# TECHNIQUES FOR STUDYING THE NUCLEAR CONDITION OF GIANT CELLS INDUCED BY *MELOIDOGYNE* SPECIES

A Thesis

by

BIN HE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2004

Major Subject: Plant Pathology

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Approved as to style and content by:

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| James L. Starr<br>(Chair of Committee) | Thomas D. McKnight<br>(Member) |
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# ABSTRACT

Techniques for Studying the Nuclear Condition of

Giant Cells Induced by *Meloidogyne* Species. (December 2004)

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Nematodes of the genus *Meloidogyne* are known as “root-knot” nematodes due to the characteristic knots or galls found on the roots of infected plants. Root-knot nematodes attack over 2000 species of plants and cause over 80 billion dollars lost annually. Giant cells are feeding sites of root-knot nematodes and are enlarged multinuclear cells induced by the nematodes in susceptible host roots and that function to provide nutrients to the nematode. This thesis presents data on two techniques of studying the nuclear condition in giant cells. Colchicine was used to arrest mitosis in giant cells in a previous study. Here we test the effect of colchicine on nematode activity. The results showed that colchicine did not affect nematode egg hatch, juvenile activity, or hatch of eggs produced by treated juveniles. These results confirm that colchicine can be used to arrest mitosis in giant cells without affecting the nematode parasite.

A major obstacle to the study of giant cells is collecting tissue samples that are specific to giant cells. Laser capture microdissection (LCM) is a technique that allows

one to sample a single giant cell. A focused laser beam was used to collect samples of giant cell cytoplasm from fixed and sectioned tissues. RNA was then extracted from those isolated samples. Using three tomato genes as test samples, specific primers were designed to measure expression level of *Rb7, LHA4, and HXK1* gene by Real-Time PCR. Expression of *LHA4* and *Rb7* increased with time after inoculation, and immature giant cells reached levels that were 3 and 6 times, respectively, that of cortical cells, but which were not different from root meristem cells. Expression of *HXK1* did not change with time after inoculation and has the same level of that in root tip and cortical tissues. These data confirmed that the techniques of LCM coupled with RT-real-time PCR can be used to quantitate expression of genes at different stages of giant cell development without contamination from surrounding cells.

*To my parents*

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# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

Nematodes of the genus *Meloidogyne* are know as “root-knot” nematodes due to the characteristic knots or galls found on the roots of infected plants. Root-knot nematodes have a very broad host range. They attack over 2000 species of plants, and most cultivated crops are attacked by at least one species of *Meloidogyne*. The annual world wide losses caused by nematodes on the life-sustaining crops, which include all grains and legumes, banana, cassava, coconut, potato, sugar beet, sugarcane, and sweet potato, are estimated to be about 11%, and losses for most other economically important crops (vegetables, fruits, and non-edible field crops) are about 14%, for a total of over 80 billion dollars annually (Agrios, 1997).

Mature females of root-knot nematodes deposit eggs, up to 1000 or more, in a gelatinous matrix, called the egg mass or egg sac, which can be observed attached to the protruding posterior end of the females on the root surface. This mass holds the eggs together and protects the eggs from dehydration. Egg development begins within a few hours after deposition, resulting in 2 cells, 4, 8, and so on, until a fully formed first-stage juvenile lies coiled in the egg membrane. After the first molt in the egg, the second-stage juvenile (J2) will hatch, emerging through a hole made in the end of the flexible egg shell by repeated thrusting with the stylet. Eggs can hatch in 48 hours in

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This thesis follows the style of Journal of Nematology.

optimized condition. Most of J2 will leave the egg mass and move through the soil, searching for suitable host root to feed on.

Juveniles explore the root surface by pressing and rubbing their lips against the epidermal cells preferentially between the root tip and the root hair zone (Mende, 1997). The preferred invasion site of J2 is at the elongation zone of growing root tip for all hosts (Endo and Wergin, 1973; Wyss et al., 1992). Juveniles penetrate the root epidermis both intra- and intercellularly (Ibrahim and Massoud, 1974; Siddiqui, 1971; Wergin and Orion, 1981; Wyss et al., 1992). Most J2 invade within 24-48 hours after inoculation. Although the intracellular invasion is more common, J2 will change to intercellular movement within the root because it is faster and seems easier (Wyss et al., 1992). Based on research using *Arabidopsis* as a host, Gravato et al. (1995) and Wyss et al. (1992) were able to reveal nematode migration within the root. After penetrating the epidermis J2 orientate themselves towards the root tip, lining up parallel to the long axis of the root. They migrate intercellularly between cortical cells until they reach the meristematic tissue. They then turn and migrate into the vascular cylinder where they stop at a suitable site in xylem parenchyma to establish a permanent feeding site. During invasion and migration, J2 do not feed and feeding sites are generally established within the differentiating vascular cylinder, within the region of cell elongation. Juveniles can locate their target cells perhaps by sensing chemical gradients through amphid receptors, such as IAA-binding proteins or by detecting plant cell-specific surface determinants (Mende, 1997). Also, the ability to invade and induce galls appears to be related

primarily to difference in the aggressiveness between nematode species, and to the age of the infective juveniles, but is also affected by temperature.

The feeding sites for root-knot nematode are called giant cells. The giant cells provide nutrients for the sedentary nematodes, which continue to feed, enlarge, and molt three times and differentiate into males and females. The male will leave the roots and become free living. Whereas the female stays in the roots, and continue to feed and produce eggs either after mating with a male or, in the absence of the male, parthenogenetically for some species. Root cells around the feeding sites are also induced to enlarge and form galls (knots), often with extensive secondary root formation.

Whether a successful parasitic relationship can be established depends on the success of initiating feeding site by J2. Induction of giant cells is mediated by J2, involving differentiation of a root cell into a giant cell in which the number of nuclei is increased by synchronous and non-synchronous mitosis or amitosis (Bleve-Zacheo and Melillo, 1997). The first change that can be observed in giant cells is their change to a multinucleate condition. Jones et al. (1978) reported that binucleate cells are found at 24 hours after nematode inoculation of a tomato root. Within 48 hours, developing giant cells show 4 to 8 nuclei. The increase in number of nuclei per giant cell occurs primarily during the first 2 weeks after infection, mitosis seldom occurs after the J2 molts. Data from pea indicate that high levels of mitotic activity occur mainly during the first 7 days of giant cell development, as the number of nuclei per giant cell nearly doubles during each of the first 4 to 6 days after infection (Starr, 1993). An increase of ground cytoplasm, cellular organelles, and small vacuoles can be observed in 3-day old giant

cells. As giant cells develop, the central vacuole is further reduced, and differentiation of abnormal vascular elements around the giant cells is advanced. A structure called "wall-membrane apparatus" was found in regions adjoining to the xylem. In this region, cytoplasmic vacuoles are absent in contrast to the presence of numerous mitochondria, which indicates a large energy requirement for selective flow across the plasma membrane. Also, the cell wall in this region increases its thickness by forming finger-like wall invaginations lined with plasma membrane (Jones and Northcote, 1972), which facilitates water transport from the xylem into the giant cell. Thus giant cells are similar to transfer cells. Normal shape of cells becomes highly amoeboid, as does the shape of the nucleus. Vacuolization of nucleoi in giant cells indicates high ribosomal RNA transcription and intense metabolic activity in of the giant cells. Giant cell enlargement continues for 2 weeks. Finally, they may reach 600 μm in length and 200 μm in diameter (Jones, 1981), occupying most of the central cylinder, where vascular elements are crushed and deformed, with severe restriction of longitudinal water movement.

