SYSTEMIC ACCUMULATION OF NOVEL PROTEINS IN THE APOPLAST OF LEAVES OF COWPEA PLANTS FOLLOWING INFECTION BY ROOT-KNOT NEMATODE, *MELOIDOGYNE INCOGNITA* AND PIGEONPEA CYST NEMATODE, *HETERODERA CAJANI*

By

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A thesis submitted to the Faculty of Post-Graduate School,
Indian Agricultural Research Institute, New Delhi,
in partial fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

IN

NEMATOLOGY

1995

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CERTIFICATE

This is to certify that the thesis entitled **"Systemic accumulation of novel proteins in the apoplast of leaves of cowpea plants following infection by root-knot nematode, *Meloidogyne incognita* and pigeonpea cyst nematode, *Heterodera cajani*"** submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY IN NEMATOLOGY is a record of bona fide research work carried out by **Mr. Rajagopal Raman** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. The assistance and help received during the course of this investigation have been duly acknowledged.

New Delhi,

Date : May 4 : 1995.

(D.R. DASGUPTA)

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ACKNOWLEDGEMENTS

Fortune strikes in many ways. For me it was through the opportunity of working under the direction of Dr. D.R. Dasgupta, Head, Division of Nematology, and Chairman of my Advisory Committee, at the most critical phase of my life. I humbly submit that he is the person who has blended my personality with the virtues, expected not only out of a meticulous and creative researcher but also out of a responsible and compassionate fellowbeing, all by setting himself as an example and never by force. Also, his valued suggestions have helped me in shaping up this manuscript.

I am thankful to Dr. A.K. Ganguly, member of my advisory committee for his constant encouragement and the helpful discussions that I had on various topics during my experimental work.. I am profoundly indebted to Drs. S.C. Dhawan and N.D. Sharma, members of my advisory committee for their help in carrying out experiments and critical review of the manuscript.

I am grateful to Dr. N. Ramakrishnan, Professor of Entomology, and member of my advisory committee for his genuine kindness and valuable suggestions throughout the study and also for providing me free access to all the facilities in his laboratory.

Thanks to Dr. Tiwari, Division of Genetics and Mr. Negi, Division of Seed Technology for providing cowpea seeds, to Dr. H.C. Kapoor (Biochemistry) and Dr. Subrahmanyam (Entomology) for their timely and sustained help by permitting me to use the required instruments in their labs. as and when needed. I acknowledge the help of Drs. S.D. Mishra, Mrs. V. Mojumder, B.K. Goswami and Mrs. S. Ganguly, Division of Nematology, for extending laboratory facilities during my research tenure.

I take this opportunity to express my affection to Drs. Anil Sirohi, Pankaj and H.K. Sharma for their good company. Special thanks are also due to Anil, for he took time from his busy schedule and poured over the manuscript editing it and producing proofs in record time.

I also acknowledge the timely and gracious help rendered by S/Shri Ali Akhtar, Satya Narayan Lal, Shashi Kant, Puran Singh, Gurbux Singh and Thakur Das during my stay at IARI.

At this stage, I revere my KG teacher Mrs. Jayalakshmi for having taken all the pains in laying the foundation of my career and introducing me to the field of learning.

I wish to record my affection to all my friends for the help and support during both good and bad times. Special thanks for Singi, Meens, Venu (Kala), Thomachan and Ramesh, V. for the help rendered during the course of the experiments, and to Ramachandran, Sudha, Jayaraj, Ganesh, Anith, Faizal, Sharad, Jiju, G.T., Nambissan, Rasheed and Senthil for all time support. My thanks to Joshna for a TVS, which made many a tasks possible.

Words fail to express my sense of respect, gratitude and affection towards my parents and my brother Krishna for sharing all my troubles. My respectful regards to my grandmothers for their affection. I am also grateful to all my uncles, aunts and cousins for the affection and concern they have showered on me.

The Director, I.A.R.I., New Delhi is thanked for awarding me the Senior Research Fellowship during the course of this study.

Place : New Delhi, (RAJAGOPAL RAMAN)

Date : May 3rd, 1995.

CONTENTS

INTRODUCTION

Plants are constantly exposed to various biotic and abiotic environmental stresses. Pathogenic bacteria, fungi, viruses and nematodes form a group of biotic menace that plants have to cope up with. Conspicuous by the absence of any specialized surveillance cells, equivalent to that of a mammalian immune system, plants have developed ingenious molecular defensive strategies to combat these pathogens.

All these defense mechanism are mediated through the production of defense related proteins. These defense related proteins can be divided into three broad classes, based on their role in defense responses (Bowles, 1990):

(a) Proteins that change the properties of the extracellular matrix, by strengthening, repairing or altering the wall environment, like hydroxy proline rich glycoproteins (HRGP), glycine rich proteins (GRPs); and enzymes like peroxidase, cinnamyl alcohol dehydrogenase, callose synthase etc. which are involved in the construction and modification of cell wall polymers like suberin, lignin etc.

(b) Proteins associated with deterrence and antimicrobial activity which act either directly as deterrents, or exhibit antimicrobial activity or may catalyse the synthesis of antimicrobial products. They include toxic proteins like thionins and lectins, hydrolases like chitinases, 1,3-glucanases, enzyme inhibitors like amylases- and protease inhibitors, and enzymes involved in production of phenolics, tannins and phytoalexins like phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL), chalcone synthetase etc.

(c) Additional defense related proteins, which include those whose appearance can be correlated with a defense response, but which are of unknown function. These include members of a family of protein called pathogenesis

related proteins(PR), Win 1 and Win 2 proteins, Wun 1 and Wun 2 proteins and elicitor induced proteins .

Pathogenesis related (PR) proteins are a group of proteins induced in plants when they are subjected to pathological and other stress related conditions. They were first discovered independently by Gianinazzi *et al.* (1970) and van Loon and van Kammen (1970) in tobacco plants that exhibited hypersensitive reaction to tobacco mosaic virus (TMV) infection. PR proteins have since been shown to be induced by other pathogens such as bacteria (Ahl *et al.*, 1981), fungi (Gianinazzi *et al.*, 1980) and nematodes (Hammond-Kossack *et al.*, 1989), in a wide range of host plants, both monocot and dicot (van Loon, 1985; White *et al.*, 1987). Several chemicals such as salicylic acid, ethephon, L-methionine, manganese chloride and ionic silver have also been shown to induce production of PR proteins (Bol *et al.*, 1990).

These polypeptides have low molecular weights, accumulate extracellularly in the infected plant tissue, possess extreme isoelectric points and can withstand digestion by a range of proteases (Kombrink *et al.*, 1988). The various PR proteins in tobacco, which incidentally is the crop on which maximum work on PR proteins has been carried out, are classified in 5 major groups (Fritig *et al.*, 1989).

Though the exact role and function of PR proteins is not yet clearly understood, they have been associated with the phenomenon of systemic acquired resistance (SAR) in plants as they are produced in large quantities in hypersensitive and resistant reactions. It has been found that some of the PR proteins, have a specific function *viz.*, PR-2 has a β1,3-glucanase activity; PR-3 has a chitinase activity and PR-5a shares similarity to the sweet tasting protein

thaumatin (Kaufmann *et al.*, 1987; Kombrink *et al.*, 1988 and Metraux *et al.*, 1988).

The induction of PR proteins by plant parasitic nematodes has been reported only on potato by the potato cyst nematode, *Globodera* spp. Hammond-Kosack *et al.* (1989) studied the induction of PR proteins in the apoplast of leaves of potato plants following root infection by *Globodera rostochiensis*, pathotypes Ro1, Ro2, Ro3 and Ro4. Another report stating the induction of PR proteins in the leaves and roots of potato plants infected with Pa 2/3 and Pa1 pathotypes of *G. pallida* has also been published by Rahimi *et al.*, 1993.

It is more than apparent, beyond *Globodera* species, no information is available in literature regarding the induction of PR proteins in various crops by other economically important nematodes. Hence, this study was undertaken in cowpea (*Vigna unguiculata* L.) as it serves as a good model host for several economically important plant parasitic nematodes. An immense treasure of information regarding molecular mechanism of host plant resistance has already been generated from this laboratory, with cowpea as the model crop (Ganguly *et al.*, 1991; Rajagopal *et al.*, 1992;. Sirohi *et al.*, 1993;). Cowpea is attacked by both the groups of nematodes viz., root-knot nematode (*Meloidogyne incognita*) and cyst nematode (*Heterodera cajani*), which cause considerable economic loss in India. Beside a susceptible host (Pusa Komal), also included in this study is cowpea cultivar C-152, a commercially grown resistant cultivar against root knot nematode *M. incognita*, but susceptible to the pigeon pea cyst nematode, *H. cajani* (Somasekhar, 1995). Keeping the above information in mind, this investigation was carried out with the following objectives :

- To study the changes in protein profile from the intercellular fluid (IF) of the leaves of cowpea cv.. Pusa Komal following infection with the root-knot nematode, *M. incognita*, when compared to healthy plants,

- to compare the changes in protein profile of IF of leaves of cowpea between a resistant cv., C-152 and a susceptible cv. Pusa Komal following inoculation with the root-knot nematode, *M. incognita*,

- to compare the changes in protein profile of IF of leaves of cowpea cv. C-152 and Pusa Komal following infection with *M. incognita* and *H. cajani*, and finally

- to study the characteristics of the novel proteins, if produced, from the above experiments .

REVIEW OF LITERATURE

Pathogenesis Related (PR) proteins are characterized by their low molecular weights (ranging from 14,000 daltons to 60,000 daltons) (Antoniw *et al.*, 1980; van Loon, 1972), accumulate predominantly in the apoplastic space (intercellular fluid) of the plants (Parent and Asselin, 1984), are selectively extractable at low pH and are highly resistant to proteolytic enzymes (Pierpoint *et al.*, 1981). Their basic counterparts were discovered later by Bol and van Kan (1988).

These proteins are induced under specific pathological conditions, locally at the site of infection and systemically in parts not directly affected by the pathogen. The PR proteins identified till date can be classified into five major groups based on their molecular weight characteristics, iso-electric point and location. Though the exact role of PR proteins in plant defense has not yet been elucidated, two groups of PR proteins viz., PR-2 and PR-3 have been found to have hydrolytic activity of β 1,3 glucanases and chitinases respectively, which may be used to degrade fungal and bacterial cell walls. Apart from being induced by pathogen attack, most of these genes can also be induced by other forms of stress like wounding, UV light and also by spraying chemicals like salicylic acid etc. Also, many of these proteins are developmentally regulated, appearing in specific plant parts as a part of normal plant development, unrelated to stress conditions. The molecular and cellular biology of PR proteins have been studied with many genes encoding these proteins isolated and characterized. Pathogen induced PR protein gene expression is not only found in incompatible interactions, but also found in some compatible plant-pathogen interactions where they are synthesized rather diffusely at a later time point.

With the above introduction, this chapter will review the major developments in PR protein research with regard to various plant pathogens till date. The presentation of the information in this chapter in the following sections is made arbitrarily for the sake of convenience, but covering the aspects which were of interest to the current investigation.

Pathogenesis Related (PR) proteins were first reported by Gianinazzi *et al.* (1970) and van Loon and van Kammen (1970) simultaneously from tobacco plants reacting hypersensitively to Tobacco Mosaic [illegible] infection. These proteins were found in the soluble leaf protein extract of the infected plants and were conspicuously absent in the healthy plants. These proteins were termed as b-proteins by Gianinazzi *et al.* (1970). Later, similar results were obtained by Antoniw *et al.* (1980), who were also the first to name these proteins as 'Pathogenesis-Related (PR) proteins'. Subsequently, a decision was taken at an international conference at Holland 1983 , to use the term PR proteins for these new protein products as these were induced under specific pathological conditions (Gianinazzi and Ahl, 1983).

Groups of PR proteins :

The maximum work on PR proteins has been carried out on tobacco and these proteins have been broadly classified into five major groups by Fritig *et al.*, 1989(Table 1).

Group 1 : This group consists of 3 serologically related acidic proteins from tobacco plant named PR 1a, PR 1b and PR 1c with molecular weights of approximately 15 kD (van Loon *et al.*, 1987). There is 90 per cent amino acid sequence similarity amongst these three proteins.

In tomato, a basic protein - P14 (molecular weight 14 kD), is produced in the extracellular space following infection by viroids and fungal pathogens (Vera *et al.*, 1989). P14 is serologically related to tobacco PR 1a, b and c proteins (Nassuth and Sanger, 1986) and its amino acid sequence is 60% similar to tobacco PR 1b (Cornelissen *et al.*, 1986). Proteins serologically related to tobacco PR 1 proteins have been detected in dicots like cowpea, potato and *Arabidopsis* and in monocots like maize and barley (Nassuth and Sanger, 1986; White *et al.*, 1987; Metzler *et al.*, 1991).

Group 2 : The PR proteins of this group are found to have a β 1,3 glucanase activity. In tobacco, upon TMV infection, 6 different proteins are induced. Of this, five are acidic in nature viz., PR 2a (PR-2), PR 2b (PR-N) PR-2c (PR-O), PRO' and PRQ'. PR-O' is not serologically related to other PR proteins in this group (Fritig *et al.*, 1989). A basic PR-2 protein, Gluc.b is another member of this group, serologically related to PR-2, N, O and Q' and is present in the cell vacuole (van den Bulcke *et al.*, 1989; Keefe *et al.*, 1990).

Group 3 : The group consists of 2 acidic PR proteins P and Q and 2 basic proteins Ch.32 and Ch.34, all having a chitinase function. All the four proteins in this group are serologically related. As in Group 2, the basic chitinases are located in the vacuole in tobacco plants.

