SOIL PHYSICAL FACTORS AFFECTING DISEASE SEVERITY OF ROOT-KNOT NEMATODE ON TOBACCO AND MORPHOLOGY AND MOLECULAR IDENTIFICATION OF TROPICAL NEMATODES IN EAST COAST MALAYSIA

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By

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

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LIST OF SYMBOLS AND ABBREVIATIONS

ng : Nanogram

µm : Micrometre

µl : Microlitre

nm : Nanometre

mM : Milimollar

ml : Millilitre

pmol : Picomolar

kb : Kilobase

bp : Base pair

se : Standard error

J2 : Second stage juveniles

DEGO : Dorsal esophageal gland orifices

DNA : Deoxyribonucleic acid

IGS : Intergenic spacer

PCR : Polymerase Chain Reaction

AFLP : Amplified fragment length polymorphism

RAPD : Random amplified polymorphic DNA

RFLP : Restriction fragment length polymorphism

AFLP : Amplified fragment length polymorphism

SCAR : Sequence characterised amplified region

BLAST : Basic local alignment search tool

DMRT : Duncan multiple range test

FAKTOR-FAKTOR FIZIKAL TANAH YANG MEMPENGARUHI TAHAP PENYAKIT HEMPEDU AKAR PADA TEMBAKAU DAN IDENTIFIKASI SECARA MORFOLOGI DAN MOLEKULAR NEMATOD TROPIKA DI PANTAI TIMUR MALAYSIA

ABSTRAK

Di Malaysia, industri tembakau adalah amat penting dalam meningkatkan taraf sosio -ekonomi petani di Kelantan dan Terengganu. Nematod hempedu-akar (*Meloidogyne* spp.) mengurangkan pengeluaran dan kualiti tembakau. Dalam kajian ini, 24 kawasan penanaman tembakau telah ditinjau untuk penyakit hempedu akar dan kepelbagaian spesies nematod hempedu-akar. Analisa fizikal tanah dan pH telah dijalankan untuk menyiasat hubunganya terhadap tahap penyakit dan bilangan bilangan nematod hempedu-akar di dalam tanah. *Meloidogyne* spp. yang diasingkan diperhatikan untuk morfologi untuk mengenal pasti genus dan analisis lanjut melalui Touchdown Polymerase chain reaction (TD- PCR) untuk mengenal pasti spesies. Dari 24 kawasan penanaman , 22 menunjukkan serangan nematod hempedu-akar. Tumbuhan tembakau dijangkiti nematod hempedu-akar menunjukkan hempedu akar, daun kuning dan pertumbuhan terbantut. Analisis ANOVA satu hala menunjukkan bahawa ciri-ciri fizikal tanah, dan pH tanah menjejaskan tahap penyakit dan bilangan *Meloidogyne* spp. di dalam tanah. Bilangan *Meloidogyne* spp. di dalam tanah juga memberi kesan kepada keterukan penyakit . Daripada analisis korelasi Pearson, terdapat hubungan yang signifikan di antara 0.01 bilangan *Meloidogyne* spp. di dalam tanah ($r = 0.753$), pH tanah ($r = 0.238$), kelembapan tanah ($r = 0.203$) dan ketumpatan pukal tanah ($r = 0.227$) kepada keterukan penyakit . Terdapat juga hubungan yang signifikan di antara 0.01 pH tanah ($r = 0.373$), kelembapan tanah ($r = 0.359$), ketumpatan zarah tanah ($r = -0.404$) dan ruang liang tanah ($r = -0.332$)

terhadap bilangan *Meloidogyne* spp. di dalam tanah. Identifikasi spesies nematod hempedu-akar telah dikenal pasti menggunakan Touchdown PCR menggunakan primer 194/195 yang mengamplifikasi rantau 5S - 18s ribosom dan spesies spesifik primer SCAR. Semua 22 sampel menunjukkan amplifikasi menggunakan primer 194/195 menghasilkan 720 bp yang dikategorikan sebagai Tropical *Meloidogyne* spp. Primer SCAR menunjukkan kekhususan tinggi kepada spesies yang berkaitan ditentukan dengan komposisi spesies , mengesan campuran populasi *Meloidogyne javanica* dan *Meloidogyne incognita* . Populasi yang diamplifikasi dengan primer SCAR Fjav / Rjav menghasilkan 720 bp produk dikategorikan dalam kumpulan *M. javanica* manakala populasi yang diamplifikasi dengan SCAR primer MIF / Mir menghasilkan 999 bp telah dikategorikan dalam kumpulan *M. incognita*. Daripada 22 sampel, 15 sampel menunjukkan terdapat *M. javanica* manakala 7 sampel menunjukkan campuran spesies *M. javanica* dan *M. incognita* .

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ABSTRACT

In Malaysia, tobacco industry is very crucial in uplifting the socio-economic status of farmer in Kelantan and Terengganu. Root-knot nematodes (*Meloidogyne* spp.) lower the production and quality of tobacco. In this study, 24 tobaccos cultivation areas were surveyed for root gall disease and species diversity of root- knot nematode. Soil physical and pH analysis were carried out to investigate their correlation to disease severity and number of root-knot nematode number in soil. Isolated *Meloidogyne* spp. was observed for morphology for genus identification and further analyses via Touchdown Polymerase chain reaction (TD-PCR) for species identification. From 24 cultivation area, 22 showed infestation of root-knot nematode. Tobacco plant infected with root-knot nematode showed root gall, yellowing leaves and stunted growth. One- Way ANOVA analysis showed that soil physical properties, and soil pH affect disease severity and *Meloidogyne* spp. number in soil. *Meloidogyne* spp. numbers in soil also affect disease severity. From Pearson correlation analysis, there was significant correlation at 0.01 between *Meloidogyne* spp. number in soil ($r = 0.753$), soil pH ($r = 0.238$), soil moisture ($r = 0.203$) and soil bulk density ($r = 0.227$) to disease severity. There was also significant correlation at 0.01 between soil pH ($r = 0.373$), soil moisture ($r = 0.359$), soil particle density ($r = -0.404$) and soil pore spaces ($r = -0.332$) to *Meloidogyne* spp. number in soil. Root-knot nematode species identification was identified using Touchdown PCR with primer 194/195 which amplified 5s-18s ribosomal region and species specific SCAR

primers. All 22 samples showed amplification using primer 194/195 yielding 720 bp which is categorized as Tropical *Meloidogyne* spp. SCAR primers showed high specificity to the related species reliably determined species composition, detecting mixed population of *Meloidogyne javanica* and *Meloidogyne incognita*. A population amplified with the SCAR primer Fjav/Rjav yielding 720 bp products was categorized in the *M. javanica* group while population amplified with the SCAR primer MiF/MiR yielding 999 bp was categorized in the *M. incognita* group. Out of 22 samples, 15 samples showed occurrence of *M. javanica* while 7 samples showed mixture of *M. javanica* and *M. incognita.*

CHAPTER 1: INTRODUCTION

Tobacco (*Nicotiana tabacum*) is considered to be one of the most important industrial crops and highly demanded throughout the world (Luc *et al.*, 2005). In Malaysia, tobacco industry is very crucial in uplifting the socio-economic status of farmer in Kelantan and Terengganu. Domestic tobacco demand and economic value of tobacco in Malaysia has enabled farmers to benefit from the lucrative crop. National Kenaf & tobacco board described the economical value in the Q & A,' Why not grow tobacco and other food crops?' It is said that the farmers who grow tobacco earn up to RM10,000 per hectare and for the farmer-curers able to earn up to RM25,000 per hectare (http://www.lktn.gov.my/page.php?140).