It has never been proven, but it is widely accepted that the induction of giant cell formation is due to glandular secretions injected via the nematode stylet (Hussey, 1989). Evidence shows that a specific tube-like structure, the feeding tube, is formed rapidly after nematode stylet penetration of the selected cell wall, and is connected with the endomembrane system of the developing giant cell (Grundler and Böchenhoff, 1997; Wyss and Zunke 1986). Observations using video-enhanced light microscopy clearly show that secretions from the gland cells are pumped into the plant prior to the onset of the changes in plant (Wyss and Zunke, 1986; Wyss et al., 1992).

*Meloidogyne spp*. synthesize secretory proteins in their one dorsal and two subventral esophageal gland cells and release these proteins through the stylet. However, the dorsal gland and subventral glands are thought to have different functions during parasitism. In preparasitic J2, the most active esophageal glands are the two subventral gland cells, whereas the single dorsal gland cell becomes the predominate source of secretions released through the stylet in subsequent parasitic stages (Davis et al., 2000; Hussey, 1989). These observations suggest the secretions of the subventral gland cells may be important during induction and establishment of the feeding site, whereas the secretions of the dorsal gland cell may have a role in maintenance of the feeding site, in the actual feeding process, or in production of the feeding tube. Knowledge of specific secretions has been limited until recently, because juveniles are too small to allow the gland cells to be separated from the rest of the body contents for analysis. Huang et al. (2003) were able to construct a cDNA library of *M. incognita* parasitic genes by microaspirating the cytoplasm from the esophageal gland cells of different parasitic stages. Thirty seven unique clones with a signal peptide for secretion were identified, including a pectate lyase gene, acid phosphatase gene, hypothetical protein code sequences from other organisms, and 27 novel protein coding sequences.

Recent research has shown that the gland cell proteins injected into plant cells though the stylet can alter plant cell development. *Meloidogyne javanica* chorismate mutase1 (MjCM-1) is one such esophageal gland protein likely to be secreted from the nematode as giant cells form. MjCM has two domains, an N-terminal chorismate mutase (CM) domain and a C-terminal region of unknown function (Lambert et al.,

1999). CM is part of the shikimate pathway, a primary metabolic route in plants and microorganisms. The end product of the common shikimate pathway is chorismate. In plants, chorismate-derived compounds (CDCs) play critical roles in plant growth, in development and defense, and in interactions with other organisms (Schmid and Amrhein, 1995; Weaver and Hermann, 1997). CM is found in bacteria, fungi, plants, and protists, but not in animals (Roberts et al., 1998). It is commonly believed that animals do not have the shikimate pathway or CM because they obtain their essential amino acids in their diet. Transgenic expression of MjCM-1 in soybean hairy roots results in a phenotype of reduced and aborted lateral roots. Histological studies demonstrate the absence of vascular tissue in hairy roots expressing MjCM-1. The phenotype of MjCM-1 expressed at low level can be rescued by the addition of indole-3-acetic acid (IAA) (Lambert et al., 1999; Doyle and Lambert, 2003). This evidence suggest CM plays an important role in establish parasitic relationship between root-knot nematodes and their host plants. The black box of how nematodes bring about such elaborate cell differentiation in the plant is starting to open.

Changes on gene expression in giant cells also can assist us understand the parasitic relationship between plant and nematode. Many techniques have been used to explore gene expression in giant cells, including protein analysis, differential screening and subtraction of cDNA, differential display, promoter-β-glucuronidase (GUS) fusions, mRNA in situ hybridization, and reverse-transcription polymerase chain reaction (RT-PCR). Many nematode-responsive plant genes have been identified using these techniques, and now can be grouped in categories related to plant developmental

pathways, including wound and defense response genes, genes involved in cell cycle and cytoskeleton, cell wall growth and degradation, physiology, water status, general and stress metabolism, transcription factors, auxin response, late-embryogenesis abundant (LEA) (Gheysen and Fenoll, 2002).

Among the genes induced early are several cell cycle genes. Giant cells are stimulated to undergo multiple rounds of mitosis without cytokinesis. Thus it is not surprising that genes involved in cell cycle control have altered expression profiles during giant cell establishment.

Giant cell expansion requires cell wall loosening and strengthening of inner supports (cytoskeleton), processes that are both reflected in molecular changes. Opperman et al. (1994) observed that the GUS fusion tobacco (*Nicotiana tabacum*) water channel gene, *TobRB7*, was upregulated in giant cells induced by *M. incognita*.

A cohort of house-keeping genes and stress response genes are up regulated in giant cells to maintain their active metabolism. The upregulation of ubiquitin E2 in tomato (Bird and Wilson, 1994) represents an active ubiqutin-proteasome pathway, which fits well with the high protein turnover that may be caused by continuous nematode feeding. Another house-keeping gene in *Arabidopsis* AtHMG1 encodes hydroxymethyl glutaryl CoA reductase (HMGR), which is the key enzyme for phytosterol biosynthesis. Both promoter induction (Fenoll et al., 1997) and protein accumulation (Bleve-Zacheo and Melillo, 1997) occur inside giant cells from very early nematode stages until the end of the egg maturation. These observation all indicate

HMGR may serve the intense vesicle traffic needed for the extensive plasma membrane and wall biogenesis.

Transcription factors shape gene expression patterns and resultant cell differentiation. A group of transcription factors are also found upregulated in giant cells, such as transcription factors in the WRKY family, which have multiple roles in plant defense and development. KNOX and PHAN are two important groups of transcriptional factors that required for meristem maintenance. Their expression in giant cells has led to a hypothesis that giant cell are a type of induced meristem, perhaps with similarities to those induced by rhizobia, which also express PHAN and KNOX.

One of the most interesting questions in gene expression in giant cells is that of which genes are crucial for giant cell initiation and maintenance. Many of the analyzed genes may be activated because they happen to have the appropriate regulatory signals that are recognized in developing giant cells, and not because they play an essential role. To investigate which genes are crucial for giant cell initiation and development, knock-out techniques have been used to mutate plants. In such experiments, plants expressing antisense ToRB7 and PHAN were less susceptible to root-knot nematode infection (Favery et al, 1998).