Group 4 : Four tobacco PR proteins induced by TMV, come under this group, which are present extracellularly. They are slightly acidic in nature with S_1 and r_1, having a molecular weight of 14.5 kD and S_2 and r_2 having a molecular weight of 13 kD. The exact function of this group of proteins is yet unknown (Kaufmann *et al.*, 1990). A basic protein from the intercellular fluid of *Cladosporium fulvum* infected tomato showing serological relationship with tobacco PR-4 has been identified. This protein shows 80-90% amino acid sequence similarity with the PR-4 proteins of tobacco (Joosten *et al.*, 1990). These proteins have been found to be chitin binding and to processes antifungal activity against *Trichoderma harzianum* (Hejgaard *et al.*, 1992).

Group 5 : Two acidic proteins of molecular weight 24 kD named R and S have been isolated from tobacco cv. Samsun NN upon TMV infection. These two proteins share 65% similarity with the sweet tasting protein from the tropical herb *Thaumatococcus daniellii* Bath. (Cornellisen *et al.*,1986). Richardson (1987) reported that these thaumatin like PR proteins act as a bifunctional inhibitor of α–amylase/protease inhibitor from maize. It has been clearly shown that these proteins do not have a direct role against insect pests, since transgenic plants constitutively expressing PR-5 were eaten by insect larvae as like control plants (van de Rhee *et al.*, 1994). The basic PR protein in this group is a 24 kD protein, called osmotin, as they are also found in tobacco cells adapted to high levels of NaCl. Osmotin shows similarity to PR-R and S (Singh *et al.*, 1987).

Table 1. PR proteins induced in Samsun tobacco (NN genotypes) by TMV infection

Group	Acidic PR proteins		Basic PR proteins		Function
	Name [a]	Mol. wt.(kD)	Name	Mol.wt.(kD)	
1	1a	15.8	16 kD	16.0	Unknown
	1b	15.5			
	1c	15.6			
2a	2	39.7	Gluc.b	33.0	β-1,3 Glucanase
	N	40.0			
	O	40.6			
	Q'	36.0			
2b	O'	25.0			β-1,3 Glucanase
3	P	27.5	Ch.32	32.0	Chitinase
	Q	28.5	Ch.34	34.0	
4	S1	14.5			Unknown
	r1	14.5			
	S2	13.0			
	r2	13.0			
5a	R	24.0	Osmotin	24.0	Thaumatin like
	S	24.0			
5b			45 kD	45.0	Unknown

[a] Nomenclature is according to Fritig *et al.* (1989).

Systemic induction and localization of PR proteins

The systemic induction of PR proteins has been demonstrated at the level of mRNA accumulation and direct gene activity measurements. Six tobacco PR mRNAs, associated with TMV infection, were shown to be induced within two days in the inoculated leaf and within 8 days in the virus-free upper leaves (Hooft van Huijsduijnen *et al.*, 1986). Transgenic plants containing chimeric genes

consisting of the PR-1 promoter fused to the β-glucuronidase (GUS) reporter gene exhibited a ring of activity around viral induced necrotic lesions 3 days after inoculation and a weaker whole leaf response 5 days later (Ohshima *et al.*, 1990). When younger transgenic leaves were infected, the expression of the chimeric gene was prominent, especially along the vascular elements. Earlier, Carr *et al.*, 1987, have shown that PR-1 proteins accumulate around the xylem of infected leaves.

The above phenomenon has been explained by the existence of a hypothetical mobile moiety/molecule produced at infection sites, which then proceeds to spread throughout the plant via the vascular system. Support for the existence of such mobile moiety/molecule comes from the observations in tobacco species *Nicotiana debneyi* and *N. glutinosa* each of which show inducible PR protein accumulation. Their interspecific cross results in a hybrid progeny that expresses PR proteins constitutively (Ahl *et al.*, 1982; Lotan and Fluhr, 1990). When the hybrid amphidiploid was used as rootstock for grafting either of these *Nicotiana* species, the scions also exhibited constitutive expression of PR proteins of their particular species (Gianinazzi and Ahl, 1983). This demonstrates that it is not the proteins themselves which are transported but rather a mobile inducing factor produced in the rootstock, which spreads throughout the plant.

The candidates which are supposed to play a role in systemic signalling are oligogalacturonides, lipid derived signals like jasmonic acid and methyljasmonate, peptides like systemin, abscisic acid and salicylic acid and also electrical signals [action potentials (Hammerschmidt, R., 1993)].

A number of plant pathogens including viruses, bacteria, fungi and nematodes induce systemic production of PR proteins in a number of plants. Also, it is found that these proteins are induced following exposure to abiotic

stress. Some of the well known chemical inducers of these proteins include polyacrylic acid, ethephon, benzoic acid, salicylic acid, acetyl salicylic acid, anti-viral agents like 2, thiouracil and dioxohexahydro triazine and barium and magnesium salts. Aspirin also induces PR protein production in potato plants. These proteins are also induced by spraying plants with elicitor preparations extracted from fungal and bacterial cultures. (van Loon, 1985; Bol *et al.*, 1990).

Of late, more interest has been shown on the cellular localization of PR proteins accumulating in the extracellular space. It is now realised that homologous protein counterparts also accumulate intercellularly. The PR chitinase from bean was shown to be present exclusively in the vacuoles (Boller and Vogeli, 1984).

In tobacco, it is found that the acidic and basic PR proteins appear to be strictly compartmentalised. The acidic PR proteins accumulate predominantly in the extra-cellular space i.e. the apoplast. This has been analysed by immunofluorescence microscopy, immuno-gold labelling and cell fractionation techniques especially for PR-1 and 3 proteins. In contrast, all the basic PR proteins are found sequestered in the vacuoles. Thus, the extracellular acidic PR proteins serve as a first line of defense, especially the (1,3)-β-glucanase is postulated to potentiate the release of oligosaccharide fragments from the pathogenic fungi. The released fragments would in turn act as elicitors, inducing defense, which get released around the pathogen only upon cell lysis, as occurring in a hypersensitive response. Similar results on the compartmentalisation of PR proteins are reported from cucumber (Bollar and Metraux, 1988) and in beans.

Molecular characteristic of PR proteins :

All known PR proteins are synthesized as precursors with N-terminal signal peptide. Little homology is observed between these signal sequences, except for their hydrophobic nature and the presence of charged residues at the preferred positions. Possibly, they function only in the translocation of PR polypeptide chains across the membrane of the endoplasmic reticulum, and it is the signals in the mature proteins that determine whether they are to be transported to vacuoles or are to be secreted through the plasma membrane.

The popular view is that a positive signal near the N-terminus of a mature protein specifies its routing to the vacuoles, whereas the absence of such a signal results in a default pathway leading to its secretion. This would mean that C-terminus has no role in the targeting of this protein (Bol *et al.*, 1990; Eyal and Fluhr, 1991).

The expression of a number of PR protein genes are developmentally regulated. It was found that Chitinases, β-1,3 glucanase and their corresponding mRNAs are present in high concentration in roots of healthy plants (Felix and Mens, 1986). Memelink *et al.* (1990) showed by northern blot analysis that genes encoding basic PR-1 proteins, basic β–1,3 glucanases and basic chitinases are expressed at different levels in roots, stems and flowers of healthy plants.

Pierpoint (1986) and Fraser (1981) have reported that PR proteins are also induced in senescing yellow leaves of flowering plants. It is possible that hormones such as auxins, absicic acid and ethylene, which may be associated with senescence, play an active role in the induction of PR proteins in mature leaves. Hence, it is possible that their induction in senescing tissue and by pathogenesis share common regulatory control elements (Eyal and Fluhr, 1991).

A careful serological analysis revealed an organ specific expression of several acidic PR proteins in different parts of flowers (Lotan *et al.*, 1989). PR-1 group appeared in sepal tissue, glycosylated PR-2-N, O type was found in pistillar parts, while PR-P, Q type was present in pedicels, sepals, anthers and ovaries.

Recently, various gene(s) encoding and controlling PR-protein gene expression have been identified. Ohshima *et al.* (1990) used a GUS reporter gene to monitor the regulation of PR-1a gene in transgenic plants.

Despite a lot of work, the precise role of PR proteins in defense response remains elusive, although increasingly, properties are being assigned to individual members. At present, it is considered more likely that the proteins are involved in general defense, and certainly their wide ranging modes of action from inhibitors of enzymes to that of structural wall component proteins make them ideally suited in defense strategies against "all-comers."

Possible functions of PR proteins :

It is seen that some of the PR proteins have a specific function. PR-2 proteins have been found to have a β 1,3 glucanase activity (Kaufmann *et al.*, 1987; Kombrink *et al.*, 1988). PR-3 type proteins were found to have a endochitinase activity (Metraux *et al.*, 1988; Kombrink *et al.*, 1988). Chitin, a 1,4-(N acetyl-B-O-glucosamine) is a major cell wall component of fungal pathogens with the exception of oomycetes. Similarly, β 1,3 glucans are cell wall constituents of many fungi and bacteria. PR 5a proteins have shown some similarity to the protein from *Thaumatococcus daniellii* ,a sweet tasting thaumatin (Cornellisen *et al.*, 1986)

These proteins might be involved in directly attacking the structural integrity of the pathogens cell wall and thus causing cell lysis and killing the pathogen. Also, the degraded products of the fungal cell wall polymers might function as elicitors of further defense response which might help in limiting the pathogen.

The PR proteins are also supposed to be involved in the phenomenon of systemic acquired resistance(SAR) in plants. SAR is the plant's ability to significantly increase the resistance of the entire plant to a wide range of unrelated pathogens by limited infection with a pathogen.

However, not all PR proteins confer the plant with defensive capabilities. It is reported that in transgenic potato plants over expressing the STH-2 gene encoding a 17 kD PR protein, which is induced by *Phytophthora infestans* shows unaltered susceptibility to this pathogen and also to potato virus X (Constabel *et al.*, 1993).

The above results obtained with *Nicotiana* spp. was followed by reports from other plants like *Cucumis sativus, Gomphrena globosa* and 14 other plant genera (van Loon, 1985). PR proteins from cowpea *Vigna sinensis* were reported by Coutts and Wagih (1981) (van Loon, 1985). Many of these proteins have been found to have a serological relationship with Tobacco PR proteins (Nassuth and Sanger, 1986). PR proteins have been identified in monocots as well. Nasser *et al.* (1988) characterised several PR proteins in maize following infection by brome mosaic virus. Based on biological activity and serological studies, the PR proteins of monocots have been divided into chitinases, (1,3)-β-glucanases and PR-1 related group of proteins. The PR-1 type protein and some of the chitinases exhibited a serological relationship to their tobacco counterparts. This showed

that these group of proteins were highly conserved between widely divergent plant species (Eyal and Fluhr, 1991).

PR proteins in relation to nematodes :

Reports of PR proteins production following TMV infection on tobacco were ensued by their systemic induction in several plants following infection by other pathogens like bacteria (Ahl *et al.*, 1981) and fungi (Gianinazzi *et al.*, 1980). Latest in the series being the root pathogenic nematodes also inducing systemic accumulation of PR proteins. Till date, two reports have come on PR protein induction in plants by nematode infection. Both these reports are on potato plant and the nematodes being *Globodera rostochiensis* and *G. pallida.*

Hammond-Kosack *et al.*(1989), published the first report of systemic accumulation of PR proteins in the leaf apoplast of potato plant following root infection with the cyst nematode, *Globodera rostochiensis.* It incidentally was also the first report stating systemic changes in the leaves following attack on the root system. The PR proteins induced by the nematode was similar to those induced by tobacco mosaic virus (TMV), other potato viruses, *Phytophthora infestans* and aspirin treatment. There was a quantitative increase in the protein content of the IF following nematode infection from 200 µg/ml in control plants to 400 µg/ml in infected plants. Eight new proteins were detected in the IF of infected plants whose molecular wt. and isoelectric characteristics were similar to those of potato PR proteins already reported. The type of novel proteins produced in both resistant and susceptible reactions were similar. Time course analysis of the induction of these proteins revealed that maximum proteins was visible 16

days following root infection. Aspirin could also elicit the production of some of these novel proteins.

Rahimi *et al.* (1993) reported the induction of several new proteins both acidic and basic from potato plants infected by *G. pallida* races Pa1 and Pa 2/3. The molecular weights of these novel proteins from the IF ranged from 14-45 kD. Newer protein bands were also detected from the leaf homogenates and the root extract.

In this experiment, it was found that a potato variety cv. P 55/7, resistant to *G. pallida* Race Pa 1 and susceptible to Pa 2/3 responded differently to the attack by these pathotypes. The intensity of PR proteins was lesser from plants of cv. P 55/7 infected by Pa 2/3 to which it was susceptible than from plants infected by Pa 1 to which it was resistant.

The foregone review is indicative of the fact that despite the importance of the subject, very little information is available in the literature concerning the investigation on PR proteins with regard to plant-parasitic nematodes.

EXPERIMENTAL PROCEDURE

Materials :

Coomassie brilliant blue R-250, coomassie brilliant blue G-250, Tris(hydroxymethyl) amino methane, glycine, sodium dodecyl sulphate(SDS), acrylamide, bis-acrylamide, ammonium per sulphate, and high molecular weight protein marker for SDS-PAGE were purchased from M/s Sigma Chemical Co. St. Louis, Missouri, USA; Sephadex G-25 from Pharmacia fine chemical , Uppasala, Sweden; bromophenol blue, β-mercaptoethanol and TEMED were obtained from Fluka AG, Switzerland; membrane filter units of 0.45μ and 100 kD obtained from Millipore India Ltd. Bangalore; electrophoresis apparatus from Atto Co. Japan and mid range SDS-PAGE molecular weight marker from Promega Chemical Co. USA. All other chemicals and reagents used in this investigation were of analytical grade.