Meloidogyne spp. (root- knot nematode) that cause root gall has been known to pose a serious threat of tobacco production in the world often lower the quality and yield (Luc *et al.*, 2005).. *Meloidogyne* spp. has been reported to cause infection on more than 5500 plant species including crop and weeds (Trudgill and Blok, 2001; Van Biljon, 2003; Adam *et al.*, 2007). Annual crop losses cause by *Meloidogyne* spp. estimated to exceed $US 50 billion (Bent *et al.*, 2008). In Malaysia, *Meloidogyne* spp. not only showed infection on tobacco but also on other crop such as guava (*Psidium guajava* L.) in Perak (Razak and Lim, 1987; Tahery *et al.*, 2011), chilli (*Capsicum frutuscen*) (Tahery *et al.*, 2011), black pepper (*Piper nigram* L.), turfgrass on golf courses (Tahery *et al.*, 2011), kenaf (*Hibiscus cannabinus*) (Tahery *et al.*, 2011) and banana (Razak, 1994; Tahery *et al.*, 2011).

Although chemical control is the most reliable method to control *Meloidogyne* spp., chemicals are toxic to human and environment (Sirias, 2011). Therefore, new strategies to control *Meloidogyne* spp. such as integrated management practice, tolerant tobacco varieties and biological control are needed (Bertrand *et al.*, 2000). Correct species identification is basic to efficient nematode control and successful plant quarantine operations. Previous studies have been carried out to identify *Meloidogyne* spp. using various morphological character but they are unpractical and insufficient (Adam *et al.,* 2007; Sirias, 2011). Besides, species identification using morphology might overlap between species (Adam *et al.,* 2007; Sirias, 2011). Therefore, nowadays, molecular method base on utilization of DNA and PCR have many advantages and have been used for reliable *Meloidogyne* spp. identification. *Meloidogyne* spp. identification could be useful to select the best strategy for their management (Adam *et al.,* 2007). Considering the importance of *Meloidogyne* spp., this study was done with the following objective:

1. To find relationship of soil physical properties and pH to disease severity and population density of *Meloidogyne* spp..

2. To observe morphology of *Meloidogyne* spp. for genus identification

3. To identify species of *Meloidogyne* spp. (root-knot nematode) via molecular method.

CHAPTER 2: LITERATURE REVIEW

2.1 Tobacco in Malaysia

2.1.1 Tobacco cultivation in Malaysia

The tobacco industry was introduced in 1959 by a private company Malayan Tobacco Company (MTC) on 20 acres land (Bek, 1977). In the late sixties, private individuals and companies that set up their own curing station increase and their influx resulted in the breakdown of the supervision and control. Cultural practices were not adhered and quality and yield deteriorated and getting worse when MTC pulling out from the curing business in 1972 (Bek, 1977). In November 1973 National Tobacco Board (now known as National Kenaf and Tobacco Board) was established following the recommendation of the Task Force appointed by the government to control, regulate and developed the tobacco industry (Bek, 1977). In 1975, tobacco industry has mushroomed into an industry with about 35000 acres producing 9 million kg of cured tobacco valued at about RM 147 million (Bek, 1977). In 1977, Kelantan is the main tobacco producing state with smaller production coming from Terengganu, Pahang, Johor, Kedah, Perlis, Melaka and Negeri Sembilan (Bek, 1977). Tobacco industry give lucrative return to farmers especially in Kelantan and in the bris soil areas where opportunities to plant other crops are limited (Bek, 1977).

Nowadays, tobacco cultivation is active in Kelantan and Terengganu (Figure 2.1). Tobacco is the short-term crops and many tobacco farmers also grow other crops after the tobacco season. Crop diversification, crop rotation, cropping systems that optimize and maximize income from agriculture is the main thrust of agricultural

development particularly with regard to small farmers (http://www.lktn.gov.my/page.php?140). Generally, tobacco is grown in relatively small scale (3-5 hectares) under the concept of individual curer growers (IEP) where cost is reduce especially when based on family labour and the expected income based on the returns to agricultural labour on par with other alternative or income from non-agricultural sources (http://www.lktn.gov.my/page.php?140). National Kenaf and Tobacco Board (NKTB) still play important roles in this industry by controlling the production and controlling the price. In addition, NKTB also providing advice from sowing until the sale of dried leaves to cigarettes manufacturers. NKTB also provide fertilizers, pesticides, plastics and other plows (http://www.lktn.gov.my/page.php?140).

2.1.2 Importance of Tobacco in Malaysia

All tobacco product produced in Malaysia is for domestic market. Three main cigarettes production companies produce around 19 billion cigars every year which required 20 million kilograms tobacco (http://www.lktn.gov.my/page.php?140). Domestic tobacco content is about 70%, meaning there is potential for import substitution and any shortfall in domestic tobacco production resulting in increased imports and reduced foreign exchange (http://www.lktn.gov.my/page.php?140). Major cigarette manufacturers also produce cigarettes and processed tobacco for the export market with an income of about RM650 million in year 1999 (http://www.lktn.gov.my/page.php?140). Due to the Malaysian tobacco not competitive in export markets, tobacco use is still being imported and Malaysia to benefit in the form of value-added activities. In 2009, tobacco industry generates RM

34.71 million which 23% goes to the farmers, 36.7% goes to farmers-curer, 9% to co-operative owned curing station, 5% to labour and the rest to the suppliers of fertilizers and plastic materials, farm machinery, firewood for fuels, transportation and others (http://www.lktn.gov.my/page.php?106).