The study of promoter regions of all these upregulated genes may give a specific promoter- nematode responsive cis element. In at least some cases, regions important for expression in giant cells can be separated from those needed for expression in other plant parts, suggesting that giant cells are equipped with a rather particular cocktail of transcription factors unique to the structure (Gheysen and Fenoll, 2002).

Not all the expression changes in giant cells are upregulated. Several promoters were found to be silenced in giant cells of transgenic plants. These are either pathogen promoters needed for infection or pathogen-induced plant promoters, such as *AtPAL1* and *AtEBP* (Goddijn et al., 1993; Hermsmeier et al., 2000), suggesting an active suppression of the defense response in nematode-infected plants. Other downregulated genes are related to general cell metabolism. Since gene silencing is as crucial as gene expression during plant development, these downregulated genes can play an important role in giant cell initiation and development.

# CHAPTER II

# EFFECT OF COLCHICINE ON ACTIVITY OF *MELOIDOGYNE JAVANICA*

## INTRODUCTION

Although a lot of research has been done on giant cell nuclear abnormalities, little is known of the relationship between the multinucleate condition of the giant cell and development of the nematode parasite. Bird and McGuire (1966) reported that DNA and RNA synthesis inhibitors, 6-azauridine and 5-bromo-2`-deoxycytidine, inhibited *M. javanica* development in tomato roots. However, they did not relate effects on nematode development to giant cell development or the nuclear condition of giant cells. Additionally, 6-azauridine was phytotoxic and the observed effects in nematode development may have been an indirect consequence of suppressed host growth.

Engler et al. (1999) used hydroxyurea (HU), which was proved not to affect nematode infectivity, to block normal cell cycle events in giant cells. Hydroxyurea treatment of seedling 3 days after inoculation with *Meloidogyne incognita* arrested gall development. However, incubation of feeding sites with HU 9 days after inoculation showed that feeding sites were apparently sufficiently developed to allow nematode maturation. However, the authors did not relate effects on nematode development to giant cell development or the nuclear condition of the giant cells.

In a recent paper by Wiggers et al. (2002), it was reported that nematode development was inhibited in roots treated with colchicine 3 days after inoculation. At this time each giant cell contained about eight nuclei. Nematode development was not affected in roots treated 7 days after inoculation, at which time there were 14 to 15 nuclei/giant cell. These observations suggest that colchicine did not affect nematode development directly but indirectly via its effects on giant cell development. However, no data were presented to confirm this conclusion. The purpose of this study was to expand upon the work reported by Wiggers et al. (2003) to determine whether colchicine has a direct effect on nematode activities, especially egg hatch, juvenile infectivity, and egg hatch of a subsequent generation.

## MATERIALS AND METHODS

*Colchicine effect on egg hatch:* The root-knot nematode *Meloidogyne javanica* was used in these experiments. Nematodes were cultured on 'Rutgers' tomato by inoculating each plant with 10,000 eggs. At 9 to 12 weeks after inoculation, the roots were cleaned with tap water and treated with 0.06% NaOCl for 4 min (Hussey and Barker, 1973) to release the eggs. Eggs were collected and washed with distilled water. Eggs were then divided into four groups and treated with aqueous solutions of 0%, 0.05%, 0.1%, and 0.2% colchicine, respectively, in 15-ml plastic tubes for 4 hours. After rinsing with distilled water three times, eggs were placed into Bureau Plant Industry (BPI) dishes with 100 to 200 eggs/dish. There were five replications for each

treatment. The eggs were incubated at 26°C. Total number of eggs in each dish was counted and hatched juveniles were counted every other day.

*Colchicine effect on nematode development* : Eggs were extracted as described and incubated at 30°C to hatch. Juveniles were collected after 48 hours, and were treated with 0%, 0.05%, 0.1%, and 0.2% colchicine in 15-ml plastic tubes for 4 hours. After rinsing with distilled water three times, juveniles were used to inoculate 2-week-old tomato seedlings with 100 to 200 J2/plant. Seedlings were grown at 27 °C with a 12 hour light/dark cycle for 3 to 4 weeks, at which time the nematodes inside the roots were stained with acid fuchsin (Bryd et al., 1972). Stained nematodes were examined microscopically to determine the effects of colchicine treatment on nematode development. Proportion of treated nematodes that reached maturity at 3 or 4 weeks after inoculation was calculated by dividing number of mature nematodes by the number of total nematodes in each root sample,

*Effect of colchicine on egg hatch of a subsequence generation*: Eggs were extracted and hatched as described, and juveniles were treated with 0%, 0.05%, 0.1%, and 0.2% colchicine. Tomato seedlings at 2 weeks of age were inoculated with colchicine-treated J2 and were grown at 27 °C and a 12 hour light/dark cycle for 5 weeks. Eggs were extracted from each tomato plant and were incubated at 30 °C for hatch. Total numbers of eggs produced and juveniles hatched from each root were recorded at 3, 7, and 13 days after harvest.

The effect of treatment in each experiment was subjected to analysis of variance, using the SAS (SAS Institute, Cary, NC) general linear model procedure. Means were separated using least significant differences at $P \leq 0.05$.

**RESULTS**

In the first experiment, a few eggs hatched by day 2 with 29% to 33% total hatch by day 9. No effect of treatment on hatch was observed ($P = 0.36$) (Fig.1). Effect of time on hatch was significant ($P < 0.01$).

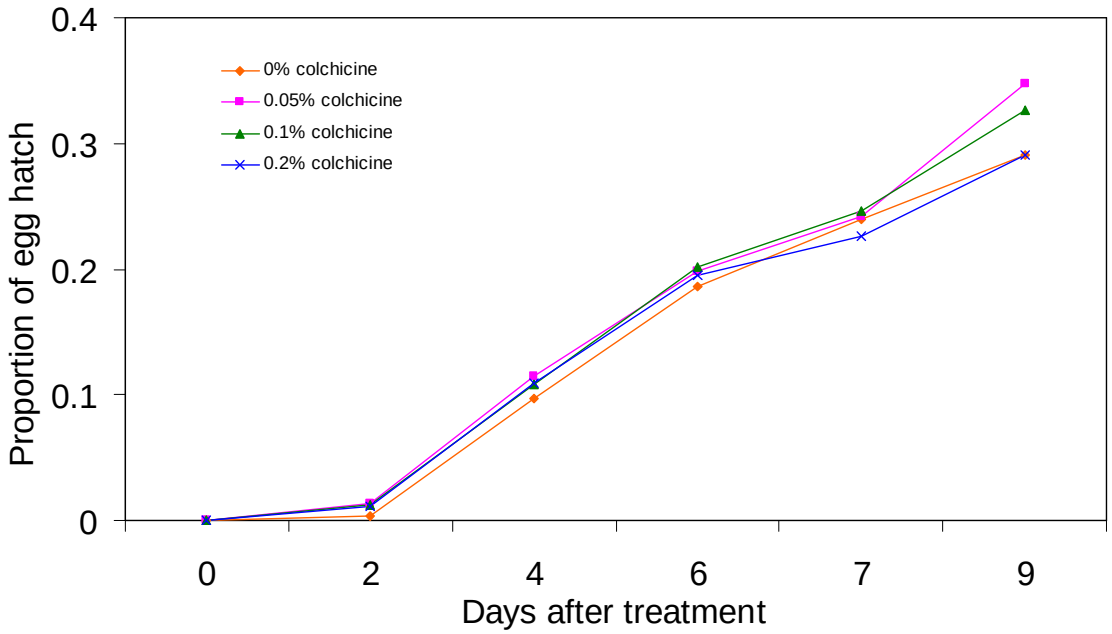


Fig.1 Effect of colchicine treatment on hatch of *Meloidogyne javanica* eggs, test 1.

