37
6.	12.0/24.0 = 0.50 x 100 = 50
7.	16.9/24.0 = 0.70 x 100 = 70

Comparing the colour given by the reagent, Rf values, the configuration and relative position of the ninhydrin positive spots on sample chromatogram with that of the mixture chromatogram the following free amino acids were identified to be present in the excretory products of *P. coffeae*. They have been arranged below serially.

1. L-serine
2. Lysine
3. L-arginine
4. L-alanine
5. L-proline
6. L-methionine
7. L-leucine

Thus, four amino acids, namely, L-serine, L-alanine, L-arginine and L-proline were found to be common to both the nematode homogenate and excretion. In addition to these, the homogenate contained DL-threonine, aspartic acid, glycine, glutamic acid and L-(+) cysteine; and the excretory materials contained lysine, L-methionine and L-leucine. In total the homogenate and the excretion showed the presence of 9 and 7 free amino acids, respectively.

CHAPTER-V
DISCUSSION

D I S C U S S I O N

Although there has not been much progress in studies relating to pathogenic association of nemic phytoparasites, yet it is generally believed that infection by any causal agent usually induces a series of biochemical changes in the host. In this respect responses produced by the plant cells appear to be equally active as that of the animal cells. Thus, in any such event involving plant parasitic nematodes, the post-infection physiology constitutes remarkable alterations in the metabolism not only of the host cells (Kanan, 1968 ; Epsten and Kohn, 1971 ; Epstein, 1972) but also of the pathogen (Asit and Riggs, 1969). Such interactions are generally responsible for the production of profound and conspicuous changes particularly in the protein synthesis and enzyme activity of the host cells. As resistant hosts show a greater rate of change in protein synthesis, this is often considered as an useful parameter to measure resistance or susceptibility of the hosts to the pathogens. Conversely, the synthesis of parasite-protein, as effected by the host, may be accepted for the determination of different pathogenic races. Thus the ability of a parasite in effecting a disease or that of the host in expressing resistance or susceptibility largely depends upon the nature of the protein synthesis mechanism, and influence of their metabolism on the

complicated host-parasite interactions.

In order to establish the existence of such a biochemical mechanism in the host-parasite relationship, a preliminary study has been made here to detect the free amino acid contents of the homogenate and discharges of a migratory endoparasitic nematode *Pratylenchus coffeae*, widely prevalent in local soils as a potential pathogen of many economic plants, such as, citrus, banana coffee etc.

The results obtained by employing the conventional paper chromatography technique (Smith, 1960) revealed the presence of the following free amino acids in the nematode homogenate: L-serine, DL-threonine, aspartic acid, glycine, glutamic acid, L-alanine, L-arginine, L-(+)cysteine and L-proline.

In the recent past, many investigators (Krusberg, 1961 ; Myers, 1963 and others summerised by Krusberg, 1971) have showed the presence of as many as 8 to 26 amino acids and amides in different plant parasitic nematodes. The aminoacids detected above have also been included in them. However, in this study, only 9 aminoacids have been identified and the presence of others could not be ascertained, probably due to their presence in very small quantities. It is also possible that the experimental procedure followed was not sophisticated and precise enough to detect their presence.

In general, the endoparasites are believed to be better adapted to parasitism. Besides, the occurrence

of free amino acids is also a very characteristic feature of the nematode species. According to Myers and Krusberg (1965), the higher plant parasitic nematodes possess and discharge greater quantities of amino acids. It is , therefore, obvious, that with only 9 amino acids, P. coffeae occupies a much lower position in pathogenicity scale than Meloidogyne, Ditylenchus and Aphelenchoides.

Some amino acids like aspartic acid, glutamic acid, serine, threonine, alanine and arginine are more common in many phytophagous nematodes ; whereas, other like proline, methionine, lysine and lucine are less frequently encountered. But in the homogenate chromatogram of the test organism, the intensity of the spot indicated the presence of proline in greater quantity.

On the excretion chromatogram of P. coffeae only seven amino acids were clearly found to be present. They were L-serine, lysine, L-arginine, L-alanine, L-proline, L-methionine and L-leucine. Here also proline appeared to be abundant and the uncommon ones observed were leucine and methionine. There were also some more faint coloured patches and strips which could be detected on the chromatogram but could not be identified with certainty. It is possible that they indicated the presence of some more amino acids or their compounds but were present perhaps in very minute quantities.

