

The Interactions of Root-Knot Nematodes and Coffee

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Abstract

Coffee is a major crop that supports the economy of over 60 countries, many of which are developing nations. A major detriment to coffee production is damage caused by plant parasitic nematodes. The most damaging species is the root-knot nematode, *Meloidogyne* spp. Many conventional methods to control plant parasitic nematodes can be inefficient and costly. This study aimed to characterise the molecular mechanisms that drive interactions of plant parasitic nematodes and Robusta coffee (*Coffea canephora*), including immune and defence responses of coffee to root-knot nematodes, and behavioural response of the nematode to root exudate of coffee, with a view to the longer-term development of improved nematode control strategies and technologies.

The Robusta coffee varieties tested showed a differential physiological response to the infection of root-knot nematodes, as well as showing subtle differences in susceptibility to attack by nematodes. Contrasting varieties in susceptibility and tolerance to nematode attack were then used to compare the transcriptomic changes induced in root and leaf tissue following infection by root-knot nematodes. Genes involved in pathogen recognition, general defence and hypersensitive responses were revealed as key mediators of the Robusta immune response to plant parasitic nematodes. Cell-wall-regulation in coffee was identified as a mechanism that could provide protection against root knot nematode infection. Genes and gene pathways that have been identified could be utilised to develop coffee varieties that have improved protection against plant parasitic nematodes. They may also be used as molecular identifiers of innate tolerance against plant parasitic nematodes.

An alternative to identifying and manipulating genetic components of coffee for nematode control would be to disrupt parasitic behaviours of root-knot nematodes to inhibit invasion and reproduction within the crop. Serotonin was immunolocalised within infective stages of root-knot nematodes and established as a key neurotransmitter mediating behaviours essential for root-knot nematode pathogenicity, including chemosensation and stylet function for invasion and feeding. Disrupting serotonin biosynthesis, using established serotonergic chemical inhibitors, also decreased the infective capability of root-knot nematodes. Serotonergic molecular components are suggested that could be targets for root-knot nematode control. Integrating novel genomic controls discussed in this project would provide crop protection against plant parasitic nematodes in coffee and reduce yield losses caused by the pathogen.

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Chapter 1

Introduction

1.1 Coffee

Coffee (*Coffea* sp.) was first cultivated in the fifth to eight centuries, with origins in the Ethiopian highlands. The beverage was introduced into and widely consumed in Egypt and Yemen where alcoholic drinks were not permitted. It was then introduced into Europe, through Venice, and its consumption within coffee houses in the 17th century over discussions of business, politics and current affairs eventually evolved into a social pastime at cafés or at home in the western world (Colby, 1920). The uptake of the beverage into society and culture is also a result of its most notable characteristic of being a stimulant. Caffeine is the main pharmacologically active compound that has a number of effects on the central nervous system to reduce the perception and effects of tiredness, as well as causing other physiological changes such as stimulating cardiac muscle, relaxing smooth muscle and diuresis (George *et al.*, 2008; Buar *et al.*, 2021).

The worldwide consumption of coffee today is hugely popular, with over 2.25 billion cups consumed every day, or 7 million tonnes per year (Ponte, 2002). The cultivation of coffee also supports both regional and national economies of over 60 tropical and subtropical countries, 90% of which are described as developing countries (Ponte, 2002). Brazil is the highest producer of coffee amounting to over 52 million 60-kilogram bags (Figure 1. 1; Ico.org, 2018).

1.1.1 The coffee crop

The genus *Coffea* belongs to the family Rubiaceae, and the two species of most economic and breeding relevance are *C. arabica* (Arabica) and *C. canephora* (Robusta). Arabica is a natural tetraploid hybrid ($2n = 44$) and autogamous, while all other species are diploids ($2n = 22$). As of 2017, Arabica is reported to make up approximately 60% of total coffee production worldwide with Robusta constituting the balance. However, interest in Robusta is growing due to issues of both climate change and pathogens. Robusta is both easier and less expensive to grow in warmer climates as well as being more resilient to pests and diseases. This has led to the demand for Robusta increasing as reported by The International Coffee Organization (International Trade Centre, 2021).

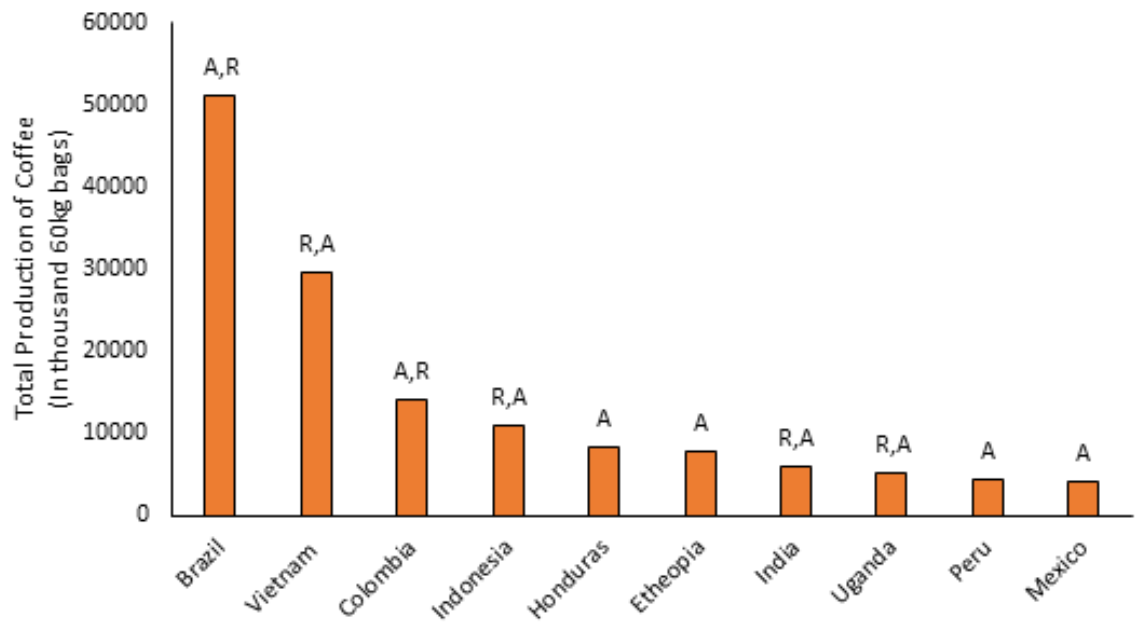


Figure 1. 1. Total production of coffee produced, in thousand 60 kg bags, in the top 10 coffee producing countries.

Data labels A and R represent the type of coffee produced, *Coffea arabica* (Arabica) or *Coffea canephora* (Robusta) respectively, with the main species produced labelled first. Data acquired from International Coffee Organisation (Ico.org, 2018).

Coffee can grow as a perennial shrub or tree depending on the species, with an orthotropic branch growing vertical from the trunk and plagiotropic branches growing horizontally where blooming and production occur (Vieira, 2008). Seedlings of the autogamous Arabica are often produced through seed but for Robusta vegetative cloning from orthotropic branches is recommended to reduce variability in the plant stand. Blooming can occur from two or three times a year up to fifteen times a year depending on latitude and rainfall pattern, occurring on plagiotropic branches grown in the previous year for Arabica and on branches grown in the current year for Robusta. The size of the fruit, or berry, depends on the *Coffea* species, and each fruit will usually produce two endosperm 'beans', varying in size depending on the variety. It is these berries which are harvested from the crop and are processed to remove and clean the beans as crop yield.

The harvesting of coffee berries can require 50% of man-hours of total cultivation and represents 25-35% of the production cost (Vieira, 2008). The harvesting season depends on both the regional climate and the variety grown. Harvesting in Brazil, for example, takes place in the dry season from June through September, but in some countries harvesting can be continuous as the plants bloom several times a year. Berries should only be harvested when fully ripe, with no more than 20% unripe berries collected for a better market price and top-quality beverage. Berries then undergo either dry or wet processing. In dry processing, the berry is washed, and any damaged berries are removed before immediately being sun dried on terraces, turning several times a day. In wet processing, berries are washed and then the outer layer is removed by fermentation, before being sun dried. This means only the bean is dried rather than the whole berry. The beans are then sold by growers to industry where further processing involves hulling, polishing, cleaning, sorting and grading, roasting and grinding.

The Arabica genome was first characterised by Lashermes *et al* (1999) using restriction fragment length polymorphism markers and genomic *in situ* hybridisation. Results indicated that the amphidiploid was formed by a hybridisation between *C. eugenoides* and *C. canephora* and revealed a low divergence between *C. arabica* genomes and its progenitors, suggesting a recent speciation. The genome was sequenced and assembled in 2017, consisting of 1.19 giga base pairs and 70,830 gene models, and is available through Phytozome (Phytozome.gov, 2018).

The genome sequence of Robusta was completed and analysed in 2014 by several teams, in particular the International Coffee Sequencing Consortium (Denoeud *et al.*, 2014). The genome showed high chromosomal gene order conservation compared to other asterid angiosperms, and no signs of whole genome triplications, which can be seen in other families of flowering plants such as *Solanaceae*. However, the genome did include several species-specific gene family

expansions including *N*-methyltransferases involved in caffeine production, alkaloid and flavonoid enzymes involved in secondary compound synthesis, and defence-related genes.

1.1.2 Challenges to coffee cultivation

The pathogens/pests of coffee most studied, reported and considered to be most important are coffee leaf rust, coffee berry borers and plant parasitic nematodes.

Coffee leaf rust is caused by the fungus *Hemileia vastatrix*. Its yellow-orange powdery mycelium covers leaves and can cause them to fall off the plant, reducing photosynthetic capability (Figure 1. 2). Coffee serves as an obligate host to this fungus, and epidemics of this disease and its severity are well reported. During the 1990s, it was thought that management of coffee leaf rust had reduced the issue to a small inconvenience, however, from 2008 a series of outbreaks commonly referred to as “the Big Rust” started to emerge across the Americas. The most recent outbreak was the epidemic of Central America in 2012, reducing the region’s output by 16% (Avelino, 2015; McCook, 2015). Since then, management of the fungus has improved, and it again serves only as an inconvenience in comparison to other issues, but lessons of vigilance should be taken from its past epidemiology (International Trade Centre, 2021).

The coffee berry borer, *Hypothenemus hampei*, is the most serious insect pest of coffee in all coffee-producing regions except China and Nepal (Figure 1. 1; Jaramillo *et al.*, 2011). Rather than affecting leaves, the small beetle damages the berry, directly reducing the yield and quality of the final crop product and increasing the berries’ vulnerability to other pathogens (Damon, 2000). Some global estimates for damage to yield caused by the pest are around 10%, or up to \$100-500 million annually (Johnson, 2020). However, Johnson (2020) argues, highlighting successful research conducted in Hawaii, that efficient and adequate management of the coffee berry borer can be achieved by improving farm sanitation practices (with better education and tools) along with threshold-based applications of biopesticides and biological control.

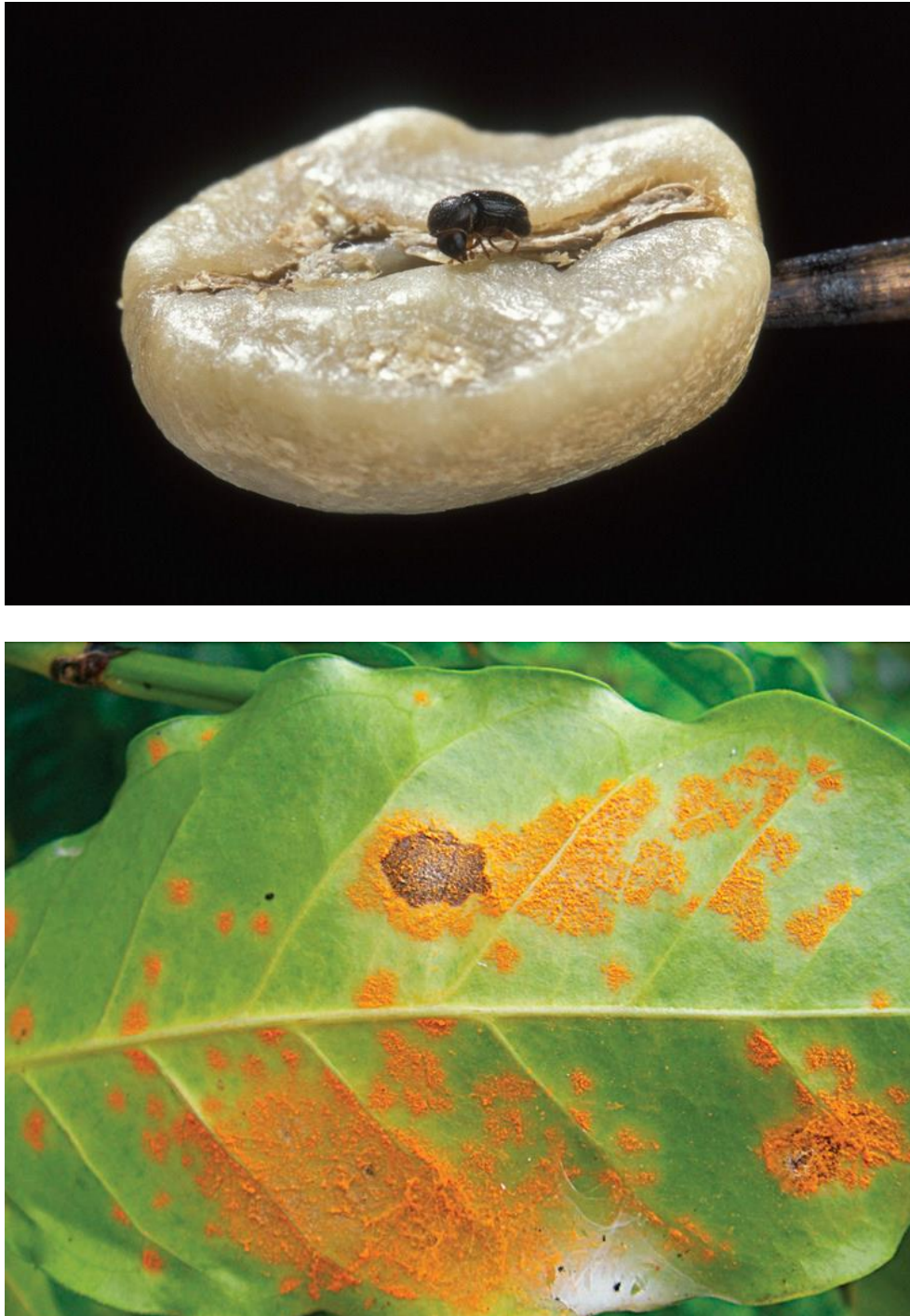


Figure 1. 2. Common pests and diseases of coffee.

Top: An adult female Coffee berry borer, *Hypothenemus hampei*, on an Arabica coffee, *Coffea arabica* berry. (©Peggy Greb, USDA Agricultural Research Service/via Bugwood.org - CC BY 3.0 US, <https://www.cabi.org/isc/datasheet/51521#toPictures>). Bottom: Coffee leaf rust found upon the bottom side of an Arabica coffee leaf (©Carvalho *et al* CC-BY <https://blog.plantwise.org/2022/03/17/coffee-leaf-rust-spotting-and-managing-hemileia-vastatrix/>)

The effect of climate change on coffee cultivation may exacerbate damage caused by pests and pathogens. Due to the sensitive nature of coffee, predicted increases in temperature and reductions in rainfall will reduce the amount of suitable land available to grow coffee for the efficient production of berries. Some reports suggest reduction of up to 70% of land available for Arabica production, and 60% for Robusta by 2050 (Läderach *et al.*, 2017; Sachs *et al.*, 2019). Furthermore, the changes in climate predicted will also favour many pathogens. Increased temperatures, for example, favour the lifestyle of the coffee berry borer, and if coffee cultivation is forced to higher elevations for optimal conditions, the coffee berry borer undergoes more generations in a shorter time, leading to higher pathogenic load during coffee growing periods (Jaramillo *et al.*, 2011; Ziska *et al.*, 2018). Climatic changes are also predicted to enable more pathways for pathogen spread globally, increasing the risk of plant pathogens entering new coffee producing regions (Ziska *et al.*, 2018; Castillo *et al.*, 2020).

As production of coffee is inevitably impeded by issues caused by climate change, directly or indirectly, it is imperative to the security of coffee production that any further potential yield losses are controlled. It is therefore important that another major detriment to coffee production, the infection from plant parasitic nematodes, is acknowledged and studied so that effective actions can be taken to control the pathogen and reduce further losses in yield.

Disease symptoms of coffee-parasitic nematodes were first described in 1878 by French naturalist Clément Jobert, but the causal agent was only extensively reported later in 1887 as the parasitic nematode *Meloidogyne exigua* by the Swiss naturalist Emil A. Göldi (Souza, 2008). Today, the problem of nematodes that parasitise and damage coffee is well known. Species of plant-parasitic nematodes have been found in all coffee growing regions, and the impacts on coffee production caused by parasitism can lead to major losses in yield. Severe infestations can even lead to the eradication of whole plantations, as was reported in Brazil in the 1970s and Vietnam in 2005 (Ferraz, 2008; Wiryadiputra & Tran, 2008). While the impact of plant parasitic nematodes on coffee is widely acknowledged, the biology of the interaction between pathogen and host is less established. Understanding the molecular mechanism of this interaction is the first step in understanding how the pest can be managed to prevent further losses in yield.

1.2 Nematoda and plant parasitic nematodes

The most common Metazoa on earth; nematodes occur in almost every habitat and can be free-living or parasites of plants and animals (Yeates, 1987; Neher, 2001). The most well studied nematode is the free-living model organism *Caenorhabditis elegans*. This small nematode, with newly hatched larvae being around 0.25 millimeters long and adults around 1 millimeter long, has a rapid lifecycle (3 days from egg to egg-laying adult), and exists primarily as a self-fertilizing hermaphrodite. These characteristics make for an ideal model for eukaryotic genetic studies, as proposed by Sydney Brenner in the 1960s (Corsi *et al.*, 2015). Research has since built-up extensive knowledge and resources on the model, including a complete cell lineage for the fate of every cell between fertilization and adulthood, and the complete reconstruction, or “wiring”, of the *C. elegans* nervous system including 302 neurons (Sulston *et al.* 1983; White *et al.* 1986; Jarrell *et al.* 2012). Due to *C. elegans* also being the first multicellular organism to have a complete genome sequenced, forward and reverse genetics have led to the molecular identification of many key genes in developmental and cell biological processes (Corsi *et al.*, 2015).

Seven orders of the phylum Nematoda contain nematodes that are parasites of invertebrates and six are parasites of vertebrate animals (Reviewed in Cross & Lindquist, 2007). In some cases, invertebrates can also function as either an intermediate host or a vector in a life cycle that includes the parasitism of a vertebrate. The nematodes that parasitise vertebrates, often characterized in a larger group of worm parasites as helminths, are a burden on the health of both domestic animals and humans. These parasitic nematodes can be found within intestines, tissue or blood, and the highest prevalence occurs in tropical countries with poor and inadequate food supplies, abundance of invertebrate vectors and unsanitary conditions (Cross & Lindquist, 2007). The widespread occurrence and disease complexes caused by animal and human parasites lead to a large economic burden through medical costs and an effect on food production (Hailu *et al.*, 2007; World Health Organisation, 2022).

Over 4000 species of nematodes parasitise plants (around 15% of total nematode species known) and they can be broadly divided into three groups according to their feeding strategy (Perry and Moens, 2013). Migratory endoparasites enter the host roots and cause extensive damage to root tissues, while sedentary endoparasites induce complex feeding structures within the roots of hosts to establish a long-lasting feeding source. Finally, ectoparasites never enter the host, instead migrate through soil and either use roots as a transient food source or feed upon an external site for prolonged periods.

Plant parasitic nematodes are a significant detriment to agriculture and food security globally. It is estimated that 10% of global vegetable production is affected by nematode infection, and damage to crops annually is estimated at around 80 billion US dollars, although these are likely to be underestimates as the presence of nematodes in soil can be difficult to identify and symptoms of infection are often non-specific (Jones *et al.*, 2013). Moreover, the damage to crops due to nematode infection can be worsened by secondary infection and disease complexes: nematodes can transmit plant viruses and bacteria (Ruark *et al.*, 2018), fungi and bacteria can invade tissue injured by nematodes (Hawn, 1963; Back *et al.*, 2002), and nematode infections have been reported to breakdown host resistance to fungi such as *Fusarium oxysporum* (Lobna *et al.*, 2016). In general, nematodes reduce the yield of coffee production by approximately 15%, but this figure can be worse in specific cases due to difficulties in assessing nematode infestations (Campos *et al.*, 1990).

1.2.1 Physiology, behaviours, and neural control of plant parasitic nematodes

Most free-living and plant parasitic nematodes are around 1 mm in length, but with a few exceptions of species which can greatly exceed this, such as *Paralongidorus epimikis* which can be as long as 12 mm. All migratory ecto- and endoparasites are vermiform throughout their lifecycle, whereas sedentary endoparasites can become fusiform and mature females become saccate. Females have a secretory-excretory pore, a vulva and an anus located ventrally, with males possessing a cloacal opening instead, and both have a mouth opening at the anterior end (Decraemer and Hunt, 2013). Another characteristic feature of plant parasitic nematodes is the specialised pharyngeal glands, consisting of two subventral and one dorsal secretory cell, which produce proteins that are secreted through the protrusible hollow mouth spear that is the stylet. Like a hypodermic needle, the stylet is used to puncture plant cells to allow the nematode to invade into and migrate through the host as well as to withdraw food (Lambert & Bekal, 2009). The stylet is connected to the pharynx that, in turn, is connected to the intestine. The intestine ends at the rectum in the female nematode and the cloaca in the male.

The behaviours of plant parasitic nematodes in host-finding and feeding are controlled by complex chemosensory systems. The understanding of these systems has been largely informed by the neurobiology of *C. elegans*. Acetylcholine is the major excitatory neurotransmitter, released by more than a third of cells in the *C. elegans* nervous system, and is involved directly or indirectly in many behaviours such as egg laying, locomotion, feeding and male mating (Rand, 2007). Four biogenic amines: octopamine, tyramine, dopamine and serotonin also modulate behaviour in *C. elegans* in response to environmental stimuli by acting at both neurones and muscles (Chase & Koelle, 2007). In contrast to vertebrates, GABA acts primarily at neuromuscular synapses, rather than at synapses in the central nervous system, to relax body

muscles during locomotion and foraging, and contract muscles during defecation (Jorgensen, 2007). Avoidance behaviours has also been demonstrated to be mediated by excitatory synaptic signalling is mediated by glutamatergic neurotransmission, specifically by the function of ionotropic glutamate receptors (Brockie & Maricq, 2006). Many behaviours will also be controlled by the function of neuropeptides, short sequences of amino acids that directly or indirectly to modulate synaptic activity, which either fall into the families of insulin-like peptides, FMRFamide (Phe-Met-Arg-Phe-NH₂)-related peptides or neuropeptide-like proteins (Li & Kim, 2008). With over 100 neuropeptide genes shown to be expressed in *C. elegans*, detailing the complexity of the role neuropeptides in behaviours is a daunting yet important task within nematology.

In plant parasitic nematodes specifically, much research on neurobehaviour has focused on chemosensation, as the driver for host-location and feeding crucial for the parasitic lifestyle. The biogenic amines have received the most interest in studies on plant parasitic nematode behaviour. Octopamine and serotonin are regularly exploited within experiments for their effect on stylet thrusting in plant parasitic nematodes, as the exposure likely directly stimulates pharyngeal muscles or indirectly through motor neurones, to cause the rapid pumping of the stylet, also known as stylet thrusting (Urwin *et al.*, 2002; Holden-Dye & Walker, 2011). Serotonin has also been linked to host-finding ability of plant parasitic nematodes through chemoreception and chemotaxis (Fleming *et al.*, 2017). Infective stage nematodes orient to hosts upon the recognition of phytochemicals released in root exudates by the plant, and move toward the host following the chemical gradient of phytochemical (Curtis, 2008). Due to the role of serotonin in food-seeking and learning behaviours of *C. elegans*, Fleming *et al* (2017) suggests this biogenic amine to have a large role in the mediation of chemoreception in plant parasitic nematodes, and such should be a topic of major importance within plant parasitic nematode research.

1.2.2 Root-knot nematodes

Species of the *Meloidogyne* genus are commonly referred to as root-knot nematodes due to their induction of galls, or root-knots, as they feed on and modify living plant cells. They are sedentary endoparasites that can infect nearly every species of vascular plant and are distributed worldwide (Jones *et al.*, 2013). Giant cells formed by root-knot nematodes are nutrient sinks for the plant as they mobilise photosynthetic products from shoots to roots (Hofmann and Grundler, 2007). Root tissues around giant cells and nematodes undergo hyperplasia and hypertrophy, which causes the characteristic galling, usually developing in one or two days after infective stage juveniles have penetrated the root but are not necessarily essential for nematode development. Damage to plants caused by root-knot nematodes can

consist of stunting, lack of vigour and wilting under water stress, although above ground symptoms are not readily apparent. The severity of symptoms will depend on the plant species and cultivar, though other factors include initial population density, crop rotations, field period and season. Although found globally, the major economic impact of root-knot nematodes is within tropical areas, due to the increased distribution of major disease causing species such as *Meloidogyne incognita*, *M. javanica* and *M. arenaria* (Ralmi *et al.*, 2016) *Meloidogyne graminicola* is also a major species that has adapted well to flooded conditions, affects both upland and lowland rice and causes up to 87% yield losses (Soriano *et al.*, 2000; Padgam *et al.*, 2004). Though Europe, several species of *Meloidogyne* have become increasingly important following the reduction of chemical nematicides (Wesemael *et al.*, 2010). Examples of economic damage caused by root-knot nematodes in Europe include *M. chitwoodi*, *M. fallax* and *M. javanica* causing severe galling on potato tubers, *M. hapla* causing damage to commercial carrots and *M. minor* being responsible for yellow patch disease that occurs in golf courses (Reviewed in Wesemael *et al.*, 2010).

There are 17 species of *Meloidogyne* that parasitise coffee and have been reported globally (Table 1. 1). Identification of root-knot nematode species has traditionally been based upon perineal pattern which can be difficult and uncertain for some populations, therefore it should be used as a complementary tool to enzyme phenotyping or polymerase chain reaction (PCR)-based assays. A high throughput PCR assay was developed for the detection and quantification of major *Pratylenchus* and *Meloidogyne* species capable of parasitising coffee in Brazil, Vietnam and Indonesia (Bell *et al.*, 2018). More recently, Sellers *et al* (2021) developed a low coverage, long-read genome sequencing technique, using Oxford Nanopore technology, to identify *Meloidogyne* nematodes to a species level using only a singular individual.

1.2.2.1 Life cycle and reproduction

The full lifecycle of root-knot nematodes is shown in Figure 1. 3. Eggs are present as masses containing up to 1000 eggs within a gelatinous matrix on root surfaces of the host plant as well as in the surrounding soil and are the main survival stage of the nematode. The first stage juvenile (J1) moults within the egg before the infective second stage juvenile (J2) hatches and is attracted to host roots (Karssen and Moens, 2013). The hatching of J2s from eggs is primarily dependant on temperature and sufficient moisture, but whilst not a requirement, root exudate can influence and encourage hatching (Curtis *et al.*, 2009). The J2 enters the root, generally directly behind the root cap, using a combination of physical damage through thrusting of the stylet and the breakdown of cell wall by enzymes. The J2 will then migrate intercellularly, through the cortex, to the apex of the root where it turns back and up into developing vascular tissue. This migration behaviour allows the root-knot nematode to avoid crossing the

endodermis. The J2 will then initiate a feeding site by injecting pharyngeal gland secretions into roots cells, causing them to enlarge and differentiate into giant cells, called so as they can become up to 100 times larger to provide a rich source of nutrient for the J2. The nematodes develop into third (J3) and fourth (J4) stage juveniles before maturing into the adult stage.

Table 1. 1. Coffee-parasitic *Meloidogyne* species.

Includes current information on mode of reproduction, chromosomal number, and the geographic distribution for where each species has been reported to parasitise coffee (Carneiro and Cofcewicz, 2008; Bell *et al.*, 2018; Stefanelo *et al.*, 2019).

Species	Reproduction and chromosome number	Geographic Distribution (Reported on Coffee)
<i>M. africana</i>	Unknown	Kenya, Zaire
<i>M. arabicida</i>	Unknown	Costa Rica
<i>M. arenaria</i> (syn. <i>M. thamesi</i>)	Mitotic parthenogenesis (36, 45 or 51-56)	Jamaica, Cuba, El Salvador
<i>M. coffeicola</i>	Unknown	Brazil
<i>M. exigua</i>	Meiotic parthenogenesis (n=18)	Brazil, Guatemala, Dominican Republic, Nicaragua, Costa Rica, Puerto Rico, Colombia, Peru, El Salvador, Venezuela, Bolivia, Honduras, Panama
<i>M. decalineata</i>	Unknown	Tanzania, São Tome and Principe
<i>M. hapla</i>	Mitotic (n=13-17) and meiotic parthenogenesis (2n=30-31, 3n=43-48)	Brazil, Tanzania, Zaire, India, Kenya, Congo, Guatemala, El Salvador, Vietnam
<i>M. incognita</i>	Mitotic parthenogenesis	Brazil, Tanzania, Jamaica, Venezuela, Guatemala, the Ivory Coast, India, Costa Rica, El Salvador, Nicaragua, Cuba, U.S.A, Vietnam
<i>M. inornata</i>	Mitotic parthenogenesis (3n=54-58)	Guatemala
<i>M. izalcoensis</i>	Mitotic parthenogenesis (2n=44-48)	Brazil, El Savador
<i>M. javanica</i>	Mitotic parthenogenesis (2n=41-48)	Brazil, Tanzania, Zaire, El Salvador, India, Cuba, São Tome and Principe
<i>M. kikueynsis</i>	Amphymitic (n=7)	Kenya
<i>M. konaensis</i>	Mitotic parthenogenesis (2n=44)	U.S.A
<i>M. mayaguensis</i>	Mitotic parthenogenesis (2n=44-45)	Cuba, Costa Rica, Guatemala
<i>M. megadora</i>	Unknown	Angola, Uganda, São Tome and Principe
<i>M. oteifa</i>	Unknown	Zaire
<i>M. paranaensis</i>	Mitotic parthenogenesis (2n=50-56)	Brazil, Guatemala, U.S.A

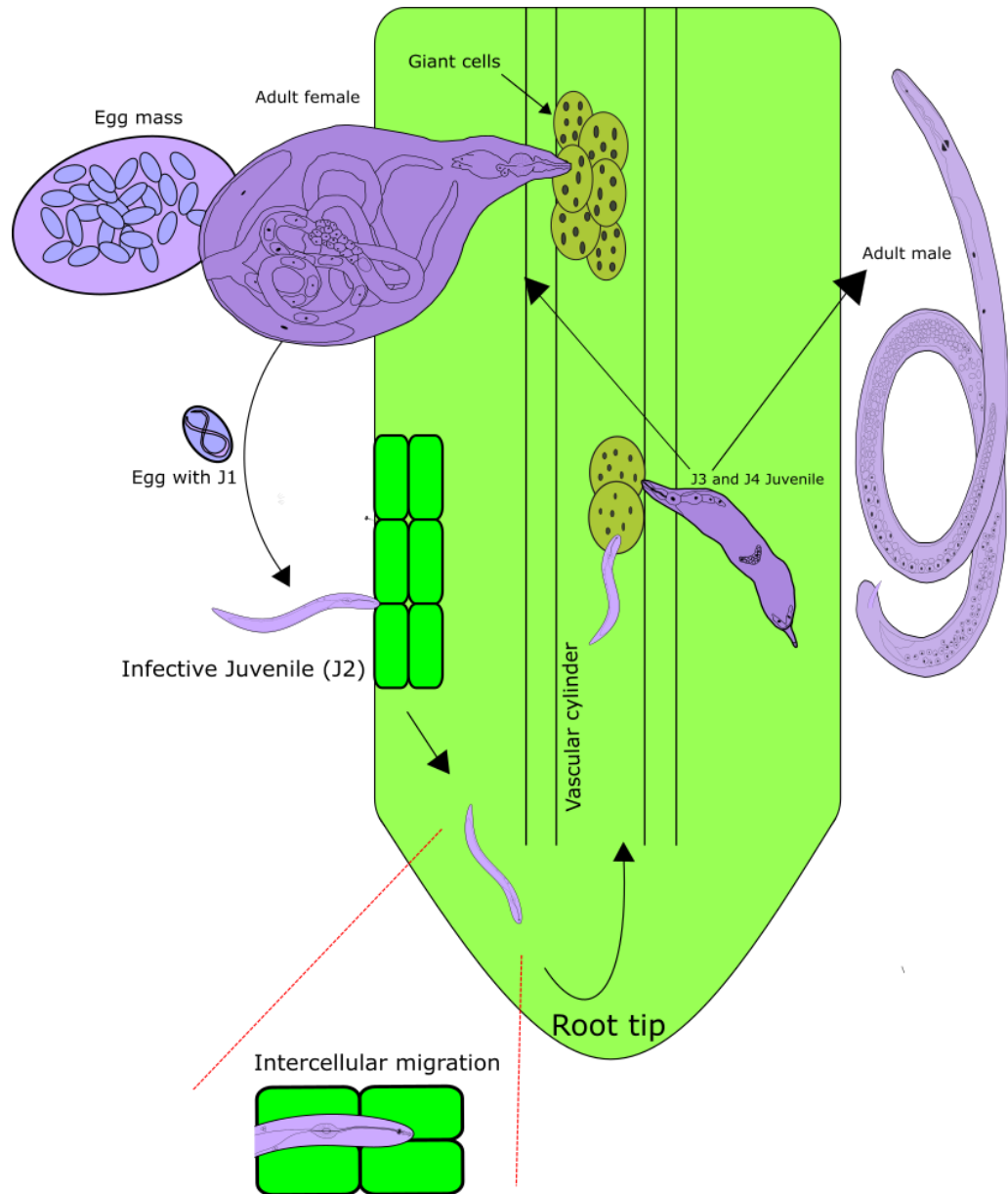


Figure 1. 3. Root-knot nematode lifecycle.

Representation of the lifecycle stages of root-knot nematodes. After hatching, juveniles (J1) develop into an infective stage (J2) which locate and invade into their plant host roots. J2s then follow a migration pattern down towards the root tip, before moving upward into the vascular cylinder. J2s will then establish feeding sites by causing the proliferation of giant cells which provide a rich nutrient source for the parasite. The nematode then develops into adult stages, of which females will produce egg masses which are released back into the rhizosphere, and males migrate to other hosts. Sizes of the nematode at each life-stage are not to scale. Figure adapted from William & Gleason (2003).

Females will then produce eggs into masses onto the surface of root galls which will have formed in response to the nematode parasitism. Unfavourable conditions such as over population, food shortage, and temperature extremes can lead to the sex reversal, as juveniles are genetically destined to be female, of *Meloidogyne* species into males (Papadopoulou & Traintaphyllou, 1982; Chitwood & Perry, 2009). Males are vermiform and become mobile, migrating back into the soil and, unlike the J2 and female, do not feed.

Species of *Meloidogyne* can have one of three modes of reproduction: (i) amphimixis, in which sperm from males fertilise female oocytes for meiotic reproduction; (ii) facultative meiotic parthenogenesis, in which amphimixis occurs in the presence of males, but in their absence parthenogenesis occurs; and (iii) obligate mitotic parthenogenesis, where males are not involved. Most *Meloidogyne* species are parthenogenic (Table 1) and the presence of males is much rarer than those that reproduce by amphimixis.

1.2.3 Root lesion nematodes

Three genera of the endoparasitic nematode family Pratylenchidae which are economically important to agriculture are *Pratylenchus*, *Radopholus* and *Hirschmanniella*. The genus *Pratylenchus* is considered to have the broadest host range of any plant parasitic nematode and is considered the third most important plant parasitic nematode in regards to crop damage (Jones *et al.*, 2013). With extensive feeding on cortical root tissue, *Pratylenchus* spp. cause cell death and necrotic regions, hence the vernacular name of lesion nematodes.

Lesion nematodes migrate through root tissue by breaking down the cell walls of epidermal and cortical cells. The feeding on and migration through root cells leads to the development of lesions, resulting in the reduction of root growth and upon extensive feeding can cause necrosis in affected cells (Fosu-Nyarko & Jones, 2016). Symptoms which correlate with lesion nematode parasitism can include leaf chlorosis, root shedding and destruction of the main root leading to stunted growth and poor shoot development. Parasitism has been shown to majorly affect the host plant's physiology as extensive root damage can lead to reduced water and nutrient uptake. *Pratylenchus coffeae* parasitised plants had significantly reduced uptake of nitrate and ammonium compared to non-inoculated plants, probably due to reduced root function following nematode damage (Vaast *et al.*, 1998). Lower concentrations of nitrogen, phosphorus, potassium, calcium, magnesium and zinc were also found in the leaves of the same coffee plants. Leaf chlorophyll content and $^{14}\text{C}_2$ fixation has also been shown to decrease in coffee seedlings parasitised by *P. coffeae*, suggesting a faster decrease in carbon assimilation in leaves as a response to lesion nematode parasitism (Mazzafera *et al.*, 2004).

The morphological identification and diagnosis of a *Pratylenchus* sp. can be difficult and unreliable due to the similarity among species as well as intraspecific variation in morphological features. Using the ratios of morphological features to distinguish between lesion nematodes was traditionally an almost essential practice for diagnosis, particularly the ratio of body length to widest body width (Siddiqi, 1997). Scanning electron microscopy is also considered a stable and reliable technique to identify *Pratylenchus* spp., with features such as the lip and face region, lateral field and tail being good taxonomic characters (Hernández *et al.*, 2000). As with the identification of *Meloidogyne* spp., PCR based techniques should provide quicker, cheaper, and more reliable species identification or verification, and are of particular use when considering soils potentially infected with multiple plant parasitic nematodes (Bell *et al.*, 2018).