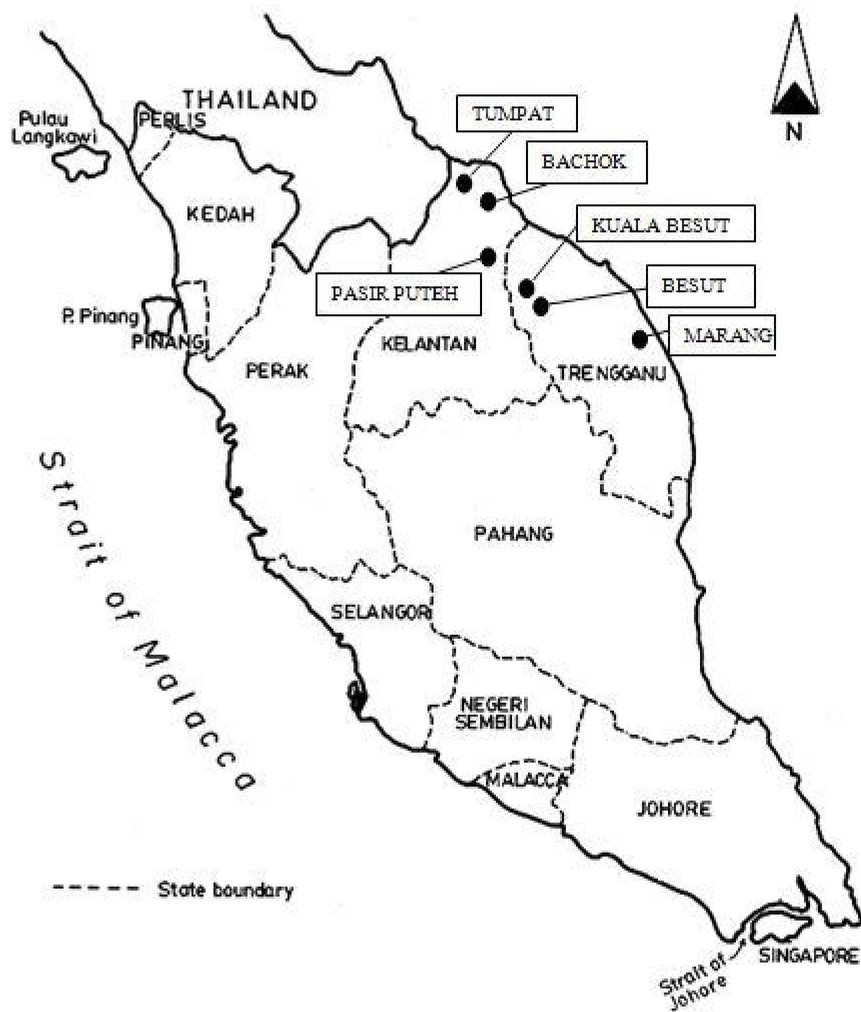

Figure 2.1: Active tobacco cultivation area in Malaysia

2.1.3 Plant parasitic nematodes related to tobacco

Dominant plant parasitic nematodes that parasitize tobacco plant were *Meloidogyne* spp. (root-knot nematode). Important species from this genus were *Meloidogyne arenaria* (Neal, 1889, Chitwood, 1949)*, Meloidogyne incognita* (Kofoid and White, 1919; Chitwood, 1949)*, Meloidogyne javanica* (Treub, 1685; Chitwood, 1949), and *Meloidogyne hapla* (Chitwood, 1949). This four species showed infection on tobacco (Barker et al., 1981). *Meloidogyne hapla* usually can be found in cooler region and higher elevation of the tropic (Taylor et al., 1982). However there was several other *Meloidogyne* species related to the tobacco for example *Meloidogyne cruciani* reported to cause infection in the US Virgin Island (Garcia-Martinez *et al.*, 1982; Muniz *et al.*, 2008). *Meloidogyne paranaensis* reported to show infection on tobacco but it has never been detected in the field (Carneiro *et al.*, 1996; Muniz *et al.*, 2008; Quénéhervé *et al.*, 2011). *Meloidogyne chitwood* also been reported to caused infection on tobacco in the southeast United States (Hirunsalee *et al.*, 1995). *Meloidogyne enterolobii* also reported to show infection on tobacco (Arens and Rich, 1981). *Pratylenchus* spp. that cause root lesion also parasitize tobacco plant but less important compare with *Meloidogyne* spp. due to did not cause significant losses (Kimpinski and Thompson, 1990).

Other species such as *Tylenchorhynchus* spp. (stunt nematode), *Globodera* spp . (cyst nematode), *Ditylenchus dipsaci* (stem nematode) and *Aphelenchus ritzemabosi* (foliar nematode) were also reported to cause infection on tobacco plant in certain restricted area. It has been found that other plant parasitic nematodes such as *Helicotylenchus* sp. (spiral nematode), *Rotylenchus* sp. (lance nematode),

Scutellonema sp., *Rotylenchulus* sp. (reniform nematode), *Tetylenchus* and *Crinomella* sp. parasitize tobacco but these nematodes normally did not cause significant losses. *Xiphinema* sp., *Longidorus* sp., *Trichodorus* sp., and *Paratrichodorus* sp. was reported to transmit viruses to tobacco (Luc *et al.* 2005). Plant parasitic nematodes also reported to form disease complex with other plant pathogens. For example *Meloidogyne* spp. proved to increase the incident of *Fusarium* wilt even when their population were incapable to cause direct damage to the tobacco plant (Mani and Sethi, 1984). *Pratylenchus brachyurus* (lesion nematode) and *Phyptopthora parasitica var. nicotianae* (cause black shank disease) also formed interaction between them. Inagaki and Powell (1969) found that *P. brachyurus* increase disease severity and cause rapid development of black shank symptom than when the fungus alone.

2.2 Root-knot nematode, *Meloidogyne* spp.

2.2.1 General morphology

2.2.1.1 Male

In general, male of root-knot nematode are vermiform, migratory and generally free living (Figure 2.2). The male develop by metamorphosis within a saccate juvenile (Luc *et al.,* 2005). The body size varies between species from 700 to 2000 µm (Eisenback, 1985). The variation in body size is usually because of environmental condition during their development. Male body usually twisted through 180 ° upon heat relaxation. The stylet length also varies in size which is about 13 to 30 µm. The location of dorsal esophageal gland orifices (DEGO) range from 2 to 13 µm posterior to the stylet knob base. Tail is short (hemispherical shape).

