DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF *MELOIDOGYNE* SPP. (ROOT-KNOT NEMATODE) ANTIGENS

Thesis

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
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ZOOLOGY
(Minor Subject: Biochemistry)

By

Mandeep Kaur
(L-2008-BS-200-M)

Department of Zoology
College of Basic Sciences and Humanities
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CERTIFICATE I

This is to certify that the thesis entitled, **"Development of Enzyme-linked immunosorbent assay for detection of *Meloidogyne* spp. (Root-knot nematode) antigens"** submitted for the degree of **M.Sc.,** in the subject of **Zoology** (Minor subject: **Biochemistry**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Mandeep Kaur (L-2008-BS-200-M)** under my supervision and that no part of this thesis/dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

_____

Major Advisor
(Dr. Jyotika Kapur-Ghai)
Professor-cum-Head
Department of Zoology
Punjab Agricultural University
Ludhiana- 141 004 (India)

CERTIFICATE II

This is to certify that the thesis/project report entitled, **"Development of Enzyme-linked immunosorbent assay for detection of *Meloidogyne* spp. (Root-knot nematode) antigens"** submitted by **Mandeep Kaur (L-2008-BS-200-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.Sc.**, in the subject of **Zoology** (Minor subject: **Biochemistry**) has been approved by the Student's Advisory Committee along with Head of the Department after an oral examination on the same.

Head of the Department
[Dr. Jyotika Kapur-Ghai]

Major Advisor
[Dr. Jyotika Kapur-Ghai]

Dean Postgraduate Studies
[Dr. (Mrs.) S. K. Mann]

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ABSTRACT

Plant parasitic nematodes are destructive pests worldwide that cause severe losses in agriculture. Root-knot nematodes (*Meloidogyne* spp.) are obligate, sedentary plant endoparasites that are extremely polyphagous in nature with a wide host range. Keeping in view the huge economic losses by this parasite, it is essential to control the disease at an early stage. For any control strategy to be effective, an early and accurate diagnosis is of paramount importance. Immunoassays have the inherent advantages of sensitivity and specificity; have the potential to identify and quantify these plant-parasitic nematodes. Hence, in the present studies, ELISA has been developed for the detection of *Meloidogyne* spp. antigens. This is based on detection of egg antigens, for which anti-*Meloidogyne* antibodies were produced by immunization of rabbits with soluble proteins of the eggs. The production of antibodies was confirmed by the appearance of precipitin lines in double immunodiffusion method. An indirect enzyme-linked immunosorbent assay (ELISA) was developed for detection and titration of these antibodies. Results indicated as high as 320 K titre of the antisera. Finally competitive inhibition ELISA was developed employing these anti-*Meloidogyne* antibodies for detection of *Meloidogyne* antigens. This is based on competition between solid phase bound antigens and free antigens for limited antibodies. Sensitivity of ELISA was 10 femtograms. This sensitivity was further enhanced to 1 femtogram with an additional step of pre-incubation, in which antibodies and free antigens are allowed to react before the competition. Competitive inhibition ELISA developed in the present studies has the potential of being used as an easy, rapid, specific and sensitive diagnostic tool for the detection of *Meloidogyne* infection.

Key Words: *Meloidogyne*, ELISA, diagnosis, antigens, antibodies.

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Signature of Major Advisor Signature of the Student

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VITA

Name of the student	:	Mandeep Kaur
Father's name	:	S. Mohinder Singh
Mother's name	:	Mrs. Amarjit Kaur
Nationality	:	Indian
Date of birth	:	6-12-1986
Permanent home address	:	V.P.O.- Hathur, Tehsil- Jagraon, Distt.- Ludhiana (142031), Punjab

EDUCATIONAL QUALIFICATION

Bachelor's degree	:	B.Sc. (Medical)
University	:	Panjab University, Chandigarh
Year of award	:	2008
OGPA/OCPA/% marks	:	65%
Master's degree	:	M.Sc. (Zoology)
OCPA	:	7.62/10.00
University	:	Punjab Agricultural University, Ludhiana
Year of award	:	2010
Title of Master's Thesis	:	Development of Enzyme-linked immunosorbent assay for detection of *Meloidogyne* spp. (Root-knot nematode) antigens
Awards /Scholarships	:	University Merit Scholarship during Master's Programme Member of PAU Science Club

Chapter I

INTRODUCTION

Crop diseases cause a significant loss to our food supply and therefore are of great economic importance. Plant-parasitic nematodes are one of the major causative agents of crop diseases. These are the agricultural pests worldwide and are limiting crop production capabilities in many developing countries, especially in tropical and sub-tropical regions (Ibrahim 2004). These result in great economic losses to the tune of about 10 billion dollars in the USA and 125 billion dollars globally (Chitwood 2003).

Nematodes, a diverse group of roundworms, are the most numerous multicellular animals on earth (Kimpinski and Sturz 2003). Since their appearance on earth, almost half a billion years ago, nematodes colonized any available trophic niche on the planet (Wall and Virginia 1999). Many nematode species are free living; some of the most important plant parasitic nematodes are quite sedentary, remaining closely associated with plant roots (Hajek 2004).

Out of the various plant parasitic nematodes, *Meloidogyne* spp. (Root-knot nematodes) are sedentary endoparasites of the economically most damaging family of plant parasitic nematodes, the *Heteroderidae* (Williamson and Gleason 2003). *Meloidogyne* belongs to:

Kingdom - Animalia
Phylum - Nematoda
Class - Secernentea
Order - Tylenchida
Super family - Tylenchoidea
Family - Heteroderidae

These are obligate parasites and distributed worldwide. These are extremely polyphagous (Trudgill and Blok 2001) with a host range of more than 2,000 plant species including major hosts like cotton, sugar beet, tobacco, tomato, pepper, peanut, even spinach, flowering and ornamental plants; and common weed species. Four major species of *Meloidogyne* worldwide are: *M. javanica, M. arenaria, M. incognita* and *M. hapla* (Eisenback and Triantaphyllou 1991); *M. incognita* and *M. chitwoodi* being the most important species since these infect almost all the cultivated plants. These biotrophic pathogens have evolved highly sophisticated parasitic relationships with their hosts (Roze 2008).

Within the root, the roundworm migrates intercellularly in a stealthy way through the cortex and induces the formation of large multinucleate cells called 'giant cells' (Koltai and

Bird 2000). These root galls are two to three times the diameter of healthy roots which give the root system a knobby appearance. Juvenile stage 2 (J2) is the most infective stage and predilection site of this juvenile stage of the worm is root tip in the zone of elongation. Females as well as the third and the fourth stage larvae of this parasite are sedentary endoparasites of many plants. Males and second stage larvae are migratory and can be found in soil. Infection with *Meloidogyne* leads to suppression of innate immunity and reprogramming of plant cells to form this feeding structure containing multinucleate giant cells (Bellafiore *et al* 2008). This parasite exhibits a complete life cycle and interaction with its host plant (Eisenback and Triantaphyllou 1991).

The economic level of crop is considered very essential for nematode management (Korayem 2006). Due to their wide host ranges, short generation times, and high reproductive rates, these root-knot nematode species are difficult to control (Trudgill and Block 2001). The polyphagous nature of this nematode makes it a difficult parasite to control with traditional strategies, like crop rotation, which are rarely effective on *M. chitwoodi* and require extremely good weed control (Den Nijs *et al* 2004).

The widespread concern about the health and environmental hazards of pesticides has led to considerable international efforts directed towards reduction of their use (Dinham 1992). Nematicides have been the most unacceptable class of pesticides and tend to be toxic, expensive to use and dangerous to the environment (Urwin *et al* 1998). Several of the most effective and widely used nematicides are being now banned due to their potential hazard to human health (Giannakou *et al* 2002). Thus, keeping in view the huge economic losses by this parasite, it is essential to control the disease at an early stage. For any control strategy to be effective, an early and accurate diagnosis is of paramount importance. There exists an increasing need in modern agriculture for rapid and large-scale identification of plant pathogens. Presently, diagnosis is mainly based on observations like visualization of galls on roots of plants, presence of eggs etc. These methods have their own limitations in terms of sensitivity and specificity. Thus, conventional methods have limited success in controlling these recalcitrant parasites resulting in the search for effective alternative control methods.

New management strategies will require simple, rapid and reliable methods for the identification and quantification of nematode pests which will enable the routine monitoring of nematode populations (Davies *et al* 1996). Qualitative diagnostic tests which require the detection of nematodes present in small numbers are essential for quarantine and certification schemes, but one of the most intractable problems in nematology is the unavailability of routine quantitative assays which are able to combine a high degree of specificity and sensitivity. Quantitative assays using molecular techniques have to rely on diagnostic probes. Further, the technology for enzyme-linked immunosorbent assay (ELISA) is more highly

developed than for quantitative DNA-based assays (Miller and Martin 1988). Hence, immunoassays are routinely used to detect and quantify plant and animal pathogens because these reduce the amount of time needed for identification and also due to enhanced reliability of diagnosis.

As *Meloidogyne* species are very similar, their precise identification requires combination of morphological and biochemical methods. The advent of molecular biology has brought with it a number of new techniques for analysing DNA, protein, carbohydrates and lipids which can be helpful in the identification of parasites. Of these, allozyme-, monoclonal antibody- and DNA-based systems are the most well developed for nematodes (Williamson 1991). These advances in biochemical and serological methods provide opportunities for identification of plant pathogens (Schots *et al* 1990). *Meloidogyne* species have been identified using polyacrylamide gel electrophoresis, immunodiffusion, immunoelectrophoresis and gas liquid chromatography (Fox and Atkinson 1986). These methods, however, are often not suitable for routine applications, being too laborious, lack desired sensitivity and specificity, require sophisticated instrumentation and are expensive. Immunoassays offer the advantage of combining specificity and simplicity at a relatively low cost. These properties render immunoassays suitable for routine applications. Out of various immunoassays, enzyme-linked immunosorbant assays (ELISAs) are the best suited due to the inherent advantages of high specificity, sensitivity and are cost effective.

Most of the serological studies have been carried out on animal-parasitic nematodes. On the other hand, only a little work has been done on plant-parasitic nematodes. Hence, keeping in view the present status of the nematode problem in agriculture, in the present studies an attempt has been made to develop ELISA for detection of *Meloidogyne* antigens. Present study was undertaken with the following objectives:

1. Collection of *Meloidogyne* from soil/roots of the infected plants.
2. Preparation of *Meloidogyne* antigens.
3. Production of antibodies against *Meloidogyne* antigens.
4. Development of indirect ELISA for detection and titration of anti-*Meloidogyne* antibodies.
5. Development of competitive inhibition ELISA for detection of *Meloidogyne* antigens.

Chapter II

REVIEW OF LITERATURE

Nematodes are a diverse group of roundworms, and are the most numerous multicellular animals on earth (Kimpinski and Sturz 2003). These are very successful animals provided with a simple body structure and efficient functioning, found in a variety of environments viz. soils, sea or fresh water, as free living, parasitic or predacious animals causing severe damage to world agriculture every year (Siddiqui and Mehmood 1996).

Plant patrasitic nematodes are ubiquitous and cosmopolitan pathogens of vascular plants and exploit all parts of the roots and shoots, causing substantial crop damage (Bird and Koltai 2000). These nematodes also inflict indirect crop damage by acting as aggravators or incitants for several pathogenic bacteria, fungi and virusesThese are found in practically every soil sample often with high species diversity and a high abundance. The economic losses due to plant parasitic nematodes have been estimated to the tune of billions of US dollars every year (Dong and Zhang 2006).

Meloidogyne (Root-knot nematodes) is one of the most destructive pests of all the major cultivated plants. Members of the genus *Meloidogyne,* the root-knot nematodes, constitute an agroeconomically important group of pathogens which establish a complex parasitic relationship with a wide range of host plants (Sasser 1980). Root-knot nematodes (*Meloidogyne* spp.) are highly polyphagous and sedentary endoparasites, which cause major crop losses in agriculture worldwide (Sasser and Freckman 1987). These parasitize monocotyledons, dicotyledons, herbaceous and woody plants causing formation of knots or galls on the roots of susceptible hosts. These nematodes have diverse parasitic relationships with their host plants in order to obtain nutrients necessary for their development and reproduction.