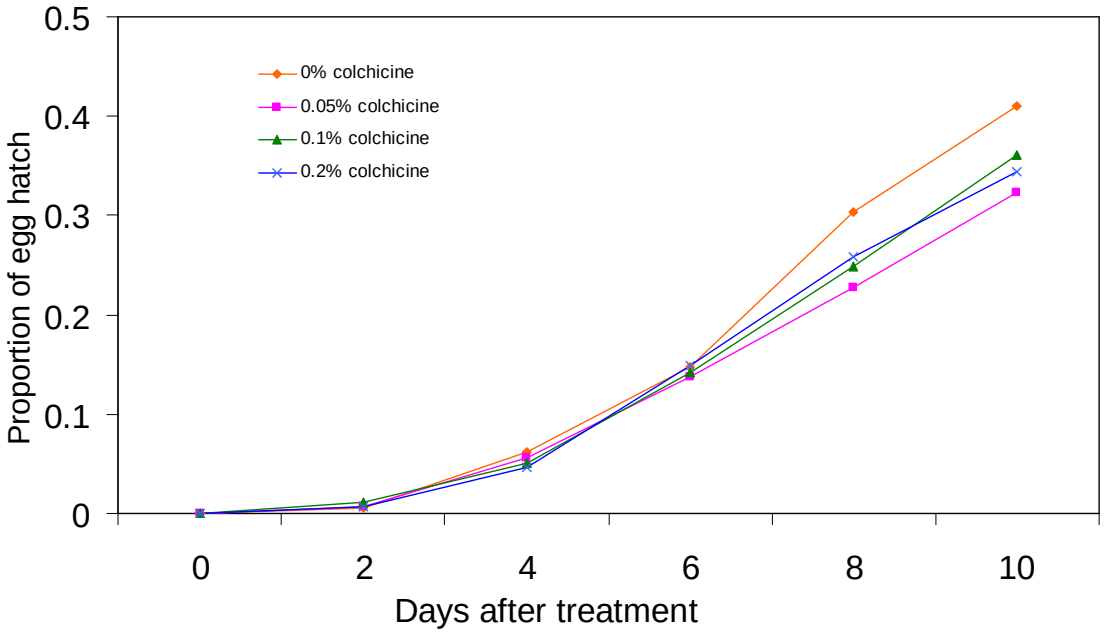


Fig. 2 Effect of colchicine treatment on hatch of *Meloidogyne javanica* eggs, test 2.

In the second test, total hatch at day 10 ranged from 34% to 41%. In this test colchicine affected egg hatch ($P < 0.01$). At days 8 and 10, hatch in 0.05% colchicine was 25% lower than hatch in 0% colchicine (Fig. 2). Percentage hatch in 0.1% or 0.2% colchicine was not different from other treatments.

The proportion of treated nematodes that reached maturity in samples harvested at 3 week after inoculation ranged from 11% to 46%, whereas samples harvested at 4 weeks after inoculation had maturity from 94% to 99%. No effect of treatment on juvenile activity was observed ($P = 0.44$) (Fig. 3). Significant effects was observed between replications ($P < 0.01$), because samples were harvested at different times.

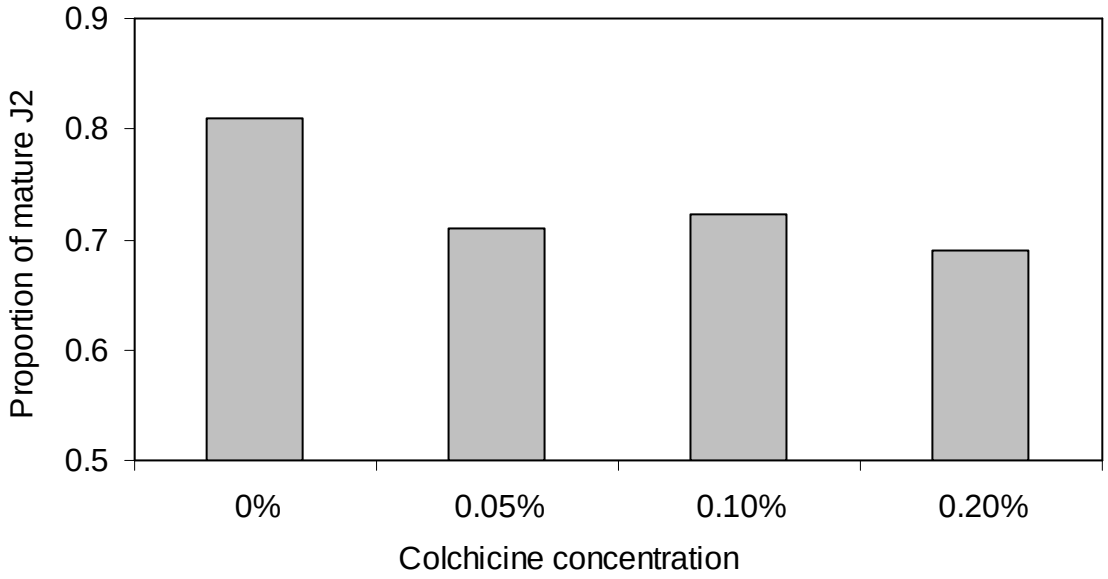


Fig. 3 Proportion of J2 treated with colchicine that reached maturity compared to untreated J2.

In the test on effect of colchicine on egg hatch in progeny of treated juveniles, a few eggs hatched on day 0 with 35% to 92% total hatch by day 13. No effect of treatment on hatch was observed at 5% level ($P = 0.33$) (Fig. 4), whereas effect of time on hatch was significant ($P < 0.01$).