Several *Pratylenchus* spp. have been reported to be parasitic to Arabica and Robusta and are of economic importance in many different regions. In Indonesia, lesion nematodes have caused yield losses to Robusta of up to 78% (Wiryadiputra and Tran, 2008). *P. coffeae* is also considered the most important parasitic nematode of Robusta in Vietnam and the most destructive nematode for Arabica in India (Palanichamy, 1973; Wiryadiputra and Tran, 2008). Other species reported to parasitise coffee include *P. gutierrezii*, *P. panamaensis*, and *P. brachyurus* in Costa Rica, Panama and Brazil respectively, though no figures for the damage on coffee or their economic importance are available.

1.2.2.1 Life cycle and reproduction

Eggs are laid individually in the root tissue where all stages of the nematode will feed. Juveniles undergo the first moult within the egg, hatching at the second stage, although some species like *P. penetrans* remain unhatched until stimulated by favourable temperature conditions to maximise survival and fitness of juveniles. In contrast to root-knot nematodes; juveniles of root lesion nematodes do not set up specialised feeding sites, instead they feed on root cells as they migrate through tissue, freely moving in and out of hosts and to neighbouring plants. Further moults occur for development into 3rd and 4th stage juveniles and eventually the adult stage. About half of *Pratylenchus* spp. lack males with a corresponding absence of sperm in the female spermatheca, suggesting they reproduce by parthenogenesis. The typical life cycle is completed in about 3-4 weeks for species in tropical, elevated temperatures and 5-7 weeks for temperate species (Duncan and Moens, 2013).

1.2.4 Other coffee-associated plant parasitic nematodes

Most if not all research into coffee and plant parasitic nematode interactions has been carried out with root-knot and lesion nematodes. There have also been many reports of other nematode genera associated with coffee plantations but there is a difficulty in interpreting these

reports, as it is not always clear whether the nematodes reported are actually parasitising the crops. Their pathogenicity and/or prevalence on coffee may be of minor importance in comparison to root-knot and lesion nematodes. Table 1. 2. summarises other plant parasitic nematodes that have been reported, under controlled conditions, to parasitise coffee crops.

Table 1. 2. Plant parasitic nematodes, other than *Meloidogyne* or *Pratylenchus* spp., which have been reported to parasitise coffee (*Coffea* sp.) under controlled conditions.

Species	Crop	Notes on infection	Reference
<i>Radopholus similis</i>	Arabica	Young plants exhibited stunted growth, undersized chlorotic leaves and enhanced susceptibility to drought	Kumar and Samuel (1990)
<i>Radopholus arabacoffeae</i>	Arabica	Populations Trinh, Ngyen, Waeyenberge, Subbotin, Karssen and Moens are more prolific and pathogenic to seedlings than <i>Pratylenchus</i> spp.	Karssen <i>et al.</i> (2004)
<i>Rotylenchulus reniformis</i>	Arabica, Robusta	Histological alterations characterised on roots. Causal agents of 'stubby root'.	Vovlas and Lamerti (1990)
<i>Hemicriconemoides</i> spp.	Arabica and Robusta	Reduced growth and weight of seedlings, and successful reproduction of the nematode on plants	Kumar and D'Souza (1969)
<i>Trophotylenchus obscurus</i>	Arabica and Robusta	Histological alterations characterised on roots	Vovlas (1987)
<i>Xiphinema ifacolum</i> and <i>X. longicaudatum</i>	Robusta	Seedlings are intolerant poor hosts	Lamberti <i>et al</i> (1992)

1.3 Plant defences against plant parasitic nematodes

Plant defences against pathogens can be classified as either constitutive, which are permanent defences which do not depend on the pathogen, or induced which are activated in response to a pathogen. As constitutive defences are always active, they incur a high cost to the plant as they are not always needed. It is theorised, therefore, that induced defences have evolved in response to pathogens to reduce the constant energy demand (Karasov *et al.*, 2017). However, the induced defences are often a stronger type of response, and the time to mount the response can also become significant drain on resources for the plant, thus creating a possible trade-off in plant fitness in the plant-pathogen interaction.

1.3.1 Secondary metabolites

Plants produce a range of biologically active compounds, or root metabolites, which are released into the rhizosphere where they can have a wide range of functions and effects on soil-borne organisms. Whilst some of these compounds can attract nematodes, many root metabolites have been studied for their effect on repelling nematodes as a constitutive defence mechanism. Wuyts (2006) studied the effect of plant phenylpropanoids on the behaviour of *Pratylenchus penetrans*, *Meloidogyne incognita* and *Radopholus similis*. Simple phenolic compounds were repellents to *R. similis* and *M. incognita* but had no effect on *P. penetrans*, as were flavanols which also had an inhibitory effect on the motility of *M. incognita* (Ohri & Pannu, 2010). Many root metabolites reported as repellents have only been demonstrated on a single taxon of plant parasitic nematode, however, which makes it difficult to draw conclusions on their contribution in constitutive defence (Sikder and Vestergård, 2020).

Metabolites exuded from plant roots can be nematicidal as well as repellent. Glucosinolates are frequently studied as a nematicidal secondary metabolite. Notably abundant in the Brassicaceae family, they are formed and released following cellular disruption, such as the wounding caused by plant parasitic nematodes, and have been found to be toxic to a range of plant parasitic nematodes (Sikder and Vestergård, 2020). Other root metabolites toxic to *Meloidogyne* spp. and *Pratylenchus* spp. include Pyrrolizidine alkaloids (Thoden *et al.*, 2009), α -terthienyl which is abundant in marigold, *Tagetes* spp., (Nježić *et al.*, 2014) and benzoxazinoids which are mainly produced by cereals (Meyer *et al.*, 2009).

1.3.2 Cell wall as a physical barrier

The cell wall, made up of cellulose, glycan, pectin and various structural proteins, presents a physical barrier to the invasion and migration of plant parasitic nematodes and is thus considered a constitutive defence. To overcome this barrier and help migration through root tissue, plant parasitic nematodes secrete a cocktail of enzymes that can degrade the plant cell

wall. Examples of these enzymes include Beta-1,4 endoglucanases that targets cellulose, pectate lyases and polygalacturonases that act upon pectin, and xylanases that will be active on certain hemicelluloses (Huang et al., 2005; Mitreva-Dautova, 2006; Ledger *et al.*, 2006).

The reinforcement of the cell wall upon infection could be an important induced defence against plant parasitic nematodes. The accumulation of lignin to reinforce the cell wall in root tissue is a common feature in resistant plants in response to plant parasitic nematodes, including resistant tomato and wheat lines (Andres *et al.*, 2001; Veronico *et al.*, 2018). Similarly, an avirulent pathotype of *M. arenaria* caused the accumulation of lignin in *Solanum torvum*, demonstrated by histochemical analyses as well as the up regulation of lignin biosynthesis genes, whilst a virulent *M. arenaria* did not (Sato *et al.*, 2019). The reinforcement of the cell wall in resistant plants following plant parasitic nematode infection indicates the key role of the cell wall as a barrier to prevent invasion, migration, and infection of plant parasitic nematodes.

1.3.3 Plant immune response

The plant immune response to nematode pathogenicity is best described by the Invasion Model, in which host receptors detect an external ligand or molecular pattern, which indicates invasion and triggers induced defences (Cook *et al.*, 2015). The wounding caused by plant parasitic nematode migration and establishment of feeding sites, for example, can trigger the release of damage-associated molecular patterns (DAMPs). These can be perceived by cell surface localised pattern recognition receptors and trigger defences. Root-knot nematodes can, however, avoid this immune response by migrating through root tissue intracellularly, reducing damage to root cells and the production of DAMPs. Immune responses can also be repressed by an invading nematode through the use of secreted effectors. Examples of effectors utilised by plant parasitic nematodes to suppress plant defences include a secreted calreticulin from *M. incognita*, which possibly prevents calcium influx (Jaouannet *et al.*, 2013), and 10A06 from the cyst nematode *Heterodera schachtii*, which disrupts salicylic acid-mediated defence signalling (Hewezi *et al.*, 2010).

The suppression of immunity by plant parasitic nematodes can also be overcome by the host plant. Following the recognition of effectors, either via receptors or metabolic changes caused by pathogen effectors, a stronger defence can be induced and often culminates in a localised cell death response (hypersensitive response) targeted on tissues on which the pathogen feeds (Jones & Dangl, 2006; Cook *et al.*, 2015). This leads to the disruption of the nematode feeding and can immobilise the pathogen. The genes which mediate this recognition and defence response have been traditionally defined as resistance genes. Examples in the plant-nematode interaction are the *Mi* group found in tomato plants (*Mi-1*, *Mi-2*, *Mi-3*, *Mi-4*, *Mi-5*, *Mi-6*, *Mi-7*,

Mi-8, *Mi-9* and *Mi-HT*) that contribute to resistance against some, but not all, *Meloidogyne* spp. (El-Sappah *et al.*, 2019). The induced immune response mediated by the *Mi* resistance genes can be characterised by necrosis of root tissue as a hypersensitive response.

For root lesion nematodes, the focus on discovering resistance genes and factors against the nematode has mostly been in cereal crops. Quantitative trait loci (QTL) for resistance against *Pratylenchus penetrans*, *P. thornei* and *P. neglectus* has been mapped and is present on all three genomes (A, B & D) of bread wheat, *Triticum aestivum*, and shown to be additive and polygenic. Later metabolomics research has shown that the reduction in nematode infection, mediated by these resistance genes or QTLs, is due to the constitutive expression of increased levels of fatty acid, glycerolipid and flavonoid classes of metabolites (Rahaman *et al.*, 2021), rather than an induced or hypersensitive response.

1.3.4 Systemically acquired resistance

In addition to local responses at infection sites, systemically acquired resistance can ensure a plant is better prepared for nematode attack, by priming low-level defence responses in remote tissue following an initial pathogen invasion (Durrant & Dong, 2004). The induction of remote defences can be mediated by signalling hormones salicylic acid, jasmonic acid and abscisic acid. However, research has shown that root-knot nematodes can also systemically suppress host defences. Pathogen response (PR) genes (*PR1-5*), which are markers for jasmonic- and salicylic-acid dependant systemically acquired resistance, are down-regulated in the leaves of infected *Arabidopsis thaliana* (Hamamouch *et al.*, 2011), and many defence related genes were also down regulated in shoots of infected rice (Kyndt *et al.*, 2012). Systemically acquired resistance can also be induced by other pathogens or symbionts to prime defence against plant parasitic nematodes. Arbuscular mycorrhizal fungi form nutrient exchanges with 80% of plants that benefits both the fungi and plant (Smith & Read, 2010). The colonization of arbuscular mycorrhizal fungi has also been shown to trigger systemically acquired resistance and reduce infection of *Meloidogyne incognita* and *Pratylenchus penetrans* in tomato plants (Vos *et al.*, 2012).

1.4 Management strategies for plant parasitic nematodes of coffee

Growers and agricultural scientists have employed a range of management strategies to control nematode infection and reduce damage to crop yields. Many conventional methods, however, can be inefficient and/or costly for the effective control of plant parasitic nematodes in coffee plantations.

1.4.1 Chemical control

Nematicides are chemical products that kill or adversely affect nematodes and can be broadly grouped as fumigants and non-fumigants. When applied to the soil, fumigant compounds reach target organisms in the form of gas that moves through the open spaces between soil particles or by dissolving into the film of water that surrounds soil particles (Chitwood, 2003). Treating soil with fumigant nematicides can effectively control plant-parasitic nematodes over a range of soil types, but they are generally more effective in coarse-textured (sandy) soils than in fine-textured (clay) soils (Chen *et al.*, 2004). Methyl-bromide was routinely used to control pests and pathogens in soils as a fumigant, until it was phased out from 2005 due to its ozone depletion effects (Zasada *et al.*, 2010).

Nonfumigant nematicides are nonvolatile toxic chemicals that can be applied prior to planting, at planting, or after planting through soil drenching, drip irrigation, or spraying onto the crop foliage to reduce population densities of nematodes and protect crops from damage. Common non-fumigants used for plant parasitic nematode control include organophosphates and carbamates (Selkirk *et al.*, 2005). These compounds are anticholinesterase inhibitors, that block the enzymes that break down the neurotransmitter acetylcholine, leading to elevation of synaptic levels of acetylcholine at the neuromuscular junction in nematodes and results in spastic paralysis. However, these compounds are also highly toxic to all organisms that utilise the neurotransmitter acetylcholine, including humans. Therefore, alternatives which are more specific to plant parasitic nematodes are sought after and encouraged.

Chemicals used today to control plant parasitic nematodes in coffee systems are almost exclusively non-fumigant products which reduce nematode populations, but do not guarantee nematode-free seedlings in nurseries or coffee plants in established plantations. Therefore, to be an efficient control, nematicides need to be used for the whole growing period of the crop to ensure maximum potential yield with no reduction due to nematode damage. Plant parasitic nematodes, however, have a high reproductive potential and populations quickly recover when the nematicides are washed out from the soil. Villain *et al.* (2000) observed that applications of the nematicide turbufos became ineffective after the second year of planting Arabica coffee in Guatemala. The use of nematicides for continuous control of lesion nematodes is therefore

difficult, especially considering the environmental concerns over the use of pesticides with high toxicity and the cost it brings to the growers.

Nematicides have also been reported as ineffective for the control of root-knot nematodes in coffee plantations and are not a recommended control strategy. *M. incognita*-infested plantations treated with nematicides show poor yield recovery compared to nematode-free plantations, and nematicides also give poor protection to coffee seedlings infected with the same species (Arita *et al.*, 2020). Nematicides are also not recommended for the control of *M. incognita* and *M. coffeicola* as the nematodes still destroy large portions of the coffee root system (Campos and Silva, 2008). The problem of using nematicides for coffee is the perennial nature of the crop; nematicides do not fully eradicate all nematodes, and so the nematodes that do survive have time to recover in a resourceful environment due to lower competition and build up to destructive levels before the crop is harvested (Villain, 2008).

Concerns for public health and environmental issues have led to the withdrawal of chemical pesticides in the market of developed countries (Schneider *et al.*, 2003). Research in plant parasitic nematode management has since focused on finding alternatives. Humic acid, for example, is traditionally used for plant nutrition but is also nematicidal to *Meloidogyne spp.* at concentrations between 0.04-2%, and thus is suggested to be used as a safe, environmentally friendly chemical control solution (Nagachandrabose, 2021). More research will be needed, however, to determine its efficacy for more nematode species such as *Pratylenchus spp.*, if used for coffee.

1.4.2 Cultural practices for control

Management of plant parasitic nematode infection has largely focused on the grafting of susceptible coffee cultivars onto the roots of a resistant species. This method has long been used for the cultivation of Arabica coffee, grafting onto *C. canephora* root stocks, in Guatemala and Indonesia to control for *P. coffeae* (Palanichamy, 1973; Villain *et al.*, 2008). The resistance in the roots of *C. canephora* is considered to be due a number of innate metabolic and physiological factors, such as high levels of polyphenols, which due to be being present even before nematode infection is likely to be a constitutive defence mechanism (Villain *et al.*, 2004).

Plant parasitic nematodes have been successfully controlled in tropical crop plantations using preplant cover crops with nematicidal properties. In coffee plantations of Indonesia, preplant cultivation of marigold suppressed *P. coffeae* populations (Wiryadiputra, 2008). Compounds exuded from marigold root and shoot tissue could either act as repellents to the parasites, or be beneficial to neighbouring plants (allelopathy) allowing them to perform better under infection (Hooks *et al.*, 2000). Other research shows that marigold can also act as a poor host, reducing

the reproduction of plant parasitic nematodes or becoming a “dead end” trap crop which arrests development of nematodes that have invaded its roots (Hooks *et al.*, 2000).

Despite these promising results, implementing cover or intercrops as a control strategy in coffee cultivation can be difficult (Villain, 2008). Firstly, seeds for such crops can be expensive, which is especially of concern to the grower if they cannot produce goods that they can sell, or not readily available. Furthermore, there is a need for a cover crop that can suppress both root-knot and lesion nematodes, integrating a cover crop which only partially controls for nematode parasitism may be perceived to be a high cost with little benefit.

1.4.3 Biological control

Nematodes are themselves infected or antagonised by many species of bacteria and fungi in the rhizosphere, and these microorganisms can be used to control plant parasitic nematodes in crop systems. Strains of bacteria *Bacillus firmus* and *Pseudomonas* spp. can be effective alternatives to chemical nematicides (Ali *et al.*, 2002; Giannakou *et al.*, 2004). The fungus *Paecilomyces lilacinus* has been shown to inhibit egg hatching and kill *M. incognita*, and increased germination whilst reducing disease severity in tomato plants (Hashem and Abo-Elyousr, 2011). The nematicidal effect of a concentrated peptidase derived from *Pseudomonas syringae* was also demonstrated on *M. incognita*, offering a potential control agent (Bashir *et al.*, 2022). However, these studies both only demonstrate the nematicidal potential of biological control agents in laboratory or greenhouse studies; the efficacy in fields and in a commercial setting has not yet been demonstrated.

Regarding coffee cultivation, nematophagous bacteria found within coffee plantations include *Pasteuria penetrans* and *Pasteuria chlamydosporia* in Brazil, and *Pochonia chlamydosporia* in Cuba (Hidalgo-Diaz *et al.*, 2000; Villain 2008). Nematophagous fungi found within coffee plantations include *Arthrobotrys conoides*, *A. musiformes* and *P. lilacinus* (Campos and Silva, 2008). These organisms have potential to be used for control of plant parasitic nematodes within coffee plantations, but it should be considered that most of these have a specificity in parasitism or antagonism to nematode species. It would probably be necessary to use a combination of organisms, or control strategies, in order to manage the particular nematodes that infect a field.

1.4.4 Genetic control

Understanding and utilising the innate genetic resistance some plants will have to plant parasitic nematodes allows an inexpensive, non-polluting control which requires little change to cultural practices. It is important to note the difference between resistance and tolerance to plant parasitic nematodes. Resistance is defined as the ability of the host plant to reduce the

reproduction and multiplication of the nematode, whereas tolerance describes the amount of injury a nematode causes the host plant and the host plant's ability to withstand or recover from plant parasitic nematode injury (Trudgill, 1991). Resistance is usually associated with a hypersensitive response in the host plant, where plant defences are implemented to isolate the pathogen and prevent further damage.

Multiple strategies can be implemented for the genetic control of coffee-parasitic nematodes. Resistance genes within the genetic resources of *Coffea* spp. can be integrated into cultivars through breeding programmes. Breeding can be assisted by the use of molecular markers near resistance genes, which are identified using gene mapping. Noir *et al.* (2003) were able to describe an inherited major gene *Mex-1*, which co-segregated perfectly with the marker Exi-11, and confers resistance to *M. exigua* within *C. canephora*, and could also be transferred into *C. arabica*. Similar to the previously mentioned 'Mi' group of resistance genes, which provides resistance against *M. incognita* in tomato, research shows that these genes only work on the one species and no other *Meloidogyne* spp.. Coffee-nematode interactions are complex especially considering the range of plant parasitic nematode species, from multiple genera with different feeding strategies, which associate with coffee. Resistance gene pyramiding is therefore an almost essential breeding strategy in which multiple resistance genes are stacked within a cultivar for combining multiple resistances in an individual and can be costly in both time and resources.

1.4.4.1 Using 'Omics' to inform plant parasitic nematode control for coffee

Recent developments in research have led to 'omics' technologies in which the structure and function of genes ('genomics'), genes that are expressed under specific conditions ('transcriptomics'), and the total metabolites or proteins that are produced or modified by an organism ('Metabolomics' and 'Proteomics') can all be analysed in a high through-put manner (Langridge and Fleury, 2011). These technologies offer new resources for understanding the molecular mechanisms of tolerance and resistance in a plant against parasitism and coupled with advances in genome engineering tools such as the CRISPR/Cas9 system, gene editing can be implemented to directly introduce genomic components that contribute to resistance or tolerance into a crop (Bortesi and Fischer, 2015).

Characterising the transcriptomic profile of a plant following plant parasitic nematode infection can reveal how plant gene expression responds to parasitism, and comparison analyses between a susceptible and resistant coffee could reveal defence related transcription patterns useful for identifying genes contributing to tolerance and resistance. However, upon identifying genes of interest from transcriptomic experiments in coffee, the functional confirmation of the role these genes play in the root-knot nematode and coffee interaction is difficult if keeping to the same

plant species. The slow growing nature of plantlets and development of new generations, combined with relatively little progress in the use of genomic technologies on the crop, such as CRISPR-Cas, means that genetic manipulation for experimentation is difficult with coffee. The use of a model plant, such as *Arabidopsis*, which has rich and accessible resources, such as genebanks to easily purchase seeds for experimental genetics, and is more easily maintained or experimentally manipulative in the lab, is recommended as a homologous system to characterise genes discovered from RNA-Seq studies (Norman & Benfy, 2009). Though considerations and caveats do need to be made regarding genetic differences between the two species; Phenotypes between *Arabidopsis* and coffee may be different despite similarities in genes and gene sequences.

Metabolomic profiling of resistant and susceptible Arabica exposed to *M. exigua* was explored by Machado *et al* (2012). Their results revealed the defensive role of phenolic compounds produced in roots, and also an increased concentration of fumaric acid by 132% in resistant cultivars after one day of infection. Fumaric acid may be acting directly on the nematodes as it has been previously described as nematocidal to *M. arenaria*. Regarding proteomics, a successful two-dimensional electrophoresis assay has been developed to identify plant proteins expressed following nematode infection in coffee and cotton (*Gossypium hirsutum*) (Franco *et al.*, 2010). This method revealed that chitinase and a pathogenesis-related protein were differentially expressed in Robusta following infection with *M. paranaensis*. Other more sophisticated methods may also be available for coffee systems including gel free systems and mass spectrometry identification and may reveal a larger or more detailed proteomic profile (Mehta *et al.*, 2008).

These technologies and examples provide new sophisticated methods for gene discovery in plant-pathogen interactions, unlocking the potential to develop novel control methods for plant parasitic nematodes of coffee. Genes which have shown to be expressed during parasitism in a more resistant or tolerant plant is of major interest to be introduced into susceptible varieties as a genetic control. Marker assisted selection can be used to locate genes which drive desirable traits and introduce them into varieties of commercial interest, but a more efficient and effective approach would be using genetic engineering; the modification of genomic DNA at the molecular level, altering the expression of a gene and thereby changing the genetic characteristics of cells to achieve specific effects (Lanigan *et al.*, 2020). As well as the introduction of genetic traits that provide protection against plant parasitic nematodes, the technologies described can be used to suppress or silence genetic components that contribute to susceptibility to nematode infection. These tools and technologies can therefore provide novel methods of control, yet research in gene discovery regarding coffee and plant parasitic

nematodes is still limited, and advances in the fundamental knowledge of this interaction, at all levels of “omics”, is required.

1.5 Aim and objectives

The overall aim of this project is to explore the interaction of root-knot nematodes and coffee as a host at a molecular level, to inform and suggest sophisticated and genetic based control methods. Root-knot nematodes were chosen as a focus due to being the most important plant parasitic nematode as a burden to crop production. Of this genus, two species will be considered, *M. incognita* and *M. paranaensis*, due to being the most studied root-knot nematode species and the most prevalent species affecting important crops globally, respectively.

To achieve the project aim, the first objective will be to diagnose and explore the phenotypic variability in resistance and tolerance in robusta coffee, in response to root-knot nematode infection. Assessing and understanding the genetic variability of Robusta, and how it effects the response to root-knot nematodes, will provide options to further study the genetic components that mediate tolerance and resistance.

The next objective will be to identify the molecular components that mediate resistance or tolerance. A transcriptomic approach will be taken to understand both the inert genetic components of Robusta coffee involved in pathogen response, as well as the induced changes in gene expression root knot nematode infection causes to different varieties of Robusta. Genes highlighted by this study will be of interest to coffee breeders for the development of new varieties that have better protection from root-knot nematodes.

The final objective of this project will be to determine the role of the neurotransmitter serotonin in the mediation of essential parasitic behaviours of root-knot nematodes and highlight the potential of serotonin-related genes in plant parasitic nematodes to be novel targets of control. This will provide another means to protect coffee from plant parasitic nematodes, to be used instead of- or as a compliment to- the utilisation of the genetic resources of coffee. The result of these objectives together will provide the fundamental knowledge required to develop sophisticated genetic control of plant parasitic nematodes for the protection of coffee crops.

Chapter 2

General Materials and Methods

2.1. Biological material

2.1.1. Plants

Coffea canephora 'Robusta' varieties FRT97, FRT101, FRT133, FRT141 and FRT 142 were supplied by Nestle R&D Tours. *Coffea arabica* 'Arabica' was purchased from Eden Project. Coffee plants was grown in compost in a glasshouse at 23 -25 °C, with supplementary lighting providing 16:8 hour light:dark conditions and 60% humidity. Tomato, *Solanum lycopersicum* (cv. Ailsa craig), plants were grown in compost in a glasshouse at 23 – 25 °C with the same light:dark conditions.

Arabidopsis seeds were ordered from Eurasian Arabidopsis Stock Centre (uNASc; table). were kept at 20 °C and 16:8 hour light:dark conditions. Seeds were sown into compost and seedlings with the first pair of true leaves were transplanted into individual three-inch pots after two weeks for collection of seed. Five cotyledons per three-inch pot were used for infection assays.

Aduki bean plants, *Vigna angularis*, were germinated from seed in petri dishes on wet 90 mm diameter filter paper. After the emergence of root and shoot, the seeds were placed at the top of soil-free pouches with both ends of the pouch open and kept upright in two-three inches tap water at 24°C and 16 hours light per day.

Table 2. 1. List of *Arabidopsis thaliana* accessions.

Purchased from the Nottingham Arabidopsis Stock Centre (NASC).

Genotype/NASC Accession no.	Gene knock-out in mutant
Columbia-0 "Col-0" (Wild type)	NA
N16534	Xyloglucan endotransglucosylase 16 (XTH16), AT3G23730.1
N576866	Irregular xylem 9 (IRX9), AT2G37090.1
N696434	Irregular xylem 14 (IRX14), AT5G67230.1
N860818	Xyloglucan endotransglucosylase 22, AT5G57560.1
N924359	Golgi apparatus-localized galactosyltransferase 18 (GT18), AT5G62220.1
N675408	Pectin methylesterase 17 (PME17), AT2G45220.1

2.2. General solutions

The following chemicals were prepared by dissolving in sterile distilled H₂O to required stock concentrations: 4-chloro-DL-phenylalanine methyl ester hydrochloride (CPA), 81mM, Methiothepin, 81mM, Serotonin (5-hydroxytryptamine), 10mM, Fluoxetine, 10mM, Salicylic acid, 10mM. Reserpine was dissolved in dimethyl sulphoxide (DMSO; 0.05% in PBS) to a concentration of 160 µM. All chemicals were prepared for each experimental procedure and used within the same day and stored at 4°C in darkness when not in use.

Pluronic gel was prepared by adding 23 grams to 8 ml of cold sterile water and stirring at 4°C overnight. The dissolved gel was stored at 4°C and aliquots were dispensed for experiments.

A 10x stock solution of Acid Fuchsin stain was prepared with 0.35% acid fuchsin in 25% glacial acetic acid.

M9 buffer was prepared by first dissolving 3 g of KH₂PO₄, 6 g of Na₂HPO₄, and 5 g of NaCl in 1 litre of distilled water. The solution was sterilised by autoclave and, after cooling, 1 mL of 1 M MgSO₄ was added.

PBS buffer was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 1.42 g Na₂HPO₄ and 1.8 g KH₂PO₄ in 1 litre of distilled water.

2.3. Coffee propagation

Cuttings from *Coffea* plants that were at least one year old were used for propagation. Scissors, scalpels, and secateurs were all disinfected before the process. Lateral branches were removed from the tree. The stem was cut within 1 cm above each node, leaving 3-5cm of stem below the node for each cutting (Figure 2. 1). Each cutting was immediately submerged into water with a few drops of detergent. Leaves on cuttings were cut in half to reduce transpiration and the cuttings were submerged into a second bucket of water (without detergent). The bottom stem of the cutting was soaked into rhizogenic powder, and after gently removing excess powder, was transferred into a 3-inch pot with pre-watered compost. Pots were placed into trays and covered by a lid to ensure humidity. Cuttings were not transplanted into larger pots, nor the lid removed, for at least 2 months.

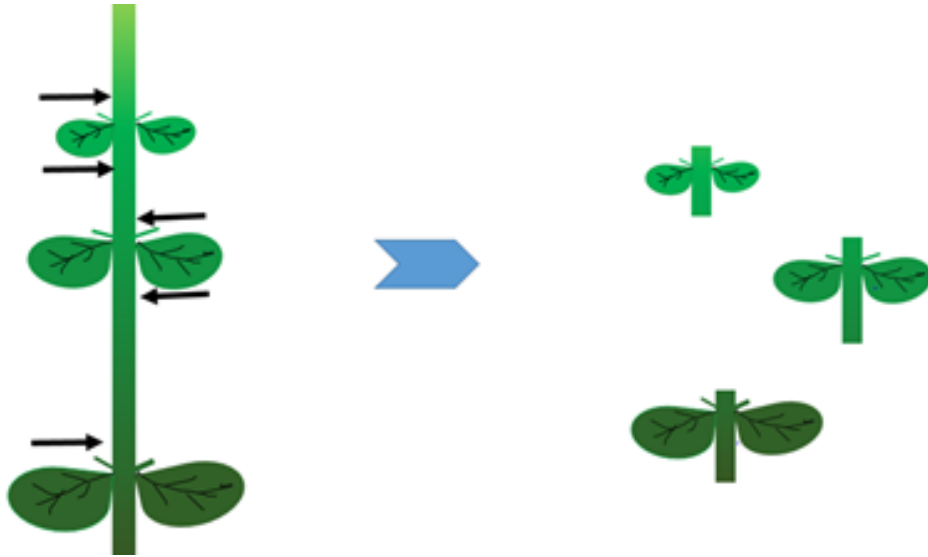


Figure 2. 1 Cuttings to be made on *Coffea* trees during propagation after lateral branch suppression.

Cuttings should be around 3-6 cm long.

2.4. Maintenance of nematode populations

Populations of *Meloidogyne incognita* and *M. paranaensis* originated from coffee fields in Brazil. Nematodes were cultured on four-week-old tomato cv. Ailsa craig in 18 cm pots containing compost with previously infected root, containing mature *Meloidogyne* females carrying egg masses, chopped up and spread evenly into the compost. Newly infected plants were grown for eight weeks before *Meloidogyne* J2s were collected, or roots were used to infect new plants. All research involving quarantine species of nematodes (*Meloidogyne* spp.) was carried out in a contained environment in accordance with Defra Plant Health Licence no. 6588-198805-4.

2.5. Extraction of nematodes

To extract mobile stages of *Meloidogyne*, roots were collected eight weeks post infection, washed to remove excess soil, and chopped into small pieces. Roots were laid on a single layer of paper tissue supported by nylon mesh held over funnels and placed in a misting chamber. A warm mist of tap water encouraged egg hatching and the movement of second stage juveniles out of the root, through the mesh and into 50 ml collection tubes. These tubes were changed every day for up to five days and collected nematodes were stored at 10 °C in tap water and washed with at least three changes of tap water before use.

2.6. Acid fuchsin staining of nematodes

Plants infected with nematodes were carefully washed in tap water to remove all soil and had above ground material removed. Roots were then soaked in sodium hypochlorite solution (1% available chlorine) for two minutes and then in tap water for 5 minutes with at least three changes. Roots were then transferred into boiling acid fuchsin stain (diluted from 10x stock) for two minutes. Roots were then rinsed briefly in tap water, placed in a petri dish containing acidified glycerol, and incubated at 65 °C overnight.

2.7. Statistical methods

Statistical analyses were performed using R programming and RStudio software. Unless otherwise stated in methods. Normality was tested on data visually by plotting as histograms and Q-Q plots, and if ambiguous a Shapiro–Wilk test was implemented. Unless otherwise stated in methods and if data was normal, ANOVA tests were performed to test for significance between groups. For experiments involving a different number of weeks of infection, each infection period (e.g. one-week post infection) was treated as individual fixed variables and not continuous. When ANOVAs were performed, fixed effects were also assumed. For non-parametric data, data were fitted onto various models, which are appropriate to the type of distribution, until a model showed good fit based on the ratio between residual deviances to

residual degrees of freedom. Tukey HSD post-hoc analysis was used following ANOVAs and Mann-Whitney post-hoc following non-parametric models.

Chapter 3

Characterising the interaction between Robusta coffee and root-knot nematodes

3.1 Introduction

Developing new coffee varieties that are genetically more tolerant or resistant to plant parasitic nematodes would be a cost effective and non-polluting control strategy against the pathogen (Bertrand and Anthony, 2008). However, this requires identification of the genetic and molecular components that contribute to resistance or tolerance in coffee. This, in turn, requires accurate phenotyping of coffee varieties for tolerance and susceptibility to plant parasitic nematodes in order to select appropriate genotypes to investigate.

Testing for resistance in a coffee variety involves inoculating the plant with a high density of nematodes and assessing the number of nematodes able to reproduce within the plant over an extended period (Villian, 2008; Trinh *et al.*, 2011; Aribi *et al.*, 2018; Carraro-Lemes, 2021). For tolerance, measurements of physiology are recorded during an infection period to understand how the plant responds to any parasitism. This could include measurements of growth such as increases in stem height, leaf weight, root weight, development of new leaves, and measurements of chlorophyll content or fluorescence as an indicator of photosynthetic capability and plant fitness (Baker and Oxborough, 2004). A more detailed and accurate reflection for tolerance would also consider how the production of berries was affected by parasitism, as Myers *et al.* (2020) did recently in a study on the tolerance of grafted Arabica coffee against *Meloidogyne konaensis*, though this is only feasible with field trials due to the size of the coffee plant and length of time to seed production. Coffee trees start to flower an average of three to four years after planting, with the flowering phase lasting for approximately two to three months. Measuring the impact of nematodes infection on fruiting is therefore not an option for higher-throughput screening.

An essential stage of the root-knot nematode lifecycle is the hatching from eggs of infective stage juveniles (J2s) and their migration towards a host root, following a gradient of chemical cues exuded from the root (Curtis, 2008). This chemotaxis behaviour allows the J2s to identify and respond to a susceptible host and food source and reduces energy spent navigating in the rhizosphere. Once a host has been found, the J2 penetrates into root tissue by thrusting its stylet, a behaviour that is triggered by root exudate and will later be used for feeding (Grundler *et al.*, 1991; Bell *et al.*, 2019). Root-knot nematode J2s do not perform these behaviours, or

perform them to a lesser extent, when exposed to the root tip exudate of some non-hosts (Zhao *et al.*, 2000). Therefore, a variety of coffee that produces exudate not attractive, or perhaps even repulsive to root-knot nematode juveniles, will be of as much interest as a tolerant variety, and may be a characteristic of the coffee plant that contributes to resistance.

Five varieties of Robusta coffee have been phenotyped according to their response to root-knot nematodes *M. incognita* and *M. paranaensis*, characterising for tolerance and resistance. For tolerance, we measured the growth of the plants over the infection period as well as photosynthetic efficiency, via chlorophyll fluorescence, as an indicator of the plants' vigour. Susceptibility of the variety to root-knot nematodes was determined by assessing the number of nematodes within roots at the end of the infection period as an indicator of how many nematodes were able to invade into the root and reproduce. We also assessed the behavioural response of root-knot nematodes to the root exudate of these Robusta varieties, measuring their chemotaxis toward root exudate and the stylet thrust response following exposure to the exudate.

3.2 Methods

All *C. canephora* (Robusta) plants were provided by J. McCarthy (Nestlé - Tours Research Centre) and *C. arabica* (Arabica) plants were purchased from Eden Project (UK). Robusta var. FRT97, FRT101, FRT133, and FRT142 were germinated somatic embryos whilst variety FRT 141 was a hybrid variety resulting from seeds of open pollination between varieties. Plants were propagated from cuttings (Chapter 2.4) and grown until 1st or second leaf stage at Tours Research Centre, shipped to the University of Leeds, immediately planted into compost in 9 cm pots and grown for at least two weeks before any further experimentation. Arabica plants were purchased from Eden Project at 1-2 leaf pairs and treated the same as Robusta.

3.2.1 Development of susceptibility and tolerance trial conditions

Six plants of Arabica at the 2nd or 3rd leaf stage were potted into 9 cm pots with either compost, 1:1 compost and sand, or 1:1:1 compost, perlite and coir. Plants were height matched across treatments as closely as possible. Measurements of stem height, leaf number and chlorophyll fluorescence (F_v/F_m : ratio of variable fluorescence and maximum fluorescence, measured using OS-30P chlorophyll fluorometer supplied by Opti-Sciences) were taken at weekly intervals for eight weeks.

Six more plants were then inoculated with 2000 infective stage juveniles of either *M. incognita* or *M. paranaensis* in each of the three mediums. After eight weeks, root systems were collected and soil carefully removed. Roots were then weighed before acid fuchsin staining (Chapter 2.7). Stained nematodes in roots were counted using a bright field stereobinocular microscope (Olympus SZX9), recording the life stage of each nematode.

3.2.2 Susceptibility and tolerance trial

Six plants of each of the *C. canephora* varieties FRT97, FRT101, FRT133, FRT142 and FRT144 were inoculated with approximately 2500 infective stage juvenile *M. incognita* or *M. paranaensis*, which were collected within the same week. The *C. arabica* variety previously tested was inoculated concurrently in each trial to ensure fitness of the nematodes and to provide a between-trial comparator as each variety was tested on separate occasions. A known susceptible variety of *Coffea arabica*, for example var. Caturra (Aribi *et al.*, 2018), and the resistant Robusta var. Nemaya were intended to be used as ideal comparisons to better understand the susceptibility of varieties. However, these were not available to us in sufficient numbers for inclusion in the trials.