Plant parasitic nematodes are important plant pests which are difficult to identify and quantify. There exists an increasing need in modern agriculture for rapid and large scale identification of plant pathogens. This need can be explained in view of the necessity to monitor alternative crop protection methods because of increasing environmental concerns with regard to chemical crop protection. These parasites are also difficult to control using conventional methods. During the past three decades, nematode control has undergone spectacular changes regarding diagnostics and management techniques largely due to advances in understanding of nematode biology and innovations in pest management approaches. Although, nematicides are quite effective in controlling these nematodes, the high cost of nematicides and their applications, toxicity to man and animals and

phytotoxicity to some plants, prevent their liberal use. The control of *Meloidogyne* spp. by chemicals with some exceptions, is now considered uneconomical (Norton 1978).

Within nematology, recent advances in biochemistry and serology may provide opportunities for such species identification. Serologically based techniques offer new ways to identify and quantify these parasites. Differences between nematode species have been demonstrated at the protein level by polyacrylamide gel electrophoresis (PAGE), immunodiffusion, immunoelectrophoresis and pyrolysis gas liquid chromatography (Fox and Atkinson 1986) and at the DNA level by restriction fragment length polymorphism (De Jong *et al* 1989). These methods, however, are often not suitable for routine applications either because they are too laborious or they lack sensitivity. In comparisons with these techniques, immunoassays, particularly Enzyme-linked immunosorbent assays (ELISAs), offer the advantage of combining specificity, rapidity and simplicity at a relative low cost. These properties render immunoassays suitable for routine applications. Literature pertaining to the present piece of research work is presented as under:

2.1 *Meloidogyne* spp. (Root-knot nematodes)

2.1.1 Occurrence and distribution

The root-knot nematodes are ubiquitous and cosmopolitan and thus are of major economic significance especially in temperate, warm and tropical regions. On top of their broad spectrum of feeding habits, nematodes also live in a wide range of ecological niches including the most extreme environments on earth (Wall and Virginia 1999). Examples of such extreme environments are hot and cold deserts, which are characterised by extreme temperatures, limited water availability and high soil pH and salinity (Freckman and Virginia 1989). These have been collected from frozen soil, tropical rain forests, arid plains and remote islands (Sasser *et al* 1983). The largest populations of *Meloidogyne* spp. are found in sandy or coarse-textured soils and in these soils these may be found up to a depth of 30 cm. Clay soils are less favorable for *Meloidogyne* spp., and the nematodes are confined to the top 10 cm. Soil temperatures that are optimum for invasion of the roots and development and reproduction of the nematodes range from 25 to 30°C.

At present, more than ninety different root-knot nematode species have been described in the literature (Karssen and Den Nijs 2004). The Columbia root-knot nematode *Meloidogyne chitwoodi* was first described in 1980 as an endoparasite of potato in the Pacific Northwest of the USA (Golden *et al* 1980). Four species of *Meloidogyne* are the major pests worldwide which include *M. incognita, M. javanica, M. hapla* and *M. chitwoodi.* A total of 1,256 populations of *Meloidogyne incognita* were collected from vegetables in eight districts

of Uttar Pradesh, India (Khan and Khan 1991). *M. graminicola* has also been reported from Bangladesh and India (Bridge *et al* 1982).

2.1.2 Morphology

Meloidogyne are small round worms typically from 300 µm to 2 mm usually not larger than 500 µm in length and 15 µm in width. Juvenile stage (J2) is the only infective stage. Second-stage juveniles are migratory and have a distinct vermiform body, tapering at both extremities, but much more posteriorly. Cephalic framework of juveniles is also present but the labial areas are not properly set off. Late second-stage juveniles are sedentary and have a swollen body (spike-tailed). Third and fourth stages occur within the second-stage cuticle and are devoid of stylet.

Female root-knot nematodes are sedentary and globose wtih a projecting neck. No pre-adult vermiform female stage is present. Its cuticle is moderately thick and annulations are present forming a fingerprint-like pattern around vulva and anus (Luc *et al* 1975). Excretory pore is present anterior to median esophageal bulb, often only slightly posterior to stylet base. Vulva and anus are present terminally and perineal region is slightly raised. No cyst stage is present in females and eggs are not retained in the female body but deposited in a gelatinous matrix (exception: *Meloidogyne spartinae* where eggs are deposited individually without gelatinous matrix). Females swell to pear shape and are incapable of moving (Taylor and Sasser 1978). Male nematodes are migratory and can be found in soil. Detailed information about nematode morphology is necessary for understanding the host-parasite interaction because it has complete life cycle and interaction with its host plants (Bird 1971).

2.1.3 Life Cycle of the root-knot nematode

The sedentary endoparasitic nematodes have a complex life cycle and it includes a period in the soil, outside the host plant and a parasitic period inside the roots of a host. These nematodes have evolved highly specialized and efficient interactions with their host plants (Lilley *et al* 1999). The root knot nematodes are obligate, cross-fertile species with six life stages: the egg, four juvenile stages and the sexually dimorphic adult stage (Opperman and Bird 1998). Adult female root-knot nematodes release hundreds of eggs into a gelatinous matrix on the surface of the root. The annual life cycle begins with the hatching of eggs in the soil. The egg develops into a first stage juvenile that moults within the cyst and emerges as an infectious, motile second stage juvenile. In a compatible interaction with a host plant, the second-stage juvenile invades the host root and migrates to the vascular cylinder in search of a permanent feeding site. The second-stage juvenile (J2) penetrates the cell in the zone of elongation of the host root. Then, it migrates intercellulary and uses a combination of mechanical force and enzymatic secretions to separate cells at the middle lamella.

Ultimately, the nematode migrates to the root tip and the zone of differentiation (Williamson and Hussey 1996).

These parasitic nematodes initiate the establishment of a complex feeding site after migration. These use their stylet to puncture five to six procambial cells surrounding their head and begin to feed on the cytosolic nutrients. As these feed, the nematodes intermittently secrete saliva into the cells, which triggers a series of dramatic changes in the feeding cells and those surrounding the feeding cells. The saliva contains digestive enzymes, including proteases, which aid in the degradation and liquification of plant cell materials. Depending on the nematode genus, these cells are either stimulated to form a system of hypertrophial multinulcleate cells or "giant cells" (Gheysen *et al* 1996). Giant cells result from the repeated nuclear divisions. The giant cells or syncyntia supply the nematode with nutrients necessary for growth by transferring nutrients from the vascular tissue to the feeding nematode (De Boer *et al* 1996). These feeding cells must remain healthy and metabolically active throughout the nematode's life cycle to ensure their survival (Gheysen *et al* 1996). The cells surrounding the developing juvenile and the giant cells typically proliferate and enlarge to form a gall, hence the common name root-knot nematodes.

Once feeding commences, the juvenile becomes immobile or sedentary and undergoes three moults developing into an adult male or female. The adult female nematode (4th stage larvae) becomes sedentary and increases in thickness during feeding. The body of the female root- knot nematode is entirely embedded in the root tissues. Adult males regain their mobility after feeding and can either fertilize the females and/or migrate through the roots to become free-living in the soil. The female can produce approximately 500 eggs after fertilization. After development, which usually takes three to five weeks, the root knot nematode can lay eggs (covered in a gelatinous matrix) either inside or outside the root. Nematode eggs may hatch immediately and further develop into second-stage juveniles that can cause secondary infections in the same host root or new infections in other host roots in close proximity. Depending upon the host and soil temperature, the entire life cycle of the root knot nematode may be completed in 17 to 57 days.

2.1.4 Gelatinous matrix around the eggs of root-knot nematodes

Root-knot nematode females lay eggs into a gelatinous matrix which is produced by six rectal glands and secreted before and during egg laying (Maggenti and Allen 1960). The matrix initially forms a canal through the outer layers of root tissue and later surrounds the eggs, providing a barrier to water loss by maintaining a high moisture level around the eggs (Wallace 1968). As the gelatinous matrix ages, it becomes tanned, turning from a sticky colourless jelly to an orange-brown substance which appears layered (Bird 1958).

2.2 Attraction, penetration and infection

Plants can affect the life cycle of *Meloidogyne* spp. prior to egg hatching. Eggs are enclosed in gelatinous egg sacs which are usually deposited on the surface of galled roots, although on certain hosts these may also occur within the galls (Godfrey and Oliveira 1932). Hatching of eggs is known to occur without any stimulus from plant roots, however, in some instances root diffusates do stimulate egg hatching (Viglierchio and Lownsbery 1960).

Second-stage juveniles (J2) migrate to the soil from host tissues and constitute the infective stage. Juveniles do not find roots by random movements but are attracted to plants in response to exudates emanating from roots (Green 1971; Prot 1980). Juveniles of *Meloidogyne* spp. could be attracted to and migrate to host roots 25 cm vertically in 10 days (Prot 1978). The rhizosphere is greatly modified by growing roots and associated micro-organisms (Russel 1977). When second-stage juveniles contact plant roots, these often penetrate immediately. Although juveniles primarily enter roots directly behind the root cap, penetration can also occur at other sites, points where lateral roots emerge and galled tissue surrounding the adult females. The penetration site of one juvenile frequently becomes an attractive site for other juveniles to enter the roots (Godfrey and Oliveira 1932). The mechanism of penetration may involve mechanical action by thrusting of stylet. Cellulolytic and pectolytic enzymes may also be involved in penetration (Bird *et al* 1975). After penetration, the root tip may enlarge and root growth is often suppressed for a brief period.

After penetration, juveniles migrate intercellularly in the cortex region of the plants. Susceptible plants respond to feeding by juveniles and undergo pronounced morphological and physiological changes including the formation of giant cells (Byrne *et al* 1977). Galls generally develop in one to two days after penetration and are the first symptoms of infection.

2.2.1 Mechanism of infestation

The species belonging to *Meloidogyne* are highly specialized plant parasitic nematodes, which cause tumor-like formations on the roots known as “galls”. This group is one of the most intensively investigated plant nematodes. These are of great economic importance and attack more than 2000 plant species. The invasion of the host is realized from the second stage larvae (J2) which penetrate the roots. The juveniles inject the special proteins secretions produced by their oesophageal glands into several root cells (usually five to seven). It moves between, rather than through, cortical cells towards root apex, turns at the meristem and migrates back to the vascular cylinder in the zone of cell differentiation**.** In this way J2 transform these cambial root cells into highly specialized feeding cells named “giant cells”. These transformed cells are the permanent feeding site for the parasite (Hussey *et al*

1994). The feeding cells become multinucleate due to the karyokinesis with no cytokinesis. The development of the giant cells depends only on the feeding activities of the juveniles which have penetrated the roots. J2 feeds from the giant cells for 10-12 days, then ceases feeding and moults three times over the next two days to form the adult (Davis *et al* 2000).

2.3 Economic Importance

Plant parasitic nematodes are destructive pests that cause severe losses in agriculture. For example, losses of over hundered billion dollars per year world-wide can be attributed to infections caused by parasitic nematodes (Urwin *et al* 1997). The majority of species within 50 genera of plant parasitic nematodes feed on root tissue with diverse modes of parasitism (Lilley *et al* 1999). These causes a significant annual crop losses, making root-knot nematodes, the most important nematode species throughout the world. These cause a considerable losses in many vegetable crops (Ravichandra and Krishnappa 2004). Several plant families, such as Solanacae, Cucurbitaceae, Leguminosae, Liliaceae, Chenopodiaceae, Compositae, Umbelliferae, Cruciferae and Malvaceae, suffer the greatest damage (Stirling *et al* 1992). It is also highly pathogenic to some staple crops such as cereals, including rice, maize, potato, soyabean, banana, plantain, sweet potato and yam or industrial crops such as tobacco, coffee, sugar cane, sugar beet, cotton and black pepper. *Meloidogyne* species also cause economic damage to fruit crops such as guava, pineapple, papaya and grapes. These also cause a severe damage to fruits (Patel *et al* 2004) and pulses (Mohanty *et al* 1997).