Maintenance and culturing of test organisms :

Cowpea (*Vigna unguiculata*) cultivars Pusa Komal (PK) and C-152 obtained from the Division of Genetics, IARI, were used in this investigation and served as the sources of compatible and incompatible hosts, respectively to the southern root-knot nematode, *Meloidogyne incognita*. The same cultivars viz., Pusa Komal and C-152 served as the hosts for the pigeonpea cyst nematode, *Heterodera cajani*.

Exploratory tests confirmed incompatible (resistant) reaction of cultivar C-152 to the *M. incognita* and the compatibility (susceptibility) of C-152 and Pusa Komal against *Heterodera cajani.*

Stock cultures of *M. incognita* were maintained and multiplied on eggplant (*Solanum tuberosum* cv. Pusa Kranti), raised in 35 cm clay pots containing sterilized sand and soil (ratio 1:1) fed with full strength Hoagland nutrient solution. For large scale harvest and constant supply of *M. incognita* second-stage juveniles (J2), the nematode population was subcultured at 45-60 days interval by periodic replacement of old eggplants with fresh seedlings. Eggs of *M. incognita* was collected by dissolving eggmasses in 1% sodium hypochlorite solution for 1 minute and sieved through a 500 mesh sieve (Southey, 1986).

Stock cultures of *H. cajani* were maintained and multiplied on cowpea (*Vigna unguiculata* cv.. Pusa Komal), raised in 35 cm clay pots containing sterilized sand and soil (ratio 1:1) fed with full strength Hoagland nutrient solution as and when necessary. For large scale harvest and constant supply of cyst of *H. cajani* J2, the nematode population was subcultured at 30-45 days interval by periodic replacement of old cowpea plants with fresh seedlings. Cysts of *H. cajani* was collected by washing the soil in a Fenwick can and collecting the filtrate in a 60 mesh sieve. The cysts were kept at 30-31° C for hatching of eggs into J2.

The procedures for storage, examination and axenization of J2 by streptomycin sulfate were same as described previously (Dasgupta and Ganguly, 1975).

Cultivation and Inoculation of Experimental Plants :

Seeds of the two cultivars of cowpea C-152 and Pusa Komal were surface-sterilized with 0.1% mercuric chloride for 5 minutes followed by repeated and

thorough washings with sterile water. Surface sterilized seeds was soaked in water for 8-10 hours and sown in plastic pots containing a mixture of sterilized river sand and loamy soil (ratio 1:1). The seedlings received usual greenhouse care and were fed with full strength Hoagland nutrient solution at regular intervals.

Three-week-old seedlings were inoculated with axenized suspension of active 5000 J2 of *M. incognita* or *H. cajani* per seedling. For inoculation, the feeder roots of the plants were exposed by carefully removing the top layer of the soil. Suspension containing the required number of J2 was poured uniformly all over the exposed root system which was then immediately covered with sand. This technique of inoculation ensured infection of the entire root system by the nematode.

The control plants were treated in the same way but with sterile water instead of the nematode suspension.

Synchronization of infection was achieved by adding copious quantity of water (150-200 ml) to each plant/pot, so that the free juveniles (J2 which were unsuccessful in penetrating roots by 48 hrs) were washed out as described by Hammond-Kosack (1989). All the experiments were replicated thrice.

Extraction of Intercellular Fluid (IF) of Cowpea Leaves :

In order to determine the post-infection period at which better yields of apoplastic protein can be realized, cowpea leaves were harvested at different intervals (2, 4, 7, 10, 14 and 21 days after inoculation) following inoculation of the plants with nematodes, and their intercellular fluid (IF) extracted.

Larger leaves were cut from the mid-rib longitudinally into two and smaller leaves were used intact. The leaves were washed for 15 min. to remove any debris from the surface and cytoplasmic contaminants from the cut edges.

These leaves were then infiltered with distilled water *in vacuo*. For this purpose, the leaves were put in a Petri-plate (20 cm dia.) which was filled with water. Over this a smaller Petri-plate (19 cm dia.) was placed, in the inverted position, so that the leaves are fully immersed in water. This was then placed in a dessicator connected to a vacuum pump. On trying various vacuum pumps, it was found that the Christ model lyophilizer chamber and vacuum pump gave the best results. Vacuum was applied for 2 minutes and air was readmitted into the dessicator, upon which the leaves appeared uniformly water soaked. All further operations were undertaken with utmost care as the infiltrated leaves get damaged easily.

The infiltrated leaves were surface dried, by placing the leaves between two layers of blotting paper. These leaves were then made into a roll. In order to collect the IF from the infiltrated leaves various methods were tried (Table-2)

Table 2: Various methods tried for the collection of IF from infiltrated leaves

S.No.	Methods
1.	Perforated aluminium screen held by glass cylinder fitting into a 50 ml centrifuge tube.
2.	Glass beads at the bottom of 50 ml centrifuge tube.
3.	Syringe barrel(20 ml) to hold the infiltrated leaves and placing this in a 50 ml centrifuge tube.
4.	Perforated plastic test tube caps which fit into 50 ml centrifuge tubes

Of these, the last procedure gave the best results (Figure-1).The infiltrated leaves were thus spun for 15 minutes at 1000g in a refrigerated (4°C) Kontron Centricon model H-401 centrifuge.

The recovery of IF was at the rate of 300-350 µl per g of leaf tissue. For each treatment, 10 g of leaf material was used which ensured 3 ml of IF harvest.

The IF had a yellowish colour. A greenish colour indicates rupture of cell wall and the such samples were discarded. Also, the IF's were assayed for Glucose-6-phosphate dehydrogenase (G6PD) to check for the presence of cytoplasmic contaminants (Hammond, 1985). The samples were either analyzed immediately or were stored at 20°C. Other details concerning the extraction of IF from leaf apoplast are given under the section 'Results'.

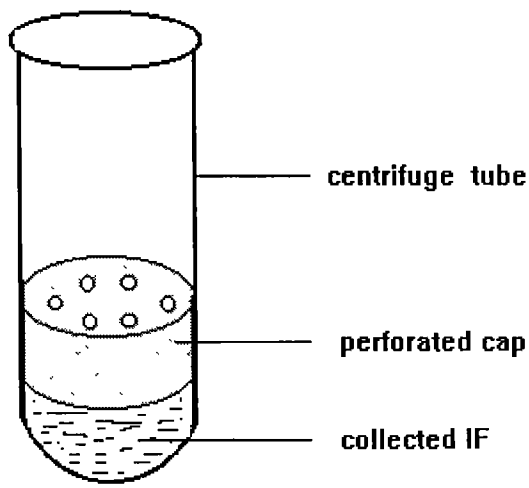


Figure 1. Diagram showing the modified apparatus i.e. use of perforated plastic test tube cap, placed in a centrifuge tube for collecting intercellular fluid (IF) from leaves infiltrated with water.

Purification of Protein from IF :

Various methods tried for purification of protein contained in the IF are as under.

1. The raw IF was passed through a mini Column 10 x 0.5 cm filled with Sephadex G-25. An Isco model Foxy 200 X-Y fraction collector was used with ISCO V4 variable wavelength absorbance detector at wave length 280 nm and the fractions with protein pooled.
2. Raw i.e. pre-swelled Sephadex was added to one ml of raw IF and allowed to swell for 1 hour with intermittent shaking. This was then spun at 2000 g for 5 min. in a refrigerated Sigma Model 3K 30 centrifuge at 4°C. The supernatant was collected (~ 800 µl) which contained the purified protein.
3. Raw IF was also passed through a 0.20µ syringe filter unit (Nalgene) and filtrate collected.
4. Raw IF was passed through a Ultrafree MC filter unit with a 0.45µm low protein binding Durapore membrane (Millipore, Model UFC3 OHU 25).

Quantitative Estimation of Protein :

The protein concentration of IF was assayed according to Lowry *et al.* (1951) and Bradford (1976) using a Ciba-Corning model spectrophotometer .

SDS-Polyacrylamide Gel Electrophoresis :

Purified IF extracts, corresponding to 50 µg. of protein were analysed by discontinuous SDS-PAGE carried out according to Laemmli (1970) .

IFs corresponding to 50 μg quantity was lyophilized in a Savant model SC-110 speed vac. lyophilizer fitted to a Savant model RVT-4104 vapour trap.

Reagents and Solutions:

The reagents and solutions used in gel electrophoresis are as under-

1. 30% Acrylamide/0.8% bis-acrylamide.
   The solution was filtered and stored in amber colored bottles at 4° C.
2. 1.5M Tris-HCl pH 8.8
   18.2 g of Tris base was dissolved in 60 ml of double distilled water. pH adjusted with conc. HCl and the volume made to 100 ml.
3. 0.5M Tris-HCl pH 6.8
   6.05 g of Tris base dissolved in 40 ml of double distilled water(DDW). pH adjusted with conc. HCl and volume made to 100 ml.
4. 10% Sodium Dodecyl Sulphate (SDS).
5. 10% ammonium persulfate (APS).
6 TEMED
7. Sample buffer: It contained
   60 mM Tris-HCl, pH 6.8
   10% glycerol
   2% SDS
   5% β mercaptoethanol
   0.01% bromophenol blue

8. Electrophoresis buffer pH 8.3 : It contained

0.025M Tris

0.192 M glycine and

0.1% SDS

Vertical gel electrophoresis apparatus from Atto Corporation was used. Glass plates (11.5 x 13.7 cm) with a spacer of 1 mm thickness was used for the preparation of denaturing SDS-polyacrylamide gel.

Preparation of separating gel :

The composition of separating gel mix for different percentages of polyacrylamide is given in Table 3.

Table 3: Composition of various reagents for separating gel preparation

Reagent	Volume to be added (ml)		
	10%	12%	15%
Sterile double distilled water	7.9	6.6	4.6
30% Acrylamide mix	6.6	8.0	10.0
1.5M Tris-HCl pH 8.8	5.0	5.0	5.0
10%w/v SDS	0.2	0.2	0.2
10% APS	0.2	0.2	0.2
TEMED	0.008	0.008	0.008

Specified amounts of each reagent except APS or TEMED for a given percentage of acrylamide were taken into a conical flask and degassed. Ten per cent APS and TEMED was then added, stirred thoroughly and poured into the

glass plate sandwich. About 0.2 ml of sterile double distilled water was carefully layered onto the gel mix and the gel was allowed to polymerise for at least 1 hr. After polymerization the overlayered water was poured off and the surface was allowed to dry before pouring the stacking gel mix.

Preparation of stacking gel (5%) :

For 6 ml of 5% gel mix, appropriate quantities of 30% acrylamide mix (1.0 ml), 10% SDS (0.06 ml), 0.5M Tris-HCl pH 6.8 (1.5 ml) and sterile double distilled water (3.3 ml) were taken into a conical flask and degassed. Required quantities of TEMED (6 µl) and 10% APS (60 µl) were added, mixed and poured on top of the already polymerized separating gel. A comb of 1 mm thickness was carefully inserted without trapping any air bubbles. After polymerization the comb was carefully removed and the wells were rinsed with double distilled water (DDW). The sandwich containing the gel after removing the gasket, was fitted in the electrophoretic apparatus. The upper chamber was constructed by inserting another sandwich into the apparatus and both the chambers were then filled with electrophoresis buffer.

Conditions for electrophoresis :

The gel was given a pre-run at 100 V for 10 minutes. The protein sample was dissolved in sample buffer and heated in a boiling water bath for 3 minutes. After cooling the samples to room temperature, they were loaded on the gel. Electrophoresis was carried out at 250 mA and room temperature till the bromophenol blue dye reached the bottom of separating gel. After electrophoresis

the sandwich was removed and disassembled . The gel was then washed with DDW and stained either by coomassie brilliant blue or silver nitrate.

Detection of proteins in gels:

Briefly, the staining and destaining solutions and procedures for detection of protein profiles employed are as follows-

Coomassie blue staining:

Reagents and Solutions :

(a) Coomassie Blue solution :
50% methanol
0.05% coomassie brilliant blue R-250
10% acetic acid
40% distilled water

Coomassie brilliant blue R-250 was dissolved in methanol before adding acetic acid and water.

(b) Destaining solution :
5% methanol
7% acetic acid
88% distilled water.

The gel after electrophoresis was placed in a glass tray and covered with coomassie blue solution. After 4 hr. the staining solution was poured out and the gel was covered with destaining solution. After 4 hrs. the used destaining solution was replaced by a fresh destaining solution. Destaining was continued until blue bands and a clear background were obtained. The destained gel was photographed.

Silver staining :

Silver staining procedure (Nesterenko *et al.*,1994) was also attempted to detect protein profiles besides the staining procedure described above. The following are the details-

Reagents and solutions :

50% Acetone in double distilled (DD) water,

50% TCA in DD water,

20% $AgNO_3$ in DD water (store in dark),

10% $Na_2S_2O_3.5H_2O$ in DD water.

1% glacial acetic acid.