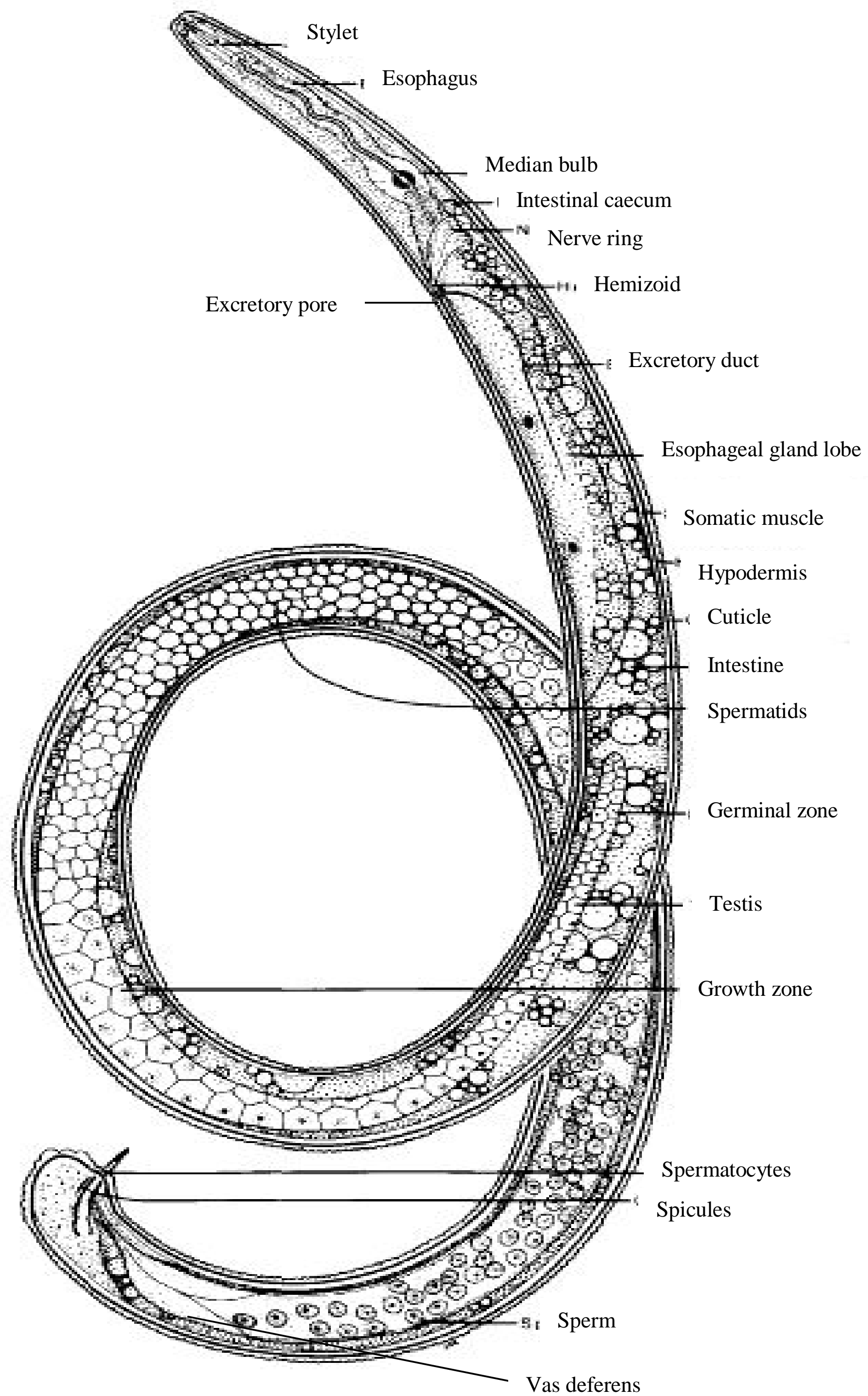

Figure 2.2: Gross morphology and anatomy of a male *Meloidogyne* spp. (After Eisenback, 1985)

2.2.1.2 Female

Adult female of *Meloidogyne* spp. have swollen, saccate bodies (pear shape like) (Figure 2.3). Female length ranges from 0.44 to 1.30 mm while width ranges from 0.33 to 0.70 mm (Eisenback, 1985). They have pearly white body with moderately thick cuticle. The neck protrudes anteriorly while vulva and anus were located terminally. Stylet were short, moderately sclerotized and protrusively hollow. The stylet length range from 10 to 24 µm which is consists of cone, shaft and knobs. The stylet functions like hypodermic needle which was moved by protractor muscles. At the posterior of stylet knobs, there was DEGO. DEGO is the two sub ventral gland orifices open into the esophageal lumen. DEGO size also varies depend on the species. The excretory pore situated anterior to median bulb valve plat and usually near stylet base. Female of root-knot nematode have two convoluted genital tracts. The major part of the body content consists of two gonads which is very long and greatly convoluted. There are ovary with germinal zone and growth zone, narrow oviduct, globular spermatotheca and long uterus in each gonad. The cuticles in the perineal region of female from this genus form a finger print-like pattern (the perineal pattern) which is use for species identification. This is due to, the perineal pattern hold most characteristic of female such as tail terminus, phasmids, lateral lines, anus, and vulva which surrounded by cuticular striae or folds. Female of *Meloidogyne spp*. have six large unicellular rectal glands situated in the posterior body region. These rectal glands were connected to the rectum and produce very large amount of gelatinous matrix material. The matrix material was excreted through the rectum and act as protective egg sac (Eisenback, 1985).