Both the homogenate and excretion had four amino acids in common, namely, L-serine, L-alanine, L-arginine

and L-proline. The presence of other five in the homogenate and their absence in the excretion suggest that they may be essential and used up in biosynthesis. It is also likely that they were not available in excess so as to be excreted out of the nematode body. But the three more amino acids obtained from the excretion chromatogram, namely L-methionine, L-leucine and lysine were perhaps incidentally released extraneously from some bound froms to meet specific requirements of this endophytic species. They may be helping the nematode species in neutralising the toxic plant metabolites or in stabilizing the osmoregulatory mechanism inside their host tissue (Musa sp.). These are, however, only speculations as it could not be confirmed here through actual experimentation and as details regarding amino acid contents of the host root are not available.

The DL-forms of amino acids, such as, DL-alanine, and DL-serine have shown antimetabolitic activities (Krishna Prasad and Setty, 1974) against root-knot nematodes but not DL-threonine. Here L-forms of serine and alanine were detected alongwith DL-form of threonine. Hence, as in the case of M. incognita on tomato, DL-threonine perhaps has no antimetabolitic effect on the test organism, P. coffeae and it might be essential for the nematode species. The high concentration of proline suggest that either this amino acid is abundantly present

in the host root as a part of the defence mechanism of the host or it is profusely synthesised inside the body of the nematode and the excess unused amount is excreted.

Presence of these free amino acids may also provide some clues on the nutritional requirement as well as the nature of protein synthesis of the parasite. However, we can not definitely say, which are essential, non-essential or which have implications in pathogenisis. On the basis of this study we can only speculate, but not conclude also, if these amino acids were directly drawn from the host or synthesised by the nematode.

Another possibility relating to the existence and function of these amino acids is to detoxify the excess ammonia accumulating in the nematode tissues thus excreting a non-toxic excretory product (Myers and Krusberg, 1965). In this aspect, aspartic acid and glutamic acid are thought to be helpful, particularly in dry soil or in decomposing host tissue where the environment may also be rich with ammonia and the nematode has to neutralise the effect by excretion of amino acids and such other materials.

Sahu and Das (1974) and Mohanty and Das (1976) have qualitatively determined the free amino acid contents in the ring nematode *Macroposthonia ornata* homogenate and in ragi roots nematised with the same nematode species, respectively. Their work indicated the presence of 6

amino acids namely aspartic acid, glutamic acid, serine, alanine, valine and L-leucine common to the nematode and the host. But 3 others, such as, glycine, L-proline and DL-phenyl alanine were produced by the host, but not found in the nematode. There were three more, like L-glutamine, L-cysteine and possibly tyrosine which were present in the nematode homogenate, but were absent in the infected or un-infected ragi root extracts. The possibility of this type of a system may hold good for our test organism P. coffeae and its prime host, banana, and the situation undoubtedly offers ample scope for further study on protein synthesis mechanism as related to pathogenicity and physiology of parasitism.

In spite of the difficulties encountered in course of this study, such as, in obtaining a large number of nematodes in uninform population at one time, homogenization under aseptic and low temperature conditions, extreamly low concentration of the aminoacids, extreme day and night temperatures and other errors which might have inadvertently affected the results, this preliminary finding provides some useful information which may have important implications for a better understanding of the pathogenisis and host-parasite interactions of this endoparasite.

CHAPTER-VI
SUMMARY

S U M M A R Y

A qualitative detection of free amino acids in the homogenate and excretions of a surface sterilised migratory endoparasitic nematode species, *Pratylenchus coffeae* (Zimm., 1898) Filip. and Stekh., 1941 ; has been demonstrated by paper chromatography.

In the nematode homogenate, the following amino acids were found to be present ; L-serine, DL-threonine, aspartic acid, glycine, glutamic acid, L-alanine, L-arginine, L-(+)cysteine and L-proline having Rf values of 17, 25, 18, 21, 26, 33, 15, 7 and 34, respectively. The nemic excretions showed the presence of L-serine, lysine, L-arginine, L-alanine, L-proline, L-methionine and L-leucine with Rf values of 19, 11, 18, 34, 37, 50 and 70, respectively. Thus 4 amino acids were common to both the nematode homogenate and excretion.

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