Coffee plants used were between the 3rd and 4th leaf pair stage, and plants within trials were size matched across treatments as closely as possible. Nematodes were added to the soil in 1

cm deep holes adjacent to the plant stem. All plants when inoculated were arranged in a latin square formation with controls within the same glasshouse during infection.

Measurements of stem height, number of nodes on the stem (tallest stem if coffee plants had multiple), number of leaf pairs, and chlorophyll fluorescence were taken one week apart for sixteen weeks for each plant. F_v/F_m measurements were taken on the youngest fully expanded leaf. After sixteen weeks, root systems were collected and soil carefully removed. Roots were then weighed before acid fuchsin staining. Stained nematodes in roots were counted under a bright field stereobinocular microscope, recording the life stage of each nematode.

3.2.3 Collection of root exudates

To collect root exudates from all plants, roots were washed and separated intact from above ground tissue. Roots were soaked in water (100 g/L) in darkness for 24 hours at four °C. Root exudates were then filter sterilised through a 0.22 µm filter and stored at four °C until use within two weeks. Root exudate was collected from Robusta (var. FRT97, FRT101, FRT133, FRT 141, FRT142 and Nemaya) and Arabica plants.

3.3.4 Chemotaxis assay

Pluronic gel was prepared by stirring Pluronic F-127 powder in distilled water at a concentration of 23% at 4°C overnight. Six ml of Pluronic gel was poured into a 50x10 mm petri plate and incubated at 20°C.

Once the gel had solidified, 10 µl of root exudate was injected directly using a pipette into the marked region on the right side of the plate, with 10 µl of distilled water injected into the left region as a negative control (Figure 3. 1). Plates were left for 40 minutes to allow the exudate and water to diffuse into the gel. Approximately 100 J2 *M. incognita* in 20 µl suspension were then injected into the centre of the plate. The number of J2s in each circle was counted under a microscope after three hours. The number of J2s that had not moved away from the centre of the plate was also counted. The chemotaxis index (CI) was calculated as:

$$\frac{J2s \text{ in treatment circle } (E) - J2s \text{ in control circle } (C)}{\text{Total J2s on plate} - \text{Non moving J2s}}$$

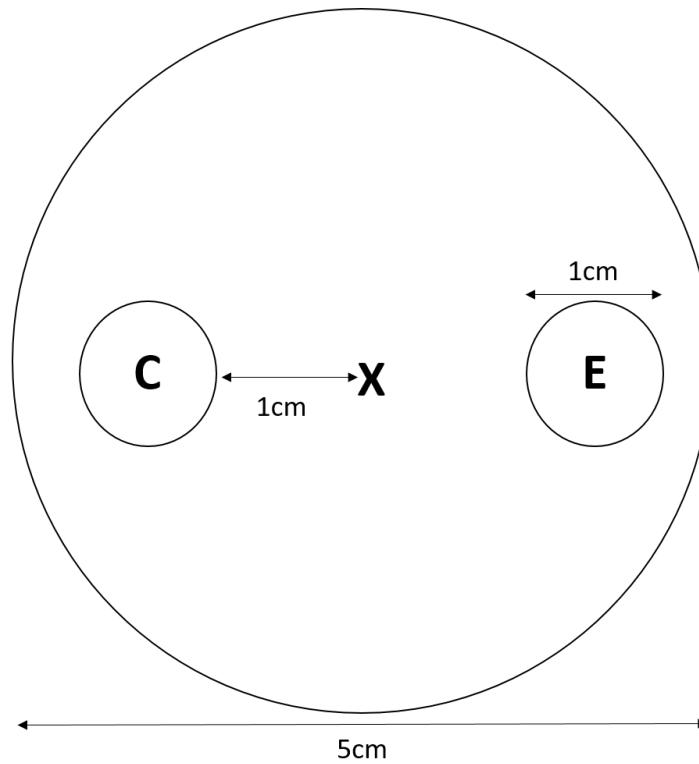


Figure 3. 1 Template design for the chemotaxis assay.

Five cm diameter plates are filled with pluronic gel. Root exudates are applied at E, distilled water is applied at C, and both are allowed to diffuse through the pluronic gel. Nematodes are then added at point X.

A CI of 1 and -1 equates to perfect attraction and repulsion respectively (Bargmann et al., 1993). A strong attraction was considered greater than 0.2 CI, between 0.1 and 0.2 for a weak attraction, between -0.1 and 0.1 for neutral or no chemotaxis response, between -0.1 and -0.2 for a weak repulsion and less than -0.2 for a strong repulsion.

Each experiment was replicated with at least two biological repeats, set up on two occasions with four technical replicates each, to account for variation between exudate and nematodes. Data was compared using one-way analysis of variance with Tukey's HSD post-hoc test. A Spearman's rank correlation was performed for comparing the susceptibility of coffee (nem.gram⁻¹) to the attractiveness of root exudate (CI), and chosen over Pearson's correlation as the test is less sensitive to the possibility of FRT97 being an outlier.

3.3.5 Stylet thrusting assay

Approximately 100 *M. incognita* J2s were soaked in 100 µl of either distilled water (negative control), 20 mM 5-hydroxytryptamine (5-HT; positive control), or root exudate (100 g/L) for 15 minutes. Upon exposure to 5HT, plant parasitic nematodes rapidly perform stylet thrusts and so the chemical can be used as a control for proper stylet thrust functioning for nematodes tested (Masler, 2007).

Ten nematodes per treatment were observed for 30 seconds at a magnification of 80x (Zeiss Axio Scope A1 microscope) and each stylet thrust was counted. A single movement of the stylet forwards and then backwards to its original position was counted as a single stylet thrust. This was repeated three times with different batches of nematodes and exudate collected from new plants. Due to non-responsive nematodes resulting in zero values, a negative binomial test was performed to test for significance between all coffee root exudate treatments, followed by Mann-Whitney post hoc analysis.

3.3 Results

3.3.1 Developing the Robusta coffee and *Meloidogyne spp.* infection system

Before inoculating coffee plants with root-knot nematodes, it was important to ensure that the growth conditions were suitable for development of healthy plants. To establish the best experimental conditions, young Arabica plants were grown for eight weeks in three different medium types. The height of Arabica coffee was significantly affected by the medium the plants were grown in ($F_{11}=4.845$, $p=0.015$). The mean (\pm SE) height increase of Arabica grown after eight weeks in the compost and sand mix was 8.34% (± 1.56), which was significantly less than Arabica grown in compost or the compost, coir and perlite mix (31.55 \pm 5.23 % and 29.21 \pm 3.34 % respectively) (Figure 3. 3). There was no significant effect of medium type alone on the chlorophyll fluorescence or number of leaf pairs of Arabica plants.

The medium in which plants are grown may also affect the ability of nematodes to invade and parasitise the plant roots, so the ability of *M. incognita* and *M. paranaensis* to infect Arabica, established at 2nd or 3rd leaf stage, was assessed in the three mediums. A generalised linear model revealed a significant difference in the number of *Meloidogyne spp.* in the roots of Arabica between medium types eight weeks post inoculation ($\chi^2 = 58.5$, d.f. = 32, $p < 0.001$), with a significantly higher number of both *M. incognita* and *M. paranaensis* in Arabica roots in compost (Figure 3.3). Reproduction factor was still low in compost. These results justified the use of compost in all subsequent trials to provide optimal conditions for both the health and growth of coffee and the infectivity of root-knot nematodes.

3.3.2 Susceptibility to root-knot nematodes

We tested the response of five Robusta varieties of coffee to infection with two root-knot nematode species, *M. incognita* and *M. paranaensis*, to understand the range of resistance and tolerance that occurs within a coffee species. Both species of root-knot nematode have a similar ability to infect Robusta, as there was no significant difference between the mean number of *M. incognita* and *M. paranaensis* in the roots of any Robusta variety ($F_{4,84}=0.058$, $p=0.925$). Robusta var. FRT97 had a significantly higher mean (\pm SE) number of nematodes within its roots compared to every other variety (Figure 3. 4; $F_{4,84}=15.798$, $p < 0.001$), with 8.49 (± 1.39) and 7.75 (± 1.02) *M. incognita* and *M. paranaensis* individuals per gram of root respectively. The number of *M. incognita* individuals at adult female life stage, and *M. paranaensis* individuals at J2, was also significantly higher in var. FRT97 compared to any other variety (Figure 3. 5; *M. incognita* $F_{4,25}=9.262$, $p < 0.001$; *M. paranaensis* $F_{4,25}=22.324$, $p < 0.001$). This could be indicative to a

different rate of development for each of the root knot nematode species depending on the host variety.



Figure 3. 2. Growth of Arabica plants in different media.

Arabica coffee was grown in, from left to right; compost, a mix of compost, coir and perlite, and mix of compost and sand. Plants were grown from 2rd or 3rd leaf stage and grown in media for eight weeks before photograph was taken.

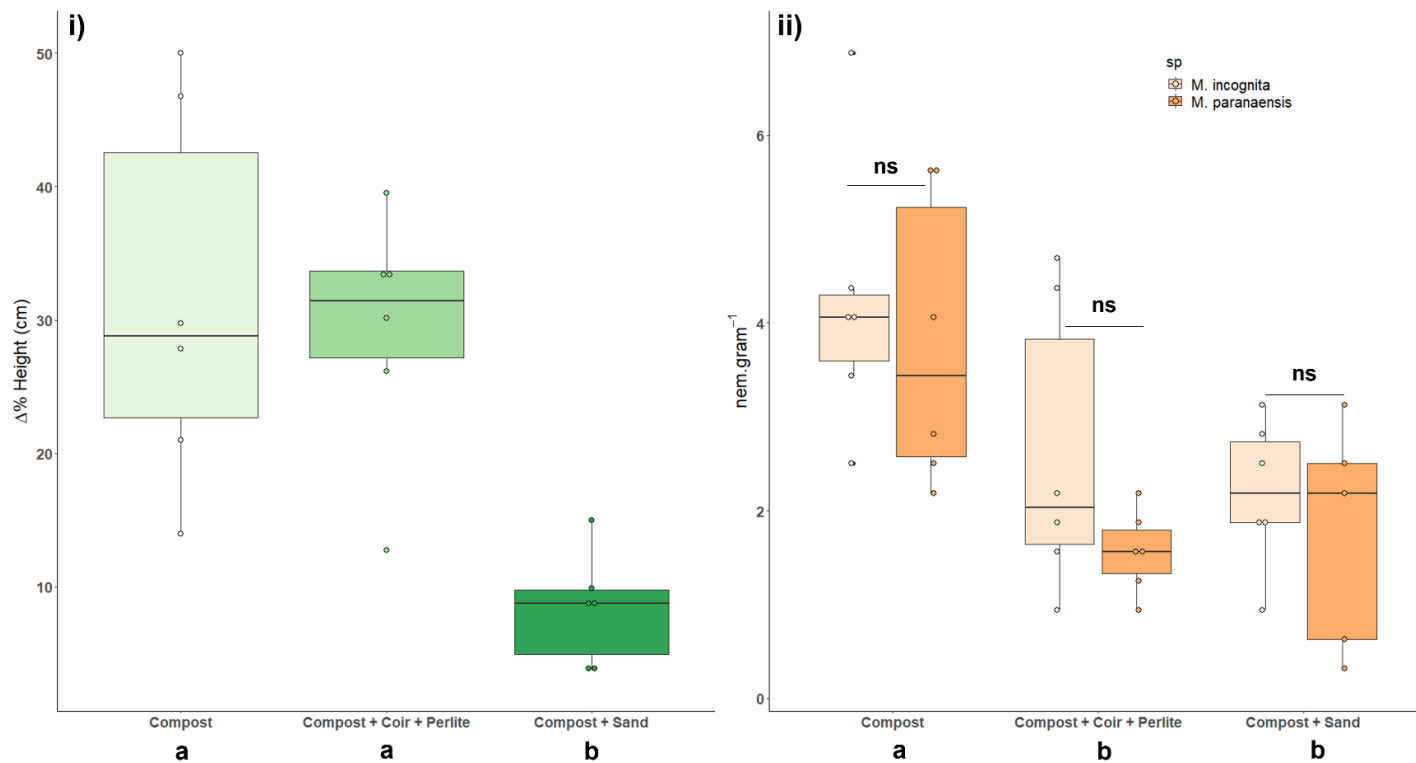


Figure 3.3. Growth of Arabica coffee, and infectivity of root-knot nematodes, in three compost mixes.

i) The percentage increase in stem height of *C. arabica* coffee (n=6) after eight weeks grown in one of three compost mixes. ii) Number of root-knot nematodes (*Meloidogyne incognita* and *M. paranaensis*), per gram of root, counted in Arabica eight weeks post infection with approx. 2000 infective stage juveniles. Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Groups not sharing a letter are statistically significant (p<0.05) following post-hoc analysis (in ii- combining *M. incognita* and *M. paranaensis* numbers. There was no significant difference between the number *M. incognita* and *M. paranaensis* individuals per gram of root for any of the treatments (t-test; p>0.05).

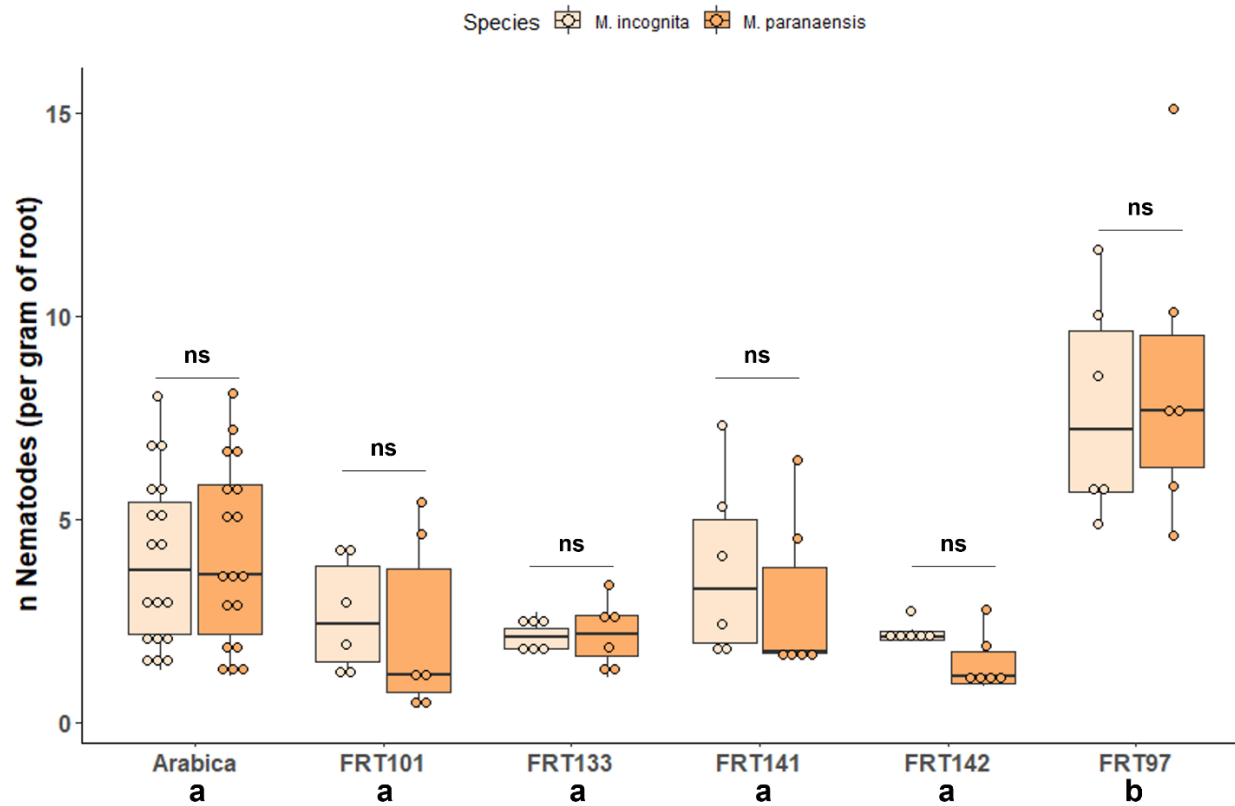


Figure 3. 4 Susceptibility of *Coffea canephora* (Robusta) var. to the plant parasitic nematodes *Meloidogyne incognita* and *M. paranaensis*.

Robusta plants (n=6) were inoculated with approx. 2500 infective stage juveniles for 16 weeks. A variety of Arabica was inoculated alongside each Robusta variety as a positive control for nematode fitness (n=18). Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters under each var. represent significantly different groups in a Tukey HSD test following a one-way ANOVA. ($p < 0.001$). There was no significant difference between the number *M. incognita* and *M. paranaensis* individuals per gram of root for any of the treatments (t-test; $p > 0.05$).

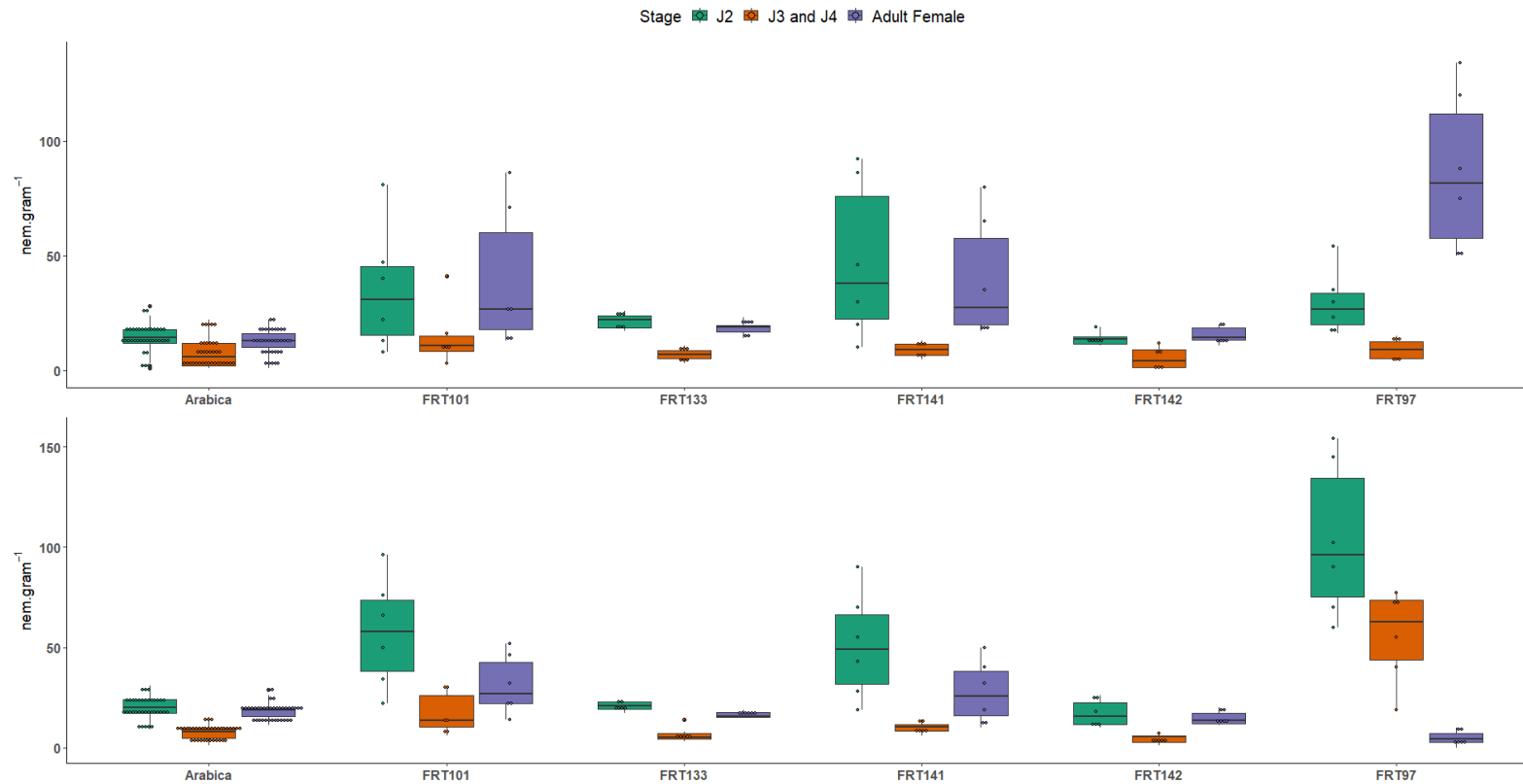


Figure 3.5 Percentage of *Meloidogyne incognita* or *M. paranaensis* individuals at each lifecycle stage counted within the roots of five *Coffea canephora* varieties; FRT97, FRT101, FRT133, FRT 141 and FRT142.

A one-way ANOVA and Tukey post-hoc analysis was performed to test for a significant difference ($p < 0.05$) in the percentage of each lifecycle stage for each and between each variety and found significantly higher number of adult female *M. incognita* (top panel) and a higher number of J2 *M. paranaensis* in FRT97 (bottom panel), compared to all other varieties. Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range.

3.3.3 Tolerance to root-knot nematodes

Many of the phenotypic and physiological measures of Robusta were not significantly affected by the infection of either root-knot nematode species (Table 3. 1). No symptoms commonly associated with root-knot nematodes, e.g. galling, were observed in infected roots of any variety. To see if longer infection would cause more severe symptoms of root-knot nematode infection, a single plant of FRT97 was inoculated and grown for a further six months, but still no physical root symptoms, including galling, or necrotic root tissue could be observed.

The growth and development of all tested varieties was unaffected by infection by either species of root-knot nematodes, except for FRT97, which had a statistically significant smaller increase in height (%) after 16 weeks in response to *M. incognita* (Figure 3. 6; $F_{5,14}=16.054$, $p<0.001$). A decrease in the height:node ratio of FRT97 was also caused by *M. paranaensis*, but not *M. incognita* (Figure 3. 6; $F_{5,14}=17.132$, $p<0.001$). This decrease in height:nodes resulted in shorter stem lengths between nodes, and therefore a stunted appearance of affected plants, highlighting the impact of *M. paranaensis* infection on the growth of FRT97.

M. incognita caused a slight, but statistically significant, reduction in chlorophyll fluorescence of varieties FRT97, FRT133 and FRT142 (Figure 3. 7; FRT97: $F_{5,14}=254$, $p<0.001$; FRT133: $F_{15}=14.33$, $p<0.001$; FRT141: $F_{5,15}=7.02$, $p<0.001$). *M. paranaensis*, however, had a statistically significant effect on the chlorophyll fluorescence of only FRT97 (Figure 3. 7; $F_{5,14}=254$, $p<0.001$). Regarding the chlorophyll fluorescence of FRT97 specifically, while neither *Meloidogyne spp.* caused a significant change in fluorescence after 16 weeks ($R=-0.077$, $p=0.55$; $R=0.21$, $p=0.51$ respectively), there was a significant increase in fluorescence for the uninfected controls ($R=0.38$, $p<0.001$) over the 16-week period. This is due to all FRT97 plants having lower F_v/F_m values at the start of the trial compared to controls or other varieties. Uninfected plants were then able to recover and produce higher F_v/F_m values over the 16 weeks, while *M. incognita* and *M. paranaensis* infected plants were not.

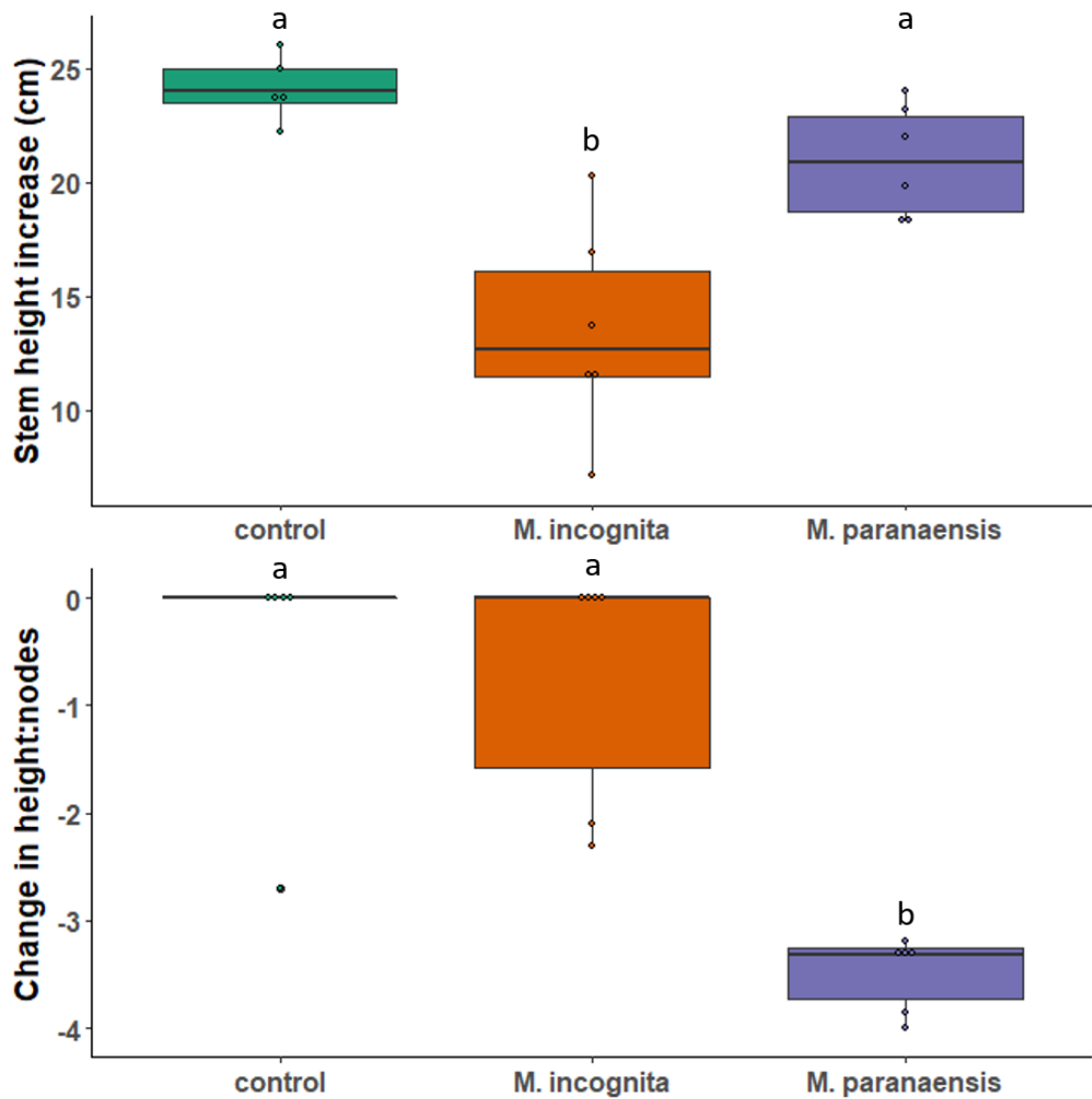


Figure 3. 6 Change in the height and height:node ratio of *Coffea canephora* var. FRT97 under infection from root-knot nematodes.

Measurements of stem height (n=6) were taken at the start of infection and 16 weeks post inoculation with approx. 2500 infective stage juveniles of *Meloidogyne incognita* or *M. paranaensis*. Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters above each treatment represent significantly different groups in a Tukey HSD test following one-way ANOVAs.

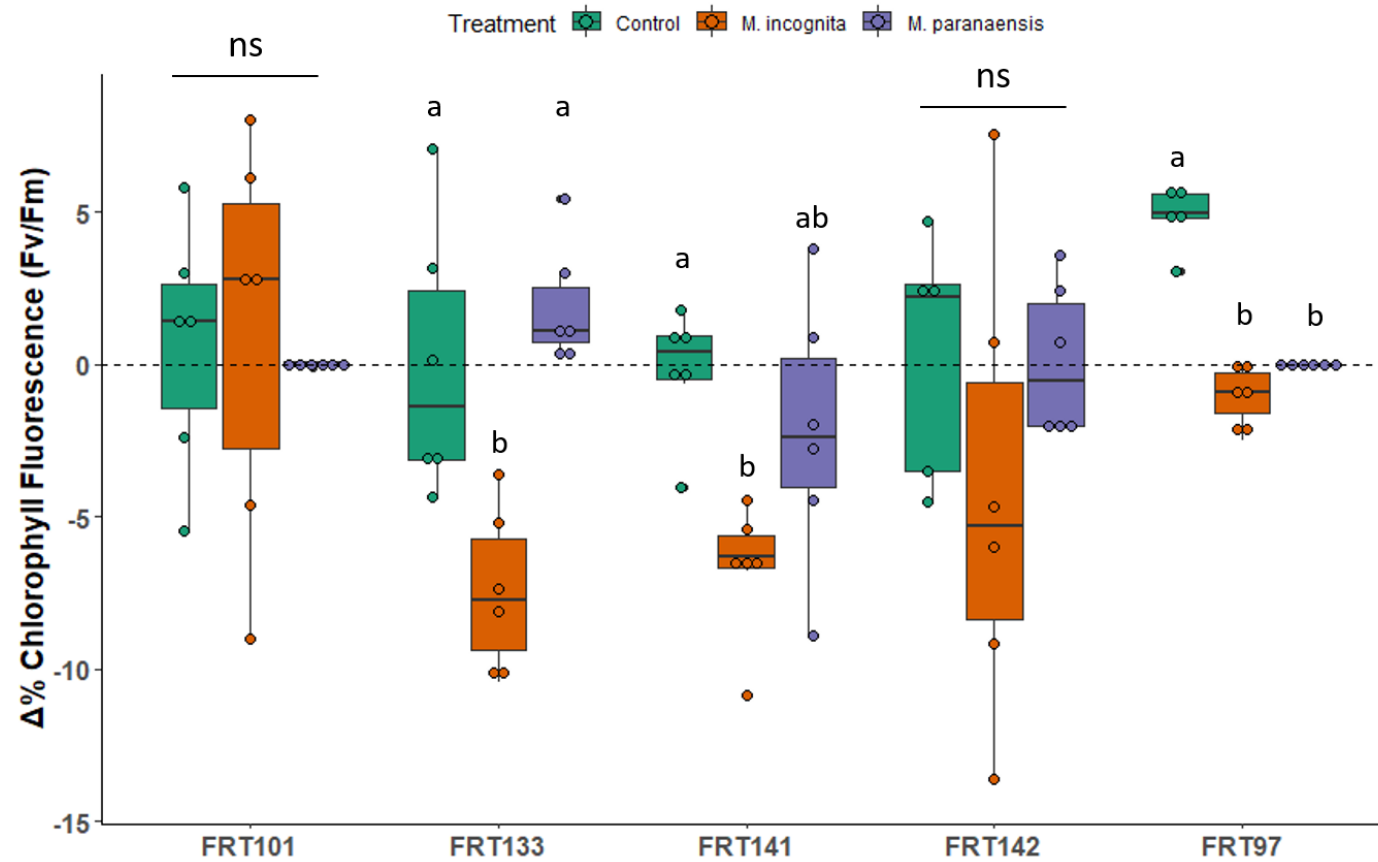


Figure 3. 7 Percentage change in chlorophyll fluorescence (F_v/F_m) of *Coffea canephora* var. FRT97, FRT101, FRT133, FRT 141 and FRT142.

Measurements were taken at the start of infection and 16 weeks post-inoculation with approx. 2500 infective stage juveniles of *Meloidogyne incognita* (n=6) or *M. paranaensis* (n=6). Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters shows significantly different groups in Tukey's HSD tests (p<0.05) within each variety.

3.3.4 Root-knot nematode chemotactic response to Robusta root exudate

The interaction between plant parasitic nematode and host begins before invasion into the host roots, and the response of nematodes to host root exudate may contribute to how susceptible the host is. We therefore wanted to see how root-knot nematodes responded to the exudate of coffee roots and if their chemotactic response could be linked to how susceptible each variety is.

Infective juveniles of *M. incognita* did show a range of chemotaxis response toward the root exudate of different Robusta varieties. The ANOVA showed a significant difference between the chemotaxis responses to all six varieties of Robusta and Arabica ($F_{6,37}=3.33$, $p=0.010$). Tukey post-hoc analysis showed that *M. incognita* J2s were significantly more attracted to the root exudate of Arabica, with a mean (\pm SE) chemotaxis index of 0.15 ± 0.02 , which was greater than the chemo-attraction toward Robusta var. FRT101, FRT142, and Nemaya ($p<0.05$, Figure 3. 8). Some nematodes also showed negative chemotaxis, possibly a repulsion, to the exudate of Robusta var. FRT101, FRT142 and Nemaya, the latter of which produced the lowest mean (\pm SE) chemotaxis index of $0.018 (\pm 0.02)$. There was no significant difference, however, when directly comparing the chemotactic response of the Robusta varieties. There also appears to be a slight trend for *M. incognita* to show the highest chemo-attraction to the most susceptible varieties, FRT97 and Arabica, as there was a positive significant correlation, between the two variables ($\rho(4)=0.89$, $p=0.033$; Figure 3. 9).

3.3.5 Root-knot nematode stylet thrust response to Robusta root exudate

Plant parasitic nematodes also respond to host root exudate by performing stylet thrusts, a behaviour which is required for moving through root cells and for feeding. The stylet thrust response to different Robusta root exudates, therefore, may reflect how suitable the plant is as a host.

For all stylet thrust assays performed, not every individual J2 responded to an exudate or to serotonin (5-HT; 5-hydroxytryptamine) which was used a positive control. 63.3 % of nematodes performed stylet thrusts in response to 5-HT during the observation period and there was variation in the percentage of individuals responding between all coffee root exudates; the highest proportion of responding *M. incognita* juveniles was 70 % to FRT97 root exudate, whilst the lowest was to Nemaya root exudate with only 20 % (Figure 3. 10).

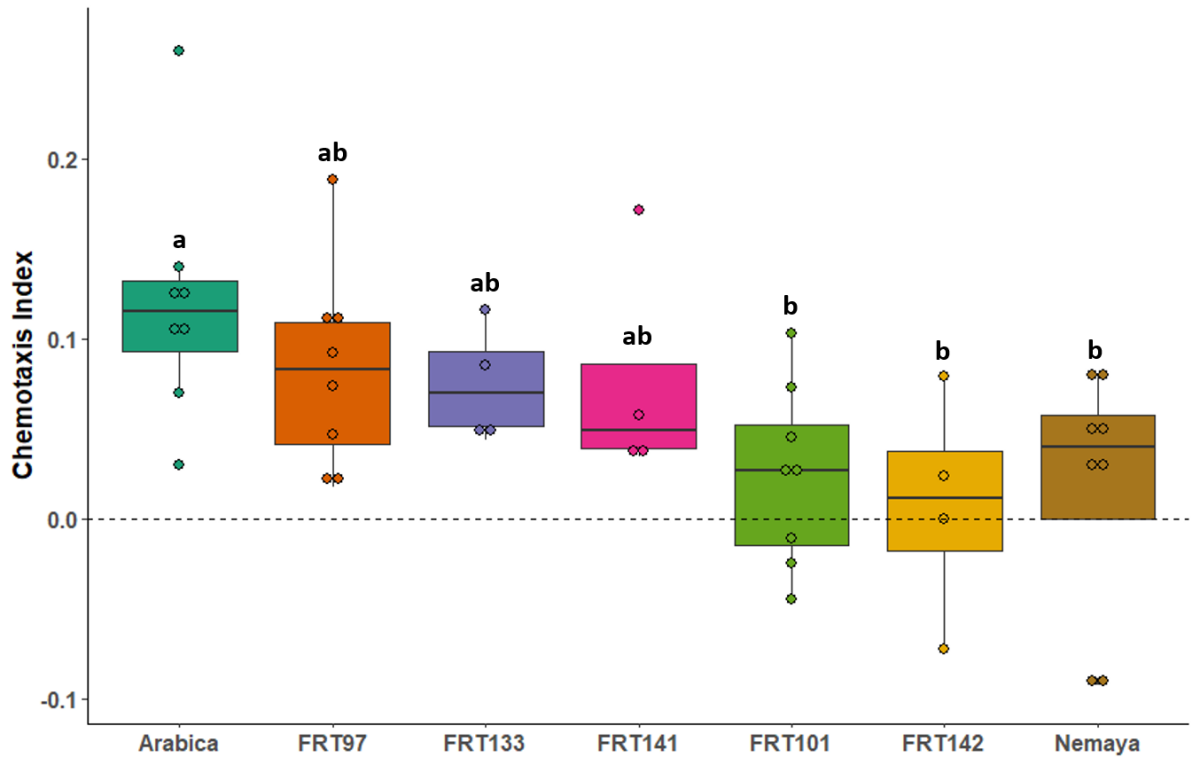


Figure 3. 8 The chemotaxis index for *Meloidogyne incognita* movement in response to the coffee root exudate.

Root exudate (100 g/L) was collected from *Coffea arabica*, and *Coffea canephora* varieties FRT97, FRT101, FRT133, FRT 141, FRT142 and Nemaya (n=8). Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters show significantly different groups in Tukey's HSD tests ($p < 0.05$) within each variety.