Summarised steps for silver staining method of detecting protein bands in gel surface

Steps	Treatment	Schedule
Fixation	60 ml acetone stock 1.5 ml TCA stock 25 µl 37% HCHO	5 minutes
Rinse	DD water	3 times x 5 seconds
Wash	DD water	5 minutes
Rinse	DD water	3 times x 5 seconds
Pre-treatment	60 ml acetone stock	5 minutes
Pre-treatment	100 µl $Na_2S_2O_3.5H_2O$ stock in 60 ml DD water	1 minute
Rinse	DD water	3 times x 5 seconds
Impregnate	0.8 ml $AgNO_3$ stock 0.6 ml 37% HCHO 60 ml DD water	8 minutes
Rinse	DD water	2 times x 5 seconds
Develop	1.2 g Na2CO3 25 µl 37% HCHO 25 µl $Na_2S_2O_3.5H_2O$ stock 60 ml DD water	10-20 seconds
Stop	1% glacial acetic acid	

All steps were performed in glass/ plastic containers on a shaker at room temperature (approximately 23°C) A brown precipitate may appear upon contact of the gel with the developer. It gets dissolved by vigorous shaking.

Molecular weight charecterization of novel proteins:

The particulars of authentic molecular weight (mol. wt.) markers used in this investigation to charecterize and determine the molecular masses of the novel polypeptides from the IF of the leaf are as under -

Mid range molecular wt. marker used in 10% acrylamide gels: (Promega)

Phosphorylase B	97.4 kD
Bovine serum albumin	66.2 kD
Glutamanate dehydrogenase	55.0 kD
Ovalbumin	42.7 kD
Aldolase	40.0 kD
Carbonic anhydrase	31.0 kD
Soyabean trypsin inhibitor	21.5 kD
Lysozyme	14.4 kD

High range molecular wt. marker used in 15% acrylamide gels :(Sigma)

Rabbit muscle myosin	205 kD
Rabbit muscle β galactosidase	116 kD
Phosphorylase B	97.4 kD
Bovine serum albumin	66.0 kD
Egg albumin	45.0 kD
Carbonic anhydrase, bovine erythrocytes	29.0 kD

RESULTS

Standardization of Procedures :

Extraction of Intercellular Fluid (IF) :

One of the major components of the experimental procedures of this investigation is the standardization of extraction of IF from leaf apoplast containing PR proteins so as to ensure optimum yield of polypeptides. In order to achieve this objective, a three-step procedure was adopted.

Step (i) :Removal of air from apoplastic space:

The commonly adopted procedure by deWit and Spikman (1982) was followed by keeping a beaker containing the leaves and filled with water (a perforated ceramic lid was placed in the beaker to prevent the leaves from floating) in a desiccator connected to a suction pump for a period of 10 minutes. The difficulty when this procedure was followed, was that only upper layer of leaves got infiltrated with water and that too in patches. So, the period of vacuum application was increased upto 1 hr, which did not improve the results appreciably. Later on, beaker was substituted with a Petri-plate (dia 20 cm). Instead of a perforated ceramic lid, a smaller Petri-plate (dia 19 cm) was used as a lid in the inverted position. This remarkably improved the infiltration.

Finally, a better and more powerful vacuum pump was used. The Petri-plates with the leaves immersed in water was placed in the vacuum hood of 'Christ' model lyophilizer and the vacuum was applied for a period of 2 minutes.

Table 4 illustrates typical experimental results on yield of IF from leaves infiltrated by various techniques. It is more than apparent that best results were obtained when a Petri-plate was placed in lyophilizer hood.

Table 4: Yield of IF (µl/gm.) from leaves infiltrated by various techniques

Time of vacuum application	Beaker without lid	Beaker with lid	Petri-plate in vacuum pump	Petri-plate in lyophilizer hood
2 minutes	0	0	10	310
30 minutes	0	14	40	cell lysis
60 minutes	15	27	75	cell lysis

Step (ii): Infiltration of apoplastic space with water :

After 2 minutes vacuum was released and air readmitted, upon which all the leaves appeared uniformly water soaked (100% infiltration rate).

Step (iii) Recovery of infiltrated IF :

Recovery of the infiltrated fluid i.e. IF from the tissue was done by low speed centrifugation (1000 g for 10 minutes) as per the modified procedure of de Wit and Spikman (1982). For best recovery of the IF, it is required that the leaves should not come in contact with the collected fluid. This requires a kind of porous barrier which allows passage of IF to the bottom of the tube and at the same time withholding the leaves from coming in contact with the collected IF. Several innovative methods were tried (Table 2).

The perforated aluminium screening could not withstand the weight of the leaves and the centrifugal force, and as a result there was negligible yield of IF.

The use of glass beads, withheld the leaves from floating on the collected IF, but the recovery of IF from the bottom of the centrifuge tubes with the glass beads was cumbersome. This also resulted in some loss of IF when compared with the extraction by perforated test tube caps.

Syringe barrels gave good results with respect to IF extraction, but the number of leaves(5-6) that could go in one syringe barrel was less.

Using perforated plastic test tube caps, fitting into 50 ml centrifuge tube gave the best results. Twenty leaves could be processed at a time with none coming in contact with the collected IF. Recovery of the IF after centrifugation from the bottom of the tube was 100 per cent and easiest of all the procedures tried.

Quantitative Estimation of Protein in IF :

Initially, the procedure of Lowry *et al.* (1951) was followed to determine the protein concentration in the IF, using BSA as a standard. Since this procedure did not yield satisfactory results in detecting the low concentration of apoplast protein, several other methods were tried. Out of several techniques experimented with, the method of Bradford (1976) was noted to be most sensitive enabling to detect upto 10 µg protein.

Qualitative Examination of IF Protein by SDS-PAGE :

The protein profiles were analyzed using discontinuous SDS-PAGE system via the method as described by Laemmli (1970).

At first raw IFs corresponding to 40 µl quantity was loaded in each well (Figure 2). SDS-PAGE of raw intercellular fluid showed diffused protein bands

which were perhaps because of artefact contamination. Hence, the IFs had to be purified before electrophoresis. The following methods were tried :

(i) Lyophilizing the raw IF and making up the volume to 20 µl.

(ii) Passing the IF through a Sephadex G-25 column.

(iii) Adding raw Sephadex G-25 beads to the IF.

(iv) Passing the IF through a 0.2 µm syringe filter unit.

(v) Passing the IF through a 0.45 µ Durapore membrane.

(vi) Passing the IF through a 0.45 µ Durapore membrane and then passing through a 100 kD Millipore filter unit.

Purification of IF by lyophilizing as well as passing the IF through a syringe filter unit did not yield sharper protein bands when compared to control (Figure 3). Passing the IF through Sephadex G-25 column or through a Durapore membrane resulted in a cleaner extract which produced sharper bands. Adding of pre-swelled Sephadex G-25 beads to the raw IF also gave comparable results.

Quantitative Estimation of Proteins from IF :

Time course of protein concentration in cowpea inoculated with *Meloidogyne incognita* and *Heterodera cajani* :

Tables 5 and 6 present the protein concentrations in the two cowpea cultivars inoculated with the nematodes, *M. incognita* and *H. cajani*, respectively. Based on these, three major points are to be stressed on . Firstly, there was an increase in protein concentration with age in the two cultivars of cowpea in both inoculated and uninoculated conditions. Secondly, the percentage increase of the

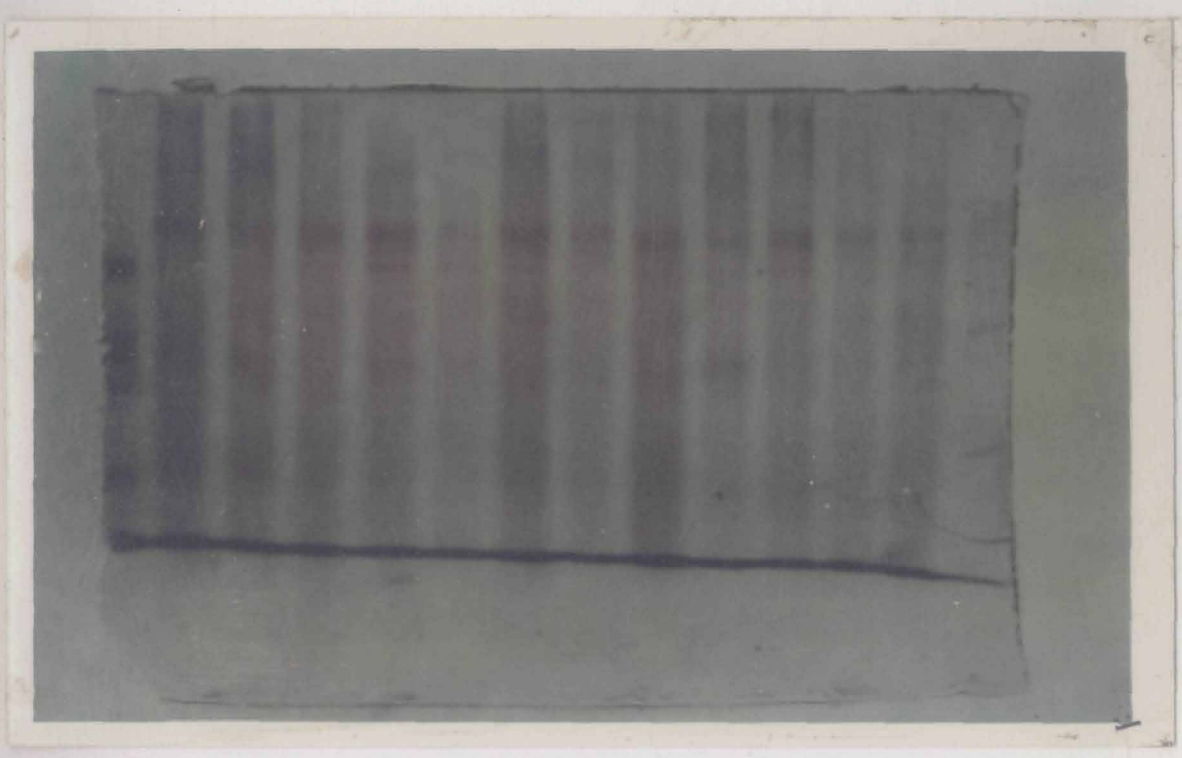


Figure 2:

SDS-PAGE profiles of proteins from intercellular fluid of leaves of cowpea cv. Pusa Komal at various times following infection by root-knot nematode, *M. incognita* and pigeonpea cyst nematode, *H. cajani*. Lanes were loaded with 30 µl of IF extract without any purification i.e. raw IF was loaded. Gel was stained with Coomassie blue. For details see text.

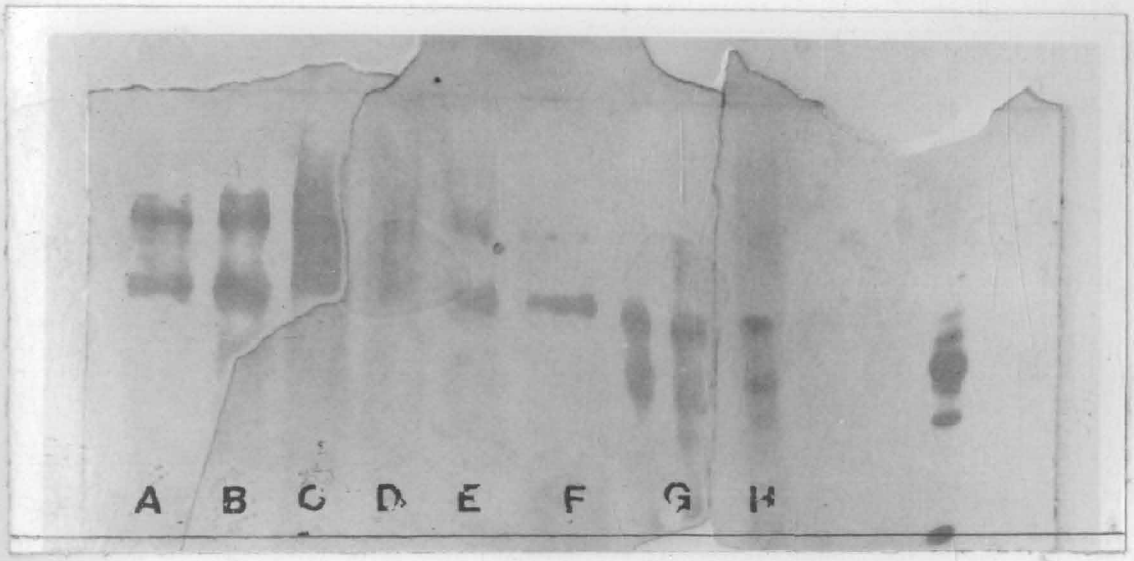


Figure 3:

SDS-PAGE profiles of proteins purified by different procedures from intercellular fluid (IF) of leaves of cowpea cultivar, Pusa Komal. Each lane was loaded with IF equivalent to 10 µg of protein. Gels were stained with silver nitrate. Lanes are indicated as below :

Lane A : Using a 0.45 u Durapore membrane (Millipore)
Lane B : Using a 0.45 m Durapore membrane followed with a 100 kD filter (Millipore)
Lane C : Using a 0.2 µ syringe filter unit (Nalgene)
Lane D : Using a 0.2 µ syringe filter, unit (Nalgene)
Lane E : Using a Sephadex G-25 column
Lane F : Using preswelled (raw) sephadex G-25 beads.
Lane G : Raw IF without lyophilization.
Lane H : Raw IF, lyophilized and made up to 20 µl.

protein concentration was greater in the case of nematode inoculated plants in both the cultivars when compared to their respective controls. Thirdly, inter-varietal comparison of protein concentration indicated that Pusa Komal is constitutively richer in protein than C-152.