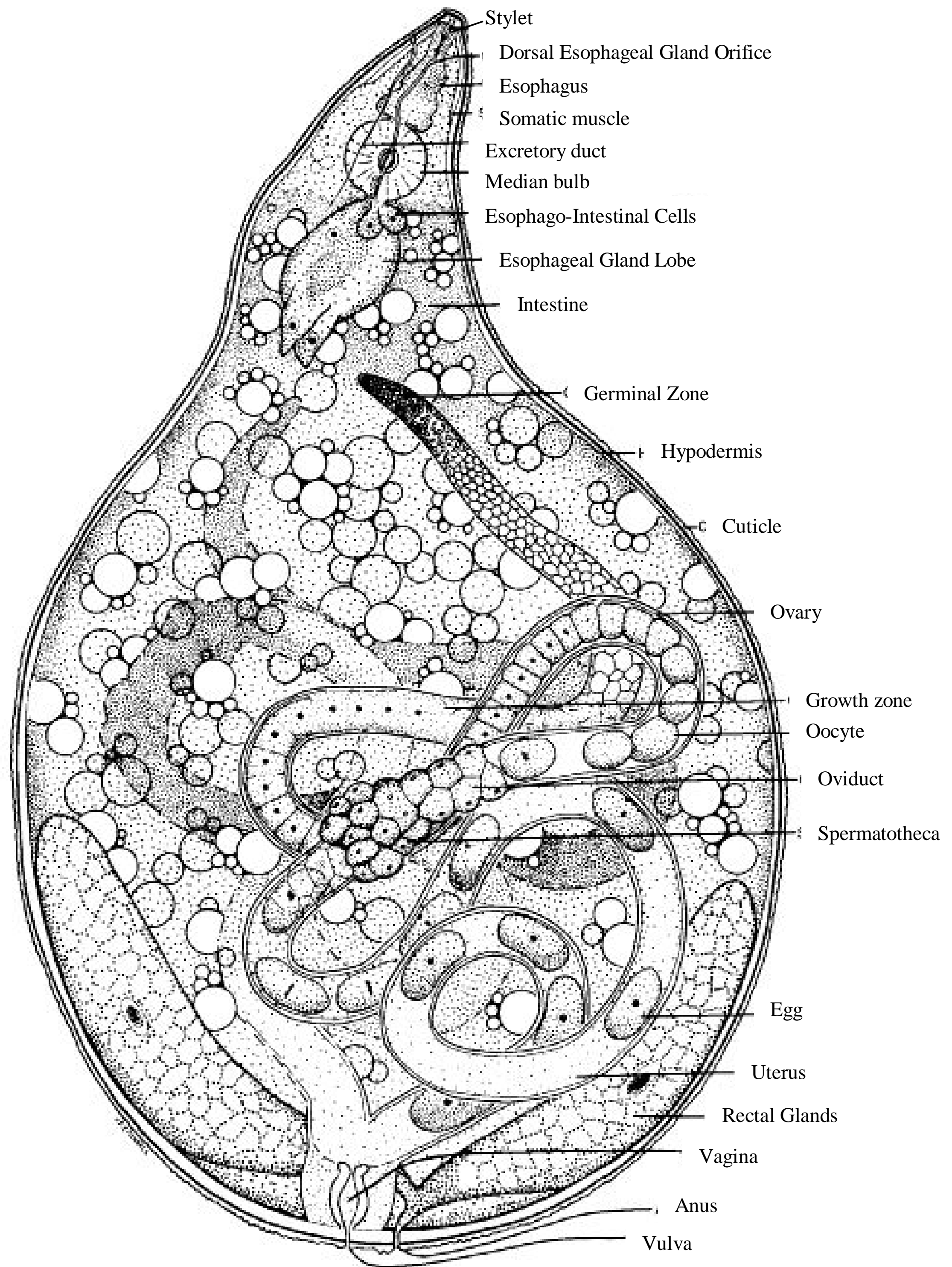

Figure 2.3: Gross morphology and anatomy of a female *Meloidogyne* spp. (After Eisenback, 1985)

2.2.1.3 Second stage juveniles (J2)

The J2 is the infective stage of root-knot nematode (Figure 2.4). The body length of this species varies from 290 to 912 µm (Eisenback, 1985). The head shape is same with the male. The stylet length range from 8 to 18 µm. DEGO distance are varies from 2 to 8 µm. The esophagus is narrow with faintly outline procorpus. Median bulb has a large plate and three long ventrally overlapping glands that are use for molting and feeding. The position of excretory pore varied. The tail length range from 15 to 100 µm depends on the species. At the end of the tail, there is hyaline terminus. There is difference in either mean tail and or mean hyaline terminus among species (Jepson, 1987). These differences can be very useful to distinguish the species.

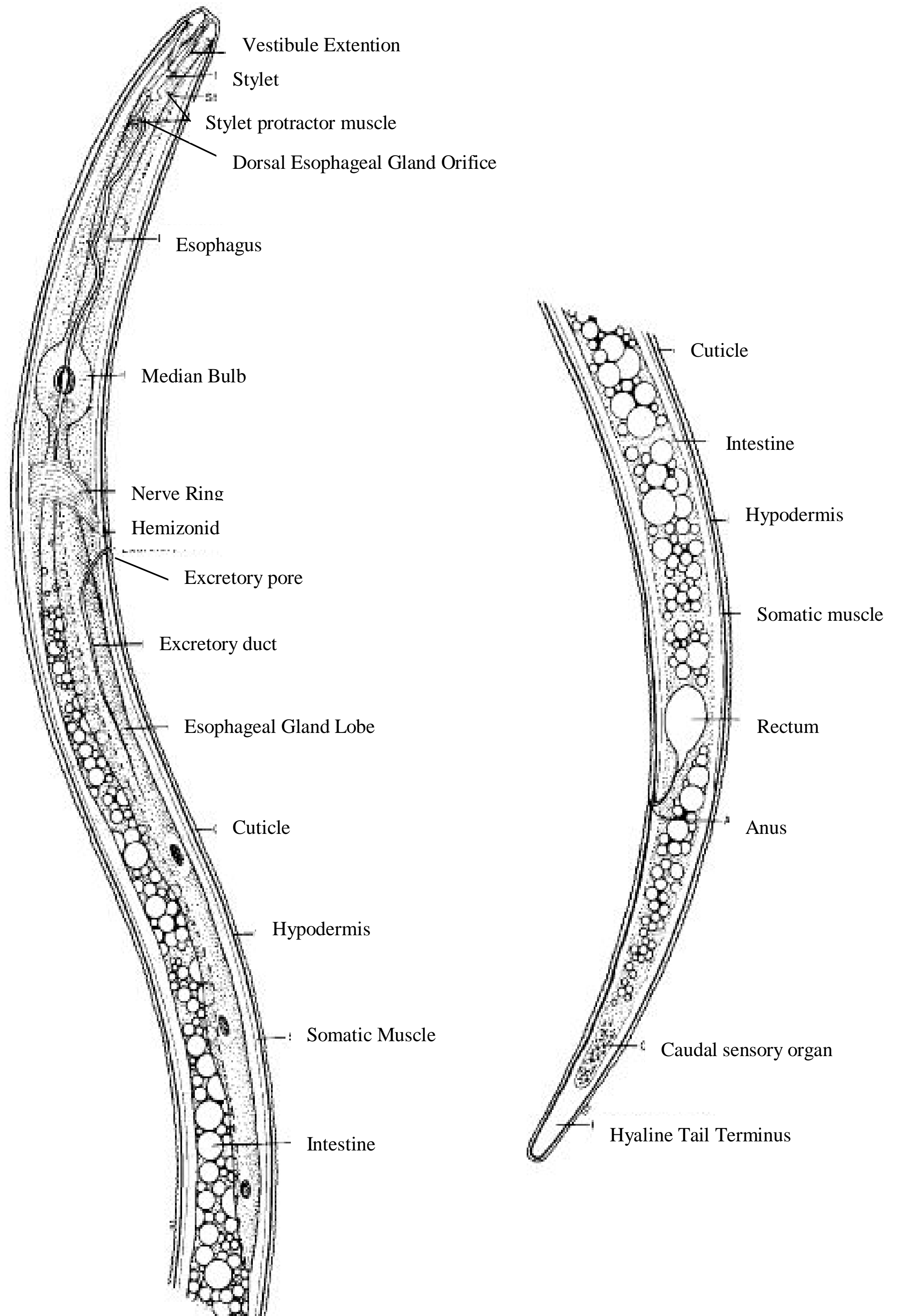

Figure 2.4: Gross morphology and anatomy of a J2 *Meloidogyne* spp. (After Eisenback, 1985)

2.2.2 Life cycle

Meloidogyne spp. are endoparasites and show sexually dimorphism, which is the female are pyriform or saccate, while the male's vermiform (Eisenback, 1985). *Meloidogyne* spp. exhibits various modes of reproduction which is sexuality (amphimixis), facultative sexuality, meitotic parthenogenesis (automixis) and mitotic parthenogenesis (apomixis) (Luc *et al*., 2005). Four major *Meloidogyne* species (*M. javanica, M. incognita, M. arenaria,* and *M. hapla*) reproduce via mitotic parthenogenesis (Oh *et al*., 2009). Development of *Meloidogyne* spp. and the biological activities in the life cycle varies from three weeks to several month depend on temperature, light, aeration, humidity of the soil and nutritional status of the host. Their population dynamics depend on both host plant and its environment such as soil types, soil moisture, different geographical region and different host (Loubser and Meyer, 1987). Temperature plays a vital role for the length of the life cycle. For example, the first adult female of *M. incognita* on tomato appear 13-15 days after root penetration at temperature approximately 29 ºC and the female laid the first egg about 19-21 days after penetration (Triantaphyllou and Hirschmann,1960). Some species of root-knot nematode are dominant in cooler region. For example *M. hapla* and some other species are dominant in the tropical and subtropical region (Karssen and Moens, 2006). Usually, for most common root-knot nematode such as *M. javanica, M. incognita* and *M. arenaria*, which thrive in tropical and subtropical regions, the optimum temperature for the reproduction and survival range from 25 ºC to 30 ºC.