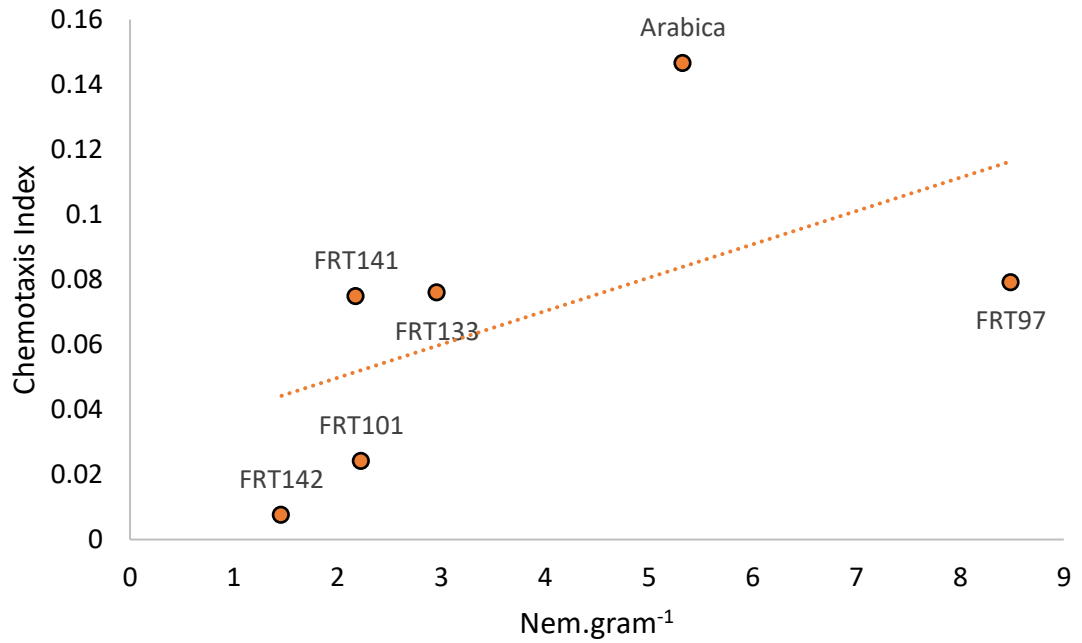


Figure 3. 9 Relationship between susceptibility and root exudate attraction to root-knot nematodes in coffee.

Correlations are shown for five *Coffea canephora* varieties (FRT97, FRT101, FRT133, FRT 141, FRT142) and *Coffea arabica* (Arabica) between the susceptibility to *Meloidogyne incognita* (number of nematodes per gram of infected root 16 weeks post infection with 2500 infective stage juveniles; nem.gram⁻¹, n=6), and attraction (chemotaxis index) of *Meloidogyne incognita* juveniles to the root exudate (100 g/L, n=8). Spearman's Rank test was performed between the two variables to show a significant positive correlation ($\rho(4)=0.89$, $p=0.033$). Linear regression between variables is represented by the dashed orange line.

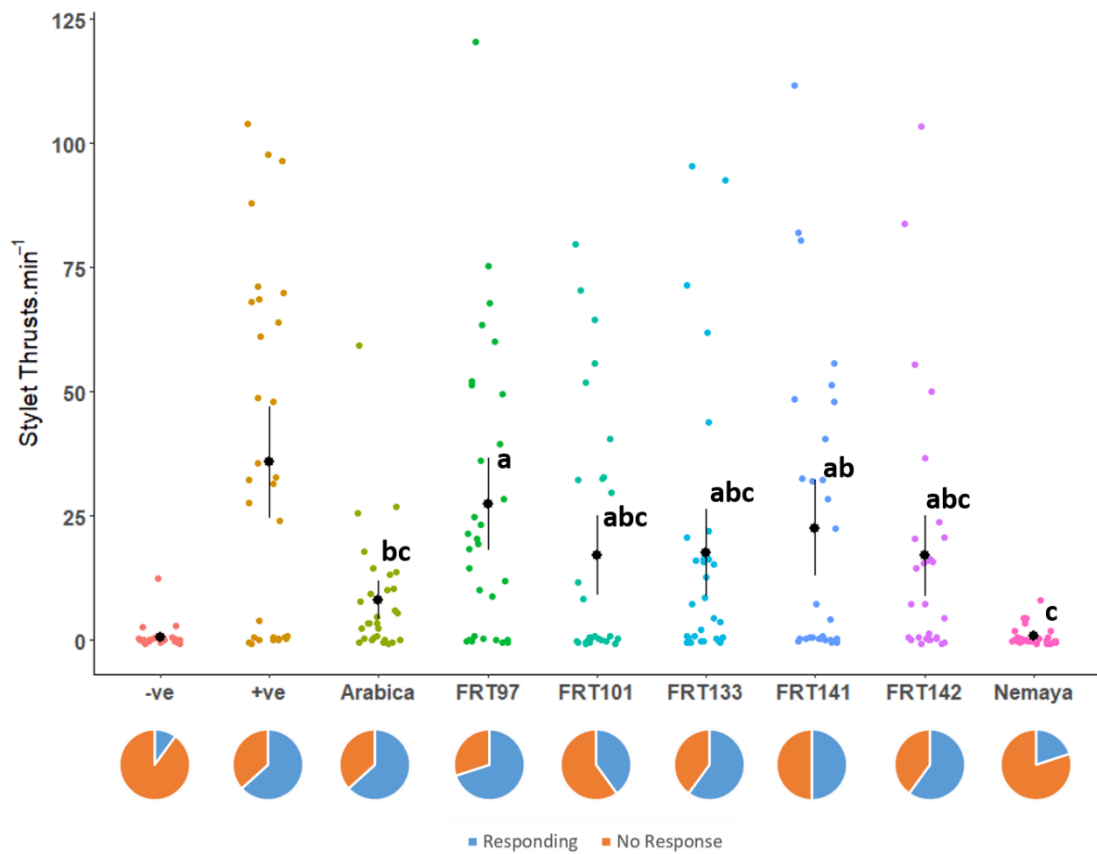


Figure 3. 10. Stylet thrust response of *Meloidogyne incognita* juveniles to the root exudate of coffee.

Both thrusts per minute and the percentage of nematodes performing thrusts (n=30) are shown for *Meloidogyne incognita* juveniles in response to the root exudate of *Coffea canephora* varieties FRT97, FRT101, FRT133, FRT 141, FRT142 and Nemaya, and *Coffea arabica*. Serotonin (5-hydroxytryptamine; 20 mM) was used as a positive control (+ve), and ddH₂O as a negative control (-ve). Black points represent the mean stylet thrust rate of all nematodes, including those that did not respond, in each treatment, with black bars showing standard error. Letters represent significantly different groups according to Mann-Whitney post-hoc analysis.

Regardless of the non-responding nematodes, differences were still observed for the rate of stylet thrusting in *M. incognita* juveniles to different coffee root exudates ($X^2=67.016$, $df=6$, $p<0.001$; Figure 3. 10). *M. incognita* juveniles responded with a mean ($\pm SE$) of only 0.8 (± 0.3) stylet thrusts per minute to exudate of Nemaya, which was significantly less than FRT97 (27.3 ± 5.3) and FRT 141 (22.5 ± 5.5). In contrast to chemotactic response, there was no significant difference between the number of stylet thrusts performed per minute in response to Nemaya and Arabica (8.1 ± 2.2). The number of stylet thrusts in response to Arabica root exudate was also significantly lower than all other Robusta varieties excluding Nemaya.

3.4 Discussion

3.4.1 Susceptibility of Robusta coffee to root-knot nematodes

Following the 16-week infection with the two root-knot nematode species, we were able to assess the susceptibility of the Robusta coffee varieties by counting the number of nematodes in the roots and thus the penetration and reproductive ability of the nematode within its host. Of the five Robusta varieties in this study, *C. canephora* FRT97 was the most susceptible, as it had statistically more *M. incognita* and *M. paranaensis* were present in the roots 16 weeks post infection. As the chlorophyll fluorescence of the leaves of FRT97 plants was lower at the start of the trial, it may mean that these plants in poorer condition were less able to respond to nematode infection, leading to increased susceptibility, rather than any innate genetic differences. However, there is no evidence to suggest that this would have a major effect. The only comparable research on the general health of the plant influencing susceptibility showed no difference in susceptibility to the cyst nematode *Heterodera sacchari* between drought-stressed and non-stressed rice plants (Audebert *et al.*, 2000).

The reproduction of *Meloidogyne* across all coffee varieties tested here was relatively low compared with that reported for other Robusta and Arabica coffee. Aribi *et al.* (2018) inoculated coffee cuttings with the same number of J2s of *M. incognita*, and after eight weeks found between one and 1819 J2 per gram of root depending on the species and variety. The lower-than-expected number of nematodes suggests that the varieties tested in this work were poor hosts in comparison to other coffee varieties. It is generally reported that Arabica is more susceptible to root-knot nematodes, whereas Robusta plants possess resistance, through resistance genes or constitutive defences that protect them from root-knot nematode attacks (Bertrand & Anthony, 2008; International Trade Centre, 2021). This led us to assume that the Arabica plants used for this work would be the most susceptible variety whilst the Robusta varieties would be less susceptible and possibly resistant. As none of our varieties were completely resistant, the differences we see in susceptibility are most likely due to other factors outside of any resistance gene. These could be constitutive defences that lead to the plants being less suitable hosts to root-knot nematodes, such as higher levels of polyphenols that are present in varieties of Robusta (Toruan-Mathius *et al.*, 1995; Villain *et al.*, 2004). It also appears that the particular Arabica variety used may share these constitutive defences that should be explored further.

An issue that may influence the ability of the *Meloidogyne* spp. to infect coffee is that the nematodes were routinely maintained on tomato plants before inoculation onto coffee plants, despite the population originating from soils of coffee fields in Brazil. This may require some adaptation to the new host, reducing the amount of infection in comparison to that observed

by Aribi *et al.* (2018), in which *M. incognita* was reared on a susceptible Arabica coffee before infection in their coffee varieties. These populations of *M. incognita*, however, were only reared on greenhouse tomato for a short time, less than five years in total, relative to the many years or decades that nematode populations are generally cultured on tomato where any change in host preference would be expected. There is also no evidence of any habituation for a plant parasitic nematode on one host leading to a reduced ability to infect another.

A meta-analysis conducted by Carraro-Lemes *et al.* (2021) attempted to correlate and consolidate multiple phenotyping methods for *M. paranaensis* resistance in coffee. By comparing 13 studies from 2008 to 2019, they established that the best practice for phenotyping was using plants at the four-leaf pair stage and inoculating with 1000 eggs for 80-90 days. In comparison, this study used a similar age of plant, inoculated with over twice the number of juveniles for a longer time. The authors of this study suggest that the higher initial inoculation number may result in a lower reproduction factor as it would introduce competition for feeding sites between nematodes, ultimately reducing development and reproduction within the host due to scarcity of resources. Therefore, reducing the initial inoculation number may allow for increased reproduction factor and show a higher contrast in susceptibility phenotypes in Robusta coffee varieties.

Whilst considering the decreased susceptibility of some of the varieties tested, more investigations will be needed to understand the ability of coffee to limit the infection and reproduction of root-knot nematodes. Firstly, comparisons should be made to a known resistant variety of Robusta, e.g. Nemaya (Aribi *et al.*, 2018), to see a full range of susceptibility and resistance within Robusta coffee, as this variety was not available to us in the number required for controls across all tests. More comparisons could also be made for the Arabica variety used, as this was less susceptible than expected. Comparing this Arabica and the Robusta varieties against varieties that are considered good hosts of root-knot nematodes would better contextualise the performance of these varieties. It should also be noted that only one race of each species of *Meloidogyne* was used and utilising a greater range of root-knot nematodes, as well as other plant parasitic nematodes which infect coffee, is recommended to understand this host-parasite interaction. Furthermore, investigations into the ability of root-knot nematodes to invade the roots of Robusta could reveal if the slightly increased susceptibility of FRT97 seen here is due to a greater number of nematodes invading the host, or if it is due to a greater ability to reproduce within FRT97. This could be achieved by counting the number of nematodes in roots after a shorter time, less than one week for example, rather than after eight or 16 weeks where multiple generations of the nematode are expected to have developed within the host.

3.4.2 Physiological detriments caused by root-knot nematode infection in coffee

Regarding below ground physiology, and considering the establishment of feeding sites (i.e. the development of giant cells and root galls that root-knot nematodes cause), it was unusual not to see phenotypic differences between infected and uninfected roots. This could be due to a low reproduction factor, although as coffee is a woody plant with thick roots a much longer and heavier infestation may be required before any visual symptoms of *Meloidogyne spp.* can be observed.

There were, however, aboveground growth and physiological detriments observed in Robusta coffee following root-knot nematode infection. Whilst the development of leaves was unaffected, both *Meloidogyne* species inhibited the growth of FRT97, but not any other variety. Stunted growth is a frequent symptom of root-knot nematode infection, due to the nematode “hijacking” nutrient uptake from roots to shoots. This is best displayed in the ratio of height to nodes; as plant height is more sensitive to stress than the number of nodes present, the ratio of height to number of nodes can provide a useful measure of source-sink balance, or a plant’s ability to allocate resources into growth and development (Kerby *et al*, 1998; King’oro *et al*, 2014). The observed significant decrease of height:nodes in FRT97 could suggest a detriment to nutrient allocation caused by the infection of *M. incognita*, as well as a physiological response of the plant caused by the parasitism. However, it may also be due to plants not being in optimal conditions at the start of the trial, as shown by lower-than-expected chlorophyll fluorescence. Meanwhile, the ability of the other varieties tested to grow unimpeded by infection suggests a potential level of tolerance, as physiologically the plants coped with similar levels of nematodes infecting them. This will become of greater relevance and interest if the coffee plants are then able to produce coffee berries similarly unimpeded by infection.

The detriment to coffee plant health caused by root-knot nematode infection is also reflected in the significant decrease observed in chlorophyll fluorescence, which will be a result of reduced water and nutrient uptake from roots to shoots due to parasitism. We measured this fluorescence as the ratio of variable fluorescence and maximum fluorescence (F_v/F_m), which directly measures the photosynthetic efficiency of plant cells, which is shown to be directly correlated to the vigour and health of a plant (Baker and Oxborough, 2004). Only *M. incognita* caused a significant change in chlorophyll fluorescence, despite *M. paranaensis* infecting each variety equally well. This highlights the increased severity of *M. incognita* presence within coffee fields and the importance of controlling this plant parasitic nematode. Though significant, the change in chlorophyll fluorescence of FRT97, FRT133 and FRT 141 appears small and may not be of great biological importance, especially in comparison to other biotic and abiotic stresses. For example, nutrient stress (insufficient nitrogen) and water stress caused a mean percentage

decrease of 22.69 and 30.45 respectively in the F_v/F_m of five Arabica varieties (de Souza *et al.*, 2020), which is considerably more than the decrease caused by root-knot nematodes here. However, coffee leaf rust was shown to have a similarly small but significant level of effect on photosystem II efficiency and can be used as an indicator of physiological status to predict the capacity of the plant to resist the disease (Toniutti *et al.*, 2017). Moreover, parasitic nematodes generally have a high reproductive potential, and damage caused to the crop usually occurs over a long period through multiple generations of the nematode (Villain *et al.*, 2008). The difference in photosynthetic efficiency therefore may worsen and cause physiological detriment to the coffee plant with increasing nematode loads and damage.

It would be important to see if these changes in physiology followed a similar trend with coffee infected with plant parasitic nematodes in longer field experiments, adding validity to the significant changes observed within these relatively short-term trials. Still, the significant effect on physiology, including stunted growth and chlorophyll fluorescence, provide measures that can easily be taken short term to identify and characterise the susceptibility and tolerance profiles of Robusta coffee.

3.4.3 Root-knot nematode response to coffee root exudate

Plant parasitic nematodes rely on chemical cues exuded from plant roots to navigate towards their host. This can be demonstrated in assays developed to measure the chemotaxis of root-knot nematodes to the root exudate of hosts and non-hosts (Yang *et al.*, 2016; Kirwa *et al.*, 2018). The host-associated behavioural response is further exemplified by host-specific gene expression changes in plant parasitic nematodes, which are relative to substrates, such as cellulose and xylan, within root exudate (Teillet *et al.*, 2013; Bell *et al.*, 2019). While the mechanistic components of chemotaxis within plant parasitic nematodes are highly conserved, with carbon dioxide released from plant tissue being a ubiquitous attractant across plant parasitic nematode species (Rasmann *et al.*, 2012), differences do also exist between nematodes with different feeding strategies and the plants they infect. For example, *M. incognita* is attracted to the root tips of marigold (*Tagetes patula*), soybean (*Glycine max*), and pepper (*Capsicum annuum*) but the soybean cyst nematode (*Heterodera glycines*), which has a narrower host range, is only attracted to root tips of soybean (Wang *et al.*, 2018).

In this study, a semi-natural method of root exudate collection was used, in which whole plant root systems were used to collect “leachate” in water. Some studies use a more natural, in field system, where exudate is collected directly from the soil (Phillips *et al.*, 2008; Zang *et al.*, 2014). While this reflects in-field scenarios more closely, access to plant roots is directly impeded by the soil matrix, and compounds unlikely to persist, as microorganisms will metabolise them

readily (Kuijken *et al.*, 2015). The system used here is also a more high-throughput method, which easily controlled and standardised across samples, allowing direct comparisons of root exudates at the same concentration. However, damage caused to the plants during collection may produce a change in chemical profile (Williams *et al.*, 2021). A hydroponic system, in which the plant is largely left unaffected during exudate collection, should therefore also be considered if a practical system were set up for coffee.

We observed that the root exudate of Robusta variety Nemaya, which is reported to be resistant to *M. paranaensis* and *M. exigua* (Bertrand & Anthony, 2008), was not an attractant for *M. incognita*, and the exudate did not stimulate any stylet thrusts in the nematodes. It is therefore possible that the nematode perceives a chemical profile from Nemaya that indicates it to be a poor host so it can migrate elsewhere to find a more suitable host. The exudate of FRT142 was similar to Nemaya in regard to its attractiveness to *M. incognita*. This may be a contributing factor to its lower susceptibility compared to FRT97, which was more attractive to the root-knot nematode, and stimulated a stronger stylet thrust response. However, the chemotaxis index of *M. incognita* toward the root exudate of FRT101, FRT133 and FRT 141 was not significantly different from FRT97, yet all had lower levels of infection in the susceptibility trial, indicating that a number of other factors undoubtedly determine differences in susceptibility between Robusta varieties. Furthermore, the root exudate of Arabica was found to be one of the most attractive to root-knot nematodes, yet stimulated a low stylet thrust response relative to Robusta varieties despite being a better host.

Low attraction and no stylet thrust response could be considered to be indicators of coffee host status to root-knot nematodes, and the composition of the root exudate of coffee should be explored further to understand what determines the differences in behavioural response and possibly even resistance and/or tolerance. However, this would first need the testing of a greater number of varieties to confirm any correlations, and with the caveat that it varies between different cultivars. Regardless, there is extensive evidence linking behavioural responses of plant parasitic nematodes to host status, so the responses to coffee root exudate are highly informative in the coffee-nematode interaction.

3.5 Key Findings

All the Robusta varieties tested showed lower susceptibility to root-knot nematodes to varieties of other reports. One of the Robusta's tested, FRT97, showed a slight, but significant increase in susceptibility to nematode infection versus the other varieties tested here at the plantlet stage. The infection of root-knot nematode also caused a detriment to the physiology of only FRT97, FRT133 and FRT141. These varieties, therefore, had lower tolerance to the parasite relative to other Robusta's tested. The differences in tolerance between Robusta varieties would be useful to explore the molecular mechanisms that determine tolerance. FRT97 and FRT101 were identified as contrasting varieties in response to root-knot nematodes and selected for further molecular characterisation. Finally, root-knot nematodes show a variety dependant behavioural response to Robusta root exudate, in which the more susceptible varieties also produce root exudate that is more attractive to the nematode. This could provide a phenotype of coffee, which can be correlated to the host response to root-knot nematodes.

Table 3.2. Summary of Chapter 3 results on the phenotypes of Arabica and Robusta coffee in response to root-knot nematode infection.

Coffee/ Variety	Root-knot nematode response to root exudate			
	Susceptibility	Physiological response (tolerance)	Chemotaxis	Stylet thrusting
Arabica	Susceptible but comparatively less so than other reports on coffee	Infection had no on physiology	Weak to moderate attraction to root exudate	Little to no response
Robusta FRT97	Relatively more susceptible than other varieties	Growth and photosynthesis efficiency impaired by infection	Weak to moderate attraction to root exudate	Exposure caused stylet thrust behaviour
Robusta FRT101	Susceptible but comparatively less so than other reports on coffee	Infection had no on physiology	No attraction to root exudate	Exposure caused stylet thrust behaviour
Robusta FRT133	Susceptible but comparatively less so than other reports on coffee	Photosynthesis impaired by infection	No attraction to root exudate	Exposure caused stylet thrust behaviour
Robusta FRT141	Susceptible but comparatively less so than other reports on coffee	Photosynthesis impaired by infection	Weak to moderate attraction to root exudate	Exposure caused stylet thrust behaviour
Robusta FRT142	Susceptible but comparatively less so than other reports on coffee	Infection had no on physiology	No attraction to root exudate	Exposure caused stylet thrust behaviour
Robusta Nemaya	Resistant (not reported by this study)	Tolerant (Anzueto <i>et al.</i> , 1995)	Slight repulsion to root exudate	No response

Chapter 4

Transcriptional responses in Robusta coffee following root-knot nematode infection

4.1 Introduction

The natural genetic variation found between varieties of the same plant species often leads to a large range of, and in some cases contrasting, observable traits, or phenotype. In Chapter 3, we observed that different varieties of robusta coffee show a range of responses to root-knot nematodes; both in susceptibility to the plant parasitic nematode, and in how much their growth and health were impacted. This range in phenotype may be a result of variations in both genomes and how these genes respond to the environment. These variations are of interest to understand mechanisms underlying the coffee and root-knot nematode interaction. Comparisons made between the genotypes of varieties with contrasting phenotypes would highlight genes that contribute to the resistance or tolerance to plant parasitic nematodes in coffee. Those genes would serve as novel sources of either resistance; which could be utilised for the control of plant parasitic nematodes that infect coffee, tolerance; which could protect the health and therefore yields of coffee despite parasitism by nematodes; or susceptibility, which aid or are even required for plant parasitic nematodes to infect coffee and should be avoided.

It is not just genomic variation that contributes to differences in phenotypes, but also the expression of genes under different environmental conditions, or under infection of a plant parasitic nematode for example. It is therefore both advantageous and more accurate to compare the transcriptomes, which is the set of all RNA transcripts, including coding and non-coding, in an individual under specific conditions, rather than just the genome. With the rapid development of next generation sequencing technologies (Lowe *et al.*, 2017), the sequencing of RNA (RNA-Seq) is now readily available and affordable. Transcriptome comparison of two coffee varieties of contrasting phenotype is therefore the ideal method to identify molecular mechanisms mediating resistance and tolerance to plant parasitic nematodes. The non-targeted approach of a transcriptomic study also allows for the identification of novel transcript networks that is ideal for less studied systems such as coffee. For example, both de Freitas Guedes *et al.* (2018) and Thioune *et al.* (2020) have used RNA-Seq to identify differentially expressed genes in coffee under water deficits to understand the mechanisms of drought tolerance in the crop in preparation for changing climate conditions.

Following transcriptomic analysis, we highlighted several genes which all had high similarity to Arabidopsis genes involved in cell wall regulation and modification. Irregular xylem 9 (*IRX9*) and irregular xylem 14 (*IRX14*) are two of four Arabidopsis genes in the GT43 family, along with their homologs *IRX9L* and *IRX14L*, which are functionally nonredundant in the formation of the xylan backbone (Wu *et al.*, 2010). Golgi apparatus-localized galactosyltransferase 18 encodes a Golgi apparatus-localized galactosyltransferase involved in the biosynthesis of xyloglucan, which allows for elasticity in the cell wall and contributes to formation and remodelling of new cell wall during growth (Cosgrove, 2000). The xyloglucan endotransglucosylase/hydrolase family cleave and reconnect xyloglucan molecules to loosen cell wall and provide plasticity, though more diverse functions, including biotic and abiotic stress responses have been proposed for many of these enzymes (Ishida & Yokoyama, 2022). Golgi apparatus-localized galactosyltransferase 18 encodes a Golgi apparatus-localized galactosyltransferase involved in the biosynthesis of xyloglucan, which allows for elasticity in the cell wall and contributes to formation and remodelling of new cell wall during growth (Cosgrove, 2000). Finally, pectin methyl transferases (PMEs) are a large multigene family which cause the de-methylesterification of the most abundant part of pectin, homogalacturonan to control pectin stiffening and cell wall modification (Mohnen, 2008).

Molecular components that drive tolerance or resistance in plants may also be a useful indicator, or biomarker, to easily phenotype a plant in its response to nematode infection. Rather than infecting a plant with a number of nematodes and waiting several weeks to measure the reproduction of the parasite or the physiological detriments caused to the plant host, the presence of a gene involved in the plant-parasite interaction could indicate how susceptible or tolerant it could be. Not only could this reduce the time required to phenotype, but also by taking an above ground piece of tissue, such as leaf, avoids the destruction of the plant required when taking root samples or measurements. Afifah *et al* (2020) have already established that both resistant and susceptible tomato respectively share the same metabolite expression in roots and in their shoots, highlighting important metabolites in the resistant mechanisms to root knot nematodes. Similar findings in coffee would be of major interest in both in the development of resistant or tolerant varieties and improve screening efficiency for nematode susceptibility in existing varieties.

From the varieties studied here, Robusta var. FRT101 was selected as the least susceptible, and FRT97 as the more sensitive variety for comparison testing. Using RNA-Seq, comparisons between their transcriptomes following infection with *M. incognita* will reveal the differences in gene expression that contribute to differences in phenotypes and will be of interest for the genetic control of plant parasitic nematodes. Both root material and leaf material were analysed

to understand the transcriptomic response locally upon both nematode invasion, feeding and reproduction, as well as any systemic changes that occur in remote tissue above ground.

4.2 Methods and Materials

4.2.1 Root-knot nematode infection

Six *C. canephora* FRT97 and six FRT101 plants were each inoculated with 2500 2nd stage juvenile *M. incognita* for one week (1wpi), six of each were inoculated with the same number for twelve weeks (12wpi), and six of each were used as non-inoculated controls. Plants were grown in compost in 4-inch diameter pots, were at the 3rd or 4th leaf stage at the start of the experiment and height matched as closely as possible between treatments. All plants were set up and arranged together in a Latin square formation in the same glasshouse in conditions stated in Chapter 3.2.2, with the end of the one-week and twelve-week inoculation period occurring at the same time (Figure 4. 1).

Measurements of stem height and chlorophyll fluorescence (F_v/F_m : ratio of variable fluorescence and maximum fluorescence, measured using OS-30P chlorophyll fluorometer supplied by Opti-Sciences) were taken for plants as they were inoculated, along with controls, and again for plants at the end of the infection period. F_v/F_m measurements were taken on the youngest fully expanded leaf.

4.2.2. RNA extraction and sequencing

At the end of inoculation periods, root systems of all plants were carefully collected, washed and flash frozen in liquid nitrogen before being stored at -80°C. One fully expanded leaf of each plant was also collected, flash frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the Omega E.Z.N.A Plant RNA extraction kit, following the provided protocol. RNA quality was assessed using a 2100 Agilent bioanalyzer, checking that RNA had an RNA integrity number (RIN) above six, and purity (A260/280) value between 1.8 and 2.2.

RNA Sequencing was performed by Genewiz, including library preparation, PolyA selection and paired end 150 bp sequencing, with 15-20 million reads per sample, using the Illumina NovaSeq™ platform. At least five independent replicate samples were sequenced for each treatment.

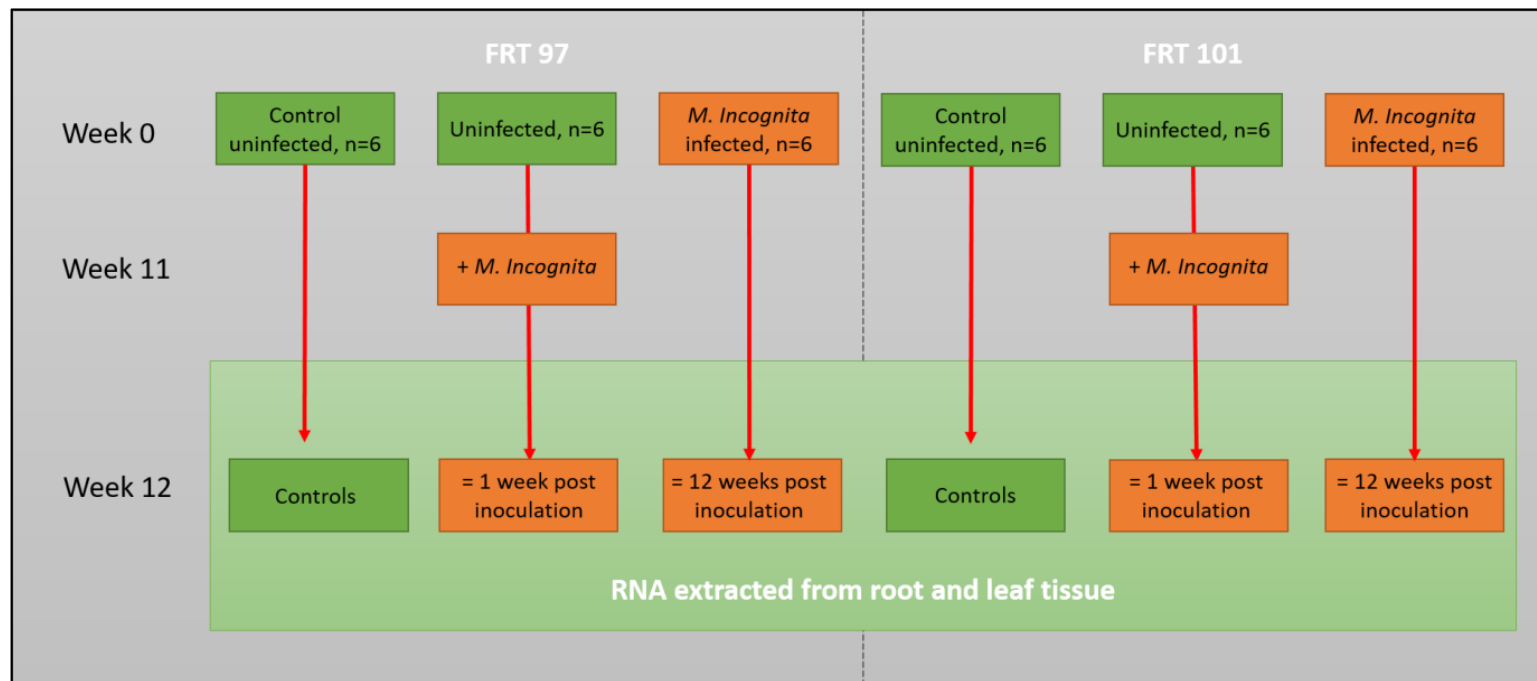


Figure 4. 1. Experimental set up for RNA sequencing of Robusta root and leaf tissue post *M. incognita* infection.

In total 18 plants each of *C. canephora* var. FRT97 and FRT101 were grown together under the same conditions for the twelve-week experiment period. Six plants of FRT97 and FRT101 were each inoculated with 2500 infective stage *M. incognita* juveniles at the start of the experiment (week 0). After eleven weeks, six more plants of each were also inoculated with 2500 infective stage juveniles. After twelve weeks, root and leaf tissue were harvested and stored at -80°C for collection of RNA.

4.2.3 RNA-Seq analysis

Transcriptomic data was prepared and analysed using software provided within Galaxy web service (<https://usegalaxy.org/>). Default options and settings were used for each tool unless stated otherwise. FASTQC (Andrews, 2010) and Trimmomatic (Bolger *et al.*, 2014) were used to check the quality of sequences, trim adapter sequences from paired end reads, and trim poor-quality sequence. Reads were then mapped onto the *C. canephora* reference genome (Denoeud *et al.*, 2014), which was downloaded from Ensembl plants (Yates *et al.*, 2022), using HISAT2 (Kim *et al.*, 2015), and quantification of the number of reads, and associated expression, for each gene was performed using HTSeq-count. Differential expression analysis was performed using DESeq2 (Love *et al.*, 2014) to statistically test for expression differences in normalised read counts for each gene, between treatments and varieties. Genes were considered significantly differentially expressed under the FDR threshold of 0.05. All significant genes were considered and included in subsequent analysis with no fold change threshold.

Coding region sequences (CDS) were retrieved for genes that were significantly differentially expressed between treatments (FDR <0.05) from Ensembl Plants (<https://plants.ensembl.org/>) and Gene Ontology (GO) IDs attributed to each gene using Blast2Go in the software OmicsBox (Götz *et al.*, 2008; <https://www.biobam.com/omicsbox>, 2019). GO terms that were over- or under-represented in a treatment compared to the reference set of expressed genes (all expressed genes across all treatments for each variety) were highlighted in enrichment analysis, using Fisher's exact test (FDR<0.05).

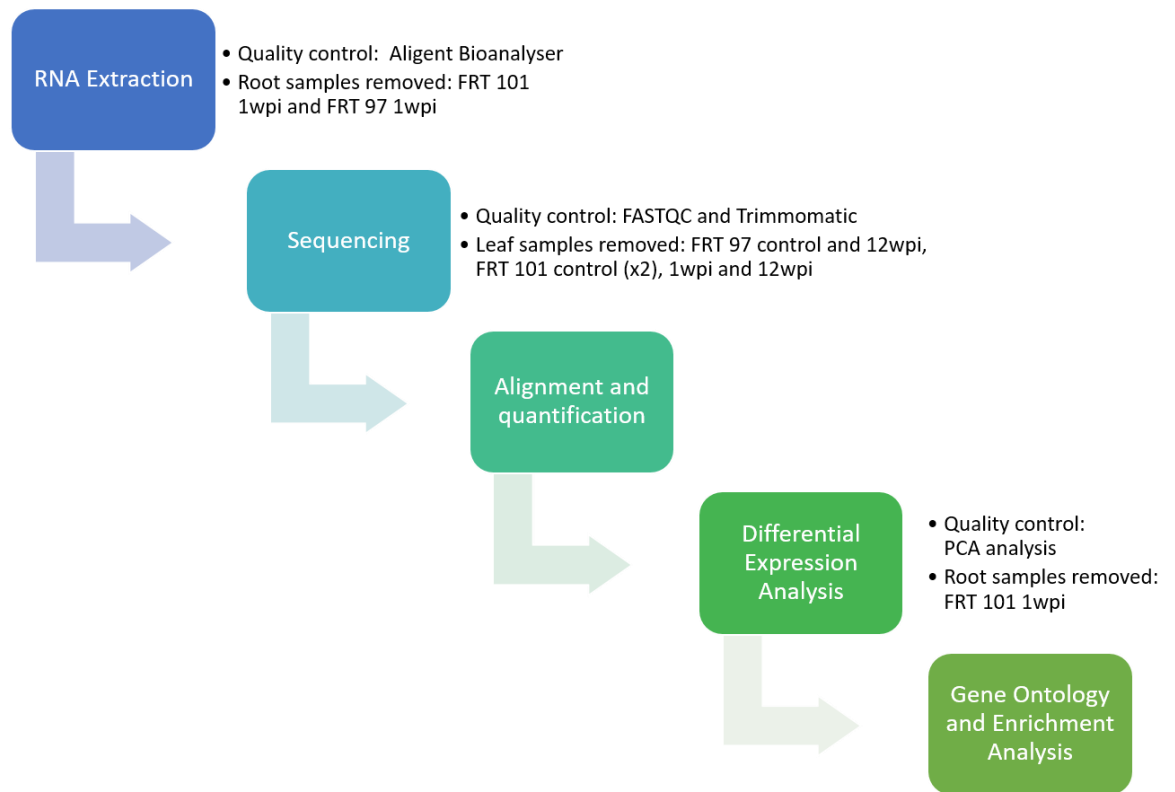


Figure 4. 2. RNA sequencing and analysis workflow.

Robusta, *C. canephora*, varieties FRT97 and FRT101 were inoculated with 2500 infective stage juvenile *M. incognita* for one (1wpi) and twelve (12wpi) weeks before RNA was extracted from root and leaf material. RNA was analysed for concentration and quality using an Agilent bioanalyser. RNA was paired end 150 bp sequenced using the Illumina NovaSeq™ platform and quality was assessed using FASTQC and Trimmomatic. RNA was aligned to the Robusta genome assembly using HISAT2 and quantified using HTSeq-count. Differential expression analysis was performed using DeSeq, which included PCA analysis. Gene ontology and enrichment analysis was performed using OmicsBox and Blast2Go.

4.2.4 Functional analysis of candidate genes using *Arabidopsis thaliana*

Germplasm for *Arabidopsis* cell wall mutants (Table 4.1) were selected from information available from The Arabidopsis Information Resource (TAIR). Mutants were only considered if homozygous for the mutation. Selected mutant seeds were ordered from The European Arabidopsis Stock Centre (NASC).

Arabidopsis seeds were germinated in compost and at approximately two weeks old were transplanted into 3 cm diameter pots in 40 % loam, 40% sand and 20% compost mix. Five cotyledons of the same genotype were transplanted into each pot. When primary roots reached the bottom of the pots, 100 infective stage juvenile *M. incognita* were inoculated into the soil around the root system of each plant for 500 juveniles per pot. Fifteen plants of each mutant were inoculated along with 15 wild type (Col-0) plants. Roots were removed from the soil at 28 days post inoculation, washed and stained with acid fuchsin (Chapter 2.7) to determine nematode number and development stage. A plant was discarded if root was lost or destroyed during washing or staining. A one-way ANOVA and Tukey-post hoc tests were used to determine significant differences in the total number of nematodes between each genotype, and a two-way ANOVA to determine if there was a significantly different proportion of each life stage per genotype.

Table 4. 1. List of cell wall *Arabidopsis thaliana* mutants.

All mutants were generated from Col-0 background.