Further, based on the data presented in Tables 5 and 6 the following observations are derived. Following inoculation with nematodes, in Pusa Komal, the intercellular protein content increased steadily throughout the experimental period, with maximum activity occurring at 21 days after inoculation (DAI). In C-152 plants, a similar increase in protein content of IF was observed only till 14 DAI where it showed maximum activity. Thereafter, it showed a decreasing trend.

Figures 4 and 5 illustrate the Inoculated/Uninoculated (I/U) ratio of protein concentration. Two points of interest are to be noted. The increase in I/U ratio with regard to C-152 for both the nematode species is biphasic. In contrast, in Pusa Komal the increase in I/U ratio with time is, in general uniphasic. Second, the I/U ratio due to *M. incognita* in C-152, as compared to *H. cajani* was of much higher magnitude two days after inoculation. When changes in protein concentration in Pusa Komal following infection by the nematodes, *Meloidogyne incognita* and *Heterodera cajani* are compared, it can be observed that infection with *M.incognita* elicited a greater increase in protein content when compared to *H. cajani* especially at 14 and 21 DAI (I/U ratio being 1.53 and 1.71 for *M. incognita*, while it is 1.37 and 1.48 for *H. cajani* at 14 and 21 DAI, respectively).

Table 5: Time course activity of protein(µg./ml) from IF of Cowpea cultivars Pusa Komal and C-152 inoculated with the root-knot nematode *Meloidogyne incognita*

Days after	PUSA KOMAL			C - 152		
inoculation	Uninoculated	Inoculated	Ratio of inoculated/ uninoculated	Uninoculated	Inoculated	Ratio of inoculated/ uninoculated
0	57.05	57.05	1.00	61.20	61.20	1.00
2	59.60	71.52	1.20	63.87	87.20	1.36
7	64.43	85.47	1.32	69.71	86.90	1.24
10	70.15	95.53	1.36	70.42	92.20	1.30
14	78.62	120.46	1.53	79.91	112.43	1.40
21	89.63	153.67	1.71	79.91	99.07	1.23

Table 6: Time course activity of protein(µg./ml) from IF of Cowpea cultivars Pusa Komal and C-152 inoculated with the pegeion pea cyst nematode *Heterodera cajani*

Days after inoculation	PUSA KOMAL			C - 152		
	Uninoculated	Inoculated	Ratio of inoculated/ uninoculated	Uninoculated	Inoculated	Ratio of inoculated/ uninoculated
0	57.05	57.05	1.00	61.20	61.20	1.00
2	59.60	70.32	1.18	63.87	77.30	1.21
7	64.43	79.32	1.23	69.71	87.13	1.25
10	70.15	93.29	1.33	70.42	92.95	1.32
14	78.62	108.24	1.37	79.91	108.41	1.35
21	89.63	133.12	1.48	79.91	89.96	1.12

Fig. 4 Protein (inoculated / uninoculated) values from IF of leaves of cowpea cultivar Pusa Komal inoculated with *M.incognita* and *H.cajani*

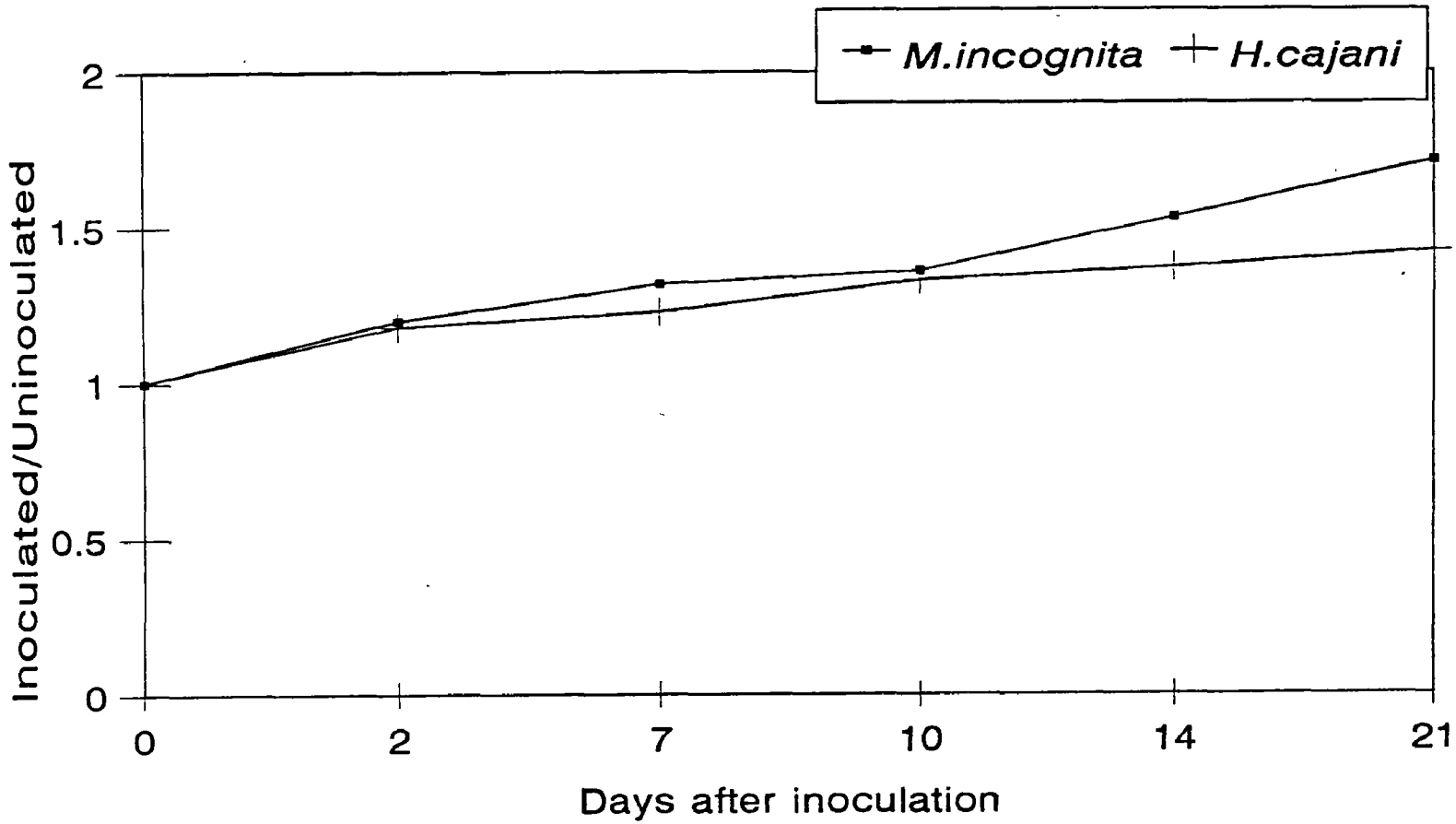
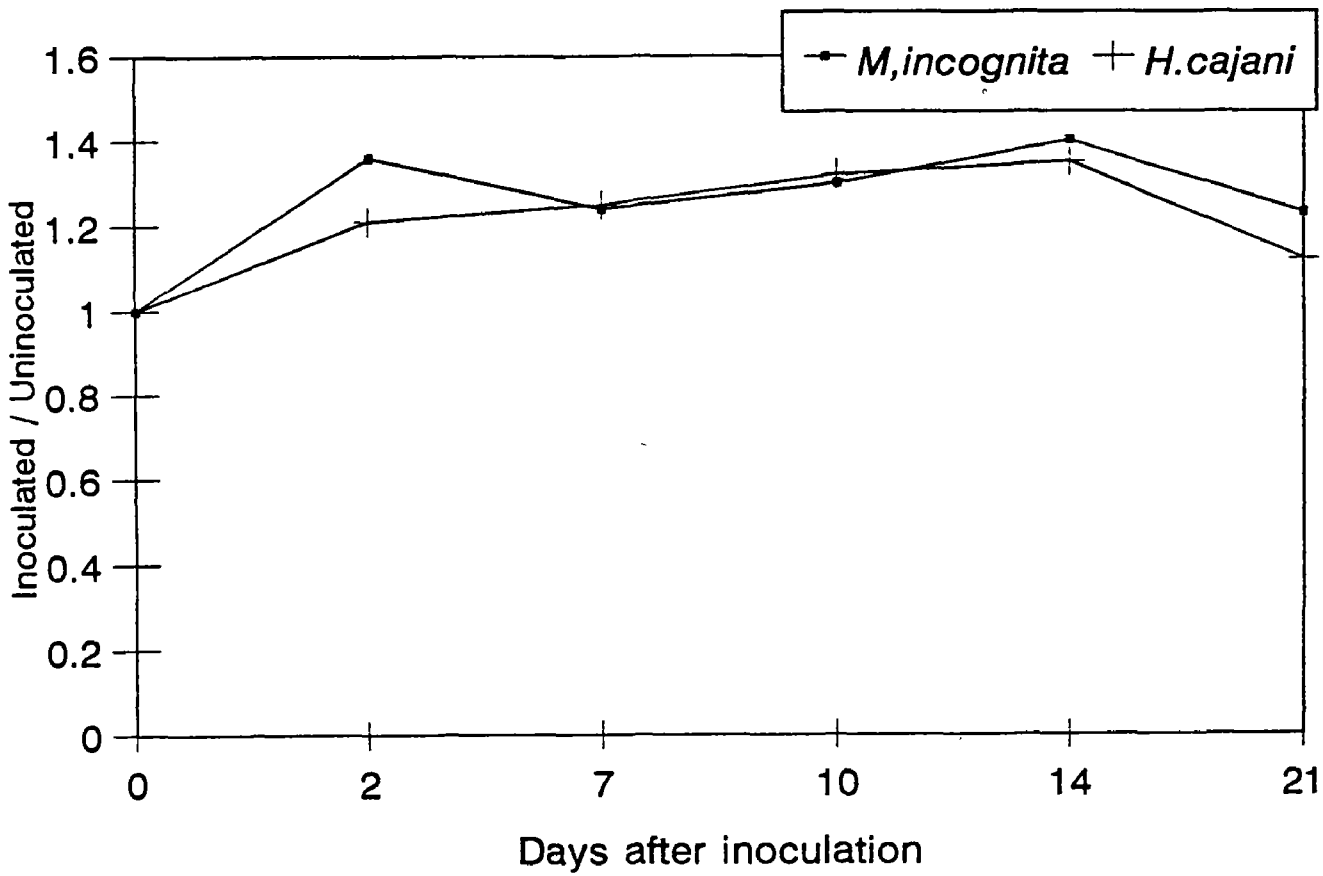


Fig. 5 Protein (inoculated / uninoculated) values from IF of leaves of cowpea cultivar C-152 inoculated with *M.incognita* and *H.cajani*.



SDS-PAGE Analysis of Proteins from IF :

One of the reasons to account for increased concentration of IF proteins, could be due to appearance of nematode-induced novel polypeptides. In order to check this possibility SDS-PAGE electrophoresis were carried out for the appearance of nematode-induced novel proteins in apoplastic space and also to characterize the protein. Variations in gel concentration were also explored to resolve maximum number of anionic protein bands. Results are presented in Tables 6 to 12 and Figures 6 to 9.

One general conclusion is that in all the treatments, as a response to nematode infection, the number of protein bands in inoculated plants were more when compared to their respective controls, with newer bands appearing at later stages of infection.

Systemic Changes as Induced by *M. incognita* on Pusa Komal :

Figure 6, Lanes C-G, shows the qualitative changes in protein of IF from the leaves of cultivar Pusa Komal inoculated with *M. incognita*. The relative mobility (Rm) values of the various bands as well as their molecular weight are reported in Table 7.

Four protein bands of Rm value 0.40, 0.58, 0.62 and 0.75 with molecular weights of 66.0, 46.0, 42.0 and 34.0 kD, respectively appeared 7 days after infection. Also, one protein band of Rm value 0.64 with a molecular weight of 40.0 kD appeared by 14 days after inoculation.

As the protein bands in the lower parts of the gel could not be resolved properly, another gel with a higher concentration of acrylamide (15%) was run so

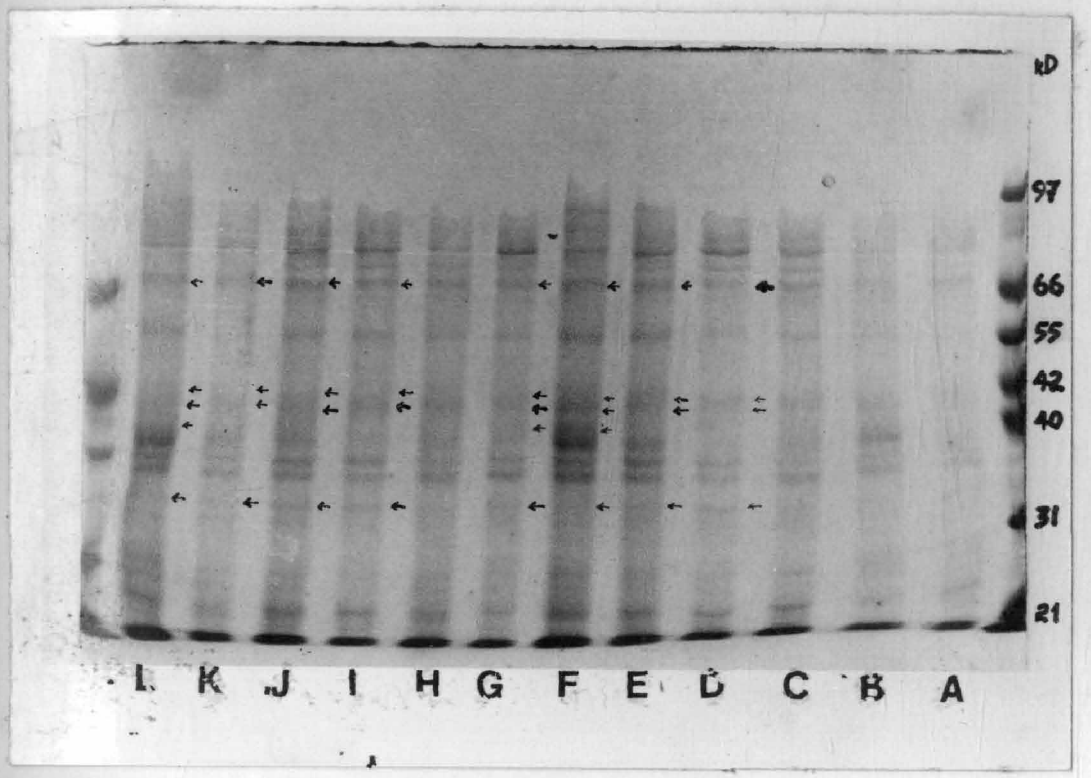


Figure 6:

Intercellular fluid (IF) was extracted from the leaves at 2, 7, 10, 14 and 21 days after inoculation (DAI) of the roots of cowpea cv. Pusa Komal with the root-knot nematode, *M. incognita* and the pigeonpea cyst nematode, *H. cajani* and at 0 and 21 days following start of the experiments from plants not infected by the nematodes. Polypeptides were separated by SDS-PAGE (10% acrylamide) and visualized using Coomassie blue. IF extracts equivalent to 50 µg of protein was loaded on each track.