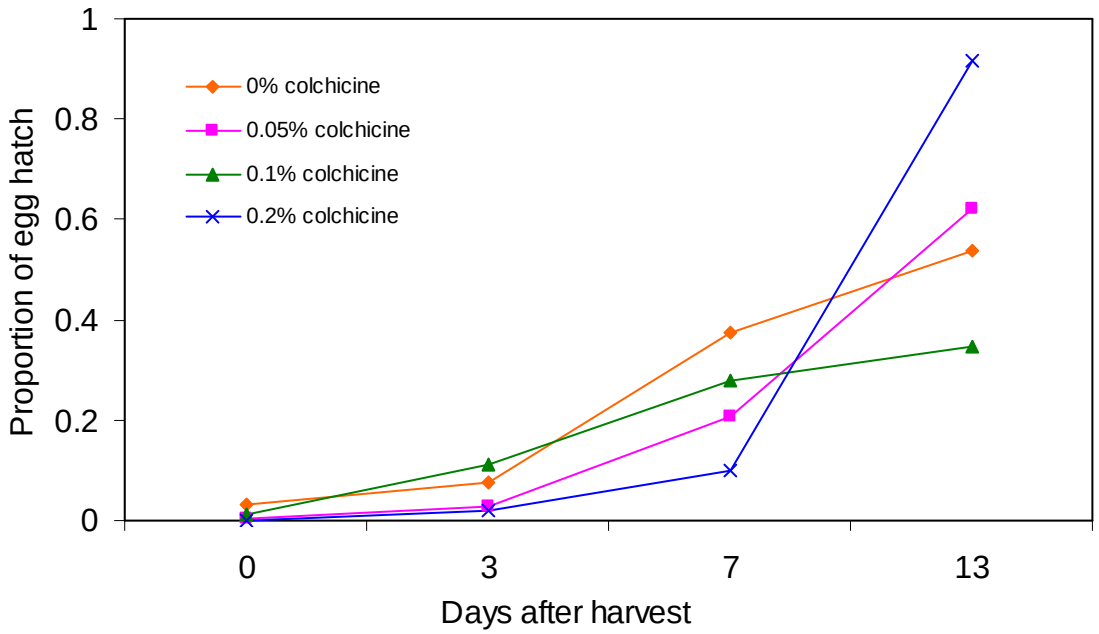


Fig. 4 Effect of colchicine treatment on hatch of *Meloidogyne javanica* eggs of the subsequent generation to that which was treated.

**DISCUSSION**

In a previous study, colchicine was used to block mitosis in giant cells and to gain insite to the relationship between numbers of nuclei per giant cell and nematode development (Wiggers et al., 2002). However those studies failed to address the question of whether the colchicine may have directly affected nematode development.

Most of the results of this study show that colchicine does not affect nematode egg hatch or juvenile activity. The only exception was on treatment with 0.05% colchicine in one of the two tests for egg hatch, where at days 8 and 10, hatch in 0.05% colchicine was 25% lower than hatch in 0% colchicine (Fig. 2). However, in this experiment percentage hatch in 0.1% or 0.2% colchicine was not different from other

treatment. Thus, the effect of 0.05% colchicine on egg hatch was likely due to random error, since if colchicine has effect on nematode egg hatch, a greater effect should have been expected with treatment with higher concentration of colchicine. These data suggest colchicine can be use to manipulate the nuclear condition of giant cells without a direct effect on the nematode parasite.

# CHAPTER III

# GENE EXPRESSION IN GIANT CELLS

## INTRODUCTION

When plant-parasitic nematodes of the genus *Meloidogyne* infect a host, they induce the formation of enlarged cells, termed giant cell, in the host roots that become the primary feeding site of the nematodes. Giant cells are densely cytoplasmic, and multinucleated. Mature giant cells from pea have about 59±23 nuclei/giant cell, lettuce has fewer nuclei, about 26±16 nuclei/giant cell (Starr, 1993). Also, nuclei from mature giant cells are polyploid. In pea and tomato, nuclei from giant cells averaged 15.8 and 14.2 times more DNA, respectively, than the 2C values from non-infected root tip nuclei (Wiggers, 1990). The 2 to 12 giant cells/feeding site (Rhode and McClure, 1974) act as transfer cells, increasing the flow of nutrients from the plant to the nematode. Giant cells are known to have elevated levels of many metabolites (e.g., ATP, glucose, and free amino acids) and some enzymes (Endo and Veech 1969; Gommers and Dropkin 1977). Potenza et al. (1994) reported that alfalfa plants infected by root-knot nematode have similar but not identical protein profiles to non-infected root tissue, based on 2-dimensional gel electrophoresis. However, most of the differentially accumulated proteins were identified as being related to the wounding produced by the nematodes during migration, rather than to the establishment of the feeding sites. These results

were most probably due to the use massively infected roots, in an attempt to obtain sufficient plant tissue for molecular studies.

A previous report by Wiggers et al. (1991) suggested that nuclear DNA increase is a systematic increase of all the genomic material. Four sequences for pea representing low (ribulose 1.5-bisphosphate carboxylase and actin), mid-level (histone 3), and highly repetitive (large ribosomal repeat) sequence DNA were tested by DNA slot blot analysis. No difference in signal strength between equal amounts of root-tip DNA and giant cell DNA was found. These results indicated that no selective DNA sequence amplification occurred in giant cells.

One aspect of giant cell activity and nematode development that has not been investigated in detail is the relationship between number of nuclei per giant cell and mRNA accumulation. A major obstacle to the study of giant cells is that of collecting tissue samples that are specific to giant cells. Wang et al. (2003) reported that transcript levels of one gene in mature giant cell cytoplasm collected by microaspiration was ca 56 times that of normal root cells. However, if an attempt was made to use only tissue samples enriched for giant cells, the measured transcription levels were only 2 times that of unaffected root cells. Further, they were unable to use microaspiration to sample giant cells that were not fully mature.

The proposed research was to test the hypothesis that transcription of genes responsible for primary cellular metabolism will increase in proportion to the number of nuclei per giant cell. The new technique of laser capture microdissection (LCM) was investigated as a means of sampling giant cell cytoplasm with increased precision. LCM

is a technique by which individual cells can be harvested from tissue sections, while they are viewed using a microscope, by moving selected cells to an adhesive film with a laser beam (Kerk et al., 2003). When a laser beam is focused on a specific cell, an adhesive film between the laser source and tissue sample is stretched by the laser beam until it contacts the sample, the sample then sticks to the film. The film with cell samples attached to it is then transferred to a microfuge tube for further processing. Using LCM, giant cells could be sampled at different stages of development.

## MATERIALS AND METHODS

*Sample collection and fixing*: Tomato (*Lycopersicon esculentum* Mill. cv. Rutgers) seeds were germinated in commercial seed-germination paper (Anchor Paper Company, Saint Paul, MN) in a 25 °C incubator for 2 days. The tomato seedlings were transplanted into sand and grown at 27 °C with a 12 hour light/dark cycle. Seedlings were inoculated at 2 weeks of age with 100 to 150 *Meloidogyne javanica* J2/seedling. Inocula of *M. javanica* were collected using NaClO (Hussey and Barker, 1973).