2.4 Diagnostics

2.4.1 Symptoms

Aboveground symptoms associated with the presence of *Meloidogyne* spp. include chlorosis, wilting, retarded maturation and reduction in growth and tillering. *M. graminicola* reduces the elongation ability of deep-water varieties. Root-knot nematodes induce hypertrophy and hyperplasia in the root meristem, cortex, endodermis and xylem and the formation of giant cells in the meristem, cortex and xylem. Primary symptom of root-knot nematode infection is the formation of typical root galls on the root of susceptible host plants (Abad *et al* 2003). Various other symptoms include yellowing, mid-day wilting, water and nutrient stress, poor, sickly growth from a few feet to hundreds of feet across, swollen root tips, root lesions, root branching, oval areas or irregular patches in the field, stunting and premature plant aging and sometimes death also, especially if interacting with other organisms. Presence of J2 and eggs in soil confirms the nematode infestation.

2.4.2 Immunodiagnostics

Plant parasitic nematodes are one of the major constraints to crop production. Keeping in view the huge economic losses by this parasite, it is essential to control the

disease at an early stage. Conventional methods currently used for their diagnosis have their own limitations in terms of sensitivity and specificity. Here is a need for ideal diagnostic tests, which can be antigen/antibody or DNA based. Immunoassys based on antigen-antibody interaction are the methods of choice due to high degree of specificity.

Potential antigens are identified using approach based on observation that B-cells are rapidly recruited to the site of infection with pathogens and to the local draining lymph nodes (Meeuseen *et al* 1989, 1990) where these migrate to target tissue and differentiate into antibody secreting cells. Most monoclonal antibodies presently used in agriculture are directed against viruses, a very few are directed against bacteria, fungi and nematodes (Halk and De Boer 1985).

Bird (1964) conducted experiments showing that injection of living larvae of the plant parasitic root-knot nematode, *Meloidigyne javanica* into rabbits results in the production of antibody. The antibody was shown by gel filteration and ultracentrifugal analysis to be located primarily in the γ-globulin fraction. Starch-gel electrophoresis of antiserum and globulin fraction containing antibody (GFCA) demonstrated that an α_2 – globulin was also involved. By using fluorescent-antibody techniques, it was shown that the antibody reacts with antigenic material exuded from the excretory pore and to a lesser extent from the buccal stylet of both larvae and adults. The gelatinous matrix exuded from the adult female appeared to have antigenic properties. As the larvae aged, a fewer produced precipitates in antiserum. These findings were discussed in the light of similar experiments with mammalian nematode parasites carried out by other workers.

Lee (1965) applied the immunodiffusion technique to the serological discrimination of *Meloidgyne* species. Antibodies raised against extracts from cysts of *Heterodera* species were used to separate, by agar-gel double diffusion, the two morphology-based groups of *Heterodera.* Gooding (1966) stated that partial purification of *Fomes annosus* antigens resulted in simplification of a precipitin pattern and permitted an easier interpretation of the interaction of precipitin bands. Gibbins and Grandison (1968) reported that antiserum to *Ditylenchus dipsaci* (isolated from *Trifolium pratense*) was to compare antigen preparations from *D. dipsaci* parasitizing *Trifolium pratense, T. repens, Medicago sativa, Narcissus* spp., and *Aphelenchoides ritzemabosi* parasitizing *Lilium* spp. The use of the serological procedures of gel diffusion and immunoelectrophoresis for differentiating biological races of *D. dipsaci* may be possible if methods are devised for separating eggs, juvenile stages and adults into large pure samples.

Webster and Hooper (1968) carried out serological and morphological studies on the inter- and intraspecific differences of the plant-parasitic nematodes, *Heterodera* and

Ditylenchus. Antibodies were produced in rabbits by inoculating saline extracts of species of *Ditylenchus* and *Heterodera*. Antigenicity of these was tested using agar gel-diffusion, in which these antisera gave a precipitate response against their homologous extracts and against extracts of some of the other nematode species and up to four precipitation bands appeared, indicating the presence of atleast four different antigens. Double immunodiffusion (DID) tests indicated that three species of *Ditylenchus* were serologically distinct and six species of *Heterodera* could be separated into two distinct serological groups.

Scott and Riggs (1971) and Riggs *et al* (1982) could not show a serological relationship between *Heterodera betule* and a number of other *Heterodera* and *Globodera* species. *G. rostochiensis* and *G. pallida* populations can be distinguished with immunoelectrophoresis (Wharton *et al* 1983) and cross-reactivities of the antisera with some *Heterodera* species were also observed. Similar results have been reported by Griffith *et al* (1982) for the serological differentiation of *H. glycines;* in races 3 and 4 only a single antigenic difference could be demonstrated.

Studies conducted by Hussey (1979) revealed that other *Meloidogyne* species, e.g. *M. incognita, M. javanica* and *M. arenaria,* are serologically closely related because they show a number of common precipitin bands. Cross-absorption of the antisera also demonstrated the presence of some species-specific antigens.

Hussey *et al* (1972) carried out the qualitative and quantitative comparison of proteins of the two *Meloidogyne* species by polyacrylamide gel electrophoresis. They observed differences in their protein composition. They observed eight to ten precipitin bands by DID, using antigens of adult females of *Meloidogyne incognita* and *M. arenaria.* Most of the precipitin bands were common to both the species. Antiserum specific for *M. incognita* was prepared by cross absorption. Two populations of *M. incognita* were serologically identical, whereas two populations of *M. arenaria* differed slightly with respect to one weak precipitin band.

Mamiya and Enda (1979) purified antigens from the homogenates of mixed life cycle stages of various populations of nematodes *Bursapheenchus xylophilus* and *B. mucronatus.* Stage-specific differences were observed in proteins of *Caenorhabditis elegans* (Cox 1981) and also in proteins associsted with the cuticle of *Meloidogyne* species (Reddigari *et al* 1986).

Fleming and Marks (1983) differentiated *Globodera rostochiensis* (Wollenweber) Behrens from *G. pallida* (Stone) Behrens by homogenising cysts containing eggs and second-stage juveniles of potato cyst nematodes (PCN) as immunogens and using isoelectric focusing (IEF) techniques to identify species specific markers.

Eisenback and Triantaphyllou (1985) reported that the identification of root-knot nematodes (RKN) using female RKN homogenates for isozymes of esterase. They employed eggs and juveniles for PCN and females for RKN as antigens for the production of antibodies, as these stages had been found to be useful in distinguishing species of PCN and RKN.

Fox and Atkinson (1985) purified species-specific antigens from potato cyst nematodes *Globodera pallida* and *G. rostochiensis* by cross-immunoelectrophoresis. Purified antigens showed limited cross-reactivity with other cyst-nematode species. The antigens were proteinaceous in nature and did not arise from micro-organisms within the cyst and were mainly hydrophilic with an acidic isoelectric point. Peptidase and acid phosphatase activity was detected in some precipitation arcs which was not species specific.

Jones *et al* (1988) produced monoclonal antibodies against *M. incognita* by immunizing Lou/Iap rats and Balb/c mice with soluble proteins extracted from adult female nematodes. Two hybridoma cells were isolated, producing antibodies with preponderant specificity for *M. incognita* antigens.

Hussey (1989) produced monoclonal antibodies to secretory granules in the dorsal or subventral esophageal glands using preparasitic second-stage juveniles (J2) of *Meloidogyne incognita* as antigens. Antibodies specific for secretory granules in the J2 subventral esophageal glands or the dorsal glands were identified by Indirect Immunofluorescence microscopy. Only antibodies that reacted with granules in the J2 dorsal gland reacted with the esophageal gland lobe of *M. incognita* adult females. The antibodies also reacted with secretory granules in both types of esophageal glands in *M. javanica* and *M. arenaria* J2 but not with granules in esophageal glands of *Heterodera glycines.*

Atkinson and Harris (1989) studied the changes in nematode antigens recognized by monoclonal anibodies during the early infection of soya beans with the cyst nematode *H. glycines.* Using an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (MAbs) whose tissue specificities had already been defined by immuno-cytochemistry, it was found that MAbs recognizing intestinal lipid droplets and granules showed a major increase in this reactivity.

Schots *et al* (1990) produced monoclonal antibodies against potato cyst nematodes using species-specific proteins purified from eggs of *Globodera rostochiensis* or *G. pallida* as immunogens. Hussey *et al* (1990) produced one monoclonal antibody that binds stylet secretions that accumulate on the lip region of *M. incognita* females incubated in perfusion chambers. They used these monoclonal antibodies to immunopurify a high molecular weight secretory glycoprotein from *M. incognita.*

De Nijs *et al* (1990) carried out quantification of *Globodera rostochiensis* and *G. pallida* in mixed populations using species-specific thermostable proteins. A method had been developed to quantify species ratios in mixed populations. The method is based on the separation of species-specific thermostable proteins by SDS-PAGE. Densitometric analyses of the 17 kD protein of *Globodera pallida* and the 18 kD protein of *G. rostochiensis* revealed a high correlation with the species ratio in the mixed samples.

Davies and Lander (1992) produced polyclonal and monoclonal antibodies to supernatants of soluble proteins from homogenates of root-knot nematodes (*Meloidogyne* spp.). The antibodies were used to investigate differences between second-stage juveniles and adults of the three major root-knot species by ELISA and immunoblotting. Most antigens revealed that the polyclonal antibodies were conserved between species but several quantitative differences were observed, mainly between developmental stages. Cluster analysis and principal coordinate analysis using 45 monoclonal antibodies, showing partial discrimination between adult females of the nematode species, divided the nematode into two groups: one group comprised *M. arenaria* and a second group comprised *M. incognita* and *M. javanica.* Three monoclonal antibodies, exhibiting partial discrimination in the order of producing coefficients of variation between the three species of nematode in excess of 40%, were selected as candidates for a diagnostic assay and immunoblotting analysis. An ELISA based assay using the three monoclonal antibodies in concomitant assays against adult females was able to distinguish the three principal species of root-knot nematodes; the same assay was unable to discriminate between the different species using second-stage juveniles.

Davis *et al* (1992) produced monoclonal antibodies against epitopes in the dorsal and subventral oesophageal glands of the root-knot nematode *Meloidogyne incognita.* Antibodies to nematode nuclei, amphids, esophago-intestinal cells, muscle and other structures were also produced. Antibody binding was confirmed by Indirect Immunofluorescence microscopy of nematode specimens. They concluded that intrasplenic immunizations required the least time, small quantities of immunogen and produced the most desirable MAbs. These MAbs provided information about the developmental expression of oesophageal gland antigens in *M. incognita* and also have been used for identification of secretory proteins in *M. incognita* (Ray *et al* 1994).

Schots *et al* (1992) developed quantitative ELISA using monoclonal antibodies to detect and quantify eggs of *Globodera pallidA* and *G. rostochiensis.* With these monoclonal antibodies, 1 to 100 eggs could be detected and identified.

Lawler and Harmey (1993) observed antigens of *Bursaphelenchus xylophilus* and *B. mucronatus* on a debarked and kiln-dried *Pinus sylvestris* section. The antigens were examined using a polyclonal antibody developed against *B. xylophilus.* The antibodies could detect the nematode proteins on the surface of the wood at a lower limit of 0.4 µg but could not discriminate between the two species examined.

Robinson *et al* (1993) described the use of MAbs for immunological differentiation of the two species of potato cyst nematodes. They produced two MAbs to potato cyst nematodes that did not have the problem of coss-reactivity since they only recognised species of the genus *Globodera.* These antibodies recognised the same diagnostic markers at pI 5.7 for *Globodera pallida* and pI 5.9 for *G. rostochiensis* as those identified by Fleming and Marks (1983).

Backett *et al* (1993) obtained monoclonal antibodies (MAbs) with specific reactivity against two developmental stages of *Globodera pallida* using either infective juveniles or females of *G. pallida.* Two MAbs (LJMAb1 & 2) were identified with high reactivity toward second-stage juveniles but showed no reactivity with either potato roots or females of *G. pallida*. One MAb (LFMAb 1) was obtained with the required specificity against only adult female *G. pallida.*

Goverse *et al* (1994) performed ELISA and immunofluorescence tests using three MAbs specific to the subventral oesophageal glands and bound to the stylet secretions from soyabean cyst nematodes (SCN) J2. These antibodies were produced using SCN J2 as antigens. Two of these three MAbs also bound to secretory granules within both the dorsal and subventral oesophageal glands of young SCN females.