Track A. Uninfected plant, 0 DAI;

Track B. Uninfected plant, 21 DAI.

Part 1. Track C-G plants infected by *M. incognita* at 2, 7, 10, 14 and 21 DAI.

Part 2. Track H-L plants infected by *H. cajani* at 2, 7, 10, 14 and 21 DAI.

Appearance of novel protein bands is indicated by arrows (←). The molecular weight of standard protein markers is given in kilo Daltons.

Table 7 : Relative Mobility(Rm) values and molecular weights of the various polypeptides on 10% SDS-PAGE gel from the IF of Pusa Komal inoculated with the root knot nematode, *Meloidogyne incognita*

Relative Mobility (Rm)	CONTROL		Days After Inoculation (DAI)				
	0	21	2	7	10	14	21
0.33	79.1	79.1	79.1	79.1	79.1	79.1	79.1
0.36	73.1	73.1	73.1	73.1	73.1	73.1	73.1
0.39	67.9	67.9	67.9	67.9	67.9	67.9	67.9
0.40	--	--	--	**66.0**	**66.0**	**66.0**	**66.0**
0.49	54.2	54.2	54.2	54.2	54.2	54.2	54.2
0.58	--	--	--	**46.0**	**46.0**	**46.0**	**46.0**
0.62	--	--	--	**42.0**	**42.0**	**42.0**	**42.0**
0.64	--	--	--	--	--	**40.0**	**40.0**
0.67	38.4	38.4	38.4	38.4	38.4	38.4	38.4
0.70	36.5	36.5	36.5	36.5	36.5	36.5	36.5
0.73	35.0	35.0	35.0	35.0	35.0	35.0	35.0
0.75	--	--	--	**34.0**	**34.0**	**34.0**	**34.0**
0.89	26.8	26.8	26.8	26.8	26.8	26.8	26.8
0.91	26.0	26.0	26.0	26.0	26.0	26.0	26.0
0.95	24.5	24.5	24.5	24.5	24.5	24.5	24.5
0.96	24.1	24.1	24.1	24.1	24.1	24.1	24.1

as to provide finer resolution. The results of the same are presented in Figure 7 and Rm values in Table 8.

Many of the newer protein bands which were detected in the earlier gel (10%) were also visible in this gel, at their corresponding molecular weight positions. Three more protein bands which could not be resolved in (10%) gel, could be detected clearly towards the lower levels of this gel. A protein band of Rm value 0.55, mol. wt. 25.0 kD starts appearing at 10 days after inoculation and was present in all the subsequent intervals. While, another protein band (Rm value 0.86, mol. wt. 15.0 kD) started developing 14 DAI and was seen in the next interval also, it was found that a 16.0 kD protein (Rm 0.82) appeared 2 DAI but could not be detected at later intervals.

Systemic Changes as Induced by *H. cajani* on Pusa Komal :

Changes in protein profile in IF of leaves of Pusa Komal inoculated with *H. cajani* are depicted in Figure 6, Lanes H-L and the Rm values with their corresponding molecular weight are given in Table 9.

Examination of the photographs and the table ostend that four new protein bands Rm value 0.40, 0.58, 0.62 and 0.75 of mol. wt. 66.0, 46.0, 42.0 and 34.0 kD started appearing 7 days after inoculation, while the 40.0 kD protein (Rm 0.64) band appeared only at 21 DAI.

A 15% SDS-PAGE gel was run so as to provide better resolutions of proteins having lower molecular weights. The results are depicted in Figure 8 and Table 10. Scrutiny of the gel reveals that the protein bands visible in the earlier gel are also visible in this gel at their corresponding mol. wt. positions. Only one protein band of Rm 0.55 and mol.wt. 25.0 kD started appearing 10 days after

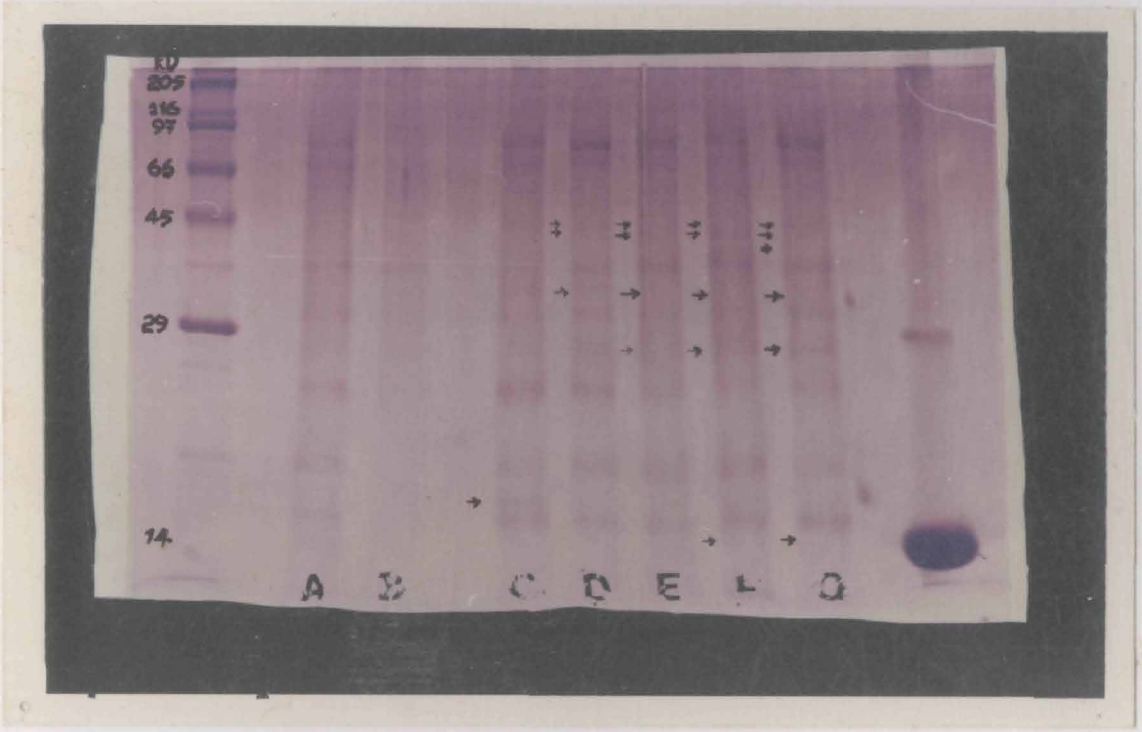


Figure 7:

SDS-polyacrylamide gel electrophoresis (15% acrylamide) of proteins from IF of leaves of cowpea cv. Pusa Komal inoculated with the root-knot nematode, *M. incognita*. IF were collected from leaves at 2, 7, 10, 14 and 21 days following root infection by the nematode. IF extracts equivalent to 50 µg is loaded in each lane and gel visualized by Coomassie blue staining. Appearance of new protein bands is indicated with arrows. Molecular weight of standard protein markers is given in kilo Daltons (kD). The lanes are indicated as below :

Lane A : Uninfected plant 0 DAI;

Lane B : Uninfected plant 21 DAI.

Lane C toG : Plants infected by *Meloidogyne incognita*.

For details, see text.

Table 8 : Relative Mobility(Rm) values and molecular weights of the various polypeptides on 15% SDS-PAGE gel from the IF of Pusa Komal inoculated with the root knot nematode *M. incognita.*

Relative Mobility (Rm)	CONTROL		Days After Inoculation (DAI)				
	0	21	2	7	10	14	21
0.15	80.9	80.9	80.9	80.9	80.9	80.9	80.9
0.17	73.3	73.3	73.3	73.3	73.3	73.3	73.3
0.18	70.0	70.0	70.0	70.0	70.0	70.0	70.0
0.23	57.0	57.0	57.0	57.0	57.0	57.0	57.0
0.29	--	--	--	**46.0**	**46.0**	**46.0**	**46.0**
0.32	--	--	--	**42.0**	**42.0**	**42.0**	**42.0**
0.33	--	--	--	--	--	--	**40.0**
0.37	36.9	36.9	36.9	36.9	36.9	36.9	36.9
0.40	35.0	35.0	35.0	35.0	35.0	35.0	35.0
0.41	--	--	--	**34.0**	**34.0**	**34.0**	**34.0**
0.46	29.7	29.7	29.7	29.7	29.7	29.7	29.7
0.55	--	--	--	--	**25.7**	**25.7**	**25.7**
0.62	21.7	21.7	21.7	21.7	21.7	21.7	21.7
0.77	17.1	17.1	17.1	17.1	17.1	17.1	17.1
0.82	--	--	**16.0**	--	--	--	--
0.85	15.2	15.2	15.2	15.2	15.2	15.2	15.2
0.86	--	--	--	--	--	**15.0**	**15.0**

Table 9 : Relative Mobility(Rm) values and molecular weights of the various polypeptides on 10% SDS-PAGE gel from the IF of Pusa Komal inoculated with the pigeon pea cyst nematode, *Heterodera cajani.*

Relative Mobility	CONTROL		Days After Inoculation (DAI)				
(Rm)	0	21	2	7	10	14	21
0.33	79.1	79.1	79.1	79.1	79.1	79.1	79.1
0.36	73.1	73.1	73.1	73.1	73.1	73.1	73.1
0.39	67.9	67.9	67.9	67.9	67.9	67.9	67.9
0.40	--	--	--	**66.0**	**66.0**	**66.0**	**66.0**
0.49	54.2	54.2	54.2	54.2	54.2	54.2	54.2
0.58	--	--	--	**46.0**	**46.0**	**46.0**	**46.0**
0.62	--	--	--	**42.0**	**42.0**	**42.0**	**42.0**
0.64	--	--	--	--	--	--	**40.0**
0.67	38.4	38.4	38.4	38.4	38.4	38.4	38.4
0.70	36.5	36.5	36.5	36.5	36.5	36.5	36.5
0.73	35.0	35.0	35.0	35.0	35.0	35.0	35.0
0.74	--	--	--	**34.0**	**34.0**	**34.0**	**34.0**
0.89	26.8	26.8	26.8	26.8	26.8	26.8	26.8
0.91	26.0	26.0	26.0	26.0	26.0	26.0	26.0
0.95	24.5	24.5	24.5	24.5	24.5	24.5	24.5
0.96	24.1	24.1	24.1	24.1	24.1	24.1	24.1

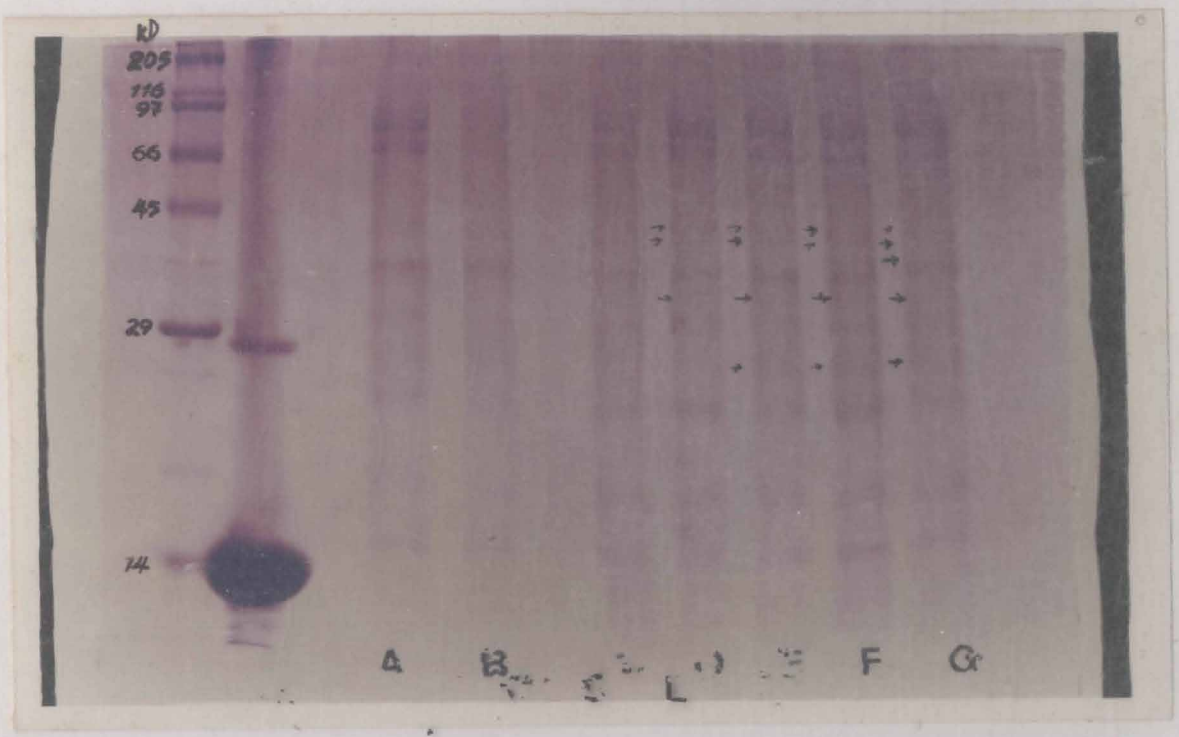


Figure 8:

SDS-polyacrylamide gel electrophoresis (15% acrylamide) of proteins from IF of leaves of cowpea cv. Pusa Komal inoculated with the pegion pea cyst nematode *H. cajani*. IF were collected from leaves at 2, 7, 10, 14 and 21 days following root infection by the nematode. IF extracts equivalent to 50 µg is loaded in each lane and gel visualized by Coomassie blue staining. Appearance of new protein bands is indicated with arrows. Molecular weight of standard protein markers is given in kilo Daltons (kD). The lanes are indicated as below :

Lane A : Uninfected plant 0 DAI;

Lane B : Uninfected plant 21 DAI.

