Development of enzyme linked immunosorbent assay (ELISA) for the detection of root-knot nematode *Meloidogyne incognita*

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Abstract Root-knot nematodes (*Meloidogyne incognita*) are obligate, sedentary plant endoparasites that are extremely polyphagous in nature and cause severe economic losses in agriculture. Hence, it is essential to control the parasite 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, enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of *M.incognita* antigens. First an indirect ELISA was developed for detection and titration of anti-*M.incognita* antibodies. Results indicated as high as 320 K titre of the antisera. Finally competitive inhibition ELISA was developed employing these anti-*M.incognita* antibodies for detection of *M.incognita* antigens. Sensitivity of ELISA was 10 fg. 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 *M.incognita* infection.

Keywords Anti-*M.incognita* antibodies · Competitive inhibition ELISA · Indirect ELISA · *Meloidogyne incognita* · Root-knot nematode

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 worldwide and are limiting crop production capabilities in many developing countries, especially in tropical and sub-tropical regions resulting in great economic losses to the tune of about 125 billion dollars globally (Chitwood 2003).

Out of the various plant parasitic nematodes, *M.incognita* (Root-knot nematodes) are sedentary, obligate endoparasites and cause maximum damage among the plant parasitic nematode family, the *Heteroderidae*. The major species of *M.incognita* distributed worldwide are *M.java-nica*, *M.arenaria*, *M.incognita*, *M.naasi* and *M.hapla*. These are extremely polyphagous in nature (Trudgill and Blok 2001) with a host range of over 2,000 plant species, major hosts being cotton, sugar beet, spinach, tobacco, tomato, pepper, peanut, flowering ornamental plants and some weed species. Within the root, the roundworm exploits intercellular spaces, invades the cortex, and secretes proteins that lead to suppression of plant innate immunity inducing large multinucleate ‘giant cells’ to be formed, involving a complex molecular interaction between nematode and the host (Bellafore et al. 2008). Due to their broad range of hosts, ability to suppress host resistance, survive extreme conditions and high reproductive rates, these root-knot nematodes are difficult to control (Trudgill and Blok 2001).

Keeping in view the huge economic losses caused 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. Presently, detection of *M.incognita* is based on gross observations

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like visualization of galls on roots of plants, presence of eggs, presence of juveniles, etc. or by determining their species and number microscopically and identification is based on merely their morphological features (Handoo et al. 2005). *Meloidogyne* species have also been identified using isozyme pattern analysis (Carneiro et al. 1996; Xu et al. 2004), RAPD-PCR (Qiu et al. 2006; El-Hady 2009) and Gas Liquid Chromatography (Calvo et al. 2005). These methods are, however, laborious, lack desired sensitivity and specificity, require sophisticated instrumentation, are cost-intensive, besides being not reliable for identification of novel discoveries. 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 immunosorbent assays (ELISA) are the best suited due to the inherent advantages of high specificity, sensitivity and are cost effective and also have an edge over molecular diagnostic methods whose success depends upon the availability of diagnostic probes, expert hands and well equipped laboratories (Blok and Powers 2009). A definite identification of the nematode species is an inevitable prerequisite for routine testing/screening of nematicide or germplasm, where an ELISA is the best option. ELISA has been employed by many workers for detection of various plant pathogens viz. Atkinson et al. (1988) developed ELISA for the detection of monoclonal antibodies of potato cyst nematode *Heterodera glycines*, Palmer et al. (1992) screened MABs raised against surface expressed antigens of *Ditylenchus dipsaci* with ELISA; Boer et al. (1996) raised MABs against J2 of *Globodera rostochiensis* and performed ELISA against their detection; Curtis et al. (1997) used double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) for the detection of anti-*H. avenae* antibodies; Kennedy et al. (1997) employed an indirect ELISA against several potato cyst nematodes *H. glycines*, using eggshells as immunogens. However, no attempts have been made in developing ELISA for detection of the root-knot nematode, *M. incognita*. Hence, the present study was undertaken with an aim to develop a sensitive ELISA as a diagnostic tool for detection of *M. incognita*.

Materials and methods

Preparation of immunogen

Stock nematode culture of *M. incognita* eggs was propagated on brinjal plants. Eggs were collected 6–8 weeks after inoculation of brinjal plants with *M. incognita*. Roots of infected plants were harvested and cut into small pieces

and washed free of debris/soil. 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

Immunogen was prepared from the homogenate of *M. incognita* eggs. These eggs were homogenized for 10 min in a motorized glass homogenizer in ice-cooled Tris–HCl buffer containing 2 mM PMSF (Phenylmethylsulfonylfluoride); followed by sonication at a pulse frequency of 7 μ per 15 s for 8 min and then cold centrifuged at 10,000 rpm for 20 min at 4°C . The supernatant thus obtained after centrifugation was used as antigen. Protein concentration in supernatant was determined by the method of Lowry et al. (1951).

Production of antibodies

The antigen prepared above was used for production of anti-*M. incognita* antibodies in Laboratory bred Soviet Chinchilla rabbits by the protocol as described in Table 1. All the rabbits were bled 15 days after each immunization and the sera thus obtained were preserved in various aliquottes at -20°C with merthiolate (0.01 %) as preservative.