ELISA and tissue print analysis revealed that level of antibody accumulation were considerably lower in root tissues of all ages when compared to equal amounts of leaf tissues as studied by Van Engelen *et al* (1994). Leaf and root extracts from plantibody producing plants were assayed for binding activity in ELISA tests using concentrated nematode stylet secretions as antigens. Both showed positive reactions, when compared to identically prepared wild type extracts (Baum *et al*, 1996).

Davis *et al* (1994) studied the developmental expression of oesophageal gland antigens and their detection in stylet secretions of *Meloidogyne incognita.* Immunofluorescence microscopy with monoclonal antibodies *(*MAbs) was used to demonstrate developmentally-regulated synthesis of oesophageal gland antigens in *M. incognita* during parasitism of plants and the secretion of some of these antigens through the nematode's stylet. Several esophageal antigens were temporally expressed in the subventral and dorsal glands of preparasitic second-stage juveniles (J2), parasitic J2 and adult life

stages. Two MAbs that bound to the subventral glands of J2, one MAb that bound to the dorsal gland in adult females and one MAb that bound to the subventral glands in adult females, bound to stylet secretions produced by adult females.

Davies *et al* (1995) produced monoclonal antibodies by using eggs and juveniles as immunogens for distinguishing potato cyst nematode and root-knot nematode species. De Boer *et al* (1996) concluded that various immunogens such as hatched J2, unhatched J2, adult females, microdissected anterior parts of females, a subcellular granule fraction from J2 and stylet secretions of females in serological studies. MAbs were produced by immunizing mice with different antigens from *Globodera rostochiensis* which bound to the subventral oesophageal glands, the body-wall muscle filaments, the genital primordium, the intestinal lumen, cell nuclei and the cuticle surface. An ELISA and dot-blot assay was developed to detect the reactivity of subventral gland MAbs with native protein homogenates from preparasitic J2 and found that with three subventral gland MAbs (MGR 14, 17 and31), antigen could be detected in the protein homogenate. Control tests were performed with four MAbs to body-wall muscle proteins (MGR 7, 13, 16 and 20) and all gave positive reactions. Similar tests with MGR 18, 24, 25, 26, 29, 33, 34 and 37 were found negative, both in ELISA and dot-blot assays.

Ibrahim *et al* (1996) identified root-knot nematode, *Meloidogyne incognita*, using monoclonal antibodies raised to non-specific esterases. The esterase isozyme from *Meloidogyne incognita* with pI$\sim$4.95 was purified using a Rotofor electrophoresis system. The purified enzyme was then used to raise monoclonal antibodies. Although the antibody was cross-reactive in western blots of denatured proteins in the presence of SDS; and in ELISA, but it was not cross-reactive in western blots of native protein. The results demonstrate that a monoclonal antibody to a diagnostic non-specific esterase enables *M. incognita* to be discriminated from *M. javanica* without the need to separate the esterases by electrophoresis first.

Baum *et al* (1996) produced three monoclonal and three polyclonal antibodies to Secreted-Excreted products of *Meloidogyne* spp. and *Heterodera avenae* that were used to examine their reactivity towards *M. incognita* and *M. arenaria* second stage juveniles and adult females. Monoclonal antibodies specific to root-knot nematode stylet secretions was expressed in tobacco and plantibodies and hybridoma derived monoclonal antibodies showed identical specificity.

Kennedy *et al* (1997) raised polyclonal antibodies against *Heterodera glycines* eggshells to determine the feasibility of developing an immunoassay for *H. glycines* eggs. An indirect enzyme-linked immunosorbent assay (ELISA) was developed from antisera

collected 10 weeks after the initial injection. From serial dilutions of sonicated eggshells or whole eggs, a sensitivity of detection to 5 ng/ml sonicated eggshells or 1 egg of *H. glycines* was determined. The method of eggshell preparation had no effect on the antibodies produced, however, the antibodies cross-reacted with sonicated J2 of *H. glycines* and eggs of *Meloidogyne incognita* and *H. schachtii*. Most of the proteins in both life stages of *H. glycines* and eggs of *Meloidogyne incognita* and *H. schachtii,* had similar migration properties when separated on SDS-PAGE gels and stained with Coomassie blue. Western blot analysis with antisera absorbed with homogenized J2 of *H. glycines* showed proteins that were specifically localized to eggshells of *H. glycines.*

Curtis *et al* (1997) suggested that Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) was better ELISA format used to identify and quantify the cereal cyst nematode (CCN) population from the processed soil samples containing up to 20% of organic matter. The threshold limit of detection was estimated by serial dilutions of the soil extracts. It was found to be approximately equivalent to 0.5 eggs or 3.6 eggs per g of soil in a DAS-ELISA, when using respectively the polyclonal antibodies or the monoclonal antibodies as the trapping antibody. The assay could be made more sensitive in soils with lower contents of organic matter.

Lopez De Mendoza *et al* (1999) studied the identification and characterization of excreted-secreted products and surface coat antigens of animals and plant-parasitic nematodes. They observed that the surface coat proteins and oral exudate of *Meloidogyne incognita, Trichinella spiralis* and *Haemonchus contortus* as antigens, were immunolocalised in the lining of the oesophagus and gut and in the exudates present during ecdysis of *H. contortus* L_3. Most of the antibodies recognized carbohydrate epitopes.

Chen *et al* (2001) demonstrated the potential for lectin- or antibody-assisted identification of nematodes with an extension of this technology, using antibody- or lectin-coated magnetic beads (Dynabeads) to recover target nematodes from mixtures of specimens. Lectins and antisera that bound specifically and reproducibly to the whole surface of second stage juveniles of *Globodera rostochiensis* and *Meloidogyne arenaria* were identified. These were then used as probes bound to Dynabeads to recover nematodes from test suspensions. While both types of probe isolated nematodes from suspension, antibody-coated beads recovered from them more efficiently than beads coated with lectins. Other factors that affected the efficiency of recovery, such as the age of the nematode samples, were analysed.

Ibrahim *et al* (2001) evaluated PCR, IEF and ELISA techniques for the detection and identification of potato cyst nematodes (PCNs) from field soil samples. Soil samples from a survey of 484 fields in potato rotations in England and Wales were used to compare the

identification and quantification of PCNs using IEF, PCR, ELISA and bait plant tests. The cyst counts and bait plant test revealed that 64.3% of field samples contained PCNs. Bait plant tests increased the detection rate of PCNs in field samples by 4–6.4%. This means that some infestations are cryptic and would not normally be detected by standard counts. IEF, PCR and ELISA methods distinguished between *Globodera rostochiensis* and *G. pallida* and were able to register mixed populations; however they were not in full agreement. All methods suggested that *G. pallida* is the dominant species in the field samples tested. The PCR results indicated that 66% of field samples contained pure *G. pallida*, 8% contained pure *G. rostochiensis* and 26% contained mixtures of the two species.

Sharon *et al* (2002) produced monoclonal and polyclonal antibodies raised against some secreted-excreted (SE) products of *Meloidogyne* species and *Heterodera avenae* were used to characterize *in vitro* binding to SC, amphids, subventral glands, phasmidal glands, metacarpus and intestine of juvenile stage 2 and adult females of *M. incognita* and J2 of *M. arenaria*.

Fioretti *et al* (2002) developed a bioassay to investigate the effect of excreted-secreted proteins (ES proteins) that were immunolocalised in the cuticle, amphids and subventral glands of second-stage juveniles of the two species of potato cyst nematodes *Globodera pallida* and *G. rostochiensis*, on the nematode movement and on their ability to invade potato roots. Monoclonal antibodies reactive with these ES proteins were used in this bioassay. These effects were temporary, since the nematodes were able to recover and infect potato plants. Nematodes treated with antibodies reactive with secretions from the amphids were temporarily unable to move towards potato roots and their exploratory behaviour was greatly affected by the antibody treatment. Abrantes and Curtis (2002) produced the monoclonal antibody IACR-CCNj.3d which was used to isolate a gene (*gp col*-8) with strong similarity to cuticular collagen from a mixed stage *Globodera pallida* cDNA library.

Chen *et al* (2003) studied the production and characterization of monoclonal antibodies against *Xiphinema index* antigens. The MAbs were used in immunolocalisation studies and antibodies recognizing epitopes present at the surface coat, inner cuticle, dorsal gland cell and dorsal duct, body wall muscle fibres, nervous system and reproductive system were identified. Although several members of the secretome have been identified from the distantly related root-knot nematode *Meloidogyne incognita*, *M. Chitwoodi* was virtually unexplored at the molecular level (Vanholme *et al*, 2004).

Karssen *et al* (2005) carried out experiments on identification of *Globodera* and *Meloidogyne* specimens. Two electrophoretic methods were modified and adapted for an automated electrophoresis system (Phast System, Pharmacia). Proteins of individual

Globodera cysts were identified using isoelectric focusing and a sensitive silver stain. Proteins of young single *Meloidogyne* females were separated using polyacrylamide gradient gels and stained for the isozymes, esterase and malate dehydrogenase.

Lima *et al* (2005) characterized secreted-excreted (S-E) products as antigens that help in the identification of nematode targets useful for novel control strategies, which interfere with the nematode infection of plants. Three monoclonal antibodies (MAbs) and three polyclonal antibodies (PAbs) to secreted-excreted products of *Meloidogyne* spp. and *Heterodera avenae* were developed, which were used to examine their reactivity towards *M. incognita* and *M. arenaria* second-stage juveniles and adult females. The three PAbs showed cross-reactivity with *M. incognita* and *M. arenaria* but reactivity of monoclonal antibodies was only tested against *M. arenaria.* Indirect ELISA was performed using different antibodies to investigate the stage specificity of the molecules they recognize. Antibodies Roth-PC 389, PC 419, MAb T46.2, MAb T116C1.1 and MAb T42D.2 reacted strongly with the J2 and poorly with the adult females. This indicates that these antibodies are recognizing epitopes which are produced in the early stages of the nematode life cycle and Western blot analysis using the antibody Roth-PC 373, revealed antigen bands in *M. incognita* and *M. arenaria* J2, with the same molecular weights.

Parera *et al* (2005) developed a simple method to identify plant parasitic nematodes, based on analysis of protein profiles of nematodes generated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). Two methods have been used: grinding and direct analysis of intact nematodes. Both methods were standardised using the nematode *Anguina tritici* (wheat seed-gall nematode) as a model. With α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix, the most effective solvent extraction was with 90% acetone and with sinapinic acid (SA) as matrix, 90% ethanol was most effective. When intact nematodes were analysed directly by mixing with the matrix solution, 40 min extraction with CHCA matrix solution generated the best protein profiles. The standardised methods were applied to analyse the seed-gall nematodes *A. tritici* and *A. funesta* and to the root-knot nematode, *Meloidogyne javanica*, which infects many horticultural crops. Typical protein profiles and diagnostic peaks were identified for these nematode species and for mixtures of *Anguina* species. The results provide 'proof-of-concept' that these nematode species can be identified by protein profiling using MALDI-TOFMS.

Davies and Carter (2008) stated that monoclonal antibodies are useful in the identification of root-knot nematode females and immunoassays using polyclonal antbodies had been developed to quantify root-knot nematodes in root systems. The development of

immunoassays for quantification of nematodes present in soil is, however, proving more difficult to accomplish. They compared several different types of immunoassays using an ELISA-based system. Antigens were released from second-stage juveniles of root-knot nematodes using Ballotini glass beads, agitated by means of a vortex shaker in a buffer containing urea. Polyclonal antibodies were highly sensitive, but these cross-reacted with other unrelated nematode genera such as *Panagrellus* spp. Although monoclonal antibodies were genus-specific, these were not sufficiently sensitive to quantify small numbers of nematodes. Antigen-coated plate assays were more sensitive than trapping assays and similarly, indirect assays were more sensitive than direct assays. Milling a suspension of nematodes extracted from soil with Ballotini glass beads in buffer containing urea showed that it was possible to quantify juveniles at the equivalent of less than one juvenile per g of soil using polyclonal antibodies.