The differences in body shaped between female and male occurred during the postembryonic development of *Meloidogyne* spp. (Figure 2.5). From the embryonic development, the egg hatches to become first-stage juvenile (J1) and then molt as J2 (Figure 2.5). The J2 is infective stage. It moves into the soil and enter the root of suiTable host plant behind the tip in the elongation zone using the piercing action of the stylet (Xue, 1991; Roze, 2008). J2 migrate intercellularly in a stealthy way through the cortex (Roze, 2008). J2 synthesize secretory protein in the dorsal and two subventral esophageal gland cells facilitate their migration through plant root and subsequent induction and maintenance of the 'giant cells' (Davis et al., 2000; Hussey et al., 2002; Roze, 2008). Then forms host-parasites relationship with the host plant when it finds prefer feeding site and release esophageal secretion which cause the formation of multinucleate feeding cells called 'giant cells' (Bird, 1996; Roze, 2008). These 'giant cells' provide constant supply of nutrients (Roze, 2008). The morphology of J2 changed to flask-shape as it feeds on the giant cells. J2 feeds from the 'giant cells' for ten to twelve days (Roze, 2008). Then, without further feeding it molt three times into the third (J3) and fourth stage juvenile (J4) , and finally become an adult (Figure 2.5).

The saccate adult female resume feeding on the giant cells shortly after the last molt and continue to do so for the remainder of her life. The reproductive system of both female and male of this genus develops into functional gonads during the postembryonic development (Triantaphyllou and Hirschmann, 1960). From the number of the gonad, we can differentiate the sexes. Females always have two gonads while males usually have one. During J4, the shape of saccate male juvenile change to the vermiform adult males. The metamorphosis occurs in which the body

elongates from saccate to a vermiform shape. Fully develops male emerges after the final molt of enclosed fourth-stage male which enclosed within the cuticles of J2 and J3 (Figure 2.5). The adult male leaves the root and move freely through the soil and it does not feed. The mode of reproduction determines the function of the male for mating. Usually for species that reproduce via amphimixis, the male enters the root searching for the female to mate (Muniz *et al*., 2008). Female of root-knot nematode laid eggs into gelatinous masses composed of glycoprotein matrix produced by rectal glands. Glycoprotein matrix keeps the eggs together and protects them against extremes environmental condition and it has antimicrobial properties (Luc *et al*., 2005). Egg mass can be found on the surface of root galls and sometimes embedded within gall tissue (Bird, 1958).

The life span of female is much longer than the male from 2 to 3 month. The J2 have sufficient stored energy to survive for a month in soil while finding the preferable host plant (Riga, 2004; Luc *et al*., 2005). The cells that surrounding the developing the developing juvenile and the giant cells forming a gall and hence the common name for *Meloidogyne* spp. is root-knot-nematode (Roze, 2008).

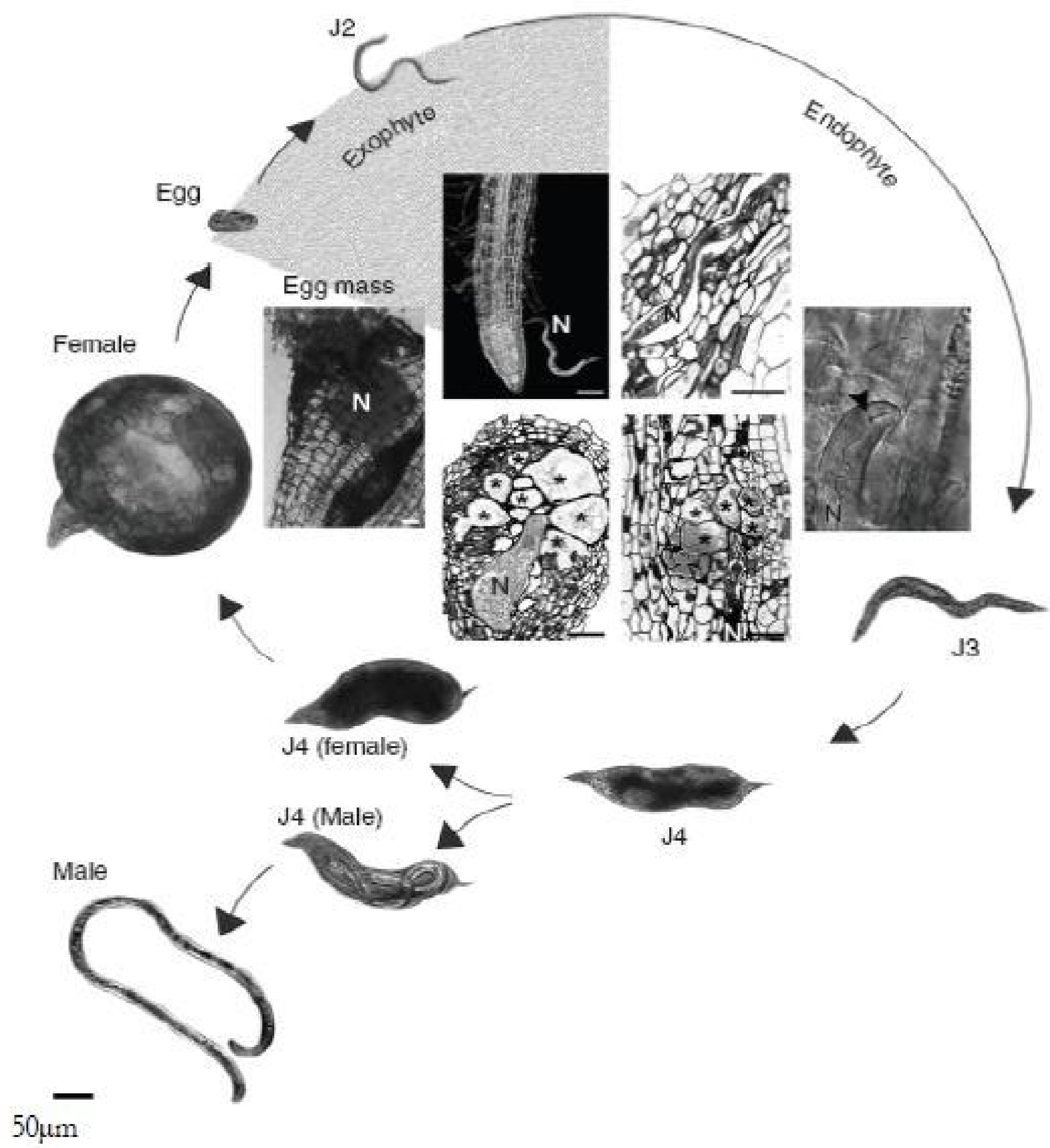

Figure 2.5: Life cycle of root-knot nematode (*Meloidogyne* species). J2: Second stage juvenile; J3: Third stage juvenile; J4: Fourth stage juvenile (Adapted from Abad *et al*., 2008)