Galled tomato roots were collected at 1, 2 and 3 weeks after inoculation. Samples were fixed for 4 to 24 hours at 4 °C in at least 10 volumes of freshly prepared 3:1 ethanol: acetic acid (Farmer's fixative) immediately after being trimmed from roots (Zhu, 2003). During fixation samples were subjected to 15 minutes of vacuum (400 millimeters of Hg) on ice to assist infiltration of the fixative. Control non-infected root tips and cortical tissue were collected at the same time and fixed using the same method.

*Sample processing and embedding*: Fixed tissue was dehydrated at room temperature in a graded series of ethanol (70% to 100% ethanol) for 30 minutes to 1 hour each (depending on sample size). Following ethanol dehydration there were three changes in pro-par (xylene substitute) for 30 minutes to 1 hour. Finally, tissue samples were embedded in paraplast (Surgipath, Richmond, IL) at 60 °C.

*Preparation of slides for Laser Capture Microdissection*: Sections of embedded tissues were cut with a microtone into 5 μm thick ribbons. The ribbons were floated in water on glass slides at 42°C to stretch. The slices were air-dried, and stored in darkness at 4°C under dehydrating condition. Before laser capture microdissection (LCM), paraffin on the slides was removed by three rinse in xylene for 3 minutes each, followed by rinsing in an ethanol serials (100%, 100%, 95%, 95%, and 70% [v/v] ethanol) for 10 to 15 dips at each concentration. The slices were soaked in diethyl pyrocarbonate (DEPC) treated water for 10 minutes and then allowed to air-dry.

*Laser Capture Microdissection*: The Pix-Cell II LCM (Arcurus Engineering, Inc., Mountain View, CA) system was used to microdissect cells from prepared samples. The laser beam was adjusted to melt the thermoplastic film on the surface of capture caps in an area of 7.5-μm-diam according the target cell size. Power settings were 50 to 60 mW, with laser pulse durations of 700 μseconds according to manufacturer's instructions.

The total captured area transferred by the laser beam to each capture cap was calculated digitally by a computer program written in MatLab as show below.

*Clear;*

```
I=imread('2WY4 CAP4_bshot4.jpg');
[m,n,p]=size(I);
area=0;
for x=1:m
   for y=1:n
      if ((I(x,y,3)>240) & (I(x,y,2)>240) & (I(x,y,1)>240))
         area=area+1;
      end;
   end;
end;
total_area=m*n
area
area=area/total_area
```

Here we compared mRNA molecules in the same volume of giant cells, root tip cells, and cortical cells. Root tip tissue represents plant tissues with relatively high metabolic level, whereas cortical tissue represents plant tissues with relatively low metabolic level. House keeping genes could not be used as controls because giant cells are such a unique type of cells, that even commonly use house keeping genes stand a good chance to of exhibiting unusual levels of expression.

*RNA extraction*: Caps with captured cells were fitted to 0.5-ml Eppendorf tubes containing 200 μl of Tri Reagent (Molecular Research Center, Inc., Cincinnati, Ohio) and the tubes were inverted and incubated on ice until extracted. When extracted, the

caps with Tri Reagent were stored at room temperature for 5 minutes. The caps were removed, 40 μl chloroform was added, and then vortexed for 15 seconds. After incubating for 5 minutes at room temperature, the tubes were centrifuged at 12,000g at 4 °C for 15 minutes. The aqueous phase was transferred to a fresh tube and 100 μl isopropanol was added to precipitate RNA. The tubes were mixed well and incubated at room temperature for 5 minutes, and then centrifuged at 12,000g for 8 minutes at room temperature. The pellet was washed with 75% (v/v) ethanol, and tubes were centrifuged at 7,500g at room temperature for 5 minutes. The pellet was air-dried for 10 to 15 minutes at room temperature. The RNA was resuspended in 20 μl of water previously treated with DEPC.

*Reverse transcript real time PCR*: Primers for real time PCR were designed by the program DNA Star (DNASTAR Inc., Madison, WI). To perform real-time PCR the primers should fit the requirements of i) the length of the PCR product 100-150 bp; ii) avoid runs of three or more G or C at the 3'-end; iii) avoid a 3'-end T; iv) the primers should be designed to flank a region that contains at least one intron; and v) have a GC content of 40% to 60%. Primers that fit all the requirements were designed for each gene to be tested. The genes to be tested and primer sequences are shown in Table 1.

The Qiagen onestep RT-PCR kit (Qiagen, Inc., Valencia, CA) was used to perform RT-PCR. The RT-PCR mixture contained 1× reaction mix (400 μM of each dATP, dTTP, dCTP, and dGTP, 2.5 mM $MgSO_4$), 0.5 μM of the forward and reverse primers, and 0.1 μl enzyme mix. Thermal cycling conditions were 50 °C for 30 minutes for reverse transcription, 95 °C for 15 minutes for HotStarTaq DNA polymerase

activation, and 10 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 1 minute for PCR. Four to 5 µl RNA (300 pg to 2 µg) of total RNA template was used for each assay.

Table 1. Primer sequences for tomato genes *Rb7, LHA4, and HXK1*.

| Gene | (Putative) gene function | Primer sequences |
|---|---|---|
| *Rb7* | Water channel | 5`-CACTGTTGCTTGCCTCCTC |
| | | 5`-CAATGGGTGCAATGGTTCC |
| *LHA4* | Plasma membrane | 5`-AGTGGAAAGGCTTGGAATAAC |
| | $H^+$-ATPase | 5`-AGGTTTGATGCTTCTGGTGG |
| *HXK1* | Hexokinase | 5`-TGGGGTAATTTTAGGTCATCC |
| | | 5`-TCTGCGTAAAATTTCTCCCAAGTA |

The QuantiTect SYBR Green PCR kit (Qiagen, Inc., Valencia, CA) was used throughout as 25 µl reactions in a Smart Cycler (Cepheid, Sunnyvale, CA). Thermal cycling condition were 95 °C for 15 minutes for HotStarTaq DNA polymerase activation, and 45 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 1 minutes for PCR. Fluorescence from SYBR Green I was detected at 494 and 521 nm wavelength by a built in fluorescent detection systems. The cycle threshold ($C_t$) values for each reaction were calculated automatically by the Smart Cycler (Cepheid, Sunnyvale, CA) detection software by determining the point in time (PCR cycle number) at which the fluorescence

exceeded 10 times the computer determined standard deviation for background. The size of the PCR product was checked periodically using electrophoresis.