Atkinson *et al* (2008) identified proteinaceous secretions involved in feeding of soya bean cyst nematode, *Heterodera glycines* and produced monoclonal antibodies (MAbs) using extracts of second stage juveniles and adult females as the immunogens. They developed a rapid, indirect immunofluorescent screening procedure that enabled the antigens for 100 MAbs/day to be localised in juveniles. A similar immunofluorescent screening procedure for adult females was less successful due to the masking of the fluorophore by autofluorescence. Thus additional approaches, including enzyme linked immunosorbent assays (ELISA), were necessary to aid in the screening process. The immunosuppressive drug, cyclophosphamide, had an effect on the range of antibodies obtained in two experiments. One was designed to favour antibodies specific to the hatched rather than the unhatched juvenile, whereas the second aimed to select those for the anterior rather than the posterior part of adult female. A large number of MAbs showed well defined specificities: the subventral pharyngeal glands (84), the dorsal pharyngeal gland (5), the amphidial pouches (10) and the excretory system (15).

Evans *et al* (2008) employed various methods of preparation of samples of cysts, eggs and larvae of *Globodera rostochiensis* and *G. pallida* in order to improve the efficiency of immunoassay as a tool for identification and quantification of nematodes in soil. Monoclonal antibodies, which specifically recognize each of the two species of potato cyst nematode and do not cross-react with other species of soil nematodes, were used in ELISA tests. It was shown that free larvae reacted less well to monoclonal antibodies than cysts and eggs and that a large proportion of the relevant antigens come from vitteline fluids of eggs. Dead eggs and non-viable components of the cyst (e.g. cyst wall, egg shell) have little or no antigenicity. Of 13 different chemical extractants tested for release of antigens from cysts, CTAB and especially 1% urea were shown to be more effective than the standard extractant

PBS. Sonication of cysts was the best of a number of chemical, physical and antigen release procedures. There was a good quantitative relationship between optical density readings; in indirect and DAS-ELISA and the number of nematodes extracted from soil.

Curtis *et al* (2008) carried out studies on two monoclonal antibodies which specifically recognise each of the two species of potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida* as these do not cross react with other species of soil nematodes. These were used successfully in an immunoassay to identify and quantify PCN species using clean cysts and mixed populations. These antibodies show reactivity only towards antigens prepared from live eggs and these also recognised antigens which are easily released from the nematodes. A significant correlation was obtained between results from immunoassay estimates and the traditional method of cyst picking and egg counting. It was found that the amount of organic matter present in the soil samples affected the sensitivity of ELISA but quantification of nematodes extracted from soil samples was possible with soils containing upto 14% of organic matter. Similar results were obtained by Lilley *et al* (1999) in case of *M. incognita.*

Schots *et al* (2009) studied the serological differentiation of the potato-cyst nematodes *Globodera pallida* and *G. rostochiensis*. Hybridomas producing antibodies which react with thermostable protein antigens, from the potato cyst nematode species *Globodera rostochiensis* (TSRoP) and *G. pallida* (TSPaP) were isolated. Three of the isolated hybridomas (WGP 1, WGP 2 and WGP 3) produce antibodies which reacted with preferent affinity with protein antigens isolated from *G. pallida* and two (WGR 11 and WGR 12) produced antibodies which bind preferentially to *G. rostochiensis*. Binding constants were determined to quantitate the differences in affinity of WGP 1, WGP 2, WGP 3, WGR 11 and WGR 12 for the protein antigens from both the nematode species and to asses the similarity in affinity for either protein antigen with respect to the other non-specific antibodies. In immunoblotting experiments, binding could be demonstrated for most antibodies to two thermostable proteins with apparent molecular weights of 20.6 / 20.8 kDa for *G. rostochiensis* and 20.5 / 21.0 kDa for *G. pallida*. The reactivity of the monoclonal antibodies with thermostable protein antigens from other common cyst nematodes was also investigated. All monoclonal antibodies, which are not specific for TSRoP or TSPaP, bind to thermostable proteins of these cyst nematode species.

Chapter III

MATERIALS AND METHODS

The materials and methods employed in the present studies are given below:

3.1 Preparation and maintenance of nematode culture

The galled brinjal roots were collected from the infested fields of Deptt. of Vegetables and Crops at Punjab Agricultural University, Ludhiana. These galls were teased under a stereoscopic binocular microscope and adult females of *Meloidogyne* were collected. Posterior part of females was dissected and the perineal pattern was observed. The identification was done according to the key given by Taylor *et al* (1955) and Eisenback *et al* (1980). Pure culture of *Meloidogyne* was maintained in brinjal plants. For this purpose, healthy seedlings of brinjal were transplanted in 12 inch pot filled with sterilized (autoclaved) soil. After one week, each pot was inoculated with egg masses of *Meloidogyne* species. Egg masses were collected by hand picking under stereoscopic binocular microscope from roots of these infected brinjal plants culture, for their further use in experiments. The culture was multiplied regularly in order to ensure regular supply of the egg masses throughout the study.

3.1.1 Preparation of soil mixture

Field soil sieved through 16 mesh sieve, sand and fine farmyard manure after autoclaved at 20 lb per square inch pressure for two hours, were mixed in the proportion of 3:1:1. The mixture thus formed was filled in the earthen pots (15 cm diameter). Prior to the filling, these pots were disinfected by dipping in 1% commercial formalin solution.

3.2 Extraction and collection of egg masses

After 6-8 weeks of inoculation, the egg masses were formed in brinjal roots, the inoculated plants were uprooted and their roots carefully washed free from adhering soil and cut into short lengths (5-10 cm) and the egg masses were isolated. These egg masses were cleared off the host's debris by washing with normal saline (0.9% NaCl in distilled water). These washed eggs were stored in phosphate buffered saline (PBS) pH 7.4, at -20°C till further use.

3.3 Antigen preparation

The egg masses collected above were used for the preparation of antigen. Antigen was prepared as per the protocol given in Flow Chart-1. Briefly, the egg masses were homogenized in Tris-HCl buffer containing 2 mM PMSF (Phenylmethylsulfonyl fluoride) for 10 min. This was followed by sonication at 7μ for 8 min. Homogenate thus obtained was centrifuged at 10,000 rpm for 20 min at 4°C. The whole procedure was then performed under cold conditions. The supernatant obtained after centrifugation was used as antigen. It was

stored in aliquots at -20°C till further use. Thiomersal (0.01%) antifungal reagent was used as the preservative.

3.4 Protein Estimation

The protein content of the antigen was determined by the method of Lowry *et al* (1951).

The reagents used were:-

- Solution A : 2% Sodium bicarbonate (Na_2CO_3) in 0.1 N Sodium hydroxide (NaOH)
- Solution B : 0.5% Copper sulphate ($CuSO_4$) in 1% Sodium potassium tartarate
- Solution C : 50 ml of Solution A was mixed with 1 ml of Solution B just before use.
- Solution D : Folin Ciocalteu phenol reagent diluted with water (1:1 v/v).
- Standard Solution : Bovine Serum Albumin (BSA) 1 mg/ml in distilled water.

Procedure:

To 1ml of diluted samples of the antigen, 5 ml of solution C was added and mixed well. Tubes were kept at room temperature for 10 min. Then 0.5 ml of solution D was added. The mixture was allowed to stand for 30 min. at room temperature and absorbance recorded at 520 nm against a blank. The amount of protein in antigen was determined from the standard curve prepared by taking known concentrations of Bovine Serum Albumin (BSA, 10 µg to 200 µg).

A

B

C

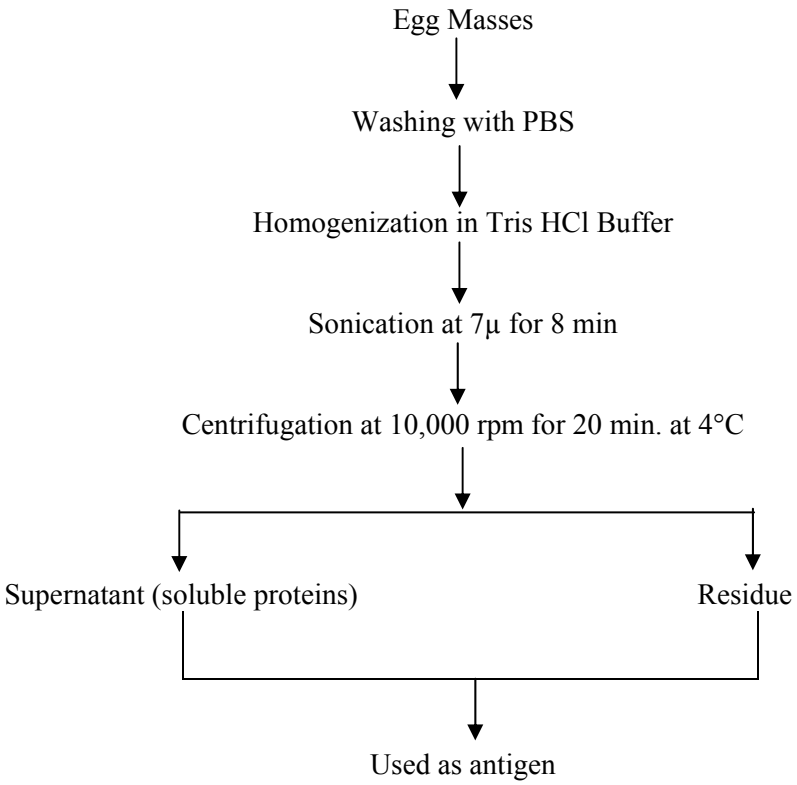
Plate 1: Brinjal seedlings transplanted in 250 ml plastic cups (A and B) Seeds beds of brinjal maintained in autoclaved soil (C)

A **B**

C

Plate 2: Roots derived from infected culture containing galls formed by root-knot nematodes (A and B), Eggs collected from infected roots (C).

FLOW CHART 1: PROTOCOL FOR ANTIGEN PREPARATION



3.5 Production of anti-*Meloidogyne* antibodies

3.5.1 Procurement and maintenance of experimental animals

Laboratory bred Soviet Chinchilla rabbits were procurred from the Department of Livestock Production and Management, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Rabbits were maintained in animal house under hygienic conditions and were provided commercial feeding pellets and water *ad libidum.*

3.5.2 Immunization of rabbits

Laboratory bred Soviet Chinchilla rabbits were immunized for the production of hyper immune sera against *Meloidogyne* spp. antigens as per the protocol given in Table 1. Four rabbits (2 months old, weighing approximately 450 g each) were employed. These were divided into two groups of two each; group I was immunized with *Meloidogyne* antigens, while group II served as control. 400 µg antigen was emulsified with Complete Freund's adjuvant (CFA) and injected into rabbits intradermally at multiple sites. Before injection, the injection sites were shaved off and sterilized with spirit. Booster injection was given in Incomplete Freund's adjuvant (IFA) after 15 days of primary immunization by intramuscular route. Third injection was given without any adjuvant, intramuscularly in the thigh region. Animals were bled 15 days after each immunization and sera thus obtained were preserved at -20º C with merthiolate (0.01%) as preservative, which was further used to test the presence of antibodies by Double immunodiffusion (DID) method.

Before immunization the animals were bled to collect preimmune sera. Rabbits were bled by puncturing suborbital plexus of veins with the help of capillaries. Blood was collected in clean glass tubes. The tubes were then placed in slanting position. Blood was allowed to coagulate for 20-30 minutes at room temperature. Then the tubes were transferred to refrigerator at 4°C for overnight. Next day, the clot was broken along the sides of the walls of the tubes to separate serum from the clot and then serum was centrifuged at 4000 rpm for 10 min. at 4°C. Serum was obtained as clear straw coloured fluid in the supernatant and preserved at -20° C with merthiolate (0.01%) as the preservative.

TABLE 1:- Immunization protocol of rabbits with *Meloidogyne* antigens

Day	Antigen	Adjuvant	Route
1	400 µg	CFA	Intradermal
15	--do--	IFA	Intramuscular
30	--do--	-----	Intramuscular

CFA- Complete Freund's Adjuvant

IFA- Incomplete Freund's Adjuvant

3.6 Detection of antibodies by Double Immunodiffusion (Ouchterlony 1949) method

Antisera collected above were tested for the presence of antibodies by Double Immunodiffusion (DID). The microscopic glass slides were coated with a layer of 1% agarose in PBS. Gels were allowed to set at room temperature. The slides were stored in petridish lined with moist filter paper at 4°C after setting. Wells were punched in the gel next day. The central well was charged with antigen and outer wells 1-2 with antisera from the unimmunized rabbits and 3-4 from the two immunized rabbits. For 2-7 days, antigen and antibodies were allowed to diffuse till no more precipitation lines were seen. To remove the unbound proteins, the slides were then washed with 0.15 N NaCl for 1 h and then further to remove the salt, the slides were kept in distilled water for 1 h. By keeping a wet filter paper over the gels, these were then dried at 37°C. The slides were then stained with 0.1% Coomassie Brilliant Blue R-250 in Ethanol : Acetic acid : Water (45:10:45 v/v) for 1 h and the destained in Ethanol : Acetic acid : Water (25:10:65 v/v) till a clear background was obtained.