Accession	Gene	Gene function	NASC Germplasm
AT3G23730.1	<i>Xyloglucan endotransglucosylase 16 (XTH 16)</i>	cell wall biogenesis, xyloglucan metabolic process	N16534
AT2G37090.1	<i>Irregular xylem 9 (IRX9)</i>	glucuronoxylan biosynthetic process, plant-type secondary cell wall biogenesis	N576866
AT5G67230.1	<i>Irregular xylem 14 (IRX14)</i>	glucuronoxylan biosynthetic process, plant-type secondary cell wall biogenesis	N696434
AT5G57560.1	<i>Xyloglucan endotransglucosylase 22 (XTH22)</i>	cell wall-modifying enzyme, cell wall biogenesis, xyloglucan metabolic process	N860818
AT5G62220.1	<i>Golgi apparatus-localized galactosyltransferase 18 (GT18)</i>	protein glycosylation, xyloglucan biosynthetic process	N924359
AT2G45220.1	<i>Pectin methylesterase 17 (PME17)</i>	Pectin remodelling, cell wall modification, pectin catabolic process	N675408

4.3 Results

4.3.1 Physiological response of Robusta to *M. incognita* prior to RNA-Seq

Measurements of height and chlorophyll fluorescence were taken for all plants at the start of the experiment, as infected plants were inoculated, and at the end of the infection period before root and leaf material samples were taken for RNA extraction. This was to ensure the coffee plants had similar responses to the infection of *M. incognita* as reported in Chapter 3. There was, as before, a significant reduction in F_v/F_m after twelve weeks in FRT97 infected with *M. incognita* (post hoc $p < 0.0027$). *M. incognita* caused a mean (\pm SE) decrease in chlorophyll fluorescence of 17.74 (± 3.9) % 12 wpi. There was no difference in chlorophyll fluorescence for both varieties after one week of infection (Figure 4. 3). In contrast to earlier findings, however, there was no significant change in the height of infected plants over the twelve weeks for both FRT97 and FRT101 compared to uninfected controls. Whilst not affecting the growth of the plants tested here, root-knot nematode did still have an impact on the health of FRT97 and so any following transcriptomic differences observed under infection between the two varieties could reveal molecular mechanisms that drive differences in susceptibility and tolerance.

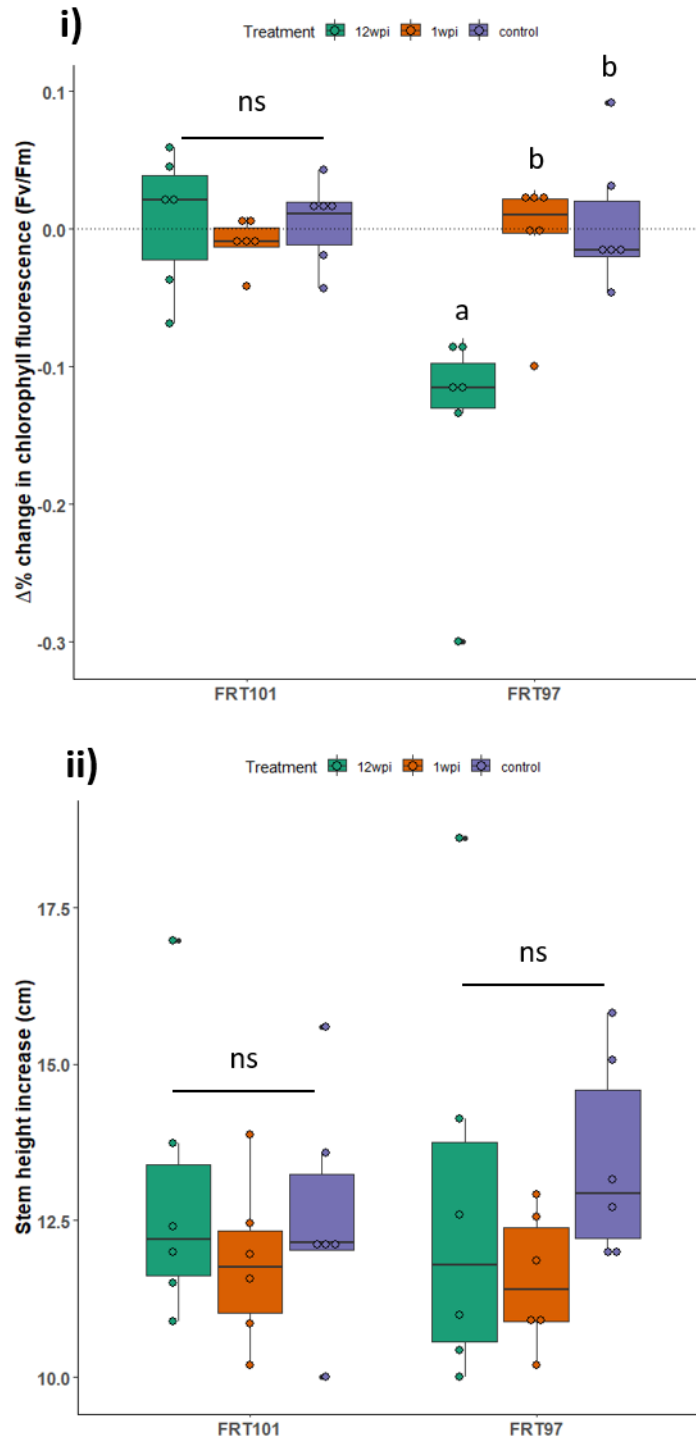


Figure 4. 3. Impact of *M. incognita* infection on photosynthesis in *C. canephora* var. FRT97 and FRT101 prior to RNA-Sequencing.

i) Percentage change, from beginning of trial, in chlorophyll fluorescence (Fv/Fm) of *Coffea canephora* var. FRT97 and FRT101 after one (1wpi) and 12 weeks (12wpi) of inoculation with approx. 2500 infective stage juveniles of *Meloidogyne incognita* (n=6). ii) Stem height change (cm) from beginning of trial of *Coffea canephora* var. FRT97 and FRT101 after one (1wpi) and 12 weeks (12wpi) of inoculation with approx. 2500 infective stage juveniles of *Meloidogyne incognita* (n=6). Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters above boxes show significantly different groups in Tukey's HSD tests ($p < 0.05$) within each variety, following an ANOVA test.

4.3.2 Sequencing of RNA from root and leaf material of Robusta following *M. incognita* infection

To identify the Robusta coffee genes that have altered expression during root-knot nematode infection, an RNA-Seq approach was utilised, using both root and leaf RNA collected from *M. incognita* infected plants, one- and twelve-weeks post inoculation. Six plants were grown as individual replicates so that low quality samples could be discarded without compromising the subsequent data analysis. Once RNA was extracted, one root sample (FRT101 1wpi) was removed due to low quality (RNA integrity number < 6) and two root samples, from two separate plants, were pooled (FRT97 1wpi) due to low RNA concentrations. Examples of root and leaf samples that were deemed good quality to sequence are shown in Figure 4. 4.

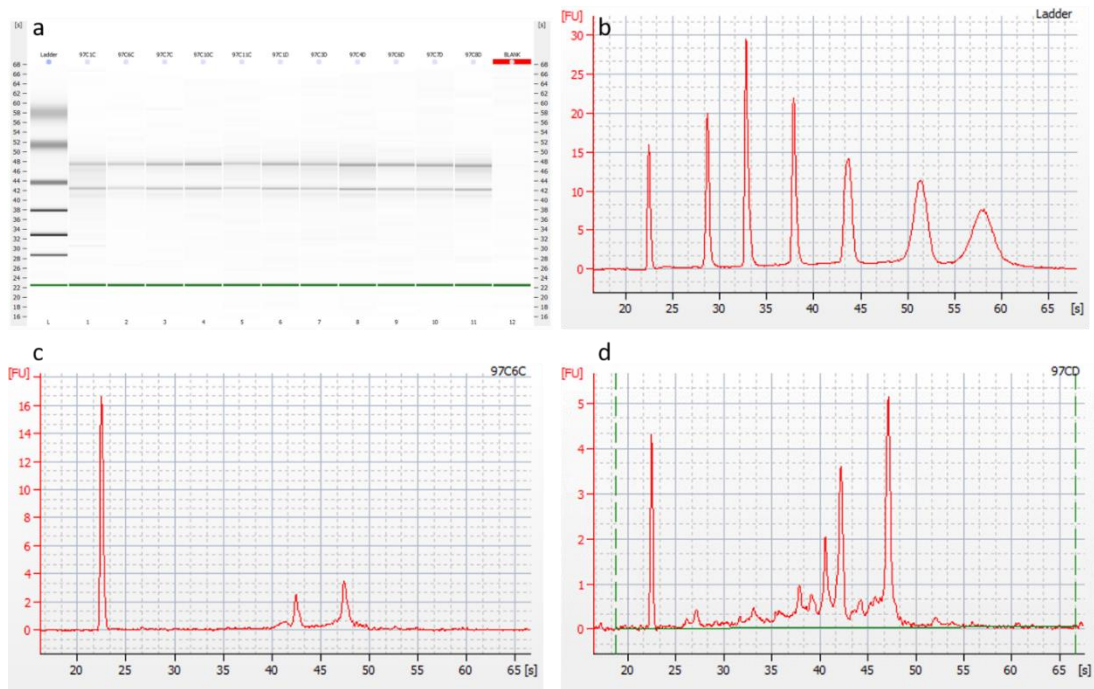


Figure 4. 4 Gel electrophoresis image and electropherograms of Robusta root and leaf RNA.

RNA was analysed using Agilent Bioanalyzer 2100. a) Bands show RNA integrity from *C. canephora* var. FRT97 root RNA as compared to an RNA ladder. b) Electropherogram of RNA ladder as a comparison for RNA integrity for Robusta RNA samples. c) Electropherogram from FRT97 root RNA sample (uninfected control). d) Electropherogram from FRT97 leaf RNA sample (uninfected control).

Once sequenced, each sample yielded over 20 million reads and over 6,000 million bases (Table 4. 2). Further “top-up” sequencing, or resequencing, was performed for all leaf samples as RNA quality and concentration was generally lower. Following further quality control (FASTQC) of the acquired reads, sequences of some leaf samples were discounted from subsequent analysis due to low quality or short reads. The removed samples were one FRT97control and one FRT97 12 wpi, two FRT101 controls, two FRT101 1 wpi and one FRT101 12 wpi. Read data from both varieties and all treatments were mapped directly to the Robusta genome assembly (Denoeud *et al.*, 2014), resulting in relative expression values (counts per million) for 21,971 genes. Overall, reads sequenced from root material mapped well to the Robusta genome assembly, whereas two samples from both FRT97 and FRT101 leaf material had less than 50% unique reads mapped to the assembly. Mapping to *M. incognita* assemblies was attempted, but was unsuccessful, probably due to a much lower concentration of nematode RNA present in samples.

After sequencing, alignment and removal of outliers, the total number of root tissue samples left for each treatment was; FRT97 Controls n= 6, FRT97 1wpi n= 5, FRT97 12wpi n= 6, FRT101 Controls n= 6, FRT101 1wpi n= 4, FRT101 12wpi n= 6. The number of samples leaf tissue in each treatment before analysis was; FRT97 Controls n=5, FRT97 1wpi n=5, FRT97 12wpi n=5, FRT101 Controls n=5, FRT101 1wpi=5, FRT101 12wpi n=5.

Table 4. 2. Sequencing and alignment of RNA from Robusta root and leaf following infection with *M. incognita*.

Robusta varieties were inoculated with 2500 infective stage juvenile *M. incognita* for one (1wpi) and twelve (12wpi) weeks before RNA was extracted from root and leaf material. RNA was paired end 150 bp sequenced using the Illumina NovaSeq™ platform, and aligned to the Robusta genome assembly using HISAT2.

Sample	Treatment_Replicate	# Reads Sequenced	Yield (Million bases)	% Uniquely mapped reads
FRT97 Root	Control_1	20,463,336	6,139	84.04
	Control_2	25,248,874	7,575	83.41
	Control_3	26,427,599	7,928	86.02
	Control_4	26,129,075	7,839	85.79
	Control_5	27,484,556	8,245	87.74
	Control_6	20,444,122	6,133	85.41
	1wpi_1	25,752,609	7,726	79.60
	1wpi_2	27,323,000	8,197	76.67
	1wpi_3	28,270,742	8,481	79.50
	1wpi_4	29,739,284	8,922	78.50
	1wpi_5	20,433,663	6,130	76.81
	12wpi_1	22,474,539	6,742	85.90
	12wpi_2	20,877,146	6,263	83.31
	12wpi_3	20,415,707	6,125	82.38
	12wpi_4	23,648,450	7,095	87.08
	12wpi_5	21,872,967	6,562	86.34
	12wpi_6	20,914,420	6,274	72.01
	FRT101 Root	Control_1	21,083,105	6,325
Control_2		22,065,828	6,620	76.40
Control_3		20,717,686	6,215	77.90
Control_4		23,782,040	7,134	64.56
Control_5		22,185,283	6,656	80.67
Control_6		21,805,444	6,542	81.93
1wpi_1		22,498,831	6,750	80.75
1wpi_2		22,542,507	6,763	78.38
1wpi_3		21,681,664	6,504	78.91
1wpi_4		21,766,654	6,530	81.38
1wpi_5		22,238,238	6,671	81.56
12wpi_1		23,157,850	6,948	77.73
12wpi_2		20,037,061	6,011	78.26
12wpi_3		23,750,640	7,125	82.97
12wpi_4		23,661,078	7,098	83.33
12wpi_5		22,549,226	6,765	82.42
12wpi_6		21,815,253	6,545	81.02

Table 4. 2 Continued. Sequencing and alignment of RNA from Robusta root and leaf following infection with *M. incognita*.

Robusta varieties were inoculated with 2500 infective stage juvenile *M. incognita* for one (1wpi) and twelve (12wpi) weeks before RNA was extracted from root and leaf material. RNA was paired end 150 bp sequenced using the Illumina NovaSeq™ platform, and aligned to the Robusta genome assembly using HISAT2.

Sample	Treatment_Replicate	#Reads Sequenced	Yield (Million bases)	% Uniquely mapped reads
FRT97 Leaf	Control_1	42,494,082	12,748	-
	Control_2	50,953,960	15,286	81.89
	Control_3	38,983,129	11,695	73.74
	Control_4	23,536,980	7,061	45.82
	Control_5	44,430,910	13,329	56.89
	1wpi_1	36,628,104	10,988	82.83
	1wpi_2	40,631,576	12,189	79.6
	1wpi_3	42,250,838	12,675	78.12
	1wpi_4	30,800,179	9,240	75.25
	1wpi_5	33,847,370	10,154	80.45
	12wpi_1	34,090,057	10,227	80.18
	12wpi_2	37,557,056	11,267	-
	12wpi_3	21,334,693	6,400	22.16
	12wpi_4	27,265,473	8,180	80.91
	12wpi_5	37,761,735	11,329	79.64
FRT101 Leaf	Control_1	27,978,621	8,394	78.5
	Control_2	38,203,473	11,461	72.65
	Control_3	35,255,119	10,577	-
	Control_4	44,683,592	13,405	-
	Control_5	34,669,415	10,401	80.26
	1wpi_1	42,660,964	12,798	77.52
	1wpi_2	27,197,891	8,159	-
	1wpi_3	47,096,528	14,129	77.03
	1wpi_4	28,097,259	8,429	76.83
	1wpi_5	31,510,136	9,453	-
	12wpi_1	31,206,757	9,362	72.6
	12wpi_2	62,540,130	18,762	45.46
	12wpi_3	55,633,143	16,690	45.46
	12wpi_4	38,658,523	11,598	-
	12wpi_5	37,033,942	11,110	74.18

4.3.3 Differential expression in Robusta roots under root-knot nematode infection

The root transcriptomes of Robusta FRT97 and FRT101 were first compared whilst not under infection with root-knot nematodes to understand their baseline genetic and transcriptomic variation that then could influence their response to the plant parasitic nematode. Differential expression analysis revealed that 1271 genes had >2-fold higher expression in FRT101 than in FRT97, and 1450 had >2-fold lower expression both with FDR <0.05. Blast2GO was used to associate gene ontology identifiers to each differentially expressed gene to understand the groups and functions of genes that are differentially expressed between the two varieties. Both defence response and plant-type hypersensitive response appeared as some of the most common biological process GO identifiers, whilst integral component of membrane and cell wall were common cellular component GO identifiers in differentially expressed genes (Figure 4. 5).

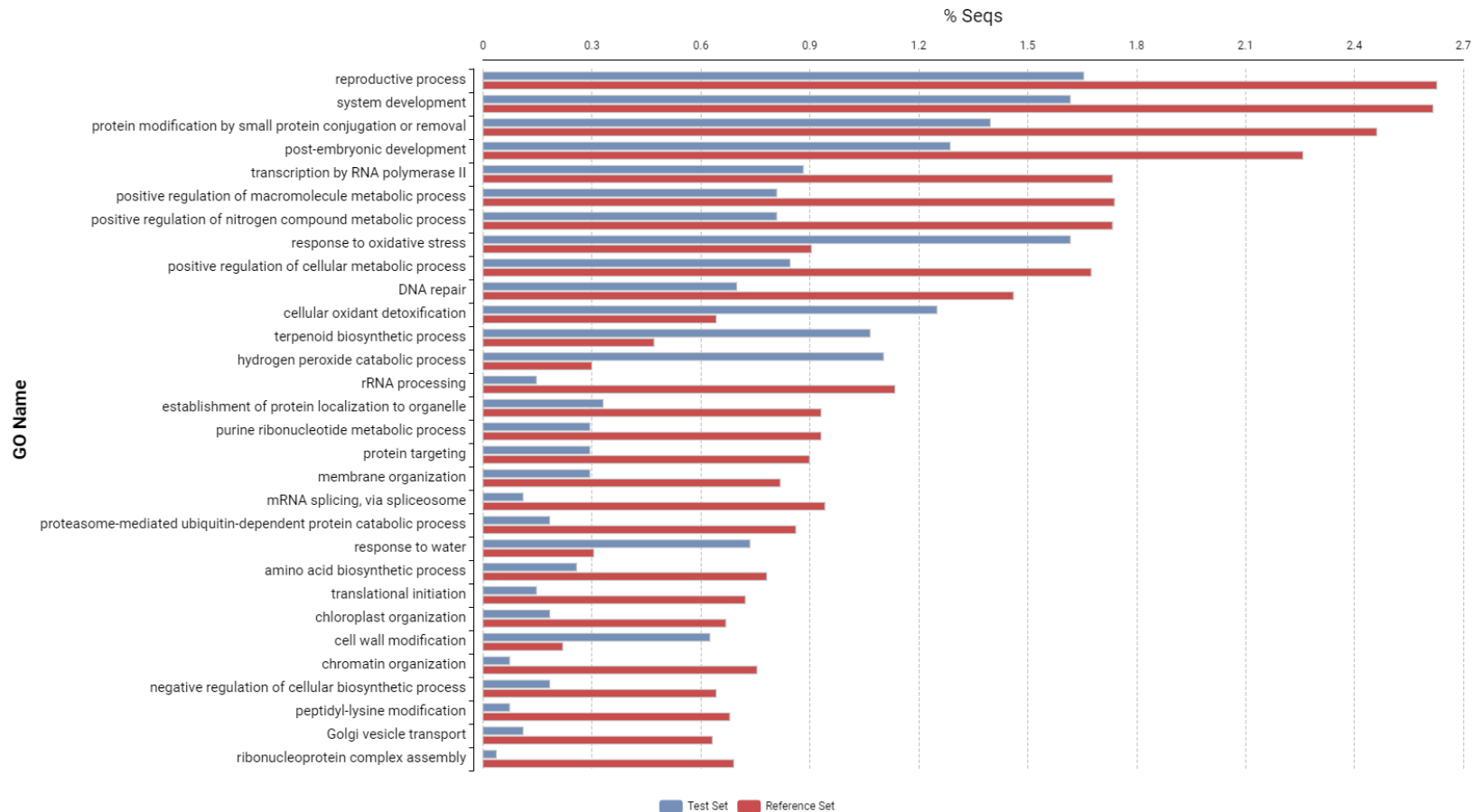


Figure 4. 5. Biological process gene ontology identifiers in differentially expressed genes between root materials of Robusta varieties.

Gene ontology (GO) IDs for biological process, retrieved using Blast2GO, of differentially expressed gene either up- or down-regulated ($FDR < 0.05$, $\text{LogFC} > 2$) between uninfected *C. canephora* var. FRT97 and FRT101. Test set represent the percentage of differentially expressed genes with corresponding GO ID to compare against the reference set of all genes in the robusta transcriptome.

As differential expression analysis was performed on either FRT97 or FRT101 including *M. incognita* infected treatments, PCA analysis highlighted one FRT101 1wpi sample as an outlier and this was removed from subsequent analysis.

The differential expression analysis showed that different sets of genes in the two varieties were regulated in response to *M. incognita* infection, and within a variety, there were also differences in gene regulation between short term and long-term infection. Most notable is the large number of genes that are differentially expressed following infection and how that differs between Robusta varieties. In FRT97, 76.25% of genes mapped from the Robusta genome assembly were up- or down-regulated from samples of the 1wpi treatment, which decreased to 51.96% in the 12wpi treatment (Figure 4. 7). In contrast, only 19.35% of FRT101 genes were up- or down-regulated 1wpi, but a much greater proportion (45.08%) of genes were differentially expressed after 12 weeks of infection (Figure 4. 8).

Comparing the differential expression between the two varieties reveals some interesting contrasts. Many genes in FRT97 were not altered by *M. incognita* between 1 wpi and 12 wpi treatments. For example, only 178 genes were down regulated 1wpi but then up in 12 wpi (Figure 4. 6). However, FRT101 had 455 genes that were up-regulated 1wpi but down-regulated at 12 wpi, and 315 genes down-regulated at 1wpi that were then up-regulated at 12 wpi. The expression of more genes in FRT101 therefore, appears to change in response to the level of infection of plant parasitic nematodes, as more nematodes will have developed between one and twelve weeks. Strikingly, many genes showed contrasting alterations in expression between the two Robusta varieties following 12 weeks of *M. incognita* infection. 1989 genes were up-regulated in FRT97 but down-regulated in FRT101, and 1420 genes were up-regulated in FRT101 but down-regulated in FRT97; the number of these genes are four to six times greater than the number of genes that show the same up- or down-regulation following infection in both varieties. Each variety, therefore, have antithetical transcriptional responses that could drive to the observed differences in susceptibility and tolerance.

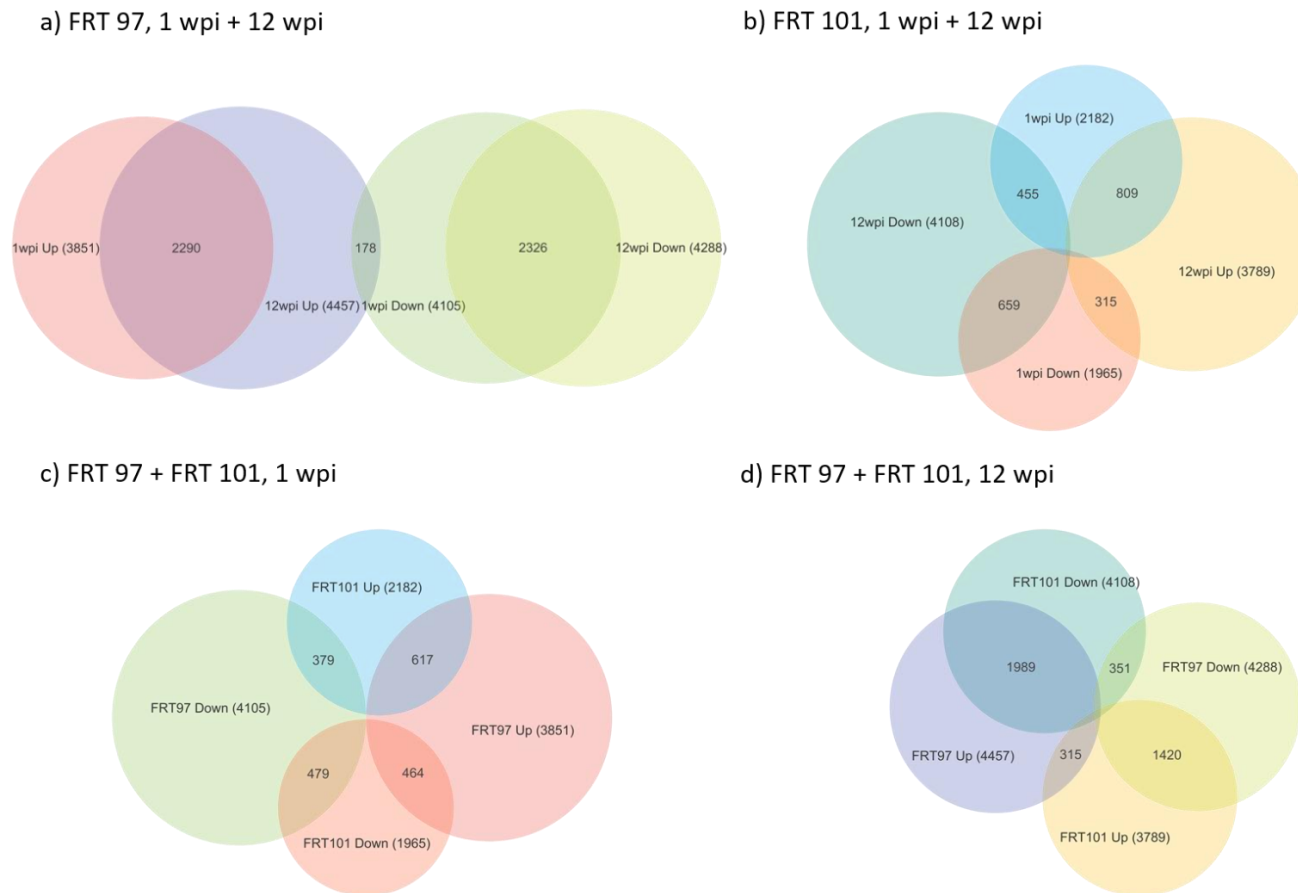


Figure 4. 6. Comparisons of genes differentially expressed in roots of *C. canephora* var. FRT97 and FRT101 at one- and twelve-weeks post inoculation of *M. incognita*.

Venn diagrams are proportional to the number of genes between treatments, split between those up and down regulated. FDR < 0.05 was used as the cut off for genes that were significantly differentially expressed. Bracketed numbers represent individual sections or genes that have no overlap into other treatments.

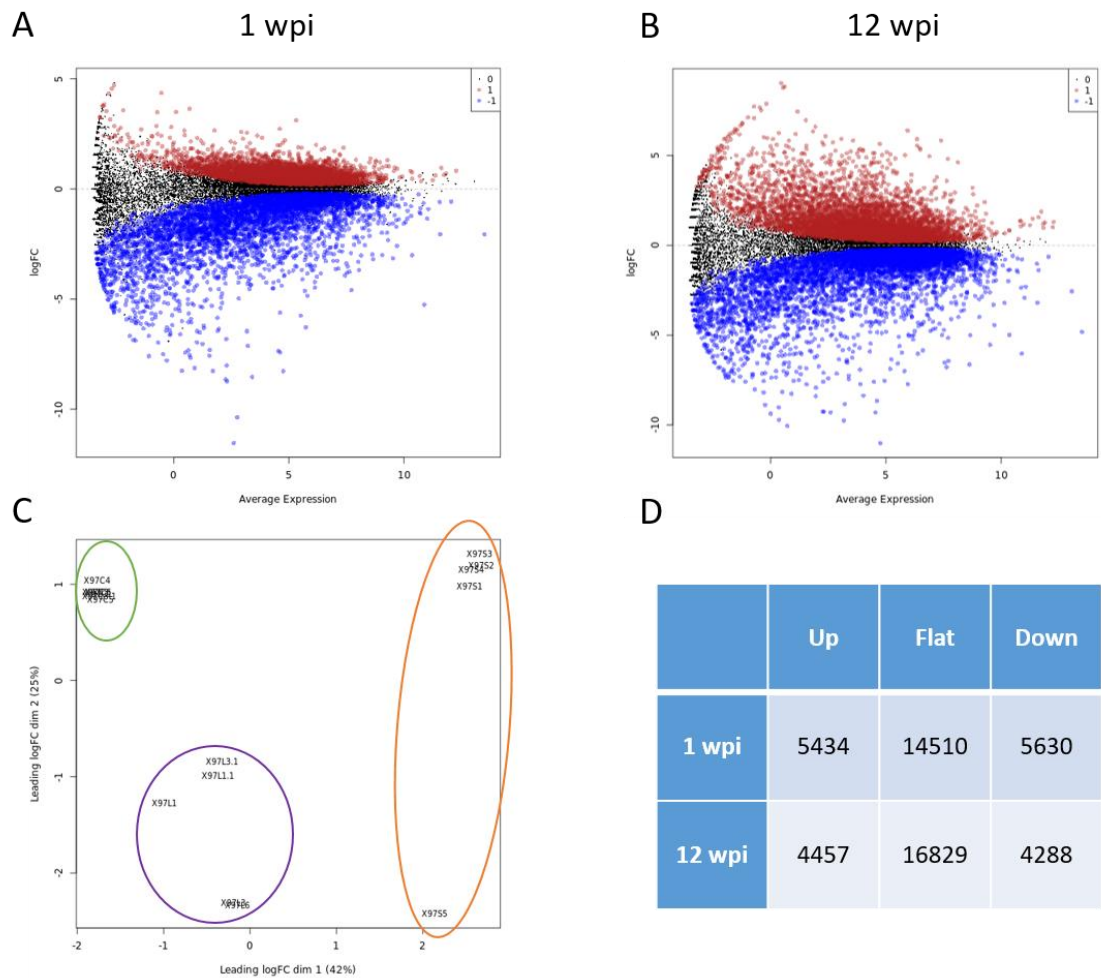


Figure 4. 7. Differentially expressed genes in the root transcriptome of *M. incognita* infected *C. canephora* var. FRT97.

Differential gene expression analysis, comparing *Coffea canephora* var. FRT97 (n=4-6) root material post inoculation with approx. 2500 *Meloidogyne incognita* infective juveniles for either one week (1wpi) or twelve weeks (12wpi) to uninfected controls. A) Log fold change and average expression of genes between uninfected controls and plants infected for one week. B) Log fold change and average expression of genes between uninfected controls and plants infected for twelve weeks. A & B; Sig. up-regulated = red, Sig. down-regulated = blue. C) Principal component analysis of sequences from all FRT97 root samples. Samples within the same treatment are circled together (Green = control, orange = 1wpi and purple = 12wpi) D) Number of genes significantly differentially expressed (FDR<0.05) between each of the treatments.

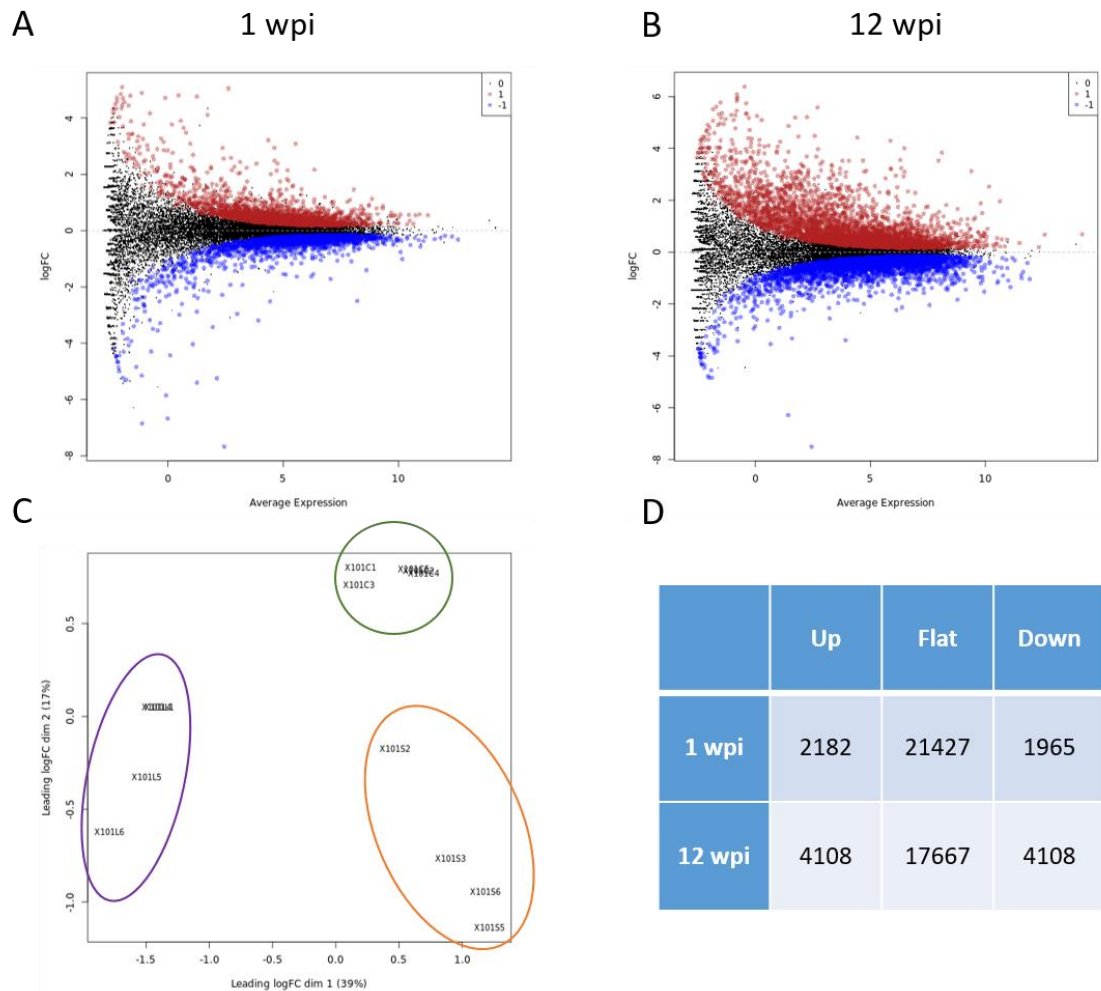


Figure 4. 8. Differentially expressed genes in the root transcriptome of *M. incognita* infected *C. canephora* var. FRT101.

Differential gene expression analysis, comparing *Coffea canephora* var. FRT101 (n=4-6) root material post inoculation with approx. 2500 *Meloidogyne incognita* infective juveniles for either one week (1wpi) or twelve weeks (12wpi) to uninfected controls. A) Log fold change and average expression of genes between uninfected controls and plants infected for one week. B) Log fold change and average expression of genes between uninfected controls and plants infected for twelve weeks. A & B; Sig. up-regulated = red, Sig. down-regulated = blue. C) Principal component analysis of sequences from all FRT101 root samples. Samples within the same treatment are circled together (Green = control, orange = 1wpi and purple = 12wpi) D) Number of genes significantly differentially expressed (FDR<0.05) between each of the treatments.

Genes that showed the highest fold changes in response to nematode infection for each variety are listed in Tables 4.3-4.6 (FDR <0.05). A notable downregulated gene from FRT97 that had a notable annotation from *A. thaliana* homologues was a root hair defective 3 protein. This gene, involved in endoplasmic reticulum (ER) stress response, had a large log fold change of -9.02, compared to uninfected controls, after one week infection with *M. incognita*. Also down regulated after one week infection in FRT97 was a LRR receptor-like serine/threonine-protein kinase (Log FC = -8.81 versus uninfected control). After 12 weeks of infection with *M. incognita*, genes which were down regulated had homologs to the *Arabidopsis* peroxisomal NAD-malate dehydrogenase 1 (PMDH1), a disease resistance protein RGA4 (-3.84) and a putative leucine-rich repeat receptor-like protein kinase (Log FC = -3.78). The putative leucine-rich repeat receptor-like protein kinase was also upregulated in FRT101 following infection after 12 weeks compared uninfected controls (Log FC = 2.98).

Up regulated genes in FRT97 included a disease resistance protein (disease resistance protein 206, log FC = -11.23) which would indicate a hypersensitive response which was absent in FRT101, as well as a putative Xyloglucan galactosyltransferase KATAMARI1 homolog (Log FC = 10.31). Twelve weeks of infection with *M. incognita* caused the upregulation of many genes in FRT97. Most notably this included a xyloglucan galactosyltransferase (Log FC = 10.37) and epoxide hydrolase (Log FC = 8.54) which was also down regulated in FRT101 12wpi (Log FC = -2.48).

In FRT101, highly differentially expressed genes with annotations from *A. thaliana* homologues included a basic helix-loop-helix (bHLH) DNA-binding superfamily protein (Log FC = -5.1) and the ethylene-responsive transcription factor ERF114 (Log FC = -5.05), both down regulated 1wpi and neither having differential expression caused by infection in FRT97. Pectinesterase inhibitor 33 (PME33) was also down regulated 12wpi (Log FC = -6.08) but up regulated in FRT97. Glucan endo-1,3-beta-glucosidase 8 was up regulated 12wpi (Log FC = 4.84). Putative G-type lectin S-receptor-like serine/threonine-protein kinase RLK1 was had a large differential in expression in FRT101 12wpi (Log FC = 12.48) but was also slightly upregulated in FRT97 12wpi (Log FC = 3.24).

These changes in gene expression suggest a strong response to *M. incognita* infection at a molecular level in both Robusta varieties, leading to a wide array of cellular and physiological changes expected from the parasitism of root-knot nematodes. This is especially highlighted by the many genes involved in defence and hypersensitive responses also showed opposite differential expression following infection with *M. incognita*.

Table 4. 3. Differentially expressed genes in *C. canephora* FRT97 root one-week post inoculation with *M. incognita*.