Development of indirect ELISA for detection of anti-*M. incognita* antibodies

An indirect ELISA (with modifications from Engvall and Perlman 1971) was developed to detect/titrate the anti-*M. incognita* antibodies produced above as per the flow chart 1. 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 intensity of colour was directly proportional to the titre of anti-*M. incognita* antibody present. The antibody titre in the rabbit sera was recorded.

Development of competitive inhibition ELISA for detection of *M. incognita* antigens

Anti-*M. incognita* antibodies produced above were employed for the development of competitive inhibition ELISA for the quantitative detection of *M. incognita* antigens as per the protocol given in flow chart 2. After antigen

Table 1 Immunization protocol of rabbits with *M. incognita* antigen. (μg = microgram)

Days	Antigen (μg)	Adjuvant	Route
1	400	Complete Freund's Adjuvant	Intradermal
15	400	Incomplete Freund's Adjuvant	Intramuscular
30	400	–	Intramuscular

coating and blocking, free antigen in the sample and anti-serum 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. Using 10 ng of *M.incognita* antigen for coating, a standard curve was prepared depicting the percentage inhibition, using known amounts (1 mg–1 fg) of *M.incognita* antigens.

Percent inhibition for any given concentration of inhibitor was calculated using the formula:

$$\% \text{ Inhibition} = \frac{A_{492\text{max}} - A_{492}}{A_{492}} \times 100$$

$A_{492\text{max}}$ is the absorbance in the absence of inhibitor (maximum absorbance); A_{492} is the absorbance at any given concentration. From this standard curve, sensitivity limit of *M.incognita* antigens detection by ELISA was recorded.

Results

Protein content of the total soluble antigen prepared from the egg masses extracted from the culture of *M.incognita* was found to be 0.75 µg/µl as calculated from the standard curve (Fig. 1). An indirect ELISA was developed for the detection of anti-*M.incognita* antibodies. From the results of checkerboard titration, the optimum antigen concentration was found to be 10 µg and optimum dilution of goat anti-rabbit HRP conjugate 1:10,000. The antisera collected from all the immunized rabbits at intervals of 15, 30, 45 days were titrated by indirect ELISA. Sera from all the rabbits immunized except the control/unimmunized group showed the presence of anti-*M.incognita* antibodies. No anti-*M.incognita* antibodies were found in preimmune sera and the highest titre of anti-*M.incognita* antibodies raised in the rabbits was found to be 320,000 in the sera collected

Fig. 1 Standard curve for protein estimation

30 days post-first-immunization. Subsequently, the antisera, so collected, were employed for detection of *M.incognita* antigens.

Competitive inhibition ELISA was developed for the detection of *M.incognita* antigens. As is evident from the standard sigmoidal curve (Fig. 2) obtained by plotting percent inhibition of binding versus concentration of antigen, there was a progressive decrease in the degree of inhibition with decrease in the amount of competing antigen, there being no inhibition with concentration lower than 1 fg. It was observed that the assay could detect as little as 1 fg of antigen.

Discussion

The inevitable need for rapid and large-scale identification of plant pests in agriculture paves the way for development of newer and faster diagnostic tools. Immunoassays are routinely used to detect and quantify plant and animal pathogens because these are cheap, reduce the amount of time needed for identification and give excellent sensitivity and specificity. In spite of many inherent advantages,

Fig. 2 Percent inhibition of binding versus concentration of antigen (µg) in competitive inhibition ELISA for detection of *M.incognita* antigens

Flow Chart 1 Development of an indirect ELISA for detection of anti-*M.incognita* antibodies

Flow Chart 2 Standardization of competitive inhibition ELISA for the detection of *M. incognita* antigens

development of ELISA has been extended for diagnosis of few plant parasitic nematodes only (Curtis et al., 1997; Atkinson et al., 1988). Though, antibodies have earlier been raised against three major *Meloidogyne* spp. (Davies and Lander 1992) and ELISA was used to investigate differences between second-stage juveniles and adult females. However, only partial discrimination between adult females of the nematode species could be made. Davies et al. (1996) and Lima et al. (2005) raised monoclonal antibodies against *M. incognita* with an aim to identify the species, but they cross reacted with some other species of root knot nematodes. Hence, in the present studies, an attempt has been made to develop ELISA for specific identification of *M. incognita*.

For raising anti-*M. incognita* antibodies, antigen was prepared from the egg masses extracted from the culture of *M. incognita* species and used for immunization of rabbits. 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 and hence, can be used for preparation of antigen.

The high titre of anti-*M. incognita* antibody (320 K) as detected by indirect ELISA gives evidence that the technique is quite sensitive in detecting anti-*M. incognita* antibody. The antibody levels were maintained till the end of experiment, which may be attributed to booster immunizations. These antisera have been successfully employed for *M. incognita* antigen detection.

For competition, with increase in concentration of *M. incognita* antigens added, the percent inhibition increases and lesser amount of antibodies are 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. 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 present study will prove to be a major breakthrough in detection of the root knot nematode and hence, preventing the loss of crop production worldwide. Keeping in view the high sensitivity of the assay, it has the potential of being applied for diagnosis of *M. incognita* infection.

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