3.7 Development of ELISA for detection / titration of anti-*Meloidogyne* antibodies

Indirect ELISA was developed for detection/titration of anti- *Meloidogyne* antibodies by checkerboard titration as per the Flow chart 2 and 3. The test antigen (concentration range 10 µg-1 ng) was coated on 96-well plates and the plate was incubated for 1h at 37°C and then kept overnight at 4°C. Next day after four washings with PBS (0.05M, pH 7.2), unused sites in the wells were blocked with lactogen (2% in PBS) by keeping at 37° C for 1h. Next, the plate was washed with PBS and then antisera from each rabbit containing anti-*Meloidogyne* antibodies (dilution range 1000-5000) were added to the wells and incubated at 37°C for 1h. Again the plate was washed with PBS four times and anti-rabbit IgG HRP conjugate was added to the wells at desired dilution. The plate was again washed 4-5 times with PBS to remove any unbound conjugate sticking to the plate. Enzymatic reaction was carried out by incubating the plate with the substrate 0.04 % o-phenylenediamine dihydrochloride (OPD) in PBS containing 0.01% H_2O_2. The reaction was allowed to occur for 2-10 mins and stopped by adding 1N H_2SO_4 to the wells. The development of sunset-orange colour indicated the presence of antibodies. The plate was subsequently read at 492 nm under ELISA reader Tecan infinite M200 Nanoquant. The antibody titre in the rabbit sera was recorded**.**

3.8 Development of competitive inhibition ELISA for the detection of *Meloidogyne* antigens

Anti-*Meloidogyne* antibodies produced above were employed for the development of competitive inhibition ELISA for the quantitative detection of *Meloidogyne* antigens as per the protocol given in Flow chart 4. After antigen coating and blocking, free antigen in the

sample and antiserum were incubated together to allow competition between sample containing free antigen and plate bound antigen for limited antibody sites. This was followed by incubation with conjugate and substrate.

To enhance the sensitivity of the test, an additional step of preincubation was incorporated. Here the test antigen and antibodies were preincubated for one hour before being allowed to bind with the plate bound antigen.

Using known concentration (20 µg – 100 ag) of *Meloidogyne* antigens, a standard curve was prepared depicting the percentage inhibition. From this standard curve, sensitivity limit of *Meloidogyne* antigens detection by ELISA was recorded.

FLOW CHART 2. PROTOCOL FOR DEVELOPMENT OF INDIRECT ELISA FOR DETECTION OF ANTI-*MELOIDOGYNE* ANTIBODIES

Antigen coating (20 µg-1 ng)

↓ Washing with PBS (0.05M, pH 7.2) X 2

Blocking with 2% (w/v) Lactogen in PBS (37 °C, 1 h)

↓ Washing

Incubation with rabbit antiserum (dilution 1K-5K, 37 °C, 1 h)

↓ Washing X 4

Incubation with secondary antibody (Goat anti-rabbit IgG HRP; 37 °C, 1 h)

↓ Washing X 4

Enzymatic reaction with substrate

(0.04 % o-phenylenediamine dihydrochloride (OPD) in PBS containing 0.01% H_2O_2)

(2-10 min)

↓

Stopping of reaction with 1N H_2SO_4

↓

Absorbance at 492 nm with ELISA reader: Tecan infinite M200 Nanoquant

FLOW CHART 3: TITRATION OF ANTI-*MELOIDOGYNE* ANTIBODIES BY INDIRECT-ELISA

Antigen coating (10 µg per well)

↓ Washing with PBS (0.05M, pH 7.2) X 2

Blocking with 2% Lactogen in PBS (37°C, 1 h)

↓ Washing with PBS

Incubation with rabbit antiserum (37°C, 1 h) (1: 500 – 1: 32000)

↓ Washing with PBS X 4

Incubation with second antibody (goat anti-rabbit IgG HRP; 10 K)

↓ (37°C, 1 h) Washing with PBS x 4

(0.04 % o-phenylenediamine dihydrochloride (OPD) in PBS containing 0.01% H_2O_2)

↓ (2-10 min.)

Stopping of reaction with 1N H_2SO_4

↓

Absorbance at 492 nm with ELISA reader: Tecan infinite M200 Nanoquant

FLOW CHART 4. STANDARDIZATION OF COMPETITIVE INHIBITION ELISA FOR THE DETECTION OF *MELOIDOGYNE* ANTIGENS

Antigen coating (10 µg)

↓ Washing with PBS (0.05M, pH 7.4) X 2

Blocking with 2% Lactogen in PBS (37°C, 1 h)

↓ Washing

Incubation with rabbit antiserum (pre-incubated with antigen) (10K, 37° C, 1 h)

↓ Washing with PBS X 4

Incubation with secondary antibody (Goat anti-rabbit IgG HRP, 10K) 37' C, 1 h

↓ Washing with PBS x 4

Enzymatic reaction (0.04% OPD in PBS containing 0.01% H_2O_2)

↓ (2-10 min.)

Stopping of reaction with 1N H_2SO_4

↓

Absorbance at 492 nm with ELISA reader: Tecan infinite M200 Nanoquant

Chapter IV

RESULTS AND DISCUSSION

Results of the present findings have been discussed under the following headings:-

4.1 Preparation of antigen

4.2 Production of anti-*Meloidogyne* antibodies

4.3 Detection of antibodies by Double Immunodiffusion (DID)

4.3.1 Development of Indirect ELISA for detection of anti-*Meloidogyne* antibodies

4.3.2 Titration of anti-*Meloidogyne* antibodies by Indirect ELISA

4.4 Development of Competitive Inhibition ELISA for the detection of *Meloidogyne* antigens

4.1 Preparation of antigen

Several antigen preparations have been used by different workers for immunological studies including the development of immunodiagnostics for nematode parasitic infections. These include antigen preparations from second-stage juveniles (J2) (Atkinson *et al* 2008; Davies and Carter 2008), whole larval and adult homogenates (Fox and Atkinson 1985, Hussey 1971), material exuded from the excretory pore and buccal stylet of both larvae and adults of *Meloidogyne javanica* (Bird 1964), secretory granules within the subventral glands of second-stage juveniles (Goverse *et al* 1994), egg shells of *Heterodera glycines* (Kennedy *et al* 1997), cysts and eggs of the parasite (Evans *et al* 2008; Backett *et al* 1993) and from the homogenate of anterior regions of *Meloidogyne incognita* adult females (Davis *et al* 1992). All these studies are limited because of difficulty in getting pure culture of larvae and adults free from all contaminants. Homogenized body wall antigens of some plant parasites have also been found to be capable of stimulating the host; and saline extract of species of *Ditylenchus* and *Heterodera* are also capable of producing anibodies (Webster and Hooper 1968).

Secreted-Excreted (S-E) products and molecules expressed at the surface cuticle of *Meloidogyne* spp. have also been used as antigen, as these molecules are considered probably to be the first signals precieved by the host (Fioretti *et al* 2002; Lopez De Mendoza 1999). Among the nematode's secretory products, which also have been used as antigens to produce antibodies, stylet secretions are believed to play a key role in the penetration and migration through the root tissue, modification and maintenance of root cells as feeding sites, formation of feeding tubes and digestion of host cell contents to facilitate nutrient acquisition by the nematodes (Hussey 1989). The secretions produced by two subventral and one dorsal oesophageal gland cells which are secreted through the stylet into the plant tissue during

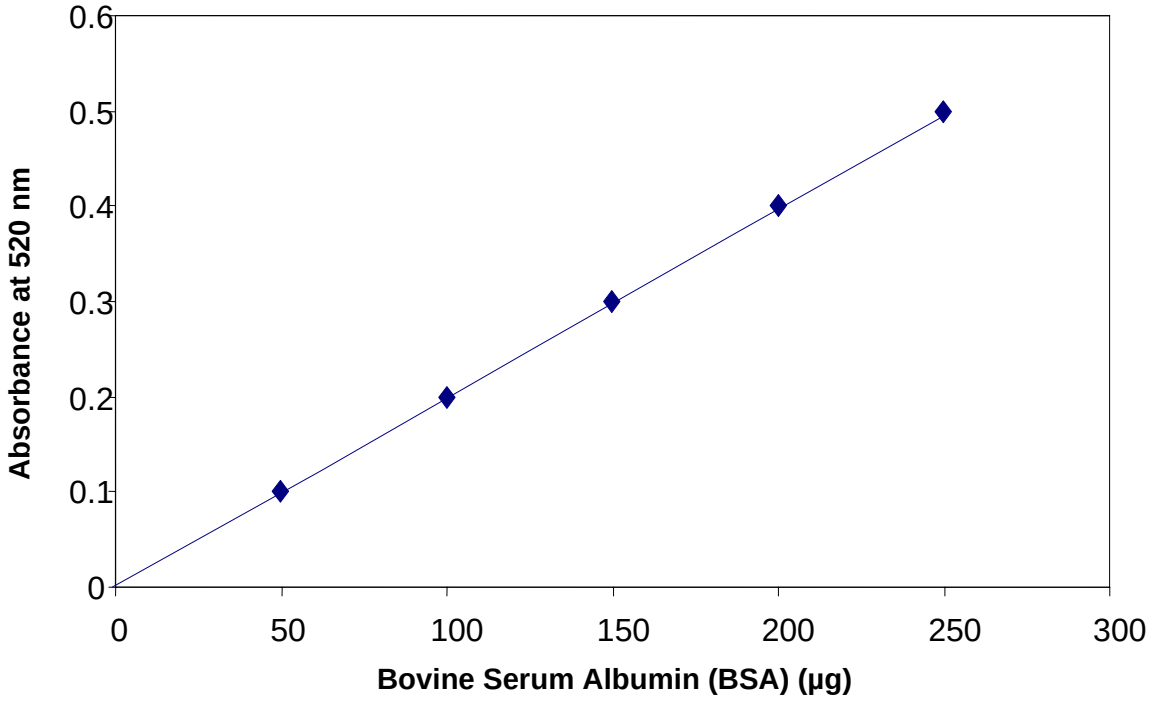


Fig 1: Standard curve for protein estimation

parasitism have also been found to be capable of producing antibodies against plant parasites (Davis *et al* 2004).

Fioretti *et al* (2001) used secretions of cuticle, amphids and oesophageal glands as antigens, which are considered to be involved in several aspects of the infection process. Lima *et al* (2005) observed that antigens present in the amphids of *Meloidogyne incognita* and *M. arenaria,* cross react in both the species and preparasitic second-stage juveniles (J2) of *Meloidogyne incognita.* These have been used as antigens for the production of monoclonal antibodies.

However no report is available regarding the preparation of antigens using *Meloidogyne* eggs. The fact that the eggs are present in the soil; and adhering on the roots of the host plants makes them easily detectable even at an early stage of the infection. Hence, in the present studies, antigen prepared from the egg masses extracted from the culture of *Meloidogyne* species have been used for immunization of rabbits. Protein content of soluble antigens calculated from the standard curve (Fig.1) was found to be 0.75 µg / µl.

4.2 Production of anti-*Meloidogyne* antibodies

For the development of an immunoassay, the production of antibodies is a prerequisite. Antibodies against various plant parasitic nematodes have been produced in a variety of animal species including mice and rabbits. Polyclonal antibodies tends to have high levels of sensitivity but are not usually discriminative, as these are highly cross-reactive across nematode genera. But these are relatively cheaper to produce than the monoclonal antibodies. Monoclonal antibodies are highly reproducible but tend to be less sensitive and very expensive to produce. Monoclonal antibodies have been shown to be more useful for diagnostic assays than polyclonal antibodies (Halk and De Boer 1985).