Top 10 significantly differentially expressed (FDR<0.05, ns = not significant) genes by log₂ fold change (both up and down) in *C. canephora* var. FRT97 root one week post *M. incognita* infection compared to uninfected controls. Descriptions of genes derive from annotations from Blast2GO descriptions. The last column represents the fold change in FRT97 in comparison to FRT101 (e.g. +ve fold change = Higher expression in FRT97).

Gene stable ID	LogFC	FDR	Description	LogFC in FRT101 1wpi	LogFC in FRT97 vs FRT101 (uninfected)
GSCOC_T00030122001	-9.02	1.41E-07	Protein of Unknown Function (DUF239)	ns	-8.66
GSCOC_T00026550001	-8.81	4.04E-08	Putative Root hair defective 3 GTP-binding	ns	-8.51
GSCOC_T00019035001	-8.76	3.6E-08	Hypothetical protein~ unknown_gene	ns	-4.88
GSCOC_T00014062001	-7.87	0.000122	Putative Probable LRR receptor-like serine/threonine-protein kinase	ns	-3.02
GSCOC_T00024362001	-7.72	5.91E-05	Hypothetical protein~ unknown_gene	4.84146	-7.29
GSCOC_T00026058001	-7.65	5.62E-07	Hypothetical protein~ At1g62810	ns	-7.36
GSCOC_T00027054001	-7.51	8.47E-06	Beta-fructofuranosidase, insoluble isoenzyme CWINV1	ns	-3.62
GSCOC_T00018841001	-7.43	1.13E-09	Putative late blight resistance protein homolog R1A-6	ns	-5.56
GSCOC_T00031441001	-7.10	2.08E-05	serine-type endopeptidase inhibitors	ns	-2.86
GSCOC_T00039570001	11.01	0.001332	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00018827001	10.05	1.63E-05	Putative pentatricopeptide repeat-containing protein At3g49142~ PCMP-H77	ns	10.01
GSCOC_T00019668001	9.75	5.49E-05	Hypothetical protein~ unknown_gene	ns	8.24
GSCOC_T00023117001	9.71	0.003675	Chlorophyll a-b binding protein 21, chloroplastic~ CAB21	ns	ns
GSCOC_T00018610001	9.37	0.000913	Hypothetical protein~ At2g22780	ns	10.16
GSCOC_T00039569001	9.30	0.000862	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00042768001	9.30	2.89E-05	Disease resistance response protein 206~ PI206	ns	6.45
GSCOC_T00023174001	9.27	0.002448	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00041471001	9.25	0.00212	Putative Protein of unknown function DUF1677	ns	ns

Table 4. 4. Differentially expressed genes in *C. canephora* FRT97 root twelve weeks post inoculation with *M. incognita*.

Top 10 significantly differentially expressed (FDR<0.05, ns = not significant) genes by log fold change (both up and down) in *C. canephora* var. FRT97 roots twelve weeks post *M. incognita* infection compared to uninfected controls. Descriptions of genes derive from annotations from Blast2GO descriptions. The last column represents the fold change in FRT97 in comparison to FRT101 (e.g. +ve fold change = Higher expression in FRT97).

Gene stable ID	logFC	Description	FC in FRT101	LogFC in FRT97 vs FRT101 (uninfected)
GSCOC_T00031456001	-4.72	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00031918001	-4.55	Putative uncharacterized protein~ unknown_gene	ns	ns
GSCOC_T00042494001	-4.36	Anamorsin homolog	ns	-7.60
GSCOC_T00031433001	-4.34	Putative Uncharacterized protein At4g06598	ns	ns
GSCOC_T00009676001	-3.84	Putative disease resistance protein RGA4	ns	ns
GSCOC_T00015513001	-3.78	Putative Probable leucine-rich repeat receptor-like protein kinase At5g49770	2.89	ns
GSCOC_T00041112001	-3.76	Cysteine-rich repeat secretory protein 55	ns	ns
GSCOC_T00033327001	-3.64	Hypothetical protein~ unknown_gene	2.70	-4.72
GSCOC_T00037738001	-3.57	Putative Probable transcriptional regulator RABBIT EARS	ns	-2.92
GSCOC_T00042768001	11.53	Disease resistance response protein 206	-5.97	6.45
GSCOC_T00019050001	10.37	Putative Xyloglucan galactosyltransferase KATAMARI1 homolog	ns	4.66
GSCOC_T00023174001	8.73	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00041471001	8.65	Putative Protein of unknown function DUF1677	ns	ns
GSCOC_T00031571001	8.54	Putative Epoxide hydrolase 2~ EPHX2	-2.48	5.17
GSCOC_T00039570001	8.27	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00036031001	8.27	Putative UDP-glycosyltransferase 74F2	-1.84	4.65
GSCOC_T00037975001	8.26	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00012028001	8.13	Putative Protein of unknown function DUF295	ns	ns

Table 4. 5. Differentially expressed genes in *C. canephora* FRT101 root one-week post inoculation with *M. incognita*.

Top 10 significantly differentially expressed (FDR<0.05, ns = not significant) genes by log fold change (both up and down), in *C. canephora* var. FRT101 roots one week post *M. incognita* infection compared to uninfected controls. Descriptions of genes derive from annotations from Blast2GO descriptions. The last column represents the fold change in FRT101 in comparison to FRT97 (e.g. +ve fold change = Higher expression in FRT101).

Gene stable ID	LogFC	Description	LogFC in FRT97	LogFC in FRT101 vs FRT97 (uninfected)
SCOC_T00042257001	-5.100047	Putative basic helix-loop-helix (bHLH) DNA-binding superfamily protein	ns	ns
GSCOC_T00027275001	-5.051339	Hypothetical protein~ ERF114	ns	-7.44
GSCOC_T00041538001	-4.896618	Putative unknown protein	4.26	-3.23
GSCOC_T00021661001	-4.85066	(R)-mandelonitrile lyase-like	ns	-2.27
GSCOC_T00037897001	-4.806818	Putative F-box/FBD/LRR-repeat protein	-3.78	3.79
GSCOC_T00032589001	-4.78929	unknown protein	ns	ns
GSCOC_T00034097001	-4.759683	Peroxidase 44	ns	-5.2
GSCOC_T00007293001	-4.724199	Putative Miraculin	ns	-4.67
GSCOC_T00026905001	-4.719773	Lignin-forming anionic peroxidase	ns	-3.42
GSCOC_T00037923001	2.12493	Hypothetical protein~ unknown_gene	ns	2.25
GSCOC_T00040894001	4.06843	Hypothetical protein~ unknown_gene	ns	ns
GSCOC_T00016611001	3.3195	Putative Major facilitator superfamily protein~ unknown_gene	ns	ns
GSCOC_T00013843001	2.8669	Putative Glutamate receptor 2.7~ GLR2.7	ns	2.23
GSCOC_T00016041001	2.28565	Predicted protein~ ALMT13	ns	ns
GSCOC_T00016560001	2.1927	Putative B3 domain-containing protein At3g19184	ns	2.61
GSCOC_T00023928001	1.92981	NAC domain-containing protein 90	ns	-1.18
GSCOC_T00029969001	1.92214	Cucumisin~ XSP1	ns	-3.98
GSCOC_T00020175001	1.79378	Putative myb domain protein 45~ LAF1	ns	2.49

Table 4. 6. Differentially expressed genes in *C. canephora* FRT101 root twelve weeks post inoculation with *M. incognita*.

Top 10 significantly differentially expressed (FDR<0.05, ns = not significant) genes by log fold change (both up and down) in *C. canephora* var. FRT101 roots twelve weeks post *M. incognita* infection compared to uninfected controls. Descriptions of genes derive from annotations from Blast2GO descriptions. The last column represents the fold change in FRT97 in comparison to FRT101 (e.g. +ve fold change = Higher expression in FRT97).

Gene stable ID	LogFC	Description	LogFC in FRT97	LogFC in FRT101 vs FRT97 (uninfected)
GSCOC_T00017218001	-6.382067	Putative UPF0481 protein At3g47200	ns	ns
GSCOC_T00020318001	-6.089473	Probable pectinesterase/pectinesterase inhibitor 33	3.08	-3.88
GSCOC_T00042768001	-5.969849	Disease resistance response protein 206	11.53	-6.45
GSCOC_T00030409001	-5.908099	Acid beta-fructofuranosidase	4.09	-3.15
GSCOC_T00029781001	-5.827809	Putative Disease resistance-responsive (dirigent-like protein) family protein	6.46	-4.10
GSCOC_T00036582001	-5.776241	Laccase-17	ns	-3.82
GSCOC_T00014035001	-5.741015	Isoflavone 2'-hydroxylase	ns	-4.34
GSCOC_T00025506001	-5.607527	Putative disease resistance protein At1g50180	4.16	-5.06
GSCOC_T00029969001	-5.584313	Cucumisin	3.48	-3.98
GSCOC_T00036471001	7.49799	Putative G-type lectin S-receptor-like serine/threonine-protein kinase RLK1	3.24	ns
GSCOC_T00029621001	6.29375	Hypothetical protein~ unknown_gene	5.30	ns
GSCOC_T00023180001	4.85131	Putative WEB family protein At5g55860	ns	ns
GSCOC_T00037554001	4.84376	Glucan endo-1,3-beta-glucosidase 8	ns	4.06
GSCOC_T00007319001	4.77574	Probable 6-phosphogluconolactonase 4, chloroplastic	5.19	ns
GSCOC_T00036644001	4.56341	Putative uncharacterized protein~ BHLH30	2.37	7.61
GSCOC_T00029361001	4.47005	(3S,6E)-nerolidol synthase 1, chloroplastic	ns	ns
GSCOC_T00023160001	4.34985	Putative Probable LRR receptor-like serine/threonine-protein kinase	ns	ns
GSCOC_T00027126001	4.34371	Putative Beta-glucosidase 11~ BGLU11	ns	ns

4.3.3.1 Enrichment analysis of differentially expressed genes in infected Robusta root

With the large number of genes differentially expressed between treatments, Gene Ontology and Enrichment analysis was used to highlight some common biological processes that are transcriptionally affected by *M. incognita* infection. Whilst there were still many significantly enriched GO terms represented by differentially expressed genes, there were some notable differences in the representation of GO terms between the two Robusta varieties under infection.

Genes with the GO ID Plant-type hypersensitive response, known to be an immediate response by plants in recognition of pathogens, were over-represented in down-regulated genes of FRT97 1wpi. This suggests an active repression of severe immune responses to *M. incognita* such as cell death. The same GO ID, however, was over-represented in up regulated genes of FRT101 12wpi, suggesting a strong immune response to the parasite (Figure 4. 9). These genes will be actively involved in the recognition of pathogens and triggering immune responses.

Common GO IDs between treatments and varieties were also those relating to the cell wall, including cell wall and secondary cell wall biogenesis, plant-type cell wall organization, and xylan/xyloglucan biosynthetic processes. The specific regulation of these genes was again in contrast between FRT97 and FRT101. In FRT97 there is a significant over-representation in up-regulated genes one and 12wpi, but a significant over-representation in down-regulated genes in FRT101 one and 12wpi (Figure 4. 10). The cell wall presents a physical barrier to the invasion and migration of plant parasitic nematodes and is thus considered a constitutive defence (Malinovsky *et al.*, 2014). However, alterations of the cell wall composition through transcriptional changes could also be an important induced defence against plant parasitic nematodes, as changes in lignin, cellulose, and hemicellulose content could lead to more rigid and firm cell walls making migration more difficult, or mitigate the damage nematodes cause to cells through cell wall degradation enzymes (Hamann, 2012).

Differentially expressed genes that were represented by these GO IDs are listed in Table 4.7 and Table 4.8, and whilst not an exhaustive list of those that might be of interest due to their differential expression in this study, they should be considered of interest and importance for tolerance and susceptibility in the interaction between root-knot nematodes and Robusta coffee.

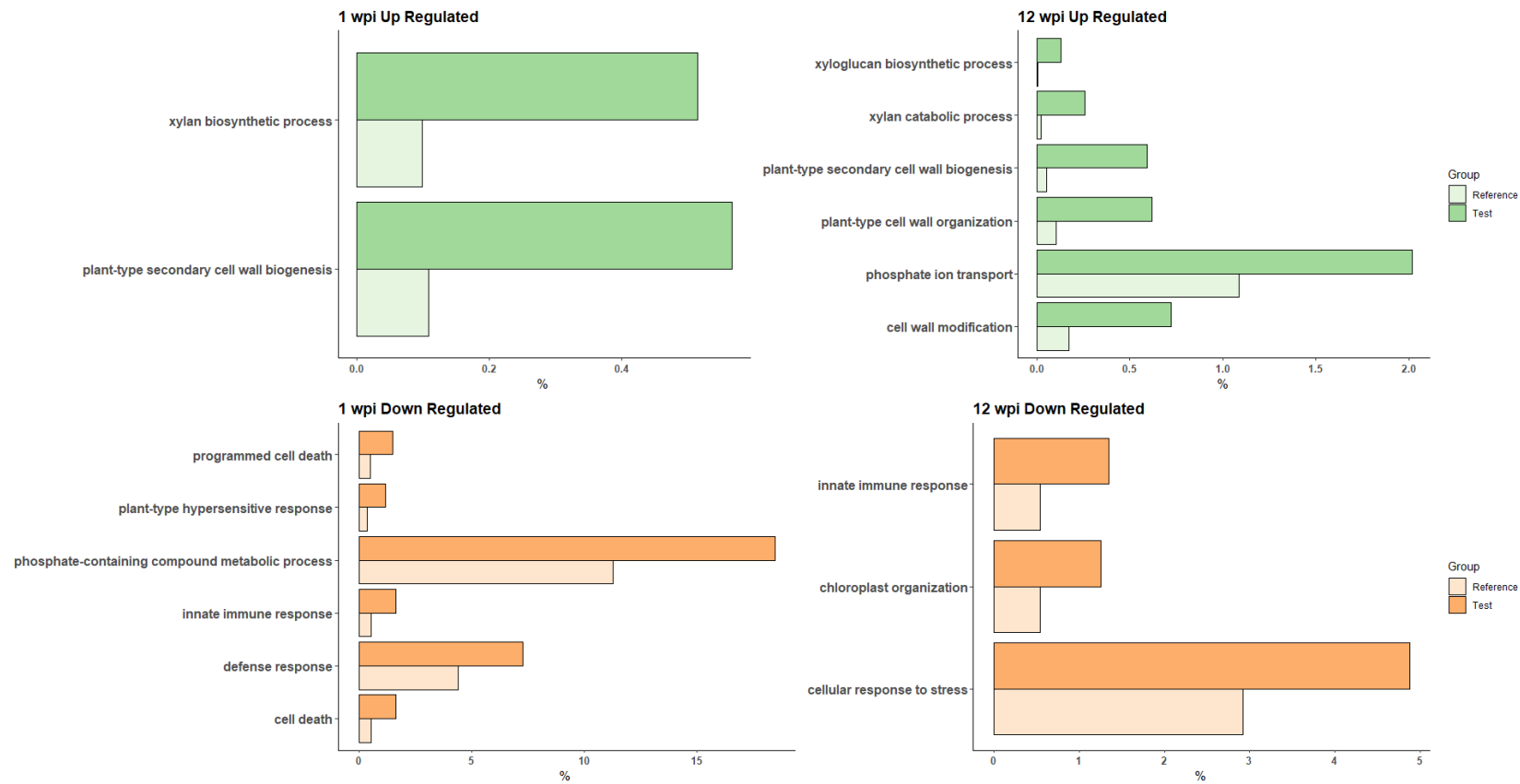


Figure 4. 9. Enriched biological processes in the transcriptome of *M. incognita* infected *C. canephora* FRT97 root.

Selected enriched biological processes, listed by Gene Ontology ID, in *C. canephora* var. FRT97 one week (1wpi) and 12 weeks (12wpi) post inoculation treatments of approx. 2500 infective stage *M. incognita*. GO IDs are presented as percentage representation in inoculated treatments (test) versus uninfected controls to show biological pathways which are significantly enriched, or over-represented by Fishers exact test (FDR <0.05).

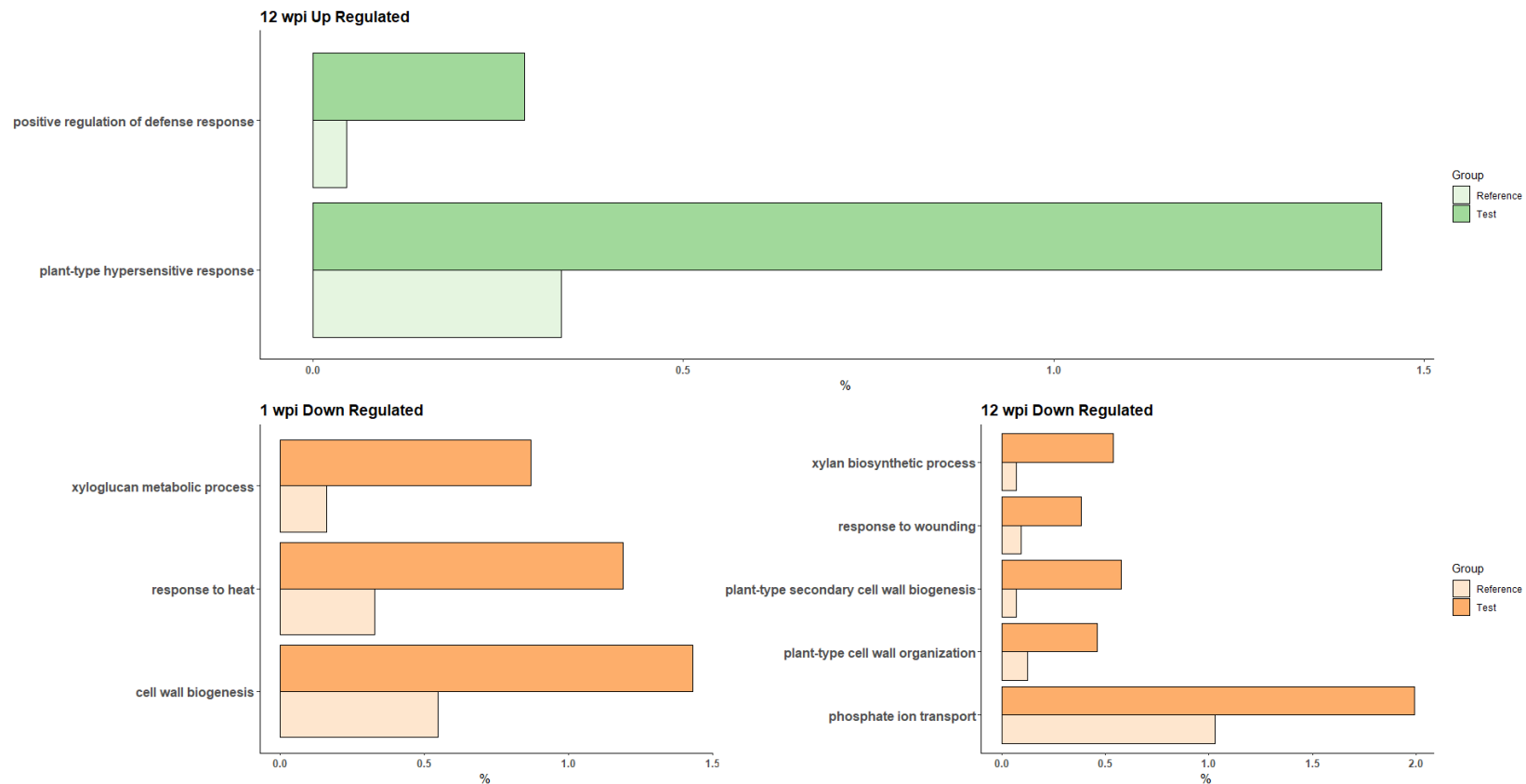


Figure 4. 10. Enriched biological processes in the transcriptome of *M. incognita* infected *C. canephora* FRT101 root.

Selected enriched biological processes, listed by Gene Ontology ID, in *C. canephora* var. FRT101 one week (1wpi) and 12 weeks (12wpi) post inoculation treatments of approx. 2500 infective stage *M. incognita*. GO IDs are presented as percentage representation in inoculated treatments (test) versus uninfected controls to show biological pathways which are significantly enriched, or over-represented by Fishers exact test (FDR <0.05).

Table 4. 7. Differentially expressed genes, within over-represented gene ontology groups, in roots of *C. canephora* FRT97 one week post inoculation with *M. incognita*.

Genes listed are selected genes that were in over-represented biological processes groups highlighted in Gene Ontology enrichment analysis (FDR < 0.05) and also showed significant differential expression in infected *C. canephora* FRT97 compared to uninfected controls. Differential expression in var. FRT101 under infection is also shown, ns = not significantly differentially expressed.

Gene Stable ID	LogFC	Description	Go ID	LogFC in FRT101
GSCOC_T00022619001	-1.68	cell growth defect factor 1	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00016739001	-2.18	recognition of peronospora parasitica 11	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00042125001	-1.57	lazarus 1	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00031057001	-2.15	cel-activated resistance 1	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00031055001	-1.7	disease resistance protein (cc-nbs-lrr class)	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00031669001	-2.07	recognition of peronospora parasitica 11	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00037420001	-1.94	nb-arc domain-containing disease resistance protein	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00027281001	-2.68	disease resistance protein (cc-nbs-lrr class)	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00042702001	-2.96	recognition of peronospora parasitica 11	Defence response, Plant-type hypersensitive response	-1.79
GSCOC_T00037456001	-2.49	nb-arc domain-containing disease resistance protein	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00001444001	-1.96	disease resistance protein (cc-nbs-lrr class)	Defence response, Plant-type hypersensitive response	-0.73
GSCOC_T00013392001	-1.56	syg1	Defence response, Plant-type hypersensitive response	ns
GSCOC_T00036829001	0.40	expansin-like b1	Regulation of defence response	-0.48
GSCOC_T00034272001	1.07	glucuronoxylan methyltransferase 1	Plant-type secondary cell wall biogenesis, xylan biosynthetic process	-1.14
GSCOC_T00023089001	0.80	reduced wall acetylation 3	Plant-type secondary cell wall biogenesis, xylan biosynthetic process	-1.07
GSCOC_T00039936001	0.69	irregular xylem 14	Plant-type secondary cell wall biogenesis, xylan biosynthetic process	-0.59
GSCOC_T00023679001	4.03	arabidopsis fasciclin-like arabinogalactan-protein 11	Plant-type secondary cell wall biogenesis	-1.16
GSCOC_T00023646001	2.80	irregular xylem 9	Plant-type secondary cell wall biogenesis	-2.58
GSCOC_T00022345001	1.10	irregular xylem 10	Plant-type secondary cell wall biogenesis	-0.68
GSCOC_T00015030001	2.92	glucuronoxylan methyltransferase1	Plant-type secondary cell wall biogenesis, xylan biosynthetic process	-2.95
GSCOC_T00013996001	3.12	cellulose synthase 8	Plant-type secondary cell wall biogenesis, Defence response to fungi	-2.34
GSCOC_T00019274001	3.80	arabinogalactan methylesterase	Plant-type secondary cell wall biogenesis, xylan biosynthetic process	-1.87
GSCOC_T00015137001	1.70	pectin methylesterase 17	Plant-type secondary cell wall biogenesis, xylan biosynthetic process	ns

Table 4. 8. Differentially expressed genes, within over-represented gene ontology groups, in roots of *C. canephora* FRT101 twelve weeks post inoculation of *M. incognita*.

Genes listed are selected genes that were in over-represented biological processes groups highlighted in Gene Ontology enrichment analysis (FDR < 0.05) and also showed significant differential expression in infected *C. canephora* FRT101 compared to uninfected controls. FRT97 under infection for 12 weeks is also shown, ns = not significantly differentially expressed.

Gene Stable ID	LogFC	Description	GO ID	LogFC in FRT97
GSCOC_T00015727001	1.7	Disease resistance protein (CC-NBS-LRR class)	Plant type hypersensitive response	ns
GSCOC_T00042125001	1.3	LAZARUS 1	Plant type hypersensitive response	-0.26
GSCOC_T00015475001	1.69	NB-ARC domain-containing disease resistance protein	Plant type hypersensitive response	ns
GSCOC_T00042068001	-3.09	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 22	Plant-type secondary cell wall biogenesis	ns
GSCOC_T00041401001	-1.88	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 16	Plant-type secondary cell wall biogenesis	4.29
GSCOC_T00042703001	-4.02	IRREGULAR XYLEM 14	Plant-type secondary cell wall biogenesis	-2.99
GSCOC_T00039936001	-1.81	GLUCURONOXILAN METHYLTRANSFERASE1	Plant-type secondary cell wall biogenesis	0.93
GSCOC_T00015030001	-2.95	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 32	Plant-type secondary cell wall biogenesis	3.38

4.3.4 Differential expression in Robusta leaves under root-knot nematode infection

Differential gene expression analysis between uninfected FRT97 and FRT101 leaf material revealed a large difference in the expression of genes between the two varieties. In FRT101 leaves, there were 2197 genes up-regulated and 1997 down-regulated in comparison to FRT97 leaf material. Acquiring GO terms for these differentially expressed genes showed that many of these genes were present in integral components of membrane and the nucleus, similar to results from root differential expression analysis, and also involved in defence response, response to stimulus and plant-type hypersensitive response (Figure 4. 11).

When performing differential expression analysis including the *M. incognita* infected treatments, PCA analysis on FRT101 samples showed a larger amount of variation in the 12-wpi treatment, compared to controls and one wpi samples. This was not observed in FRT97 analysis, as samples within each treatment clustered tightly together and separately from other treatments, indicating low variation between samples within a treatment. The high variance in FRT101 12wpi samples may be due to the low percentage of reads that mapped onto the reference Robusta transcriptome for two of the four samples (45.46% each; Table 4. 2).

Root-knot nematode infection again caused a large number of genes to be differentially expressed within the leaf material of Robusta var. FRT97. Over 6000 genes were differentially expressed at 1wpi and 12wpi respectively when compared to uninfected controls (Figure 4. 12). However, only a small number of genes were differentially expressed in FRT101 following infection; one up regulated and nine down regulated 1wpi (Figure 4. 13). The small amount of significant differential expression may be due to poor sequencing reads from all FRT101 samples and the large variation between samples for the 12-wpi treatment. The significantly differentially expressed genes in FRT101 following infection are listed in Table 4. 9. A homologue of the RNA-mediated anti-viral immunity gene *dicer-2* was significantly down-regulated, as well as a homologue of a gene encoding phospholipase D alpha 4, involved in cellular responses to nutrient starvation and cellular growth. Only one gene was significantly up-regulated 1wpi, a homologue of *A. thaliana* gene encoding a Mannose-binding lectin superfamily protein.

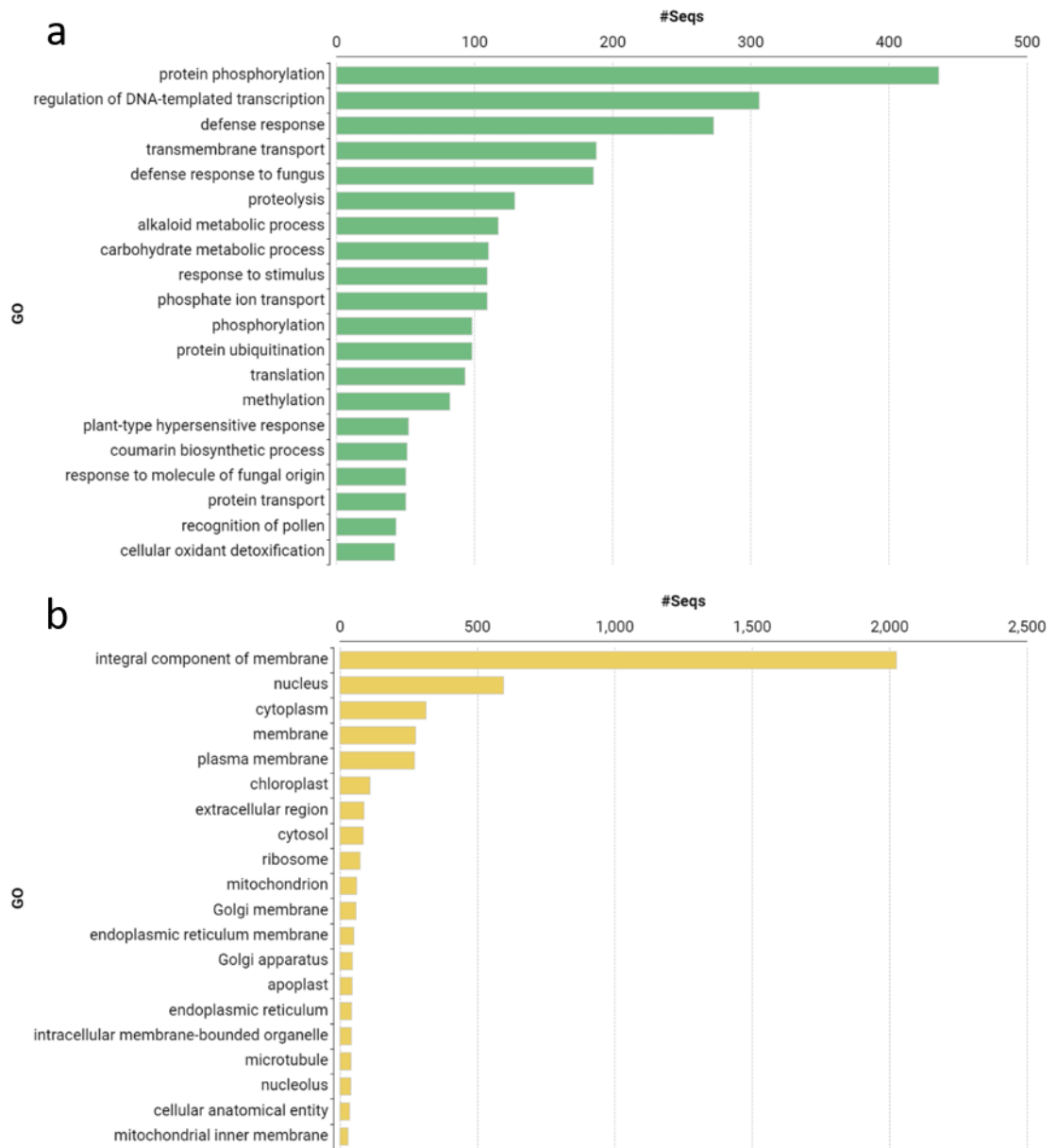


Figure 4. 11. Gene ontology identifiers in differentially expressed genes between leaves of Robusta varieties.

The top 20 most common gene ontology (GO) IDs retrieved using Blast2GO, of differentially expressed genes (FDR<0.05) between uninfected *C. canephora* var. FRT97 and FRT101 leaf material. GO IDs are listed as (a) biological process and (b) cellular component.

Table 4. 9. Differentially expressed genes in leaves of Robusta coffee var. FRT101 following *M. incognita* infection.

All genes significantly differentially expressed (FDR<0.05, ns = not significant), and their log fold change, in *C. canephora* var. FRT101 leaf one (1wpi) and twelve (12wpi) weeks post-*M. incognita* infection compared to uninfected controls. Descriptions of genes derive from annotations from Blast2GO descriptions.

Gene stable ID	Treatment	LogFC	Description	LogFC in FRT97
GSCOC_T000218 73001	1wpi	-2.94	phospholipase D alpha 4	-1.51
GSCOC_T000190 66001	1wpi	-2.76	Uncharacterized conserved protein UCP015417	1.09
GSCOC_T000343 98001	1wpi	-2.69	Rhamnogalacturonate lyase family protein	0.99
GSCOC_T000174 80001	1wpi	-2.14	dicer-like 2	-1.44
GSCOC_T000283 08001	1wpi	-1.28	eukaryotic translation initiation factor 3G1	-0.26
GSCOC_T000247 36001	1wpi	2.17	Mannose-binding lectin superfamily protein	2.32
GSCOC_T000243 00001	12wpi	-2.95	alternative oxidase 1B	ns
GSCOC_T000174 80001	12wpi	-2.66	dicer-like 2	-1.74
GSCOC_T000218 73001	12wpi	-2.54	phospholipase D alpha 4	-1.73
GSCOC_T000190 66001	12wpi	-2.33	Uncharacterized conserved protein UCP015417	0.47
GSCOC_T000160 28001	12wpi	-2.21	Phenazine biosynthesis PhzC/PhzF protein	0.62
GSCOC_T000412 62001	12wpi	-1.60	NAD(P)-binding Rossmann-fold superfamily protein	0.19
GSCOC_T000408 24001	12wpi	-1.54	RING/U-box superfamily protein	-1.42
GSCOC_T000386 88001	12wpi	-1.38	BTB/POZ domain-containing protein	0.26
GSCOC_T000283 08001	12wpi	-1.19	eukaryotic translation initiation factor 3G1	-0.28
GSCOC_T000167 58001	12wpi	-0.81	Nucleic acid-binding, OB-fold-like protein	ns
GSCOC_T000259 40001	12wpi	1.68	pyruvate orthophosphate dikinase	0.60
GSCOC_T000207 79001	12wpi	2.87	amino acid transporter 1	1.06
GSCOC_T000295 74001	12wpi	3.03	UDP-Glycosyltransferase superfamily protein	-1.34
GSCOC_T000373 41001	12wpi	3.22	NAD(P)-binding Rossmann-fold superfamily protein	0.54

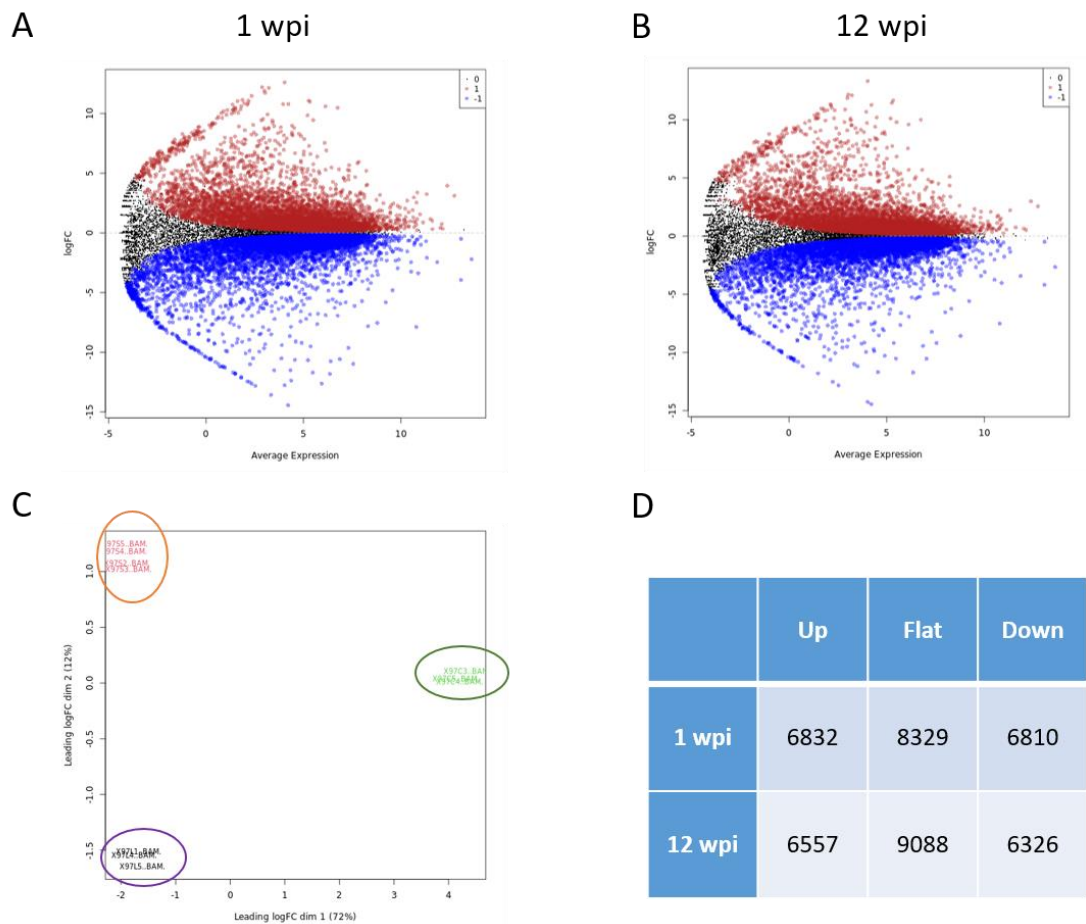


Figure 4.12. Differentially expressed genes in the transcriptome of *M. incognita* infected *C. canephora* var. FRT97 leaf tissue.

Differential gene expression analysis, comparing *Coffea canephora* var. FRT97 (n=4) leaf material post inoculation with approx. 2500 *Meloidogyne incognita* infective juveniles for either one week (1wpi) or twelve weeks (12wpi) to uninfected controls. A) Log fold change and average expression of genes between uninfected controls and plants infected for one week. B) Log fold change and average expression of genes between uninfected controls and plants infected for twelve weeks. A & B; Sig. up-regulated = red, Sig. down-regulated = blue. C) Principal component analysis of sequences from all FRT97 leaf samples. Samples within the same treatment are circled together (Green = control, orange = 1wpi and purple = 12wpi) D) Number of genes significantly differentially expressed (FDR<0.05) between each of the treatments.