*Standard curves for quantitative real time PCR*: The DNA sequences identical to expected RT-PCR products were purified from RT-PCR products using a Gel Purification Kit (Qiagen, Inc., Valencia, CA). The fragments were cloned into pCR 2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen, Inc., Carlsbad, CA). The inserts were sequenced to confirm identity with RT-PCR products. The plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen, Inc., Valencia). The plasmid concentrations were quantified by spectrophotometry, and 10-fold serial dilutions were made. The $C_t$ values were calculated by the Smart Cycler software. Calculations for each dilution set included standard deviation and average $C_t$. Regression analysis was completed on the average $C_t$ values.

## RESULTS

To quantitate RNA molecules in test samples, a standard curve for the relationship between Ct and $\log_2$ of number of RNA molecules for each gene (primer pair) needs to be made. Samples with known template molecule numbers were used to run real-time PCR and Ct values were determined. Analysis of linear regression gave the regression equations listed in Table 2. Their $r^2$ values were between 0.97 and 0.99.

Table 2. Standard curve formula for *LHA4, Rb7, and HXK1*.

| Gene Name | Standard Curve Formula |
|---|---|
| *LHA4* | $\log_2$(number of template molecules) = 43.04-1.40Ct ($r^2 = 0.98$) |
| *RB7* | log2(number of template molecules) = 43.35 -1.24Ct ($r^2 = 0.97$) |
| *HXK1* | log2(number of template molecules) = 44.21-1.255Ct ($r^2 = 0.99$) |

The giant cells of a single feeding site were used as the source of RNA to study gene expression. LCM was used to collect tissue samples form four to six serial sections of each feeding site. Four to six sections of tissue samples from root tip and cortical tissue with a similar area as the feeding site were similarity collected for each RNA sample. Each samples from giant cells yielded about 1μg total RNA.

Expression level of *LHA4* in giant cells was very high, about 1890 molecules per $10^{-6}$ $cm^3$. In root tip cells expression level of LHA4 was similar than that of giant cells, with an average about 2037 molecules per $10^{-6}$ $cm^3$. Whereas *LHA4* expression in cortical cells was only 354 molecules per $10^{-6}$ $cm^3$ (Fig.5). Analysis of variance shows that in giant cells, *LHA4* had level of expression that was 6 times that of cortical cells, but which was not different from root meristem cells.

Expression level of *Rb7* was higher in giant cells than in root tip and cortical cells (Fig.5). Analysis of variance shows that in giant cells, *Rb7* had level of expression that was 3 times that of cortical cells, but which was not different from root meristem cells. Expression level of *HXK1* was low in giant cells, root tip cells and cortical cells. Analysis of variance shows that there is no different in expression levels among all types.

Because of the high variety of the data, we did log transformations for all the data and re-ran the analysis. For *LHA4* and *HXK1*, log transformated data gave the same result as that of the original data. For Rb7, the analysis of variance by using log transferred data showed that the expression levels are different among all three tissue types ($P$ = 0.0029).

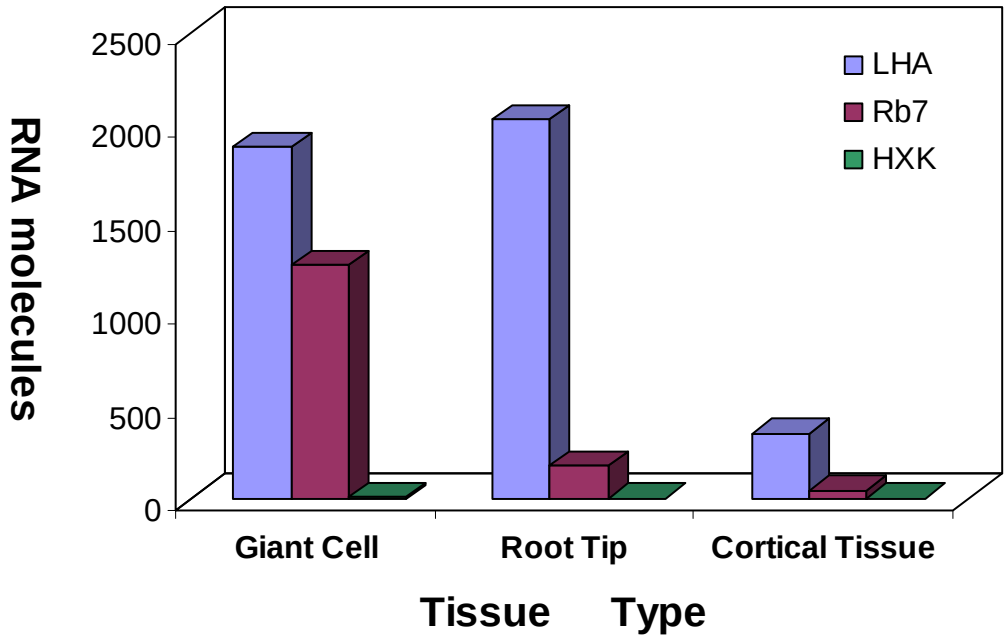


Fig. 5 Gene expression in giant cells, root tips, and cortical tissue. Giant cells, root tip cells and cortical cells were collected 21 days after inoculation. Values are number of molecules per $10^{-6}$ $cm^3$.

Secondly, the effect of time after inoculation on gene expression in giant cells was examined. Giant cells samples were collected 1 week, 2 weeks, and 3 weeks after

inoculation. For *LHA4*, analysis of variance indicated that expression of *LHA4* increased ($P = 0.02$, $r^2 = 0.528$) (Fig.6). For *Rb7*, analysis of variance indicated that expression of *Rb7* increased ($P = 0.10$, $r^2 = 0.211$) (Fig.7). Expression of *HXK1* on 1, 2, and 3 weeks after inoculation showed no difference. It did not increase with time after inoculation ($P = 0.24$).

Root tip and cortical tissue were collected at the same time as giant cells. Expression of *LHA4*, *Rb7* and *HXK1* in root tip did not change with time ($P = 0.73$, 0.37 and 0.77 respectively). Expression of the three genes in cortical tissue also did not change with time ($P = 0.73$, 0.44 and 0.39 respectively).

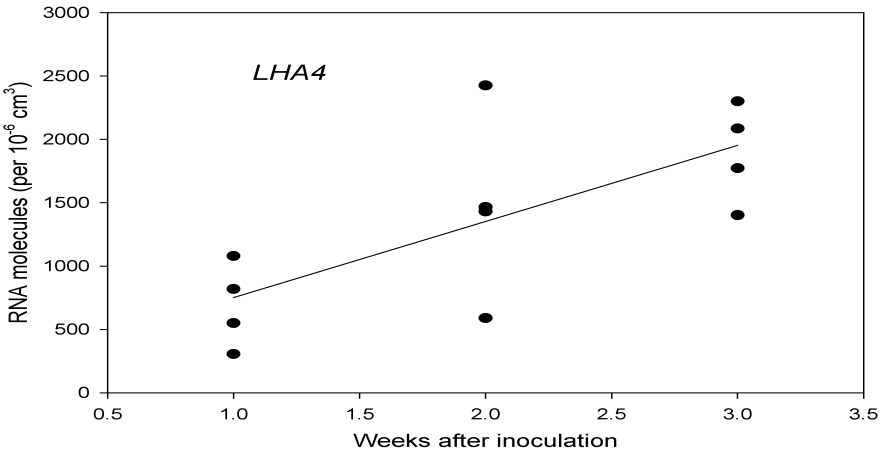


Fig. 6 Expression of *LHA4* at 1, 2, and 3 weeks after inoculation. Values are number of molecules per $10^{-6}cm^3$.