Balb/c mice have been used for the production of antibodies against secretory granules in the dorsal and subventral esophageal glands of preparasitic second-stage juveniles (J2) of *Meloidogyne incognita* as immunogens (Hussey 1989), homogenates of the anterior regions of *Meloidogyne incognita* adult females (Davis *et al* 1992). De Boer *et al* (1996) immunized mice with different antigens from *Globodera rostochiensis* to produce monoclonal antibodies and Backett *et al* (1993) immunized mice to produce stage-specific monoclonal antibodies to the potato cyst nematode *Globodera pallida* (Stone) Behrens.

Rabbits have been immunized to produce antibodies against *Heterodera glycines* eggshells (Kennedy *et al* 1997), *Meloidogyne incognita* and *M. arenaria* adult females (Hussey 1971) and secreted-excreted (S-E) products of *Meloidogyne* spp. (Lima *et al* 2005).

Though various animal species can be immunized for the production of antibodies, rabbits are considered to be the most suitable animals as these are easy to handle and respond quickly, require small amounts of immunogens and these also produce desired amount of

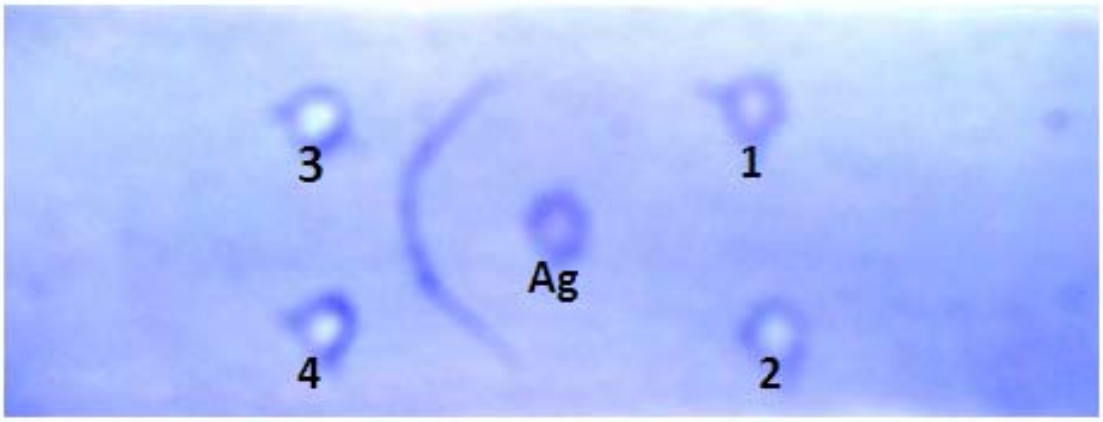


Plate 3. Double immunodiffusion test (DID) for detection of anti-*Meloidogyne* antibodies

Central Well: *Meloidogyne* antigens

Well 1 & 2: Preimmune sera

Well 3 & 4: Immune sera of rabbits

antibodies. Hence, in the present study, rabbits were used for the production of anti-*Meloidogyne* antibodies.

4.3 Detection of antibodies by Double Immunodiffusion (DID)

Various immunological methods have been employed for the detection of antibodies including Immunofluorescence (Goverse *et al* 1994; Backett *et al* 1993; Davis *et al* 1992), Western blotting (De Boer *et al* 1996; Lima *et al* 2005), Immunoelectrophoresis (Gibbins *et al* 1968), Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Kennedy *et al* 1997) and Immunoblotting (Schots *et al* 1990). Hussey (1971) used Double immunodiffusion (DID) technique to study serological relationship of *Meloidogyne incognita* and *M. arenaria.*

In the present study, DID was carried out to detect the presence of anti-*Meloidogyne* antibodies in the rabbits. Sera collected from different rabbits were tested for the presence of anti-*Meloidogyne* antibodies by Double Immunodiffusion method (Ouchterlony 1949). Double immunodiffusion owes its success to the unique nature of antibody-antigen interactions. When polyvalent antibodies are mixed with antigen, lattices of antibody-antigen complexes form and become visible as precipitin lines. In the present study, DID was employed for the detection of anti- *Meloidogyne* antibodies in rabbits.

From the results given in Plate 3, it is evident that sera from all the rabbits immunized with the *Meloidogyne* antigens showed precipitin lines and no such line was seen in preimmune sera and sera collected from the control/unimmunized group. This confirms that all the rabbits immunized with *Meloidogyne* antigens, produced anti-*Meloidogyne* antibodies. Though DID is a simple test but it is not as rapid, sensitive and specific as ELISA. Here, DID was used as a preliminary test for the detection of antibodies since it is one of the simplest techniques to check antisera for the presence of antibodies for a particular antigen.

4.3.1 Development of Indirect ELISA for detection of anti-*Meloidogyne* antibodies

For detection of antibodies, ELISA is the most commonly used assay and has been used by several workers. Atkinson *et al* (2008) developed ELISA for the detection of monoclonal antibodies of potato cyst nematode, *Heterodera glycines.* Backett *et al* (1993) developed ELISA for anti-*Globodera pallida* Stone (Behrens) antibodies. Curtis *et al* (1997) used double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) for the detection of anti-*Heterodera avenae* antibodies. De Boer *et al* (1996) used dot-blot assays for the detection of antibodies against potato cyst nematode, *Globodera rostochiensis.* Schots *et al* (1992) also developed an immunoassay for the detection of eggs of *G. rostochiensis* and *G. pallida.* Davies and Carter (2008) observed that indirect assays were more sensitive than direct assays.

In the present study, Indirect ELISA has been developed for the detection of anti-*Meloidogyne* antibodies. Results of checkerboard titration are given in Table 2. In the plate 5, it can be observed that the optimum antigen concentration is found to be 10 µg and optimum dilution of goat anti-rabbit HRP conjugate is 1: 10,000.

Table 2. Checker board titrations for Indirect ELISA

Antigen range	20 µg-1ng
Optimum antigen concentration	10 µg
Goat anti- rabbit Ig HRP dilution range	1,000-10,000
Optimum goat anti- rabbit Ig HRP dilution	10,000

Several other reports on the use of ELISA as immunological assay for the detection of plant parasitic antibodies have also been reported. ELISA being highly sensitive, specific, rapid and easy to perform, in the present studies, Indirect ELISA has been developed for the detection of antibodies against *Meloidogyne* antigens.

4.3.2 Titration of anti-*Meloidogyne* antibodies by Indirect ELISA

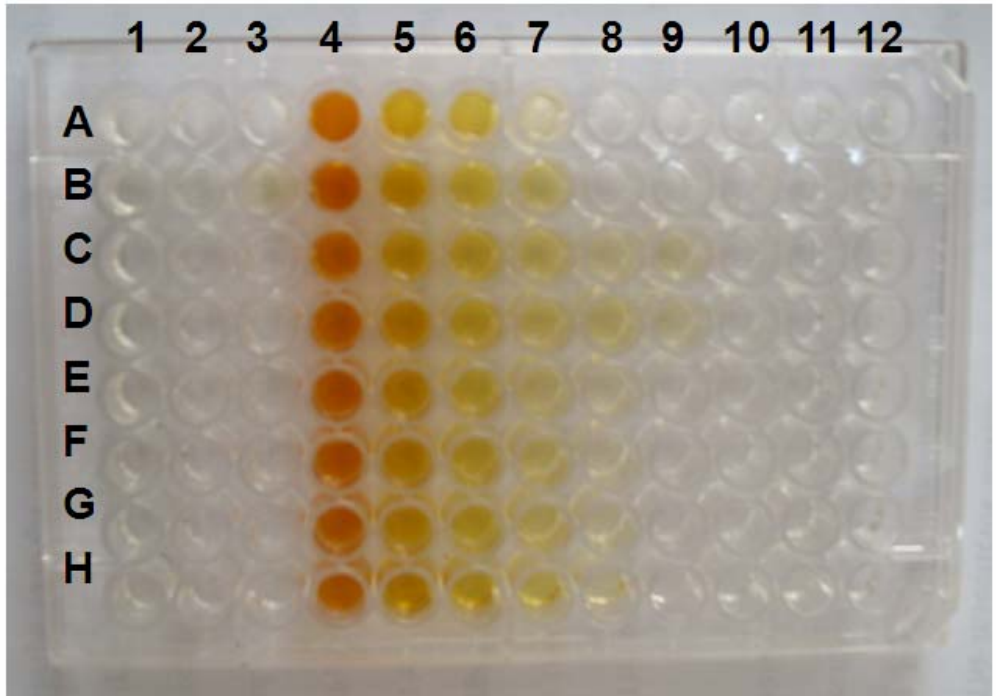
ELISA has been employed for the titration of antibodies against several potato cyst nematodes *Heterodera glycines*, using eggshells as immunogens and antibody titres of 16,000 have been reported (Kennedy *et al* 1997).

In the present study, antibody levels of sera collected at different intervals were checked by Indirect ELISA. The sera collected from all the immunized rabbits at intervals of 15, 30, 45 days were titred by Indirect ELISA and the highest titre of anti-*Meloidogyne* antibodies raised in the rabbits was found to be 320,000 as shown in plate 4. *Meloidogyne* antibodies levels were maintained till the end of experiment, which may be attributed to the booster immunizations These antisera have been successfully employed for *Meloidogyne* antigens detection.

4.4 Development of competitive inhibition ELISA for detection of *Meloidogyne* antigens

In the present studies, competitive inhibition ELISA has been developed for the detection of *Meloidogyne* antigens by employing anti-*Meloidogyne* antibodies and known amounts (20 µg-100 ag) of *Meloidogyne* antigens. This test is based on competitive inhibition of the binding of anti-*Meloidogyne* antibodies to the solid phase bound antigen by the antigen present in the test sample. Decrease in absorbance indicated the presence of antigen in the sample.

Results of competitive inhibition ELISA are given in Table 3 and plate 6. It was observed that the assay could detect as little as 1 fg of antigen. Competition with higher



Row A-B : IRS 15 DPI

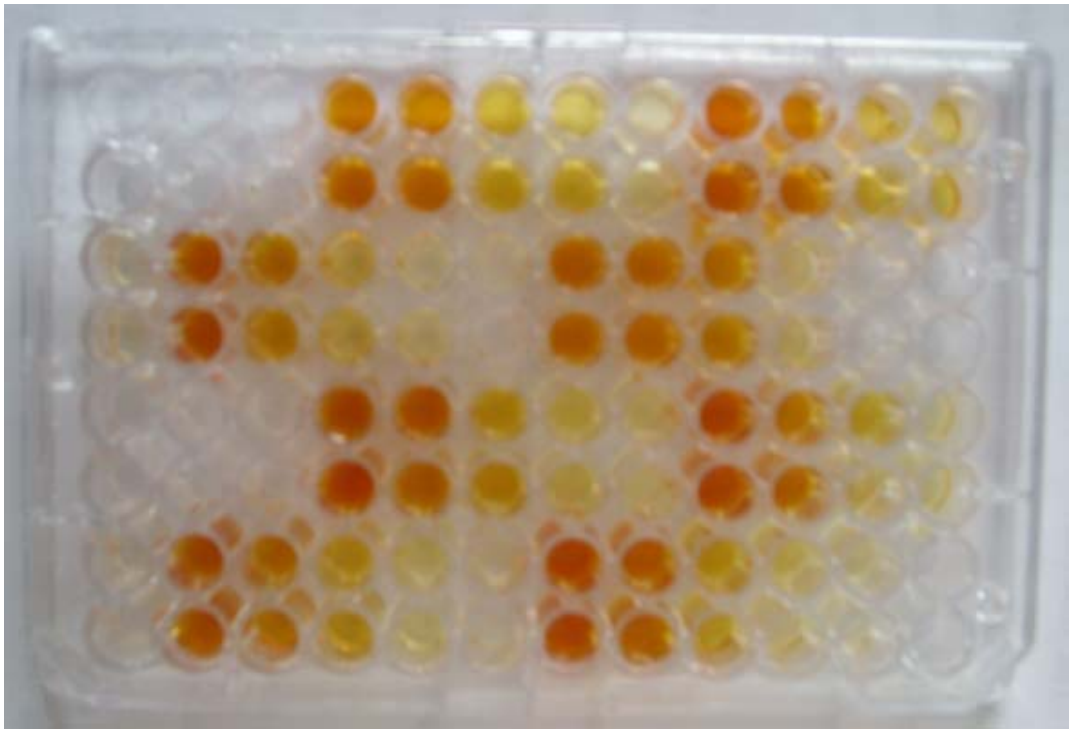
ROW C-D: IRS 30 DPI

ROW E-F: IRS 45 DPI

ROW G-H: IRS 60 DPI

PLATE 4. Indirect ELISA for titration of anti-*Meloidogyne* antibodies

	1	2	3	4	5	6	7	8	9	10	11	12
Antigen	-	-	-	10 µg								
Antibody	-	NRS	IRS	1:10K	1:20K	1:40K	1:80K	1:160K	1:320K	1:640K	1:1280K	1:2560K