Lane C toG : Plants infected by *Heterodera cajani*.

For details, see text.

Table 10 : Table listing the Relative Mobility(Rm) values and the corresponding molecular weights of the various polypeptides resolved on 15% SDS-PAGE gel from the IF of Pusa Komal inoculated with the pigeon pea cyst nematode *Heterodera cajani.*

Relative Mobility (Rm)	CONTROL		Days After Inoculation (DAI)				
	0	21	2	7	10	14	21
0.15	80.9	80.9	80.9	80.9	80.9	80.9	80.9
0.17	73.3	73.3	73.3	73.3	73.3	73.3	73.3
0.19	67.0	67.0	67.0	67.0	67.0	67.0	67.0
0.23	57.0	57.0	57.0	57.0	57.0	57.0	57.0
0.29	--	--	--	**46.0**	**46.0**	**46.0**	**46.0**
0.32	--	--	--	**42.0**	**42.0**	**42.0**	**42.0**
0.33	--	--	--	--	--	--	**40.0**
0.37	36.9	36.9	36.9	36.9	36.9	36.9	36.9
0.40	35.0	35.0	35.0	35.0	35.0	35.0	35.0
0.41	--	--	--	**34.0**	**34.0**	**34.0**	**34.0**
0.46	29.7	29.7	29.7	29.7	29.7	29.7	29.7
0.55					**25.0**	**25.0**	**25.0**
0.62	21.7	21.7	21.7	21.7	21.7	21.7	21.7
0.77	17.1	17.1	17.1	17.1	17.1	17.1	17.1
0.85	15.2	15.2	15.2	15.2	15.2	15.2	15.2

infection and was present in all the subsequent intervals. The two new protein bands of Rm values 0.82 and 0.86 detected in Figure 7 could not be detected.

Systemic Changes as Induced by *M. incognita* in C-152 :

Changes in protein profile in IF of leaves of C-152 inoculated with *M. incognita* are depicted on Figure 9, Lanes C-G and the Rm values with their respective molecular weights are given in Table 11. On analysis, we find that only one protein band, Rf 0.68, mol. wt. 42.0 started appearing 7 days following inoculation with the nematode. Four additional bands out of which two having higher molecular weight (98 and 95 kD) with Rm values 0.30 and 0.31; and two having medium molecular weight (37 and 34 kD) with Rm values 0.74 and 0.78 started appearing 10 DAI. These bands were present in the subsequent intervals also. Since no band was present at low mol. wt. positions, a higher concentration gel was not run.

Systemic Changes Induced by *H. cajani* on C-152 :

Changes in protein profile in IF of leaves of C-152 inoculated with *H. cajani* are shown in Figure 9, Lanes H-K and the Rm values with their respective mol.wt. are given in Table 12. The results indicate that only one protein band of Rm 0.68, and mol. wt. 42.0 kD start appearing 10 days after infection. Two protein bands of high mol. wt. (Rm 0.30 and 31, mol. wt. 98.0 and 95.0 kD, respectively) could be resolved only by 14 days after inoculation, while the same proteins started appearing from the 10^{th} day post-infection when inoculated with *M. incognita*. Also two protein bands of middle mol. wt., Rm 0.74 and 0.78 (37.0 and 34.0 kD) could be resolved only 21 days after inoculation. These could be detected right from the 10th day post-infection with *M. incognita*.

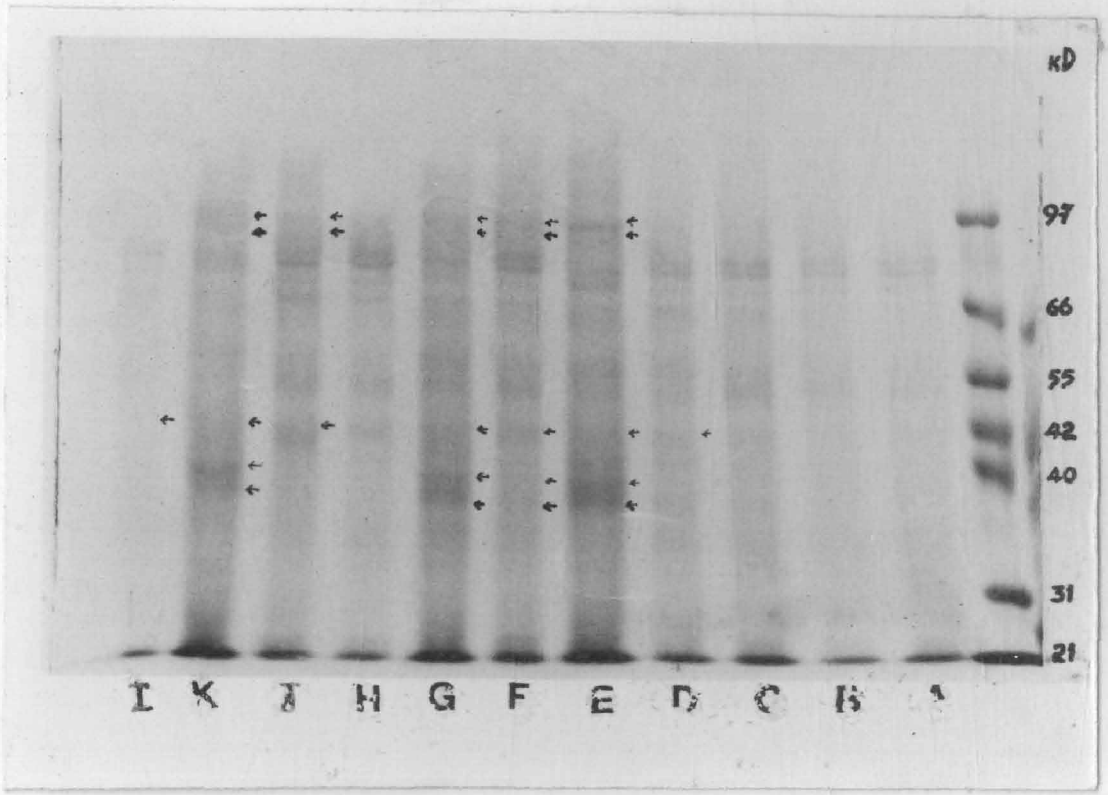


Figure 9:

Intercellular fluid (IF) was extracted from the leaves at 2, 7, 10, 14 and 21 days after inoculation (DAI) of the roots of cowpea cv. C-152 with the root-knot nematode, *M. incognita* and the pigeonpea cyst nematode, *H. cajani* and at 0 and 21 days following start of the experiments from plants not infected by the nematodes. Polypeptides were separated by SDS-PAGE (10% acrylamide) and visualized using Coomassie blue. IF extracts equivalent to 50 µg of protein was loaded on each track.

Track A. Uninfected plant, 0 DAI;
Track B. Uninfected plant, 21 DAI.
Track C toG plants infected by *M. incognita* at 2, 7, 10, 14 and 21 DAI.
Track H to K plants infected by *H. cajani* at 2, 7, 10, 14 and 21 DAI.
Appearance of novel protein bands is indicated by arrows (⇐). The molecular weight of standard protein markers is given in kilo Daltons.

Table 11 : Relative Mobility(Rm) values and molecular weights (kD) of the various polypeptides on 10% SDS-PAGE gel from the IF of C-152 inoculated with *Meloidogyne incognita*

Relative Mobility	CONTROL		Days After Inoculation (DAI)				
(Rm)	0	21	2	7	10	14	21
0.30	--	--	--	--	**98.0**	**98.0**	**98.0**
0.31	--	--	--	--	**95.0**	**95.0**	**95.0**
0.36	83.3	83.3	83.3	83.3	83.3	83.3	83.3
0.39	77.3	77.3	77.3	77.3	77.3	77.3	77.3
0.41	73.7	73.7	73.7	73.7	73.7	73.7	73.7
0.43	70.3	70.3	70.3	70.3	70.3	70.3	70.3
0.55	54.0	54.0	54.0	54.0	54.0	54.0	54.0
0.59	49.8	49.8	49.8	49.8	49.8	49.8	49.8
0.68	--	--	--	**42.0**	**42.0**	**42.0**	**42.0**
0.74	--	--	--	--	**37.0**	**37.0**	**37.0**
0.78	--	--	--	--	**34.0**	**34.0**	**34.0**

Table 12 : Relative Mobility(Rm) values and molecular weights of the various polypeptides on 10% SDS-PAGE gel from the IF of C-152 inoculated with *Heterodera cajani.*

Relative Mobility (Rm)	CONTROL		Days After Inoculation (DAI)			
	0	21	2	10	14	21
0.30	--	--	--	--	**98.0**	**98.0**
0.31	--	--	--	--	**95.0**	**95.0**
0.36	83.3	83.3	83.3	83.3	83.3	83.3
0.39	77.3	77.3	77.3	77.3	77.3	77.3
0.41	73.7	73.7	73.7	73.7	73.7	73.7
0.43	70.3	70.3	70.3	70.3	70.3	70.3
0.55	54.0	54.0	54.0	54.0	54.0	54.0
0.59	49.8	49.8	49.8	49.8	49.8	49.8
0.68	--	--	--	**42.0**	**42.0**	**42.0**
0.74	--	--	--	--	--	**37.0**
0.78	--	--	--	--	--	**34.0**

DISCUSSION

The major goal of this investigation was to look for evidence of nematode mediated leaf apoplastic novel proteins. Despite the fact that, a large number of research groups over the world have concentrated on PR proteins induced by fungal, viral and bacterial pathogens, in order to gain more insight into its molecular mechanism, but virtually a beginning is yet to be made in this area of investigation involving plant-parasitic nematodes. The significance of this investigation under report, against this backdrop, is to be realized.

For the sake of convenience the discussion is organized into two sections. The first section proposes to discuss the experimental procedure used in the investigation and in the subsequent part it is intended to judge the significance of the findings of this investigation.

The apoplast represents a key domain within the plant. It is a continuum with both the external environment and the plant cell, and therefore, its contents can potentially be modified by both internal and external stimuli. As the molecular events occurring peripheral to the plasma membrane are important to study plant-pathogen interaction, cell-to-cell interaction and cell wall metabolism; the extraction of apoplastic fluid gains immense importance. Furthermore, various defence related proteins like chitinases, β 1,3 glucanases, peroxidases, hydroxyproline rich glycoproteins and several pathogenesis related (PR) proteins of unknown function have been isolated from IF of leaves of plants infected by various pathogens. Hence, it is needless to emphasize the importance of successful extraction of apoplastic proteins in order to have meaningful information about the role of PR polypeptides in determining the role of plant - nematode relationship. It will be in order and appropriate at this juncture to discuss the experimental

procedures employed in this investigation prior to our focusing attention on conceptual molecular and functional aspects of PR proteins synthesized in leaf apoplast on the basis of this report and those cited in contemparary literature.

EXPERIMENTAL PROCEDURES :

Keeping in view the importance of the isolation of IF as the first major step in studying PR proteins, considerable attention was paid in this investigation on standardization of procedures so as to ensure good harvest of apoplast proteins and to obtain reproducible results. Several procedures for the extraction of apoplast protein including the commonly adopted method of deWit and Spikman (1982) were followed. Since the procedure given by deWit and Spikman(1982) did not yield good results certain minor modifications were carried out. Apparently, when the beaker was substituted with a Petri-plate and the leaves covered with a smaller Petri-plate, instead of a perforated ceramic lid, the extent of infiltration increased. The reason might be that the surface area directly exposed to vacuum increased. Also, the smaller Petri-plate, which was used as a lid, exerted more uniform pressure thus improving the efficiency of the vacuum in the container with leaves.