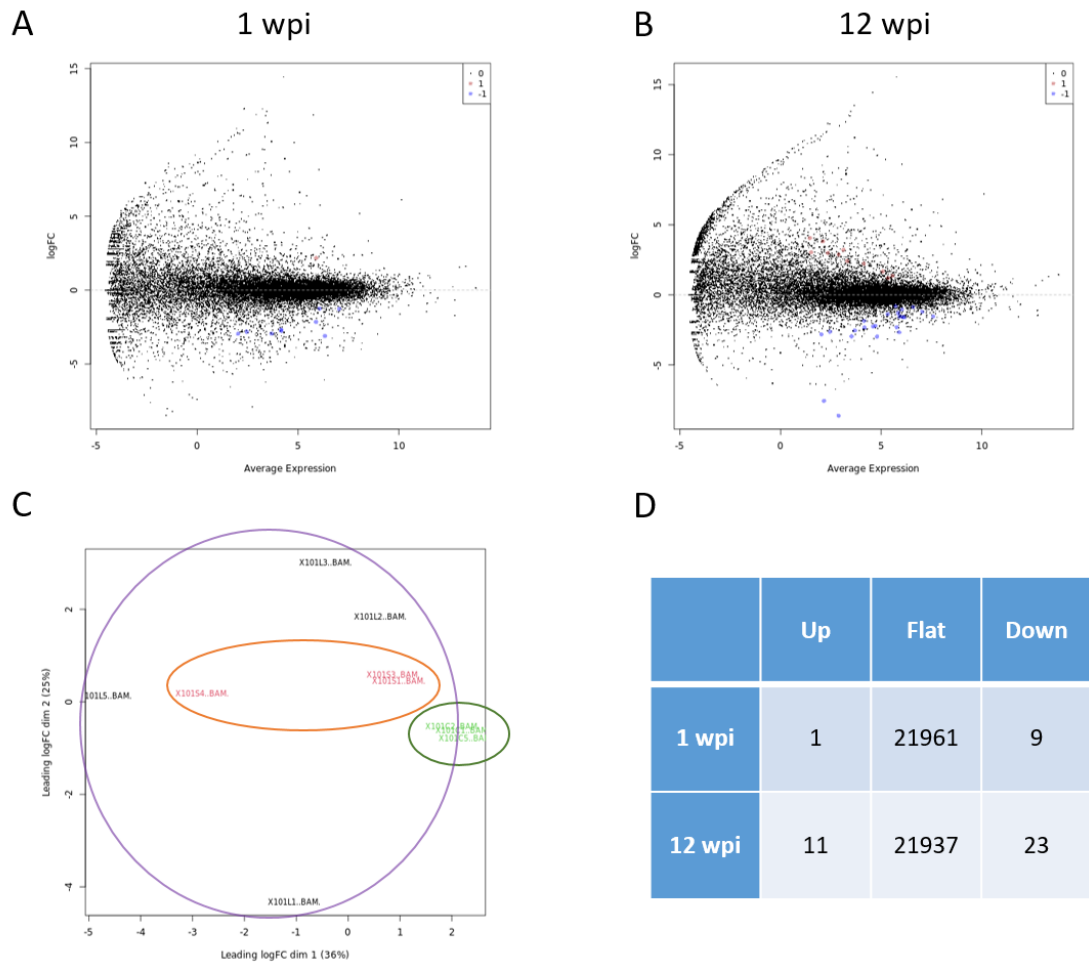


Figure 4. 13. Differentially expressed genes in the transcriptome of *M. incognita* infected *C. canephora* var. FRT101 leaf.

Differential gene expression analysis, comparing *Coffea canephora* var. FRT101 (n=3-4) leaf material post inoculation with approx. 2500 *Meloidogyne incognita* infective juveniles for either one week (1wpi) or twelve weeks (12wpi) to uninfected controls. A) Log fold change and average expression of genes between uninfected controls and plants infected for one week. B) Log fold change and average expression of genes between uninfected controls and plants infected for twelve weeks. A & B; Sig. up-regulated = red, Sig. down-regulated = blue. C) Principal component analysis of sequences from all FRT101 leaf samples. Samples within the same treatment are circled together (Green = control, orange = 1wpi and purple = 12wpi) D) Number of genes significantly differentially expressed (FDR<0.05) between each of the treatments.

4.3.4.1 Enrichment analysis of differentially expressed genes in leaves of infected Robusta plants

Due to the large amount of differential expression observed in FRT97 leaves, a gene ontology approach was again used to understand broader transcriptional changes occurring following root-knot nematode infection. Enrichment analysis of gene ontology groups showed a significant over-representation of genes involved in defence response to fungus and plant-type hypersensitive response that were significantly down-regulated in the transcriptome of Robusta FRT97 leaves from plants infected with *M. incognita* (Figure 4. 14). These were the only over-represented gene ontology groups that were down-regulated at both 1wpi and 12wpi. Significantly differentially expressed genes in these groups included orthologues to receptor kinase like protein Xa21, receptor-like protein EIX2 and the putative late blight resistance protein homolog R1A-3 (Table 4. 10).

After 1wpi, genes involved in photosynthesis and light harvesting were up-regulated in leaves of infected FRT97 plants, as well as those associated with xenobiotic transport. However, no genes sharing the same GO ID were over-represented in the transcriptome of FRT97 leaf tissue 12wpi compared to uninfected controls. Significantly differentially expressed genes in these groups shared homology to a chlorophyll a-b binding protein 13, photosystem I chlorophyll a/b-binding protein 5 and a large number of detoxification proteins (Table 4. 10, Table 4. 11). Changes in photosynthesis and light harvesting regulation in FRT97 concurs well with the decreased chlorophyll fluorescence seen in FRT97 after 12 weeks of infection with *M. incognita*, and the transport of detoxification proteins may be in response pathogen recognition in roots.

Up-regulated genes involved in phosphate ion transport were significant enriched both 1wpi and 12wpi (Figure 4. 14). There was also a significant enrichment of cellular carbohydrate metabolic processing genes up-regulated 12wpi. The below ground infection of *M. incognita*, both short and long term, induces transcriptomic changes in the leaf tissue of FRT97, potentially for the movement and processing of carbohydrates and nutrients.

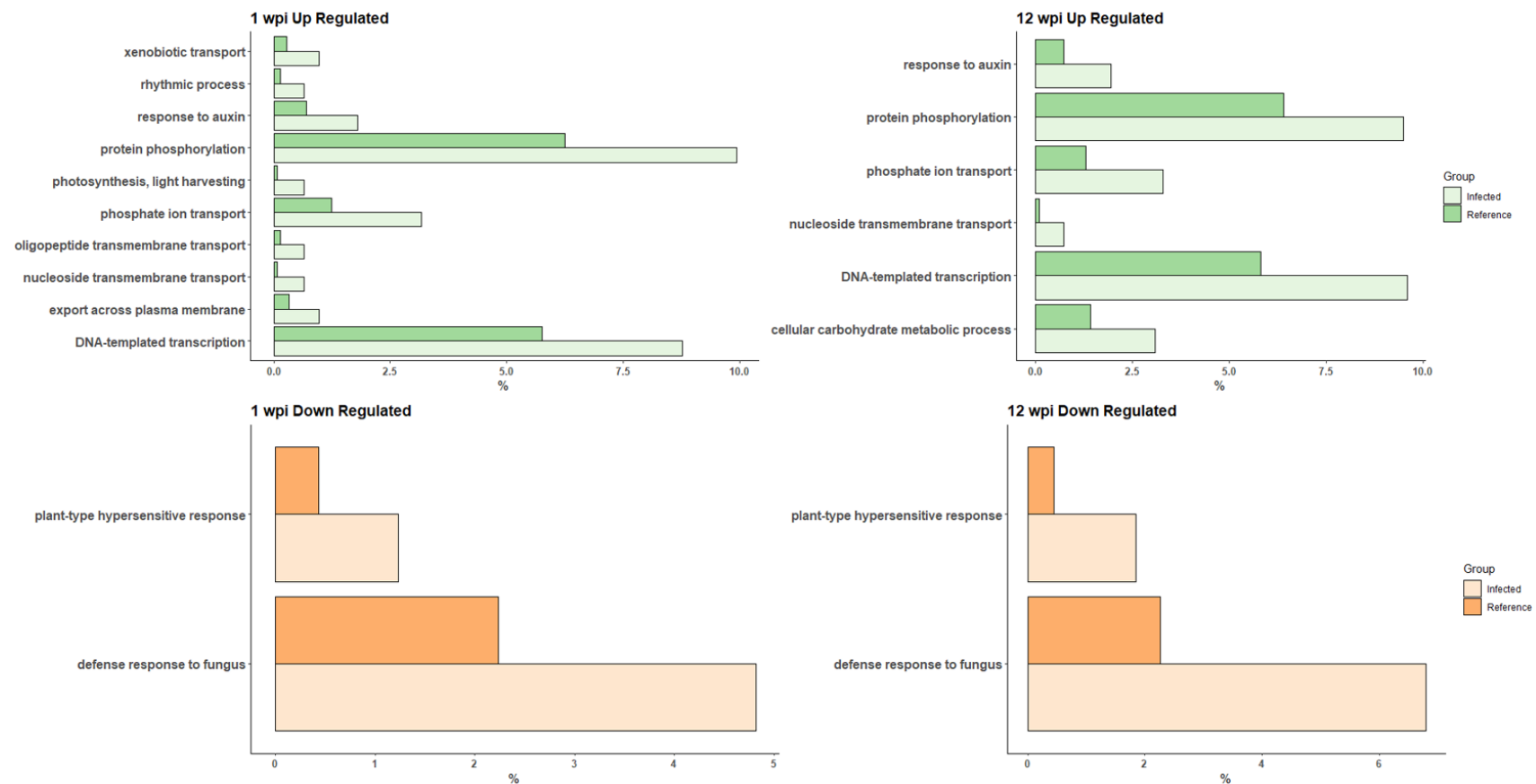


Figure 4. 14. Gene ontology groups over-represented in leaves of *M. incognita* infected FRT97 plants.

Selected enriched biological processes, listed by Gene Ontology ID, in *C. canephora* var. FRT97 leaves one week (1wpi) and 12 weeks (12wpi) post inoculation treatments of approx. 2500 infective stage *M. incognita*. GO IDs are presented as percentage representation in inoculated treatments (test) versus uninfected controls to show biological pathways which are significantly enriched, or over-represented by Fishers exact test (FDR <0.05).

Table 4. 10. Differentially expressed genes in Robusta FRT97 leaves 1 week post inoculation with *M. incognita*.

All genes were significantly differentially regulated (FDR<0.05) in infected plants versus uninfected controls. Descriptions and GO attributes were generated by BLAST2GO. Not all sig. dif. expressed genes, with corresponding GO names, are listed. All genes listed were not sig. dif. expressed in leaves of infected var. FRT101 compared to uninfected controls.

Gene stable ID	Description	GO Name	LogFC
GSCOC_T00042692001	receptor kinase-like protein Xa21	Defence response to fungus	-12.5224
GSCOC_T00020765001	receptor kinase-like protein Xa21	Defence response to fungus	-10.6538
GSCOC_T00018049001	receptor-like protein EIX2	Defence response to fungus	-9.19643
GSCOC_T00018046001	receptor-like protein EIX2	Defence response to fungus	-8.64739
GSCOC_T00027449001	receptor-like protein EIX1	Defence response to fungus	-8.34917
GSCOC_T00015704001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-10.4022
GSCOC_T00020642001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-7.78526
GSCOC_T00018841001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-6.8526
GSCOC_T00037414001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-4.8281
GSCOC_T00037453001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-4.82397
GSCOC_T00021155001	chlorophyll a-b binding protein 13, chloroplastic	Photosynthesis, light harvesting	0.634381
GSCOC_T00004154001	protein DETOXIFICATION 42-like	Xenobiotic transport	2.006566
GSCOC_T00014205001	protein DETOXIFICATION 46, chloroplastic-like	Xenobiotic transport	0.276909
GSCOC_T00025153001	protein DETOXIFICATION 43	Xenobiotic transport	1.2692
GSCOC_T00023279001	protein DETOXIFICATION 44, chloroplastic	Xenobiotic transport	1.428571
GSCOC_T00041021001	protein DETOXIFICATION 45, chloroplastic isoform X1	Xenobiotic transport	0.726252

Table 4. 11. Differentially expressed genes in Robusta FRT97 leaves 12 weeks post inoculation with *M. incognita*

All genes were significantly differentially regulated (FDR<0.05) in infected plants versus uninfected controls. Descriptions and GO attributes were generated by BLAST2GO. Not all sig. dif. expressed genes, with corresponding GO names, are listed. All genes listed were not sig. dif. expressed in leaves of infected var. FRT101 compared to uninfected controls.

Gene stable ID	Description	GO Name	LogFC
GSCOC_T00015704001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-10.4022
GSCOC_T00020642001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-7.78526
GSCOC_T00018841001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-6.8526
GSCOC_T00037414001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-4.8281
GSCOC_T00037453001	putative late blight resistance protein homolog R1A-3	Plant-type hypersensitive response	-4.82397
GSCOC_T00042692001	receptor kinase-like protein Xa21	Defence response to fungus	-12.5224
GSCOC_T00020765001	receptor kinase-like protein Xa21	Defence response to fungus	-10.6538
GSCOC_T00018049001	receptor-like protein EIX2	Defence response to fungus	-9.19643
GSCOC_T00018046001	receptor-like protein EIX2	Defence response to fungus	-8.64739
GSCOC_T00027449001	receptor-like protein EIX1	Defence response to fungus	-8.34917
GSCOC_T00034966001	photosystem I chlorophyll a/b-binding protein 5, chloroplastic	Photosynthesis, light harvesting	0.489327
GSCOC_T00014205001	protein DETOXIFICATION 46, chloroplastic-like	Xenobiotic transport	0.276909
GSCOC_T00041021001	protein DETOXIFICATION 45, chloroplastic isoform X1	Xenobiotic transport	0.726252
GSCOC_T00023279001	protein DETOXIFICATION 44, chloroplastic	Xenobiotic transport	1.428571
GSCOC_T00023280001	protein DETOXIFICATION 44, chloroplastic-like isoform X1	Xenobiotic transport	1.872326
GSCOC_T00004154001	protein DETOXIFICATION 42-like	Xenobiotic transport	2.006566

4.3.5 Functional analysis of genes of interest using *Arabidopsis thaliana* as a homologous system

Six genes of interest were selected which were highly differentially expressed between the roots of infected FRT97 and FRT101, all of which have gene ontology classifications relating to cell wall regulation (Table 4. 12). *Arabidopsis thaliana* lines carrying homozygous mutations for these genes were infected with *M. incognita* for further understanding of the role that these genes have in the root-knot nematode and host plant interaction.

Fewer nematodes were able to infect and develop in five of the *A. thaliana* mutants. There was no significant difference in the number of nematodes in the roots of *xth16* plants compared to the wild type controls four weeks post infection. *xth22* (mean = 5.44, SE = 1.08), *irx9* (mean = 4.14, SE = 0.47), *irx14* (mean = 3.64, SE = 0.60), *pme17* (mean = 5.42, SE = 0.53), and *gt18* (mean = 4.75, SE = 0.55) mutants, however, all had significantly fewer nematodes in the roots compared to the wild-type Col-0 (mean = 9.6, SE = 0.85) ($F_{6,76} = 17.192$, $p < 0.001$; Figure 4. 15). Furthermore, both *irx9* and *gt18* had many plants that had no infection with any *M. incognita* juveniles. The mutation and disruption of these genes appeared to have a strong effect on the invasion ability of *M. incognita*.

When comparing the number of nematodes at each life stage between the genotypes, there was no difference between the proportions of each life stage compared to the wild type (Figure 4. 16). This suggests that those nematodes able to establish parasitism in the roots of the mutants then developed normally at the same rate as those in the wild type roots.

Table 4. 12. Gene expression changes in cell wall related genes in Robusta FRT97 and FRT101 following *M. incognita* infection.

Gene	Gene stable ID	<i>A. thaliana</i> orthologue	% Similarity to Arabica orthologue	LogFC FRT97 (12wpi)	LogFC FRT101 (12wpi)	LogFC FRT101-FRT97 (uninfected)
Xyloglucan endotransglucosylase 16 (<i>XTH16</i>)	GSCOC_T00042703001	AT3G23730.1	84.5	-2.99	1.39	ns
Irregular xylem 9 (<i>IRX9</i>)	GSCOC_T00023646001	AT2G37090.1	100	-2.80	2.58	4.07
Irregular xylem 14 (<i>IRX14</i>)	GSCOC_T00039936001	AT5G67230.1	86.3	-0.60	0.69	1.04
Xyloglucan endotransglucosylase 22 (<i>XTH22</i>)	GSCOC_T00041401001	AT5G57560.1	84.1	ns	3.09	ns
Golgi apparatus-localized galactosyltransferase 18 (<i>GT18</i>)	GSCOC_T00019050001	AT5G62220.1	86.2	-10.37	ns	4.67
Pectin methylesterase 17 (<i>PME17</i>)	GSCOC_T00015137001	AT2G45220.1	85.2	-1.71	ns	ns

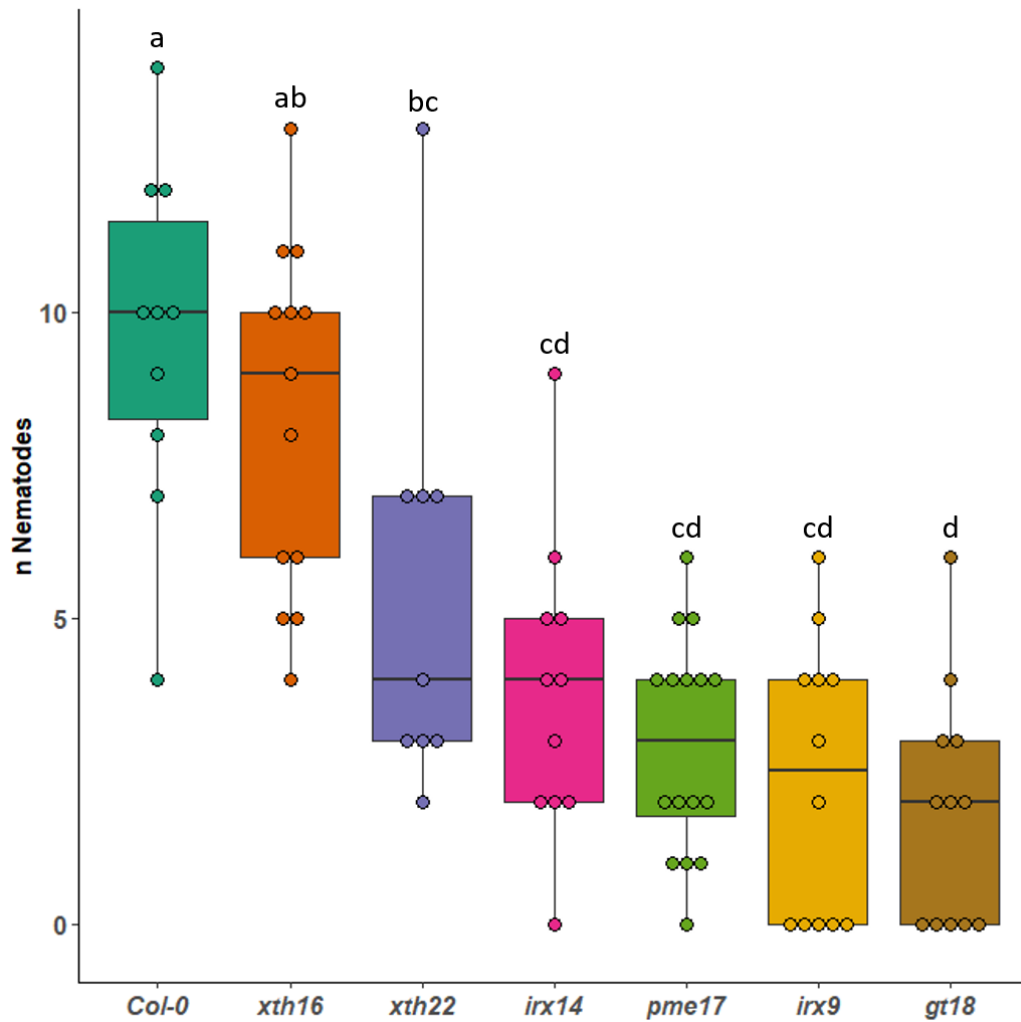


Figure 4. 15. Susceptibility of *A. thaliana* cell wall regulation mutants to *M. incognita*.

Plants of each genotype ($n > 8$) were inoculated with approx. 100 infective stage juvenile *M. incognita* for four weeks before the number of nematodes were counted. Col-0 was used as the wildtype control as the background genotype for each mutant. Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters above each boxplot show groups that are significantly different ($p < 0.05$) in Tukey's post-hoc analysis.

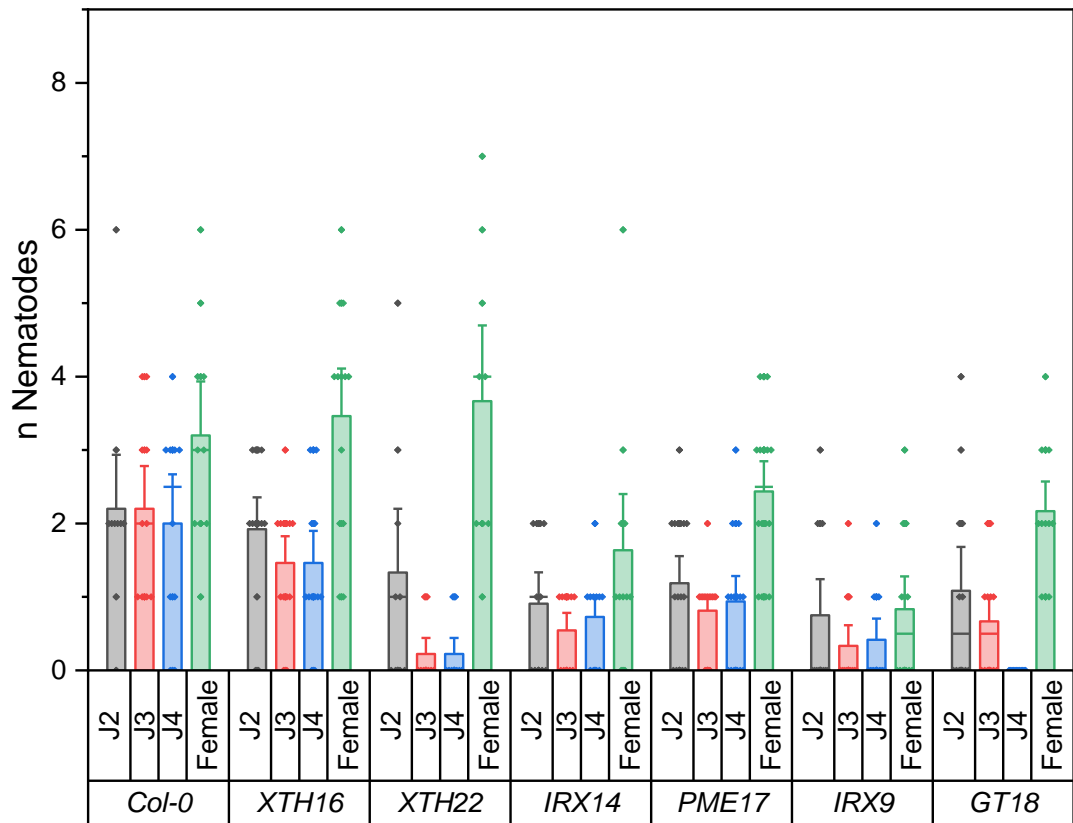


Figure 4. 16. Number of *M. incognita* of each life stage counted within *Arabidopsis thaliana* mutants for genes involved in cell wall biosynthesis and maintenance.

Plants of each genotype ($n > 8$) were inoculated with approx. 100 infective stage juvenile *M. incognita* for four weeks before the number of nematodes were counted. Col-0 was used as the control as the background genotype for each mutant. All data are shown as points for each treatment. Bars represent the mean with error bars showing standard error.

4.4 Discussion

The impact of *M. incognita* infection on Robusta varieties FRT97 and FRT101 was investigated at a transcriptomic level to understand the mechanisms that could drive the physiological differences observed in Chapter 3. However, the FRT97 Robusta plants infected to extract RNA for sequencing and analysis did not suffer any detriment to their growth over the twelve weeks. This highlights some variation in physiological response that could be seen between experiments involving the same variety of Robusta coffee. Therefore, any transcriptional changes induced by root-knot nematode infection will be considered only for differences of susceptibility and the impact on photosynthesis between the two varieties.

Before analysis of infected root and leaf material, the “baseline” or innate transcriptomic difference between the two Robusta varieties were explored. Groups of enriched differentially expressed genes, defined by their gene ontology identifiers, were those related to general defence and immune responses, as well as cell wall biosynthesis and regulation. Although no direct link can be established between the innate expression differences of these genes resulting in differences of susceptibility to root knot nematodes, the differences observed could still be considered as different levels of constituent defence. A coffee variety that expresses a relatively higher level of a gene that provides protection to plant parasitic nematodes should perform better during nematode attack, due to a stronger first line of defence (Holbein *et al.* 2016). The specific role of cell wall regulation and immune-responsive genes are discussed in detail in relation to infected robusta root and leaf gene expression.

4.4.1 Root-knot nematode-induced transcriptomic changes to Robusta root tissue

The infection of *M. incognita* in FRT97 led to a down-regulation of many defence-related genes, some of which have been described to be involved in plant parasitic nematode interactions in other plants. For example, a putative leucine-rich repeat receptor-like kinase was down regulated by *M. incognita* in FRT97. These receptors are required for the recognition of nematodes to induce innate immune responses (Mendy *et al.*, 2017), and could be actively repressed by *M. incognita* upon invasion to increase susceptibility in the Robusta variety. A homologue of the nucleotide binding and leucine-rich repeat domain RGA4, one of two resistance proteins required for recognition of the rice blast fungus *Magnaporthe oryzae* in rice (Cesari *et al.*, 2013), was also down-regulated. These transcriptional changes induced by root-knot nematode infection demonstrate an invasion strategy and host manipulation that the pathogen utilises for successful pathogenicity, and results in the greater susceptibility of FRT97. Furthermore, as these genes are not down regulated in FRT101, they demonstrate how the regulation of these genes is integral for mediating decreased susceptibility to root-knot nematodes in coffee. Whilst the genes not differentially expressed in FRT101 may be due to

lower numbers of the nematode present in the roots, the large number of genes overall differentially expressed in FRT101 still suggests that the small number of nematodes infecting the plant has significant impact on transcriptional regulation.

Defence response genes involved in recognition and immune responses to fungal pathogens were up regulated in FRT101 following root-knot nematode infection. Examples include: ERF114, which mediates fungal pathogen effector PevD1-induced disease resistance in cotton and tobacco (Li *et al.*, 2022), a basic helix-loop-helix transcription factor, which regulates fungal pathogen recognition as well as salt stress tolerance (Zang *et al.*, 2019; Liu *et al.*, 2020), and a receptor like kinase (RLK1), which are key components in broad-spectrum recognition of pathogens in induced immune response (Tang *et al.*, 2017). Whilst many of these genes may have not been demonstrated to be involved specifically in an immune response to plant parasitic nematodes, the up-regulation of these defence response genes could be the evidence of the recognition of root-knot nematodes as a pathogen and may be responsible for inducing immune responses leading to the decreased levels of nematodes compared to FRT97. Adachi and Kamoun (2022) review how the expansion of a family of intracellular immune receptors-nucleotide-binding domain and leucine-rich repeat proteins, risen from the co-evolution of plants and pathogens, have allowed plants to adapt to a broad-spectrum of pathogens and can redundantly recognise multiple pathogens. It is therefore likely, that the NLRs that recognise fungi could also be utilised by coffee to recognised plant parasitic nematodes. Broad-spectrum resistance against cross-kingdom pathogens has also been previously demonstrated for plant parasitic nematode resistance genes. The tomato gene *Mi-1.2* confers resistance against not only root-knot nematodes, but also some isolates of potato aphid, *Myzus persicae*, and the whitefly *Bemisia tabaci* (Nombela *et al.*, 2003). The infestation of aphids and whitefly causes an accumulation of salicylic acid as a defence response to the pathogens, which is mediated by the *Mi-1* gene and demonstrates a broad-spectrum defence response that resistance genes can have against a wide array of pathogens (Jesse *et al.*, 1998; Zarate *et al.*, 2007). It is therefore noteworthy to identify genes within the transcriptomic analyses, which have any defence response role, as they will be likely to be induced upon the infection of nematodes and could contribute to any successful immunity.

The identification of genetic sources of resistance to multiple pathogens is of great interest, particularly for growers and breeders who can introduce fewer sources of pathogen resistance into crops of interest whilst still providing protection against multiple pathogens.

4.4.2 Role of the cell wall in the interaction of root-knot nematodes and Robusta coffee

As well as defence response genes, there was a contrast in the regulation of genes involved in cell wall regulation between FRT97 and FRT101 following infection. Root-knot nematodes are known to secrete effectors that target the cell wall; for example, Beta-1,4 endoglucanases that targets cellulose, pectate lyases and polygalacturonases that act upon pectin, and xylanases that will be active on certain hemicelluloses (Huang *et al.*, 2005; Mitreva-Dautova, 2006; Ledger *et al.*, 2006). These cell wall enzymes would therefore cause a large transcriptomic response from the host plant. The difference in transcriptome between the two varieties also suggests a role of cell wall regulation in resistance or tolerance of coffee to root-knot nematodes, though this relationship is not clear. For example, pectin methylesterase 33 (PME-33) is part of a super family of PMEs which is not only involved in cell wall modification but can also respond to pathogenicity to induce pectin stiffening, as well as trigger the release of damage-associated molecular patterns and hormone signals for systemic acquired resistance (Coculo & Lionetti, 2022). PME33 and other cell wall regulatory genes, however, were down regulated in FRT101 following infection, whilst other genes with the same GO identifier were up regulated in FRT97. The expected function of the gene, in inducing defences against pathogens utilising the cell wall, was counter to the regulation seen in the less susceptible FRT101 and more susceptible FRT97.

A selected number of cell wall regulatory *Arabidopsis* genes, homologues to genes expressed in coffee roots under infection and identified in both differential gene expression and GO enrichment analysis, were selected for further investigation of their role in root-knot nematode susceptibility. *Arabidopsis* lines carrying mutations in cell wall biogenesis (*gt18*, *irx9*, and *irx11*) and cell wall modification (*pme17*, *xth22*) were less susceptible to root-knot nematodes, indicating their importance for successful root-knot nematode parasitism of coffee. The up regulation in uninfected FRT97 compared to uninfected FRT101 suggests a constitutive role of these genes relating to susceptibility, though the mechanism behind this will need to be explored further.

XTH16 and *IRX9* have previously been shown to be up-regulated in root-knot nematode induced gall tissue of the model tree species *Populus tremula* (Baldacci-Cresp *et al.*, 2020). *XTH16* is also up regulated in a susceptible apple rootstock in comparison to a tolerant rootstock (Reim *et al.*, 2022). Results here, though, show the constitutive expression and regulation of these genes as also being important for successful root-knot nematode parasitism, rather than just a result of the infection.

GT18 encodes a Golgi apparatus-localized galactosyltransferase involved in the biosynthesis of xyloglucan, which allows for elasticity in the cell wall and contributes to the formation and

remodelling of new cell walls during growth (Cosgrove, 2000). T-DNA insertion mutations in *gt18* also lead to a reduction of the monosaccharide galactose in the cell walls of *Arabidopsis* (Li *et al.*, 2004). Previous research has shown *GT18* to be necessary for *M. incognita* resistance in a resistant genotype of tomato, *Solanum lycopersicum* (Scaff *et al.*, 2007), yet the infection of *gt18 Arabidopsis* mutants show that the glycotransferase increases susceptibility in the model plant organism. Furthermore, *PME17* has shown been to have a role in the recognition of the fungal pathogen *Botrytis cinerea*, to induce resistance in *Arabidopsis* (Del Corpo *et al.*, 2020), but its role in defence was shown to not translate to the recognition of plant parasitic nematodes, and mutation in the gene led to a decrease in *M. incognita* infection. The contradiction between these results highlights the different functions and roles possessed by orthologues of the same gene between plant species. Genes with sequence similarity between organisms, like those identified in Robusta as orthologues to *Arabidopsis* genes, may not be function-orientated orthologues, in that they do not perform the same role in different species. Function, therefore, cannot always be translated between different plant species, and in specific situations, orthologues can have an inverse effect on plant parasitic nematode pathogenicity.

4.4.3 Systemic changes in coffee leaf tissue induced by root-knot nematodes

The infection of root-knot nematodes led to not only a large transcriptional change in root tissue of FRT97, but also in leaf tissue. The same was not true for the leaf tissue of FRT101. This could be due to a lower number of nematodes feeding within the roots of FRT101 leading to little or no nutrient detriment in the whole plant. It is also possible, though, that the molecular mechanisms that occur in roots that differentiate FRT101 from FRT97, for example the differences in regulation regarding cell wall organisation, may prevent any systemic changes occurring in above ground tissue, thus providing tolerance for the host against the nematode. As the mechanisms that drive tolerance to plant parasitic nematodes are less known than sources of resistance, these constitutive differences between varieties of different tolerance levels are an important resource to explore further.

Many different transcriptional processes were altered in FRT97 leaf tissue due to the infection of *M. incognita*. Many genes involved in defence and immune responses were down-regulated. This may be because of the active suppression of immune responses that *M. incognita* can achieve through secreted effectors. Calreticulin, for example, has been shown to be secreted by *M. incognita* to prevent calcium influx and disrupt calcium signalling which would induce immune response in a host plant (Jaouannet *et al.*, 2013). The mechanisms of receptor kinase-like proteins and late blight resistance proteins down-regulation, as observed in this study on FRT97, is not clear however, and would need to be studied further.

In a more resistant plant, the infection of plant parasitic nematodes can lead to the up regulation of defence and immune response genes via systemic acquired resistance. This priming can ensure that the plant can induce low-level defences in remote tissue to protect from further nematode attack (Durrant & Dong, 2004). This phenomenon is well studied in other plant-pathogen interactions. Pathogen-related (PR) proteins and antimicrobial peptides (AMPs) encompass broad ranges of protein families that accumulate not only at the site of pathogen attack, but systemically remote from the pathogen, and occur throughout the plant domain (van Loon *et al.*, 1994; Ali *et al.*, 2018a). These proteins and peptides are induced by the hormonal cross talks of salicylic, jasmonic and abscisic acid to provide immune response to pathogen invasions throughout the plant; as demonstrated by the SAR induced via PR1 in *Brassica juncea* against the blight fungus *Alternaria brassicae* (Ali *et al.*, 2018b). The lack of activity of either hormonal signalling, or PR protein and AMP induction in FRT97 leaf tissue may demonstrate a lack of immune response priming. Previous work on *Meloidogyne* spp. infections on Arabidopsis and rice also show an active down-regulation of PR and AMP encoding genes, which could be an evolved strategy of root-knot nematodes to evade plant immune responses (Hamamouch *et al.*, 2011; Kyndt *et al.*, 2012).

Considering the reduction of chlorophyll fluorescence seen in FRT97 12 weeks post infection with *M. incognita*, it was expected to see large transcriptomic changes in leaves compared to uninfected controls. It was interesting then, to see genes involved in photosynthesis and light harvesting only differentially expressed in the one-week post inoculation treatment where there was no change in chlorophyll fluorescence observed. Under infection with the root-knot nematode *M. graminicola* rice shoots also had an up-regulation of photosynthetic and chloroplastic genes *1wpi*, but rice shoots under infection with the migratory nematode *Hirschmanniella oryzae* had a suppression of the same genes (Xie *et al.*, 2019). This highlights the different effects on metabolism that plant parasitic nematodes will have on a crop depending on their feeding strategy. The up-regulation of photosynthetic activity in host plants therefore appears to be a key part in the parasitism of root-knot nematodes specifically, perhaps related to a unique aspect of their lifestyle such as the proliferation of giant cells induced in roots.

The few genes that were differentially expressed in FRT101 leaves following *M. incognita* infection do still suggest a systemic defence response to the pathogen. Glycosyltransferase, which was up regulated, has been identified to play a role in *M. incognita* resistance in tomato mediated by the resistance gene *Mi* (Schaff *et al.*, 2007), though the function of the expression in leaf material is not clear. Phospholipase D is a stress responsive gene encoding a lipid-degrading enzyme and is involved in cell signalling pathways. It has been shown to be up

regulated in rice leaves under *Heterodera sacchari* infection (Blouin *et al.*, 2005). However, multiple genes, which are putative homologs to Phospholipase D gene, were down-regulated in both FRT97 and FRT101 leaves. The gene is considered a marker for plant health, in that the expression is strongly associated with stress intolerance (Bargmann & Munnik, 2006). The down-regulation, therefore, could be suggestive of tolerance, though that would be contradictory to the impact of *M. incognita* on photosynthesis of FRT97 despite also being down-regulated in that variety. A caveat to conclusions drawn from FRT101 leaf transcriptomic data is the low percentage of sequenced reads that were able to map to the Robusta reference genome for some samples. This is probably due to errors in sequencing because of low quality RNA, especially considering that FRT97 sequenced reads were able to map well to the reference genome. As sequences from FRT101 root material was able to map well onto the Robusta reference genome, the poor mapping of some leaf tissue samples must not be due to with issues of polymorphisms between genomes. Further research analysis and on the systemic transcriptional response in FRT101 leaf tissue following root-knot nematode infection should therefore be considered.

4.5 Key Findings

The Robusta varieties FRT97 and FRT101 show large basal transcriptomic differences in both root and leaf material that could be a major influence on their response to subsequent plant parasitic nematode infection. *M. incognita* induces the down regulation of defence related and immune responsive gene in Robusta var. FRT97 that could contribute to its increased susceptibility relative to FRT101. In contrast, defence-related and immune responsive genes were up regulated in var. FRT101, possibly contributing to its lower susceptibility.

There was a differential response of cell wall biosynthesis and maintenance genes between each variety in response to *M. incognita* infection. Infection assays using *A. thaliana* confirms the role of these genes in the host to affect *M. incognita* infectivity. The infection of *M. incognita* also causes systemic transcriptional changes in the leaf tissue of Robusta var. FRT97, but not in the less susceptible FRT101.