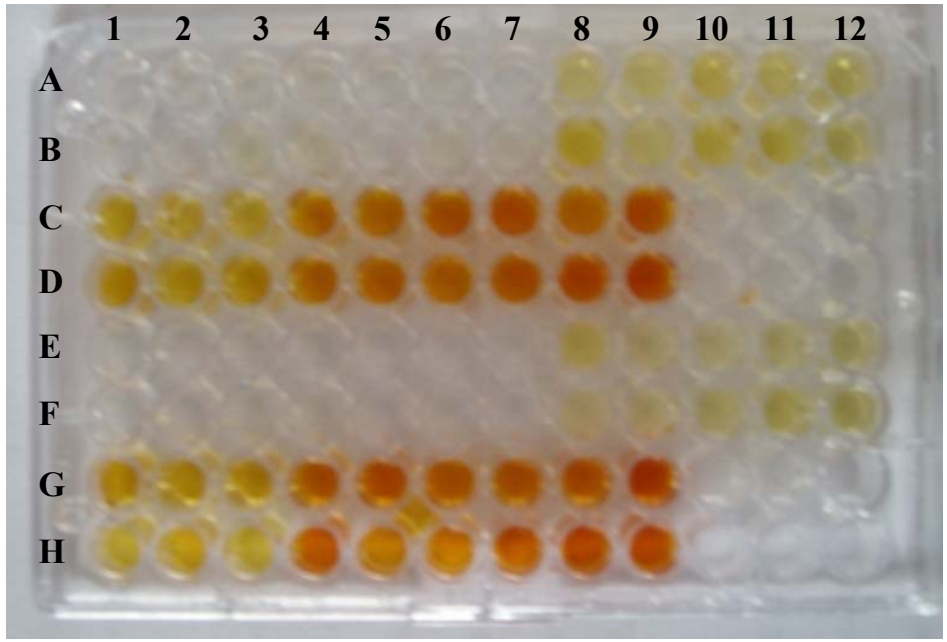


Row A-D: IRS rabbit 1

Row C-D: IRS rabbit 2

PLATE 5. Indirect ELISA for detection of anti-*Meloidogyne* antibodies

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A-B, E-F	Antigen	-	-	-	20 µg	10µg	5µg	2µg	1µg	20 µg	10µg	5µg	2µg
	Antibody	-	NRS	IRS	1:1000					1:2000			
C-D, G-H	Antigen	1µg	20 µg	10µg	5µg	2µg	1µg	20 µg	10µg	5µg	2µg	1µg	
	Antibody	1:2000	1:5000					1:10000					



Row A-D: IRS rabbit 3

Row E-H: IRS rabbit 4

PLATE 6. Competitive inhibition ELISA for detection of *Meloidogyne* antigens
Antigen coating = 10 µg in all except A, B: 1-7 and E, F: 1-7
Conjugate antibody dilution 1:10,000

	1	2	3	4	5	6	7	8	9	10	11	12
A-B, E-F	-	-	-	no Ab	NRS	IRS Rabbit 3	IRS Rabbit 4	20 µg	10 µg	1 µg	100 ng	10 ng
C-D, G-H	1 ng	100 pg	10 pg	1 pg	100 fg	10 fg	1 fg	100 ag	IRS			

concentrations of the antigens resulted in complete inhibition. However, at low concentration (1 µg, 100 ng, 1 ng) there was a progressive decrease in degree of inhibition, there being no inhibition with concentration lower than 1fg.

For competition, with increase in concentration of *Meloidogyne* antigens added, lesser amount of antibodies is available for binding with the bound antigen. There is a decrease in absorbance which can be observed as decrease in intensity of sunset-orange colour in the wells. A standard sigmoidal curve (Figure 2) was obtained by plotting % inhibition of binding versus concentration of antigen, showing that with increase in concentration of antigen, the % inhibition increases thereby decreasing the binding of the antibody to the bound antigen. At a concentration of 1 fg or above, no colour developed, indicating 100% inhibition. The sensitivity of the assay was found to be 1 fg. The additional step of preincubation of free *Meloidogyne* antigens (20 µg–100 ag) with antibody prior to competition, showed a significant improvement in the sensitivity of the assay, it being 1fg.

However, the range of concentration of antisera that can be employed for the quantification of eggs extracted from a soil sample must be too narrow to develop a useful diagnostic assay. Also, the greater range in sensitivity is seen when the eggs were stored at 4º C as compared to frozen eggs. ELISA has been employed for the detection of a number of plant-parasitic antigens. Atkinson *et al* (2008) developed ELISA for the detection of second-stage juveniles and adult females of *Heterodera glycines*. Evans *et al* (2008) developed Indirect ELISA and DAS-ELISA for the detection of antigens prepared from cysts, eggs and larvae of *Globodera rostochiensis* and *G. pallida* and Curtis *et al* (2008) used cysts and eggs for developing a highly sensitive ELISA for detection of *G. rostochiensis* and *G. pallida*. Kennedy *et al* (1997) determined the sensitivity limit of detection to the range of 5 ng/ml sonicated eggshells or 1 egg of *H. glycines.* Curtis *et al* (1997) observed the sensitivity of 0.5 eggs or 3.6 eggs per gram of soil in DAS-ELISA for the detection and quantification of *Heterodera avanae* from the processed soil samples. Lawler and Harmey (1993) detected the sensitivity limit of 0.4 µg of *Bursaphelenchus xylophilus* and *B. mucronatus* antigens.

Keeping in view the high sensitivity of the assay, it has the potential of being applied for diagnosis of *Meloidogyne* infection. Further, it needs to be checked for specificity and validation with field samples.

Table 3: Percent binding inhibition versus antigen concentration (ng) in competitive inhibition ELISA for detection of *Meloidogyne* antigens

Concentration of antigen	% inhibition	% Binding
1 fg	0	100
10 fg	7	93
100 fg	15	85
1 pg	25	75
10 pg	43	57
100 pg	65	35
1 ng	78	22
10 ng	83	17
100 ng	87	13
1 µg	90	10
10 µg	93	7
100 µg	96	4
1000 µg	100	0

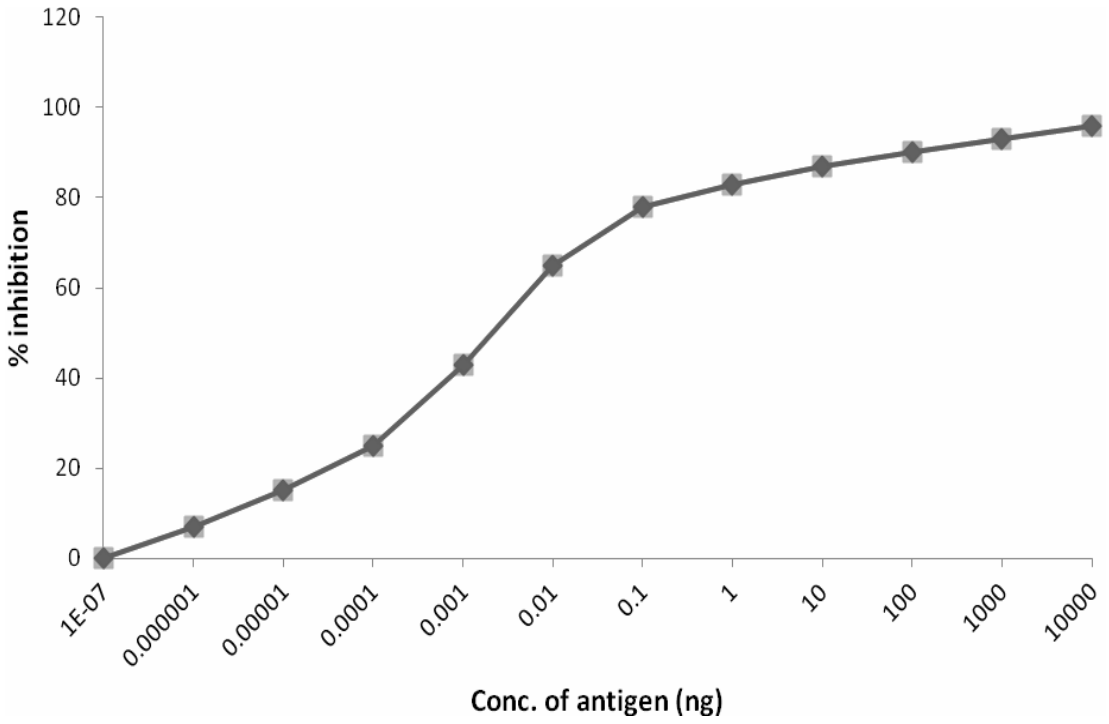


Fig 2: Percent inhibition of binding versus concentration of antigen (ng) in competitive inhibition ELISA for detection of *Meloidogyne* antigens

Chapter V

SUMMARY

Plant parasitic nematodes are one of the major causative agents of crop diseases. These limit crop production capabilities, resulting in colossal economic losses. Some of the most widespread parasitic nematodes limiting crop production are the root-knot nematodes of the genus *Meloidogyne,* attacking a wide range of crops. Four major parasitic species of *Meloidogyne* prevalent worldwide are: *M. incognita, M. javanica, M. hapla* and *M. chitwoodi.* Primary symptoms of this infection are the formation of typical root galls on the roots of susceptible host plants. These root galls are 2 to 3 times the diameter of healthy roots which give the root system a knobby appearance. Infection with *Meloidogyne* leads to the suppression of innate immunity and reprogramming of plant cells. Due to their wide host range, short generation times and high reproduction rates, these root-knot nematodes are one of the major constraints in crop production. Hence, it is essential to control these parasites. For any control strategy to be effective, an early and accurate diagnosis is essential. Conventional diagnostic methods have their own limitations in terms of specificity and sensitivity. Hence the present research work was undertaken to develop a rapid, simple, specific and sensitive method to identify root-knot nematodes at an early stage of infection. Since immunoassays, particularly enzyme linked immunosorbent assays (ELISAs) me*et* all these demands, an attempt has been made to develop ELISA for detection of *Meloidogyne* antigens.

Culture of *Meloidogyne* spp. was established following inoculation of roots of the host plants with egg masses of *Meloidogyne* spp. obtained by hand picking from roots of infected plants. Antigen was prepared from the eggs collected from the established culture. These eggs were then homogenised, sonicated and centrifuged to obtain soluble proteins. Anti-*Meloidogyne* antibodies were produced by immunization of rabbits with these soluble proteins. Production of antibodies was confirmed by appearance of the precipitin lines by Double Immunodiffusion (DID) method.

Being more sensitive, ELISA was developed for detection and titration of anti-*Meloidogyne* antibodies. From the checkerboard titrations, optimum antigen concentration was found to be 10 µg and optimum conjugate dilution 1:10,000. Titration of antisera by ELISA indicated as high as 320,000 titre of these antibodies.

Competitive inhibition ELISA was developed for the detection of *Meloidogyne* antigens using these anti-*Meloidogyne* antibodies. This is based on competition between the solid phase bound antigens and free antigens in the test sample for limited antibodies. Sensitivity of the assay was found to be 10 fg.

To enhance the sensitivity, an additional step of pre-incubation was included where the sample containing free antigen was allowed to react with antibody prior to competition. This resulted in enhanced sensitivity of 1 fg.

ELISA technique reported in the present studies has the potential of being employed as an easy, rapid, specific, sensitive and a high throughput tool for the detection of *Meloidogyne* antigens. It is also cost effective and requires less time while increasing the reliability of diagnosis. The sensitivity of the assay can be further enhanced by purification of antigen / antibodies. Further, this test can be developed into dot-ELISA format for onsite diagnosis at the field level.

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