2.2.3 Distribution and importance of *Meloidogyne* spp.

The first report of root-knot nematode was in the 1855 when M.J. Berkley observed galls on cucumbers growing in a garden frame at Nuneham, England. The genus name of *Meloidogyne* was proposed in 1887 when Goldi described *Meloidogyne exigua* Goldi isolated from galls on coffee roots in Rio de Janeiro state, Brazil (Luc *et al.*, 2005). Only 8 valid species had been described between 1880 and 1960. 18 species had been described in the 1960's, six in the 1970's, 30 in the 1980's, 22 in the 1990's and 12 in the 2000's. Until June 2009, there were 97 valid species in the *Meloidogyne* genus (Arens and Rich, 1981; Muniz *et al.*, 2008; Vivian & Thomas, 2009; Brito *et al.*, 2010).

Genus *Meloidogyne* has been interest to nematologist due to their widespread distribution and parasites of economically important crops and therefore considered as one of the most important genera of plant parasitic nematode (Dong *et al.*, 2001; Trudgill and Blok, 2001; McK Bird and Kaloshian, 2003; Adam *et al.*, 2007; Kamran *et al.*, 2010). The importance of root-knot nematode can also be reflected through large volume of literature with several books published on the *Meloidogyne* genus (Taylor and Sasser, 1978; Lamberti and Taylor, 1979; Sasser and Kirby,1979; Barker *et al.*, 1985; Sasser and Carter, 1985; Karssen, 2002).

Meloidogyne spp. considered to be the most economically important often lowered both the quantity and quality crop yield (Adam *et al.*, 2007; Bent *et al.*, 2008). Their infection depends on the climate. Most species of this genus occur in tropical region (Tesarová *et al.*, 2003; Adam *et al.*, 2007). Frequency of occurrence

of *Meloidogyne* species worldwide was found to be in following order: *M. incognita* 52%, *M. javanica* 31%, *M. hapla* 8%, *M. arenaria* 7% and others 2% (Sasser, 1982). Therefore, *M. incognita, M. javanica, M. hapla* and *M. arenaria* considered to be major *Meloidogyne* species and economically important while other species usually locally important. Sassers (1982), state that, no agricultural program of nematode control or integrated pest control could be successful if these four major species were not in consideration. Usually *M. javanica* had a higher tolerance towards high temperature compared with *M. incognita* (Taylor *et al*., 1982). Some of them (*M. hapla, M. chitwoodi* and *M. fallax*) occur in cooler region (Taylor *et al*., 1982; Tesarová *et al*., 2003; Adam *et al*., 2007). *M. incognita* reported to be found in Central Europe living in glasshouses only (Tesarová *et al*., 2003).

Tropical root-knot nematodes have short life cycle depending on temperature which range from 25-56 days. There may be 1 to 5 generations per year. Therefore, the population can rise to very damaging level. The eggs also can survive in dry soil and it makes them very dangerous parasites of plants (Tesarová *et al*., 2003; Luc *et al*., 2005). Report from fields' survey in Florida showed *M. javanica* was found in 65% of fields' survey area, *M. incognita* (33%) and *M. arenaria* was rarely found (Rich and Garcia, 1985).Field survey in Philippines showed that there were 64% of *M. incognita* and 29% of *M. javanica* (Madamba, 1981).

Meloidogyne spp. has been proved to increase the incident of other disease. For example in Fusarium wilt disease (Chen *et al*., 1994,). Not only Fusarium wilt disease, they also increase damage cause by *Verticillium, Rhizoctonia, Pythium* and

Phytophthora (Xue, 1991; Chen *et al*., 1994). In addition, root-knot nematodes also increase disease severity inflicted by bacteria such as *Corynebacterium* on tobacco, on tobacco and tomato, and *Agrobacterium* on raspberry (Taylor, 1979; Xue, 1991). Because of their importance, there is ongoing research on *Meloidogyne* genus worldwide and record of new species and host (Sirias, 2011).

2.2.4 Effect of *Meloidogyne* spp. on tobacco

Root-knot nematode (*Meloidogyne* spp.*)* caused formation of galls on tobacco root. Infections of J2 lead to formation of 'giant cells'. 'Giant cells' serve as sourced of nutrients for their development (Jones, 1980). These giant cells caused extensive distortion of tobacco root and blocked the vascular tissue which slowed water and nutrient transport. Thus, the absorption of nutrient and water greatly reduced. Therefore, plant growth and yield may be suppressed (Quénéhervé *et al*., 2011). Above ground symptoms showed temporary wilting (premature wilting) when water stress occurred usually during drought or sunny day and chlorosis of foliage. Tobacco plant becomes stunted and the leaves were yellow and thin. The root gall was observed because the root tissues around nematode and giant cells undergo hyperplasia and hypertrophy. Despite that, secondary larval invasion may occur and caused the gall to coalesce and finally the root begins to decay (Luc *et al*., 2005). *Meloidogyne* spp. also had the ability to form disease complex with other plant pathogens. The giant cell was very suiTable for development of *Fusarium wilt* (Porter and Powell, 1967). *Meloidogyne* spp. infection increase severity not only by fungi it also by bacteria for example *Pseudomonas* and *Corynebacterium* on tobacco (Taylor, 1979; Xue, 1991). Besides that, there is also interaction between *Meloidogyne* species to the other genus of plant parasitic nematodes such as

Heterodera spp., *Rotylenchus* spp., *Pratylenchus* spp. There is also evidence shows interaction among species in *Meloidogyne* genus (Hirunsalee *et al*., 1995; Perez and Fernandez, 1998; Opoku-Asiama and Yeboah, 2003). However, the interactions between two sedentary endoparasites are generally suppressive due to competition for available feeding sites (Vivian and Thomas, 2009).