Further, when a more powerful vacuum pump, especially that of a lyophilizer was used, the period of vacuum application got reduced from the normal 1 hour to 2 minutes (Table 4). Apparently it means that, a vacuum pump which is normally used in laboratory might not yield quick extraction of IF and in particular, during summer when the temperature reaches 40-43^0 C, the proteins in the leaves might get denatured/altered. Hence, a more powerful vacuum pump, as

mentioned above, should be used which improves the efficiency as well as the accuracy of IF extraction.

Following infiltration of the intercellular space with water, we need to recover the same through low speed centrifugation. For this too, a further modification and improvisation of the original procedure by deWit and Spikman (1982) and Zoltan Klement (1965) was necessary. The basic idea in this step is the construction of a perforated barrier which would allow the passage of IF to the bottom of the tube but withhold the leaves from floating on the collected IF. Four different procedures (Table 2) were experimented of which the use of a perforated plastic test tube caps (Fig.1) provided the best results. This met all the requirements by withholding the leaves from floating on the collected IF and accommodating upto 20-25 leaves in one centrifuge tube. These caps could be reused as well as autoclaved. These caps were also cost effective when compared to the 20 ml syringes. Caps being of polypropylene material did not react with the contents of the IF, thus retaining the original chemical composition. Recovery of the IF following collection also became easy as there was no other impending matter when compared to the procedure involving the use of glass beads. A 95-100% recovery of the infiltrated fluid is possible with this improvisation.

Because of the low concentration of protein in IF, quantitative estimation by the procedure of Lowry *et al.* (1951) did not give proper results. Hence, a more sensitive procedure of Bradford (1976) was followed in this investigation. This procedure gave reproducible results.

The major goal of this investigation was to study the changes in protein profile of the IF of leaves through SDS-Polyacrylamide gel electrophoresis. Exploratory studies were first made using the raw IF sample but the bands thus resolved were not sharp but became diffused. One possible explanation for this

kind of results could be the presence of artefacts in crude preparation which interfere with the proteins during electrophoresis as well as during staining.

It was experienced in this investigation that best and reproducible results were obtained with regard to PR proteins resolved on gel surface when partially purified preparations were used instead of crude samples. Several procedures were expremented to obtain partially purified IF protein samples.

Sephadex G-25 column filtration in normally adopted to purify the samples from major salts and other phenols (Scopes ,1994). Also, this is the standard preliminary step followed in all major protein purification process. This yielded good results. But this method has its own disadvantages too that need to be commented on. The time taken for this process is relatively longer i.e. one sample takes about half an hour to 3/4 of a hour to be purified. Also, the sample gets diluted and needs further concentration. This procedure requires associated instruments like a fraction collector with a spectrophotometer for easy working.

To reduce this time gap as well as the need to eliminate other associated instruments, purification of the proteins by adding pure/raw Sephadex G-25 beads to the IF sample was followed. This procedure gave results comparable to that of passing the sample through the Sephadex G-25 column. This procedure is simple, quick takes just 30 minutes and more number of samples can be processed at the same time. The problem of dilution of raw sample nor the use of associative instruments is encountered. The only disadvantage being that the recovery of proteins is 80%.(Pohl, 1990)

Syringe filter units are commonly used in all laboratories for sterilizing liquid samples. An attempt was made to see as to whether these filter units helped in purifying protein samples from other contaminants. Such procedure did not provide desired results and hence further studies with this method was abandoned.

Several new products are now available commercially which are designed to purify proteins from very low volume. The Millipore ultrafree-MC filter unit is one such product. The filter used for sample purification and desalting prior to SDS-PAGE was 0.4 μ Durapore microporous membrane which is low protein binding. Passing the IF sample prior to SDS-PAGE through this filter unit gave very good results. The process was found to be time saving with no loss in protein and the problem of dilution is also not encountered.

Following the passage of the IF sample through the 0.4 μ Durapore filter, it was also passed through a polysulfone filter with a protein mol.wt. cut off of 100,000 daltons. This also gave similar results as the earlier samples.

As the procedure involving addition of raw (pre-swelled) Sephadex G-25 beads gave the best results, in a short time this procedure was followed for purification of proteins in further experiments. The forgone discussion leads one to conclude that rather than following one well adapted technique, a combination of several standard procedures may be required to obtain reproducible and better results for analytical studies of IF.

NOVEL PROTEINS

One major conclusion of this work is that irrespective of host reaction, compatible or incompatible, nematode infection of root system leads to elevated levels of apoplast protein. This indicates that perhaps some signal is generated in the roots following root infection, which passes to the shoot/leaves resulting in an increased level of protein. Also, it can be seen that the protein content of IF increases with age, but in infected plants the quantity is more when compared to control. These observations are in accord with those reported by Hammond-

Kosack *et al.* (1989) from IF of potato inoculated with *G. rostochiensis*. It will be logical to maintain that the increase in quantity may be due to the induction of defense related proteins like pathogenesis related (PR) proteins.

One of the interesting observations of this investigation is the biphasic increase in I/U ratio with regard to cowpea cultivar C-152 following infection by *M. incognita* against which it is resistant. This is in contrast with the steady increase in I/U ratio in Pusa Komal, a cowpea cultivar susceptible to *M. incognita* and *H. cajani*. Due to lack of relevant information, it is extremely problematic to explain the significance of such phenomenon in the terms of host-parasite relationship. One may be tempted to advance an argument that cultivar Pusa Komal was in a constant effort to modify itself during the entire post-infectional period which is reflected in the terms of enhanced protein synthesis. In contrast, C-152 may present an entirely different picture especially with reference to *M. incognita*. A meaningful conclusion can only be arrived at when we know more about the secretory granules from nematode esophageal glands.

Qualitative analysis of proteins in leaf IF from plants infected by *Meloidogyne incognita* and *Heterodera cajani* reveals that systemic changes are induced in the leaves by root infection by both these nematodes. The induction of new proteins is not immediate, but starts occurring a week after inoculation. The possible reason for this is that the messenger compound from the roots that initiates induction of defense protein in uninfected plant parts takes about 5-6 days to be transported from the site of infection. These results are corresponding to the earlier results obtained in plant (potato)-nematode (*Globodera sp.*) systems by Hammond-Kosack *et al.* (1989) and Rahimi *et al.* (1993). Also, similar results are reported from other plant-pathogen systems especially with viruses and bacteria (Pierpoint *et al.*, 1981 and Cornelissen *et al.*, 1986). As the period after

inoculation increases new proteins are synthesized. Some occurring as late as 21 days after inoculation. This is possible, as the plants might still be responding to the parasites presence, which is diverting the resources of the plant from the source (leaves) to itself. Nonetheless the validity of such arguments needs to be substantiated by experimental results.

Before proceeding further, one important point that has to be borne in mind is that a constitutive intervarietal difference exists in the IF proteins from C-152 and Pusa Komal.

Inoculation of Pusa Komal roots with *Meloidogyne incognita* induces the formation of proteins in the range of 15,000 daltons to 66,000 daltons. Five proteins - 66,000, 46,000, 42,000, 34,000 and 25,000 are produced 7 days after infection (DAI) . Two proteins with characters of 25,000 and 15,000 daltons are produced 14 DAI. Surprisingly, one protein with a molecular weight of 16,000 daltons which appears immediately following inoculation is not seen at later intervals.

When these results are compared with the resistant variety to root-knot nematode C- 152, inoculated with *M. incognita*, we find that only one protein - mol. wt. 42,000 daltons is induced 7 days post-infection. Also, two high molecular weight proteins with mol.wt. 98,000 and 95,000 daltons are produced from 10 DAI, which are not seen in Pusa Komal. Two polypeptides with a molecular weight of 37,000 and 34,000 daltons are produced 10 days post-infection. Thus, we find that only 2 proteins 42,000 and 34,000 are common among the 2 varieties inoculated with *M. incognita*.

As experienced previously by Rahimi *et al.*(1993) this investigation also revealed that nematode inoculated susceptible host exhibited more apoplast protein bands than the resistant host. In contrast no such differences was

observed by Hammond-Kosack *et al.* (1989) . Whether or not such observed variations in experimental results may be due to differences of nematode species and host crop used remains to be seen. Obviously, more information needs to be generated before any meanigful conclusion is drawn.

An analysis of SDS-PAGE results prompts us to stress on one point concerning early and consistent appearance of two novel proteins having medium molecular masses (37 and 34 kD) which are expressed much earlier in a resistant response than in a susceptible response. This might be helpful to the plants in avoiding further infection by the nematodes. In a susceptible response, though the proteins produced are of similar molecular weight, they are induced at a later stage. The observations on C-152 infected with *H. cajani* provides support to this assumption.

It remains to be seen as to whether the new proteins resolved in this investigation have similar properties to the ones produced following infection by other pathogens in various plants. Such relationships can be documented by conducting serological studies with the antibodies of tobacco PR proteins. An attempt was made in this investigation to determine the molecular weight characteristics of the novel proteins. A comparison of molecular mass of the new proteins produced with those reported in other plant pathogen interactions indicates that the 42 and 34 kD produced are similar to the PR 2 (β 1-3 glucanases) and PR 3 (Chitinases) proteins respectively. It may be mentioned in this connection that PR 2 and 3 have been assigned to play an active role in plant defence especially against fungal pathogens. As these proteins are highly conserved and present both in monocots and dicots, their presence in both the variety as well as their inducement by both the nematode pathogens is not surprising. One of the major problems in this area of investigation is our inability

to assign exact functions and role to PR proteins in plant nematode interactions. Although to a limited extent the role of PR 2 and 3 has been postulated with regard to fungal pathogens their role in defence against bacterial, viral and nematode pathogens remains to be explained. But the fact remains that these proteins are systemically induced in the whole plant following infection with a pathogen at a local site and also they help in limiting further infection. It probably functions in a way similar to the human immune system, though the latter is highly advanced and complex.

Another interesting information generated from this study is that when a variety of cowpea is attacked, the response is similar to both the nematode species with regard to the new proteins that are being produced. This is true in both the varieties - Pusa Komal and C- 152. This possibly shows that the general defense mechanism of the plant might be similar. On attack by a pathogen, similar set of genes are set into action and only one or probably a set of similar messenger compounds are activated, which, in turn, brings about a similar systemic response.

In summary, this investigation conclusively documented the evidence for nematode-induced novel proteins synthesised in leaf apopolast. Some of these nematode-induced novel proteins have similar molecular masses as reported in other plants due to infection by other pathogens.

Further, work on elucidating the exact functions these proteins in the defense response has to be undertaken. Many of the major laboratories in the world are working in this direction. Most have gained success in cloning the genes involved in production of these proteins. Another important area to be investigated is whether these proteins impart resistance against organisms different from those that induce them. Various pathogen combinations can be tried following which we can say for sure as to whether these proteins have a broad

spectrum of activity. Also, antibodies raised in different plant species following inoculation with various pathogens can be studied for their similarity and if found almost similar the incorporation of these genes as transgenic plants can be experimented.

When these questions are answered, it will not only help in understanding the resistance response further, but also will help in understanding the messenger systems involved in the plant.

SUMMARY

Pathogenesis related proteins (PR) are induced in plants under specific pathological conditions, upon infection by various plant pathogens like fungi, bacteria and viruses. Very little information is available regarding PR protein gene expression following nematode attack on plants. This investigation studies the induction of PR proteins by the root-knot nematode, *Meloidogyne incognita* and pigeonpea cyst nematode, *Heterodera cajani* on cowpea. The cultivars used in the study were Pusa Komal, a variety susceptible to both the test nematodes and C-152, a commercially grown variety resistant only against *M. incognita.*

This investigation has led us to develop a more efficient method of infiltration and extraction of intercellular fluid (IF) from the apoplastic space of leaves.

The protein content of the IF changed both quantitatively and qualitatively upon inoculation by both the nematodes. It was found that the protein concentration increases in both the cultivars when inoculated with the two groups of nematodes under study, when compared with their respective controls. While Pusa Komal showed a steady increase in protein concentration, with maximum protein concentration appearing at 21 days after inoculation (DAI), C-152 showed a biphasic increase in protein concentration with maximum protein content occurring at 14 DAI, and decreasing in the next interval. *M. incognita* infection resulted in a greater increase in protein content than that by *H. cajani.*

SDS-PAGE analysis of IF extract of the leaves following root infection by the nematodes showed the appearance of novel protein polypeptides. The induction of new proteins by both *M. incognita* and *H. cajani* were similar in Pusa Komal but for a 15 kD protein that was induced by *M. incognita* at 14 and 21 DAI,

but not by *H. cajani* infection. Infection by these two nematodes on Pusa Komal, induced production of 66.0 kD, 46.0 kD, 42.0 kD and 34.0 kD proteins at 7 DAI. and a 25.0 kD protein 10 DAI. A 40.0 kD protein is induced by *M. incognita* at 14 DAI and only at 21 DAI by *H. cajani*.

Qualitative examination of IF of C-152 infected by the two nematodes showed that a 42 kD protein got induced 7 DAI . Whereas, 2 proteins *viz.* 98.0 and 95.0 kD, appeared 10 DAI by *M. incognita* and 14 DAI by *H. cajani* respectively. Two proteins viz., 37.0 kD and 34.0 kD got induced by *M. incognita* on the 10^{th} DAI were detected only on the 21^{st} DAI by *H. cajani*. Thus, similar protein changes are induced by both the nematodes on C-152, with only the time of induction varying, *M. incognita* inducing newer protein production at an earlier stage.

The molecular weights of the novel proteins induced by nematodes being similar to those induced by other pathogens in other crop plants, make us conclude that they might share a similar function. Two proteins detected fall in the range of β-glucosidase and chitinase respectively which have earlier been shown to have a role in plant defense against fungal pathogens.

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Originals not seen