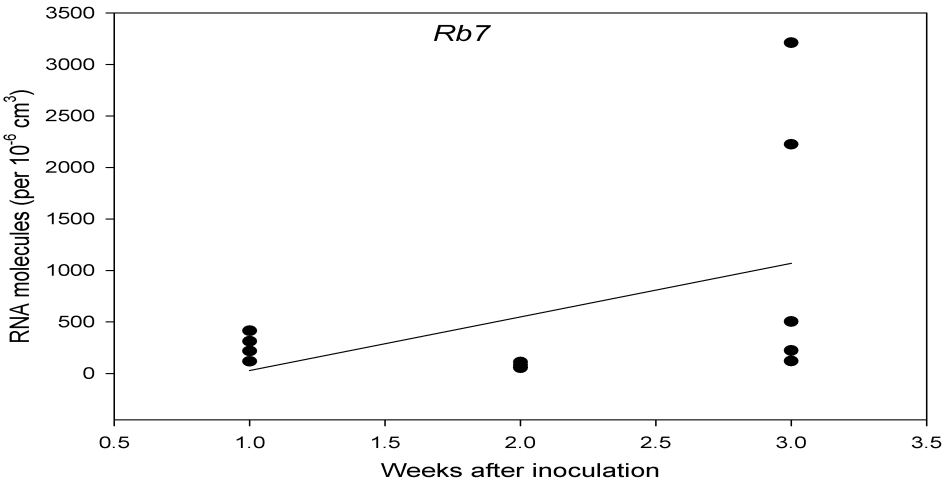


Fig.7 Expression of *Rb7* of 1, 2, and 3 weeks after inoculation. Values are number of molecules per $10^{-6}cm^3$.

## DISCUSSION

Expression of the three genes in root tip and cortical tissue did not change with time, which suggests that expression the three genes was relatively stable in normal plant tissue. However, in giant cells, two of the three genes showed increased expression over time post inoculation. From a previous study, we know that the number of nuclei in giant cells increase with time after inoculation. In *M. incognita* infected tomato, there are about 30 nuclei/giant cell at 1 week after inoculation, and 50 nuclei/giant cells at 2 weeks after inoculation. In giant cells 3 weeks after inoculation, the number of nuclei is not known because nuclei clump by this stage in giant cells. It seems that expression of LHA4 and RB7 in giant cells increase as the number of nuclei in giant cells increase.

In our results, expression of *HXK1* in giant cells has the same level as that in root tip and cortical tissues and it did not change over time after inoculation. However, this result did not mean that giant cells have the same molecule number of *HXK1* mRNA because here we only compared the number of RNA molecules in the same volume of cells. The size of one mature giant cell may be thousands of times that of one root tip cell. Thus, giant cells still have much more *HXK1* mRNA molecules than that of root tip and cortical cells.

Average expression level of *Rb7* in giant cells was measured to be about 10 time of that in root tip, however, statistically they are not significantly different with each other. This is because of high variation of *Rb7* expression measured in giant cells (Fig. 7). The huge variability may derive from the sampling technique LCM-RT-Real Time PCR has not previously been used to study gene expression in giant cell leaving many

possible places to introduce error. Both LCM and RT-Real Time PCR are newly developed technique and still show some experiment of instability, especially, in dealing with such a small amount of sample. In order to reduce the effect of on our final conclusion, more replicates are needed or identify steps where mRNA is being lost.

In other studies of gene expression change in particular cells (such as cancer cells) house keeping genes were used as a standard. In our research here, we did not use house keeping genes, but instead use root tip and cortical tissue as control to study expression of the same gene in these three different tissue cells. Root tip tissue represents plant tissues with relatively high metabolic level, whereas cortical tissue represents plant tissues with relatively low metabolic level. The reason we do not chose housing genes as controls is because giant cells are such a unique type of cells, house keeping genes stand a good chance to have expression different from normal cells. If we can find a gene whose expression does not change in giant cell at all, it will be a good candidate for a standard.

# CHAPTER IV

# CONCLUSIONS

This thesis presents data on two techniques for studying the nuclear condition in giant cells. The use of colchicine to arrest nuclei number in giant cells and LCM-RT-Real Time PCR to study gene expression levels in giant cells. Colchicine is a microtubule poison that depolymerises already formed microtubules, thus affecting any cellular functions dependent on microtubules, including mitosis. From a previous study we know that colchicine treatment of developing giant cells blocks further mitotic activity (Wiggers et al., 2003). However that study failed to consider possible direct effects of colchicine on the nematodes. The experiments reported herein were designed to test the possible effects of colchicine on different stage of nematode development, including egg hatch, juvenile activity, and egg hatch in progenies of treated nematodes. The data indicated that colchicine did not affect any of these three aspects of nematode activity. That means if nematode development in colchicine treated roots was inhibited, the inhibition came indirectly through colchicine effect on giant cells but not from the effect of colchicine on nematodes themselves.

This is the first time that laser capture microdissection coupled with RT-real-time PCR was used to study gene expression in giant cells. LCM allowed specific and precise sampling of giant cells in a manner heretofore not possible. RT-real-time PCR is a technique that allows one to estimate the quantity of a specific RNA in a sample. In

the present study, it was shown that mRNA could be quantitated from as few as six tissue samples collected by LCM.

The procedure of capture microdissection coupled with RT-real-time PCR allowed examination of the expression of three different genes (*LHA4*, *Rb7*, and *HXK1*). These three genes show different expression profiles in giant cells at different development stages. Expression of *LHA4* and *Rb7* increase with time after inoculation, and in mature giant cells they reach levels that are 3 and 6 times, respectively, that of cortical cells, but which were not different from root meristem cells. Expression of *HXK1* does not change with time after inoculation and has the same level of that in root tip and cortical tissues. One problem noted in this study was the amount of variability between samples. Because of variability in the data, more replicate samples will be needed in further studies.

The combination of colchicine to manipulate nuclei per giant cell, LCM to sample giant cell cytoplasm, and real-time PCR will allow testing of numerous hypothesis about the giant cell development, giant cell activity, and their relationship to parasite development. Further studies will include the use of colchicine to arrest mitosis in giant cells in early stages of development, then to correlate level of gene expression using LCM-RT-Real Time PCR in giant cells with reduced numbers of nuclei with rates of nematode development.

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# VITA

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