2.2.5 Identification of *Meloidogyne* spp.

Precise identification of root-knot nematode species is becoming very important for the effective nematode management practices such as crop rotation and development of plant resistant and also very important for quarantine purposes (Zijlistra, 2000; Zijlistra and Van Hoof, 2006; Brito *et al*., 2010). The need for reliable root-knot nematode species identification has also been crucial due to the reduced availability of broad spectrum chemical nematicides and increased reliance on the usage of non-chemical method for the sustainable nematode management strategies. However, identification and differentiation of specific and sub specific of root-knot nematode is far from simple. Traditionally, identification of *Meloidogyne* spp. was based mainly on female perineal pattern morphology and other phenotypic traits of J2 , female and male such as stylet structure and body length. The usage of preferable host range test also used to determine the species and race of *Meloidogyne* spp. (Chitwood, 1949; Xue, 1991). However, all these method have certain limitations and the key of the species descriptions often vary between species and among race. According to Whitehead (1968), the female perineal pattern of *Meloidogyne* spp. varies in natural populations and even within single egg-mass lines created ambiguities. There was also varied expertise of the persons describing perineal patterns which limit the accuracy of species identification (Karssen,

2002).The sizes of the root-knot nematode are affected by feeding on different host plants. For example, *Meloidogyne* sp. that feeds on the roots of resistant plants or non preferable host plant was usually smaller than those that feed on susceptible plants and had significant difference in growth (Power *et al*., 1991). Other than that, male of *Meloidogyne* spp. are hard to find while J2 are smaller and difficult to prepare for microscopic examination (Eisenback, 1985). Therefore, species identification based on morphology required a lot of skills (Hooper *et al.,* 2005). Furthermore, when using morphology characteristic, mixed population are not easily detected as almost similar in morphology and large numbers of specimens need to be examined for reliable identifications thus time consuming (Xue, 1991). The host range test also has been used to differentiate the race of *Meloidogyne* spp., but the diagnosis is time consuming, and sometimes can be uncertain and unreliable.

Host range test also known as North Carolina differential host test widely used when a number of nematologist and plant breeders notes inconsistencies in host response for the same species. This led to the discovery of host races diffrentiation (Hartman and Sasser, 1985). This test involves the inoculation of six standard host plants which is cotton 'Deltapine 61', tobacco 'NC 95', pepper 'California wonder', watermelon 'Charleston Gray', peanut 'Florunner', and tomato 'Rutgers'. This method is able to distinguish the four commonly occurring *Meloidogyne* spp. (*M. incognita, M. javanica, M. arenaria,* and *M. hapla*) and races for *M. incognita* and *M. arenaria* based on the host susceptibility or resistance (Taylor and Sasser, 1978). However, when many populations of the same species were studied, their resistance or susceptibility to a given host cannot be predicted and certain populations were not able to be determined (Netsher, 1983; Xue, 1991). Host resistance may affected by

environmental conditions such as temperature (Omwega *et al*., 1990; Xue, 1991). This test is time consuming and is not sufficient to determine mixed populations or rare populations (Sasser and Carter, 1985).

The limitation of traditional method led to search for other method to identify *Meloidogyne* species. Reproductive and cytological characters of root-knot nematodes were studied for species identification and have been used to differentiate the races based on the mode of reproduction and chromosome numbers (Triantaphyllou, 1985). Besides that, the use of carbohydrates, lipids, protein and enzyme composition pattern visualized through gel electrophoresis also been used for species identification (Hussey, 1985; Esbenhade and Triantaphyllou, 1990; Xue, 1991). Research by Dickson *et al.* (1971) proved to be able differentiates *M. hapla, M. incognita* and *M. arenaria* by using soluble proteins and profile of eight enzymes. Further studied by Esbenhade and Triantaphyllou (1985) which used sixteen *Meloidogyne* species had obtained species specific phenotypic pattern for non-specific esterase, malate dehydrogenase, superoxide dismutase and glutamate oxaloacete transaminase for *M. javanica, M. incognita,* and *M. hapla*. However, out of the four, esterase have been the most useful to separate the major *Meloidogyne* species (Cofcewicz *et al.,* 2004). However, biochemical approaches also have some limitations and drawback (Xue, 1991). The cellular expression of protein, lipids and others are often change by the influence of ontogenic and environmental factors on root-knot nematodes. Besides, it is also proved that the result may not be present consistently as they may not be present uniformly in all individuals or populations. The expression of esterase phenotype depends on specific physiological stages of the root-knot nematode (Dalmasso and Berge, 1983; Xue, 1991). For example, isozyme

analysis (usage of enzyme) is only performed with single females. Since the female stage is unavailable in soil samples it is very hard to diagnose the availability of root-knot nematode in soil (Powers and Harris, 1993; Perez and Fernandez, 1998; Adam *et al*., 2007). Biochemical method also may not be easily differentiating closely related species as biochemical component are products of genetic expression and they tend to be highly conserved in function and structure. Besides, only a small fraction of the potentially useful variation available due to accumulated macromolecules such as protein and surface antigens represent end products derived from small fraction of the cell's genetic material (Devran and Söğüt, 2009). However combination of morphology method and biochemical method increase the accuracy of species identification (Hirschman, 1986). This study was very useful on host response and parasitism mechanism (Abad *et al.,* 2003).

Diagnostic technique for identification should not be limited to the availability of certain development stage. Therefore molecular approach was used for species identification. Molecular approach was probably started when Curran *et al.* (1985) analysed fragments of genomic DNA with restriction enzyme to separate *M. arenaria* from *M. javanica* and other non-plant parasitic nematodes. This method also has a drawback as it was time consuming, not sensitive and required a substantial amount of DNA (Abrantes *et al*., 2004; Adam *et al*., 2007). Advance in DNA technology such as polymerase chain reaction (PCR), restriction enzyme analysis and nucleotide sequencing allow direct exploitation of DNA sequence polymorphism. Unlike previous methods (protein and enzyme analysis), DNA characterization do not rely on the expressed product of the genome therefore it does not influenced by environmental condition and applicable to all life stages (Hooper *et*

al., 2005). First report for root-knot nematode identification using PCR was when Harris *et al*. (1990) successfully amplified mitochondrial DNA from a crushed single J2 in a drop of sterile water. PCR allows amplification of small quantities of DNA, rapid and reliable (Harris *et al*., 1990; Oh *et al*., 2009).

DNA based method used mitochondrial DNA (Harris *et al.,* 1990; Powers and Harris, 1993), ribosomal DNA (Ziljstra *et al*., 1995; Petersen and Vrain, 1996; Petersen *et al*., 1997; Ziljstra, 1997) and randomly amplified polymorphism DNA fragments (RAPDs). Different methods based on PCR such as Random amplified polymorphic DNA (RAPD), Restriction fragment length polymorphism (RFLP), Multiplex PCR, Amplified fragment length polymorphism (AFLP) and Sequence characterised amplified region (SCAR) have been developed and proved to be able identifying a large number of *Meloidogyne* species (Oh *et al*., 2009). PCR method can be used to amplify specific regions of *Meloidogyne* species genome offers a highly method to detect inter and intra specific variation. A pair of specific oligonucleotide (forward and reverse primer) used to amplify the target gene. Identification and characterization of *Meloidogyne* species by observing variation in size or nucleotide sequence of the amplified PCR product. Power and Harris (1993) amplified mitochondrial DNA genes to differentiate *Meloidogyne* species. However variation in nucleotide sequence of different species can only be further detected by using restriction enzyme digestion of the PCR product (PCR-RFLP) or sequencing. Other genomic regions also have been proven to identify *Meloidogyne* species. The ribosomal DNA repeats unit (rDNA) consisting internal transcribed spacer (ITS 1 and ITS 2) which located between the repeating array of nuclear 18S and 28S ribosomal RNA genes and separated by the 5S ribosomal RNA gene used to