Chapter 5

Serotonin signalling pathway offers novel targets for plant parasitic nematode control

5.1 Introduction

Compounds exuded from plant roots provide plant parasitic nematodes with a chemical gradient along which to orientate and move toward their host (Prot, 1980). Exposure to host root exudate is also known to stimulate stylet thrusting, which is used to penetrate cell walls and for feeding (Doncaster 2012). One of the ways to interfere with the infectivity of plant parasitic nematodes is to selectively disable behaviours such as locomotion and stylet activity that are intrinsic to their parasitic life cycle. Disabling these behaviours leads to early arrest of the lifecycle, which in turn leads to a reduction of established nematodes in the host and prevents population build-up, protecting the crop from water loss and nutrient detriment. Importantly, targeting the pre-parasitic stage would also prevent the root damage caused by nematodes on invasion and migration that can provide an entry point for subsequent bacterial and fungal pathogens. These behaviours will be the output of the nematode nervous system, and the neurotransmitter serotonin has already been established to be integral for locomotion, feeding and reproduction in cyst and root lesion nematodes (Han *et al.*, 2017; Crisford *et al.*, 2020). Serotonin signalling therefore represents an ideal target for intervention to disrupt essential parasitic behaviours and deliver effective control.

Traditional chemicals used for control of plant parasitic nematodes, which typically paralysed nematodes by cholinesterase inhibition or were metabolically poisonous (Holden-Dye & Walker, 2014), have become unusable due to their toxicity towards off-target organisms (EU regulation EC 1107/2009). If drugs targeting serotonergic signalling that were specific to plant parasitic nematodes could be developed, based on unique motifs found within molecular components of the nervous system, they would also have the advantage of avoiding damage to off-target organisms.

In both *C. elegans* and *G. pallida*, the serotonin-signalling pathway (Described in Figure 5. 1) can be interrupted with specific chemicals, which act on selected component steps of the pathway from biosynthesis to receptor activation. The synthetic enzyme for serotonin, tryptophan hydroxylase TPH-1, is irreversibly inactivated by 4-chloro-DL-phenylalanine methyl ester hydrochloride (CPA), reserpine has been shown to disrupt vesicular monoamine transporter function (In mammals = VMAT, nematodes = CAT-1), and post-synaptic serotonin activity can be

inhibited by the serotonin receptor antagonist, methiothepin (Jéquier *et al.*, 1967; Erickson *et al.*, 1992; Crisford *et al.*, 2020).

Testing the effect of pharmacological blockers on plant parasitic nematode behaviours, such as locomotion and feeding, is a useful investigative tool for the role of serotonin signalling in nematodes. Behavioural assays for chemotaxis, stylet thrusting, and invasion of the susceptible host aduki bean, with the addition of chemicals known to inhibit serotonin signalling were conducted to demonstrate potential roles for serotonin signalling in the parasitic behaviours of *M. incognita*.

Plant parasitic nematodes can be difficult organisms to study and maintain for research. Their parasitic nature means maintenance requires the upkeep of a host, as well as adding difficulty in designing and quantifying experiments in their natural habitat. Forward and reverse genetics on plant parasitic nematode species is also comparatively primitive or difficult compared to other organisms (Costa *et al.*, 2007). The well-studied free-living organism, *Caenorhabditis elegans* is therefore often used as a 'model' organism for molecular genetic techniques and mode of action studies for anthelmintics and nematicides. Here, we use information about *C. elegans* serotonergic biology as a reference point for discovery in root-knot nematodes. From these comparisons potential targets for the control of the nematode are suggested, which could be developed to protect coffee from damage.

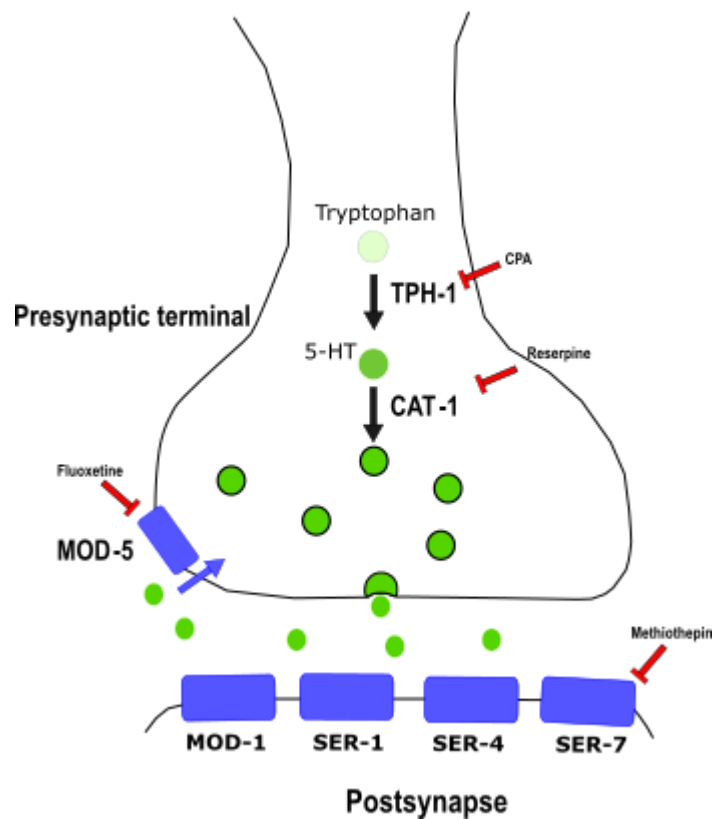


Figure 5. 1. Serotonin biosynthesis and signalling in nematodes.

Tryptophan hydroxylase (TPH-1) catalyses the conversion of tryptophan into 5-Hydroxytryptophan which matures into the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT). Serotonin is then loaded into a vesicle and transported to the synapse by the vesicular monoamine transporter CAT-1. Released serotonin bind to three G-protein coupled receptors, SER-1, SER-4 and SER-7, and a 5HT-gated chloride channel MOD-1 and activate down stream signalling. MOD-5 is a re-uptake transporter, returning serotonin to the pre-synapse. Pharmaceutical blockers can be used to inhibit serotonin signalling at different points; 4-chloro-DL-phenylalanine methyl ester hydrochloride (CPA) irreversibly inhibits the function of TPH-1, reserpine acts upon CAT-1 activity and methiothepin irreversibly blocks serotonin receptor function.

5.2 Methods

The preparation of all chemicals used in these methods are described in Chapter 2.2.

5.2.1 Motility assays

Approximately 100 *M. incognita* J2s were incubated for 24 hours in 200 μ L of either CPA (1-50 mM), methiothepin (1-50 mM), reserpine (1-168 μ M) or sterile distilled water (control). J2s were then observed under a bright-field microscope (Wild Heerbrugg microscope, magnification 25x). The total number of nematodes in each treatment were counted, and after five minutes nematodes were described as either non-motile; in which they were straight-shaped, heavily coiled which suggests a physical impairment from the chemical or visibly dead; or motile, which were all other nematodes which either showed movement or showed no straight-shaped positions. The procedure was repeated using a range of concentrations of each chemical for incubation of nematodes (Total number of measurements of any concentration; methiothepin & CPA n=12, Reserpine n=17). A sigmoidal curve was fitted onto reserpine data within Origin software (Levenberg Marquardt algorithm), and linear regressions were made for CPA and methiothepin data as they failed to fit sigmoidal curves, on the percentage of motile nematodes versus the concentration of each chemical. The IC_{50} was calculated for the concentration of reserpine that exerts half of its maximal inhibitory effect, and linear regressions used to calculate the concentration of methiothepin and CPA that reduced the percentage of *M. incognita* juveniles to below 85%.

5.2.2 Stylet thrust assays

Approximately 100 J2 *Meloidogyne incognita* were incubated for 24 hours in either methiothepin (0.1-10 mM), CPA (1-81 mM), reserpine (0.1-10 μ M) or sterile distilled water (positive control for regular stylet function; +ve). After 24 hours incubation and prior to assays, *M. incognita* in treatment chemicals were centrifuged at 4000 rpm for 2 min, test chemicals were removed and replaced with 100 μ L of either distilled water (negative control; -ve), 10 mM 5-HT or 10 mM fluoxetine (FCH) for 15 minutes. Ten nematodes per treatment were observed for 30 seconds each at a magnification of 80x (Zeiss Axio Scope A1 microscope) and each stylet thrust was counted. A single movement of the stylet forwards and then backwards to its original position was counted as a single stylet thrust. This experiment was repeated with a different batch of nematodes with fresh chemicals being prepared prior to each test. One-way ANOVAs were performed for to detect significant differences between concentrations of each chemical, followed by Tukey's post hoc analysis.

5.2.3 Chemotaxis

Approximately 1000 J2 *Meloidogyne incognita* were incubated for 24 hours in either methiothepin (0.03-3 mM), CPA (10-160 μ M), reserpine (0.5-8 μ M) or sterile distilled water (negative control). Pluronic gel in 50x10 mm petri plates were set up as described in (chapter 3 methods), with 10 μ l of sterile distilled water pipetted into the left circle as a negative control, and 10 μ l of 10 mM salicylic acid pipetted into the right circle. Plates were left for 40 minutes to allow the salicylic acid and water to diffuse into the gel.

Prior to assays, *M. incognita* in treatment chemicals or water were centrifuged at 4000 rpm for 2 min, test chemicals were removed and replaced with 100 μ l of sterile distilled water. Approx. 100 J2s in 20 μ l suspension were then injected into the centre of the chemotaxis plate, with four replicates per treatment. The number of J2s in each circle was counted under a microscope after three hours and chemotaxis was calculated as described in Chapter 3.3.4. Each experiment was replicated with at least two biological repeats, set up on separate occasions. Data were compared using one-way analysis of variance with Tukey's HSD post-hoc test.

5.2.4 Invasion pouch assays

Aduki plants were chosen as a host system as they can be quickly grown in a shorter period, hydroponically in a pouch system, which is ideal for staining of roots, as it requires no washing of roots. Nematodes can be easily introduced onto roots, and after one-day, the roots can be visualized for the number of nematodes that were able to successfully invade.

Approx. 100 infective stage juvenile (J2) *M. incognita* were exposed to CPA (0.3-9 mM), methiothepin (0.3-9 mM) and reserpine (1-20 μ M) using sterile H₂O as a negative control. Ten J2s were then pipetted onto the root tips of four aduki plants, grown in soil-free pouch systems (Atkinson & Harris, 1989) at two points per plant, for a total of 20 J2s inoculated per plant (Figure 5. 2). After 24 hours, roots of each plant were stained with acid fuchsin (Chapter 2) and the total number of J2s that had invaded the roots were counted. The experiment was repeated twice to provide eight replicates of each condition.

5.2.5 *M. incognita* serotonin immunolocalisation

M. incognita juveniles were collected in, and pelleted, into a 1.5ml microcentrifuge tube and fixed in 1 ml of 2 % paraformaldehyde in M9 buffer for 18 hours at 4°C. Nematodes were suspended in 200 μ l of M9 buffer and cut with a single edge razor blade on 3 cm length glass slide. Once cut, nematode segments were washed twice with M9 buffer before incubation in 0.5 ml proteinase-K solution (0.5 mg/ml proteinase K in M9 buffer) for 30 minutes, rotating at room temperature. Nematode sections were washed again with M9 buffer, which was removed after centrifugation so that the nematode pellet in the 1.5 ml tube could be frozen on dry ice for 15

minutes. Nematode segments were then suspended in 1 ml of pre-chilled methanol at -20°C for 30 seconds followed by 1 minute in pre-chilled acetone at -20°C. Acetone was removed and nematode sections were slowly rehydrated with distilled water. Nematode sections were washed twice with maleic acid buffer and then blocked in 1% blocking reagent (Roche; diluted in maleic acid buffer) for 30 minutes at room temperature. After blocking, nematode sections were incubated overnight at 4°C with rotation in a 1:200 dilution of rabbit-derived anti-serotonin primary antibody (Sigma-Aldrich). Nematode segments were then washed three times, for 15 minutes each, in maleic acid buffer with 0.01 % Tween-20, reblocked in 1% blocking reagent and incubated in fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit serum (Sigma-Aldrich) for two hours at room temperature. Finally, nematode sections were washed three times, for 15 minutes, in maleic acid buffer with 0.01% Tween-20 and suspended in anti-fadent (PBS/glycerol; Citifluor).

Negative controls were set up exactly the same but without the primary anti-serotonin antibody in the overnight incubation step. Samples were observed using a Leica DMRB microscope with GFP filter set, and images acquired with a QI camera (QImaging) and Q-Capture software.

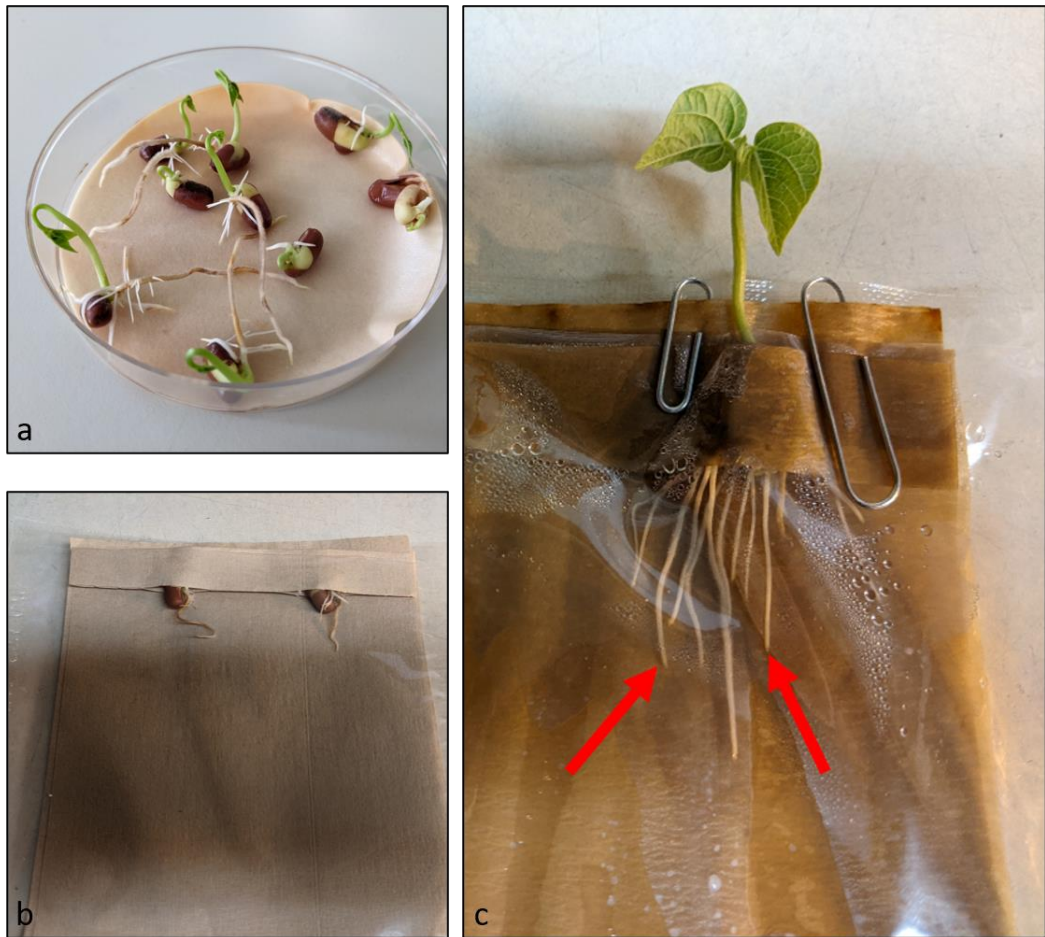


Figure 5. 2. Growth pouch system used to infect aduki plantlets with *M. incognita*.

Seeds are germinated in petri dishes on wet filter paper (a) until roots and shoots emerge. Two seeds per pouch are then placed in a trough at the top of the pouch (b), with both ends of the pouch open, which are then held upright and kept in a growth chamber at 25°C and 16 hours light per day. After 3-5 days, roots are inoculated with *M. incognita* at the tips by carefully pipetting the 10 infective stage juveniles in 10-30 μ l of water (c). Plants are stored in the same growth chambers for 24 hours, before roots are stained with acid fuchsin to allow the enumeration of nematodes that have invaded root tissue.

5.2.6 Orthologue BLAST searches

A list of molecular components involved in the synthesis and metabolism of serotonin of *C. elegans* was collected from recent literature on serotonin signalling in *G. pallida* and from serotonin-related genes described in Wormbook (Chase & Koelle, 2007; Crisford *et al.*, 2020). Protein sequences were obtained from Wormbase (<http://www.wormbase.org>, 2022). BLASTP and tBLASTn searches were then performed against the genomes and predicted proteomes of root knot nematodes available on Wormbase Parasite (Opperman *et al.*, 2008; Szitenberg *et al.*, 2017; Somvanshi *et al.*, 2018 <https://parasite.wormbase.org>, 2022), and against the genomes of *Pratylenchus coffeae* (Provided by Opperman upon request) and *Radopholus similis* (Matthew & Opperman, 2019).

Reciprocal BLAST hits back to the *C. elegans* proteome were then performed on the top five hits, and genes were only considered true one-to-one orthologues if they returned the original *C. elegans* protein sequence. The top hits for each gene remaining were then compared across plant parasitic nematodes to identify unique and conserved genes across the phylum.

5.3 Results

5.3.1 Motility of *M. incognita* following exposure to serotonin signalling inhibitors

Reserpine, methiothepin and CPA have all been shown to have inhibitory effects on the serotonin biosynthesis and signalling pathway in *G. pallida* and *Pratylenchus penetrans*, causing inhibition of behaviours such as stylet thrusting. We explored the effect of these chemicals on the behaviours of *M. incognita* to establish if they are also mediated by serotonin in this species. It was important to establish what range of dosage for each chemical would be sufficient to inhibit neurological function, but not so high as to be lethal or severely impede motility and paralyse the nematodes.

J2s of *M. incognita* were incubated in the chemicals for 24 hours, and the effect of a range of concentrations on motility was observed. All three pharmacological interventions inhibited motility in J2 *M. incognita*. Increasing concentration of reserpine significantly ($p < 0.001$) reduced the percentage of motile *M. incognita* by 0.63% per μM . Increased concentration of methiothepin also caused a significant ($P < 0.001$) reduction of motile J2s by 0.90% (± 0.08) per mM, and CPA caused a reduction of 1.4% (± 0.16) per mM (Figure 5. 3). The IC_{50} ($\pm 95\%$ confidence interval) of reserpine on the motility of *M. incognita* was 45.31 (± 17.94).

The linear regression models predicted that concentrations of 9 mM for both CPA and methiothepin is the threshold before total number of motile J2s to reduced to below 85%. These concentrations were therefore considered as the upper limits in further experiments involving more subtle behaviours before they began to have a paralysing effect on *M. incognita* J2 nematodes.

5.3.2 Role of serotonin in stylet thrusting of *M. incognita*

Exogenous application of either 5-hydroxytryptamine (5-HT) or fluoxetine (Prozac) causes plant parasitic nematodes, including J2s of *M. incognita*, to rapidly perform stylet thrusts. These two compounds have different modes of action in stimulating stylet thrusts. Fluoxetine selectively blocks the synaptic plasma membrane serotonin transporter MOD-5. By preventing re-uptake of serotonin, fluoxetine increases serotonin concentration in the synaptic cleft, which in turn activates the postsynaptic receptors. Therefore, fluoxetine stimulation of stylet thrusts still relies on endogenous serotonin, whereas exogenous application of 5-HT directly triggers the activation of post-synaptic receptors and therefore does not require endogenous serotonin signalling. Coupled with the exposure to pharmacological blockers, which target molecular components of the serotonin-signalling pathway, the involvement of serotonin signalling molecular components that regulate stylet thrusting by *M. incognita* can be assessed.

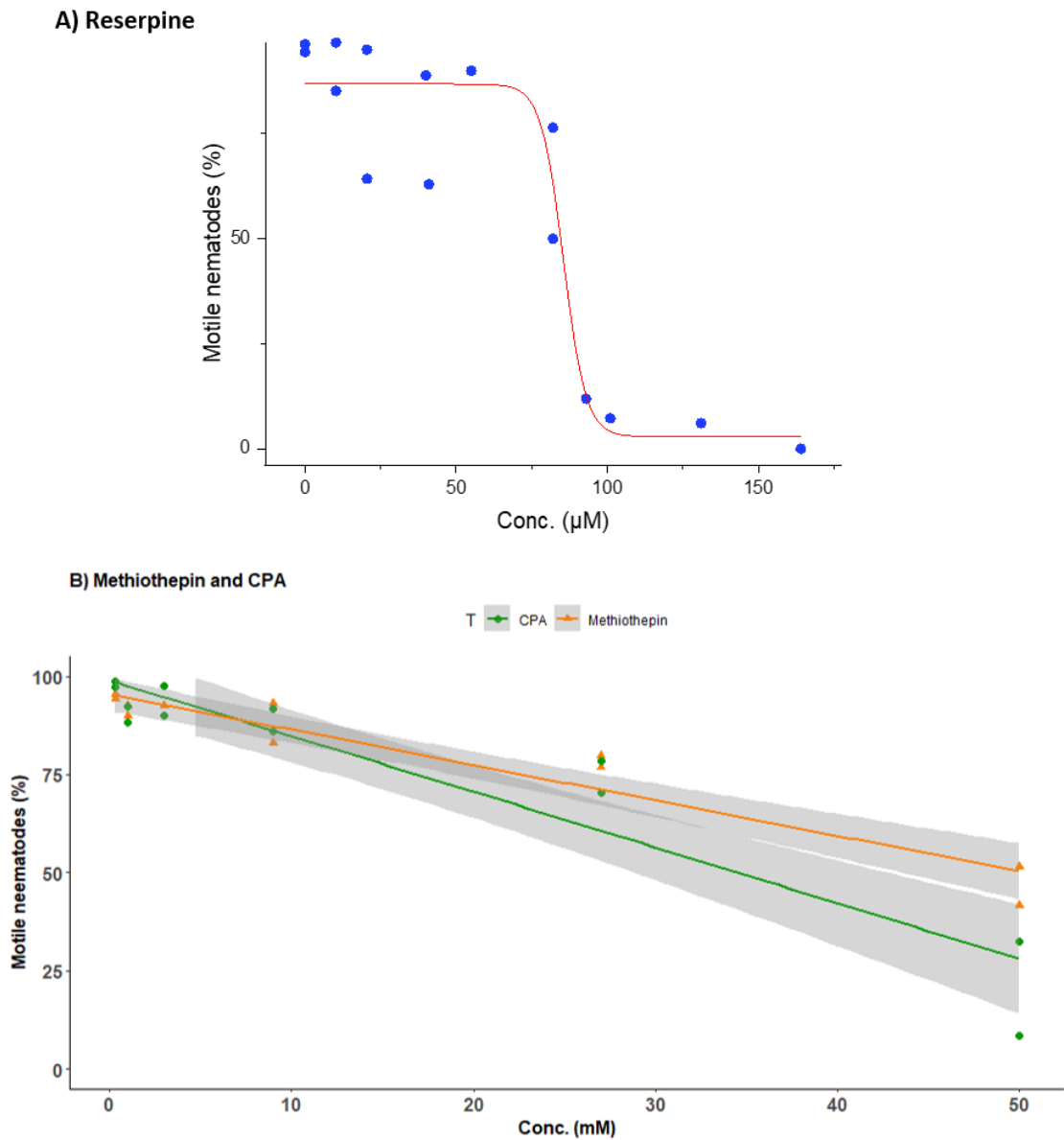


Figure 5. 3. Effect of reserpine, methiothepin and CPA on the motility of *M. incognita*.

Approximately 100 J2 *M. incognita* were incubated in; A) reserpine in concentrations up to 164 μM and B) methiothepin and CPA in concentrations up to 50 mM for 24 hours J2s that showed movement, and were not straight shaped or coiled, were counted as mobile. Data plotted on A) were fitted to a sigmoidal curve using Levenberg Marquardt algorithm. Linear regressions are shown for Methiothepin and CPA treatments, with grey areas representing the 95% confidence interval.

The inhibitory effects of reserpine, CPA and methiothepin are shown in Figure 5. 4. Incubation for 24 hours in reserpine inhibited fluoxetine-stimulated stylet thrusting in *M. incognita* ($F_{5,114}=16.5$, $p<0.001$), even at 0.1 μM ($p<0.001$) where the mean ($\pm\text{SE}$) number of stylet thrusts was reduced to 11.85 (± 3.53) per minute compared to the control rate of 34.1 (± 6.4). However, the same concentration did not have any significant effect on the rate of stylet thrusts when stimulated by 5-HT ($p>0.05$). Reserpine, therefore, was shown to inhibit *M. incognita* stylet thrusting triggered by endogenous, but not exogenous, serotonin.

The endogenous stimulation of stylet thrusting by serotonin was also more sensitive to CPA than was exogenous stimulation. CPA significantly inhibited fluoxetine-stimulated stylet thrusting from 27 mM ($F_{5,114}=4.93$, $p<0.001$), reducing the thrusting rate from 30.05 (± 7.0) to 5.5 (± 2.0) thrusts per minute, but a higher concentration of 81 mM CPA was required to significantly reduce the mean number of stylet thrusts, stimulated by exogenous 5-HT, to 13.55 (± 4.3) ($F_{5,114}=3.13$, $p<0.001$). This reduction in stylet thrusting stimulated by 5-HT, however, could be due to the higher concentrations of CPA having a significant effect on motility and thus paralyzing the nematode beyond normal functioning (Figure 5. 3).

Finally, J2s incubated in any of the concentrations of methiothepin performed significantly fewer stylet thrusts (Tukey post hoc; $p<0.05$) regardless of endo-or exogenous stimulation by fluoxetine or 5-HT ($F_{5,114}=41.24$, $p<0.001$ and $F_{5,114}=9.35$, $p<0.001$).

5.3.3 Role of serotonin in chemotaxis of *M. incognita*

The ability of *M. incognita* juveniles to move toward an attractant chemical was also inhibited after exposure to each of the pharmacological blockers (Figure 5. 5). A 24-hour exposure to increasing concentrations of reserpine ($F_{5,42}=8.68$, $p<0.001$), methiothepin ($F_{5,42}=12.94$, $p<0.001$) and CPA ($F_{5,42}=12.94$, $p<0.001$) all caused a significant reduction in chemotaxis to the attractant salicylic acid (One-way ANOVA). Concentrations of 4 μM reserpine, 3 mM methiothepin and 160 μM CPA all reduced the mean chemotaxis index to within a standard error of 0.03 around zero, which was significantly less (Tukey post-hoc; $p<0.001$) than the control indexes of 0.13 (± 0.01), 0.17 (± 0.01) and 0.10 (± 0.01) for each chemical treatment respectively. The reduced chemotaxis will also not be due to just reduced motility alone, as there was no significant difference in the number or juveniles that had moved from the inoculation points from controls. The inhibition of the serotonin biosynthesis or signalling pathway, therefore, impaired the chemotaxis of *M. incognita* toward attractant chemicals.

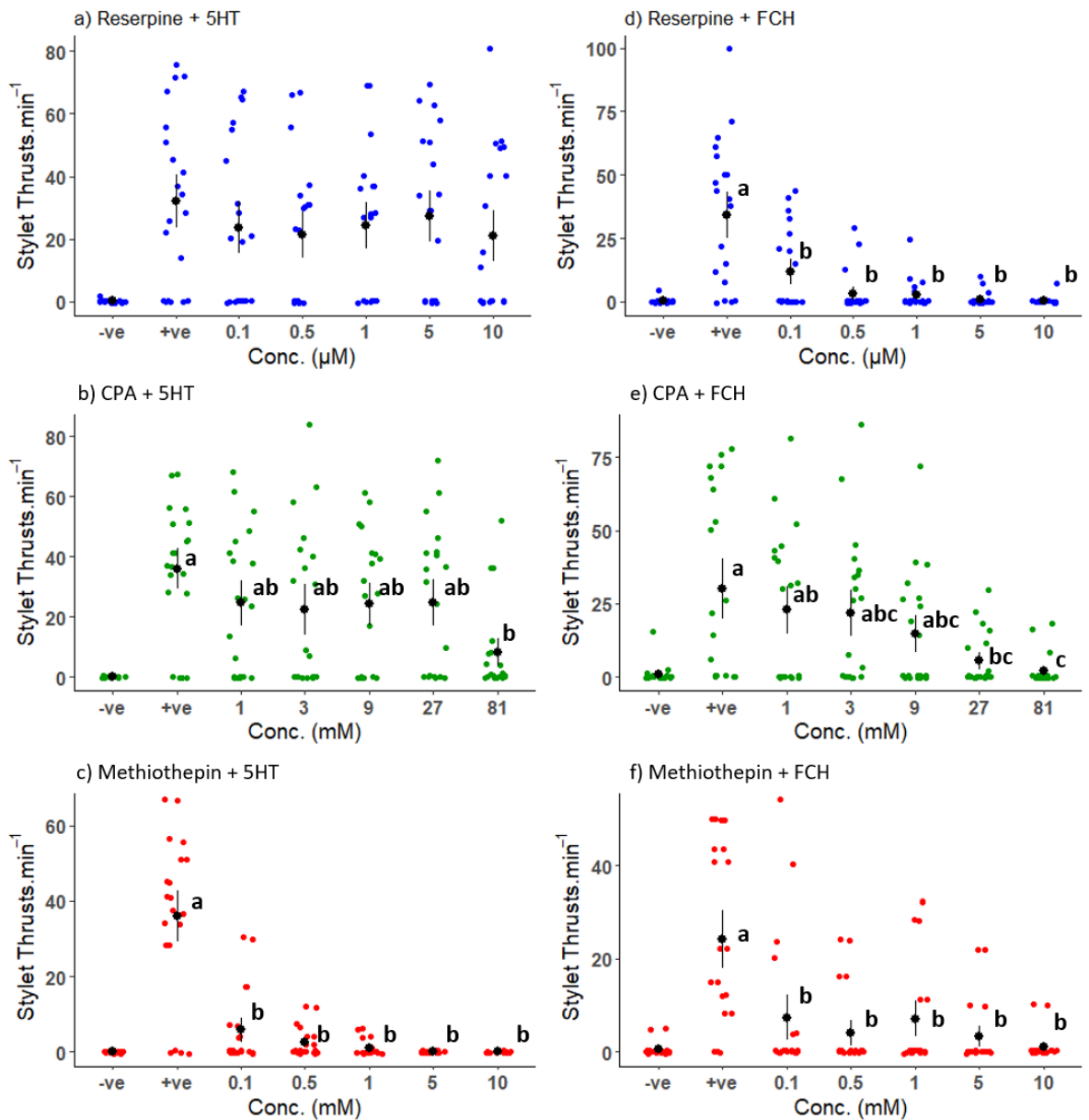


Figure 5. 4. Inhibition of stylet thrusting in *M. incognita* by reserpine, CPA and methiothepin.

Mean stylet thrusts per minute of 20 *M. incognita* J2s, stimulated by 15 minutes incubation in 10 mM 5-HT (a, b, c) or 10 mM fluoxetine (FCH) (d, e, f) and after 24 hours incubation in reserpine (a + d), CPA (b + e) or methiothepin (c + f). Positive controls (+ve) were J2s stimulated by 5-HT or FCH after 24 hours incubation in sterile distilled water, with negative controls (-ve) being J2s incubated in sterile distilled water for 15 minutes instead of 5-HT or FCH. Black points represent the mean stylet thrust rate of all nematodes in each treatment, with black bars showing standard error. Letters represent significantly different groups according to Tukey post-hoc analysis.

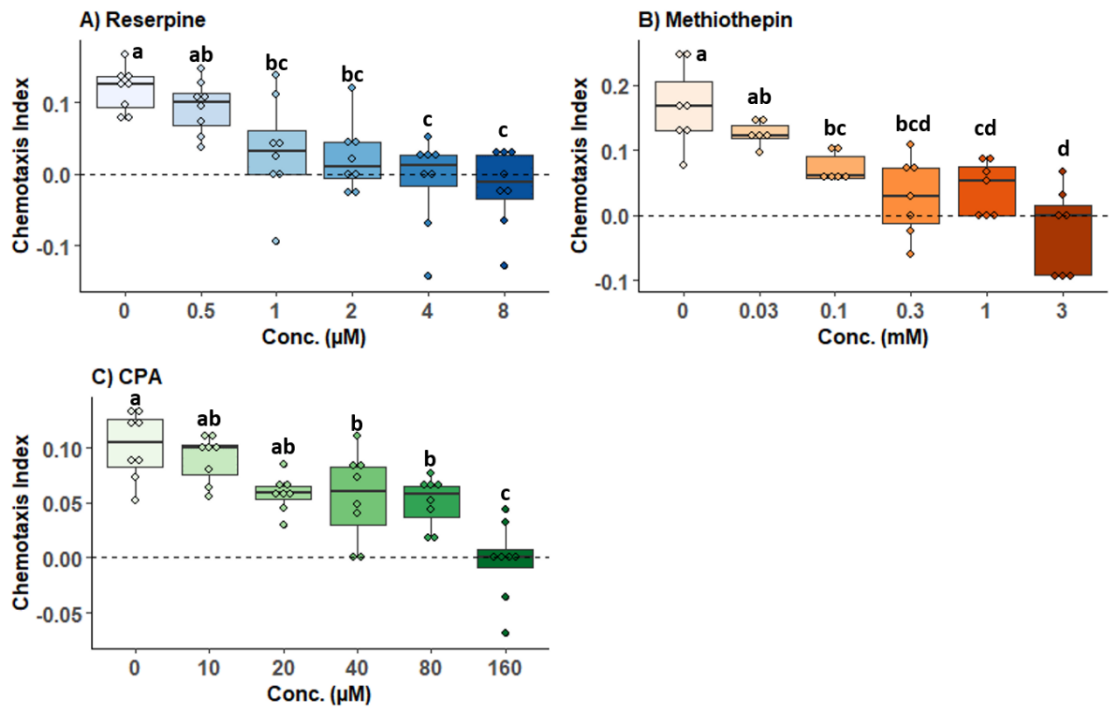


Figure 5. 5. Effect of reserpine, methiothepin and CPA exposure on *M. incognita* chemotaxis.

The chemotaxis index of *M. incognita* J2s, incubated for 24 hours in pharmacological blockers, toward 20 mM salicylic acid. Approximately 100 J2s per treatment were assayed using the pluronic gel-based chemotaxis assay (Chapter 3.3.4) following 24-hour incubation in a) reserpine b) methiothepin and c) CPA, using sterile distilled water as a negative control (0 mM). Eight replicates were used per treatment, performed in two biological repeats of nematode cultures. Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters represent significantly different groups according to Tukey post-hoc analysis.

5.3.4 Inhibition of infective ability of *M. incognita* by serotonin signalling inhibitors

The inhibition of serotonin signalling, and thus the proper functioning of parasitic behaviours, subsequently reduced the ability of *M. incognita* to infect root tissue of aduki beans. All three pharmacological blockers caused a significant reduction in the number of nematodes that were able to invade into aduki roots (Figure 5. 6). At 0.3 mM CPA, there was a significantly lower median (\pm IQR) number of J2s in roots of 2.5 (\pm 1.25) compared to the control of 4 (\pm 1.25), and significantly lower again at 9 mM with a median (\pm IQR) of 0.5 (\pm 1) J2s ($\chi^2 = 21.05$, $df=4$, $p < 0.001$). Methiothepin treatments at 1, 3 and 9 mM were all significantly lower than the control at 2 (\pm 1), 1 (\pm 1.25) and 0 (\pm 0.25) J2s respectively compared to the control of 4 (\pm 1.25) ($\chi^2 = 27.50$, $df=4$, $p < 0.001$). Finally, reserpine treatments at 1, 5, 10 and 20 μ M had significantly fewer median (\pm IQR) nematodes than the control with 1 (\pm 0.75), 1 (\pm 2), 0 (\pm 1) and 0 (\pm 0) respectively compared to the control of 3 (\pm 2) ($\chi^2 = 19.67$, $df=4$, $p < 0.001$). The highest concentration treatments of CPA (9 mM) methiothepin (9 mM) and reserpine (20 μ M) all had a median of less than one nematode counted in the roots of aduki plants. The decreasing number of invaded nematodes with increasing concentration of each chemical shows the gradient effect that the pharmacological blockers have on the ability of *M. incognita* J2s to invade into the aduki roots. As these chemicals are known pharmacological blockers of the serotonin pathway in other plant parasitic nematodes, it is likely that the same neurological components have also been blocked in *M. incognita* leading to the inhibition of locomotion and stylet thrusting necessary for the successful invasion into a host.

5.3.5 Immunolocalisation of serotonin

We used an anti-serotonin antibody to determine the presence and localisation of endogenous serotonin in juvenile *M. incognita*. The anti-serotonin antibody showed strong immunoreactivity at multiple points in the anterior half of *M. incognita* juveniles (Figure 5. 7). The strongest and most consistently highlighted structures were cell bodies behind the metacarpus; two pairs of putative neurones directly behind the metacarpus and another pair further posterior, located around the nerve ring. The pair of neurones directly behind the metacarpus appear to be homologues of the undesignated neurones positioned in a similar area as in *Pratylenchus penetrans* (Figure 5. 9). The second pair of neurones could be putative homologues of ADF neurones based on the positioning and strength of fluorescence in comparison to *P. penetrans* and *C. elegans* ADFs. Two unpaired neurones were also inconsistently immunoreactive to the anti-serotonin antibody posterior to the nerve ring, which do not appear to have any obvious homologues in *C. elegans* or *P. penetrans*. Finally, a pair of strongly staining neurone cell bodies were inconsistently immunoreactive around the mid-section of the *M. incognita* juveniles, which would most likely be putative HSN homologues.

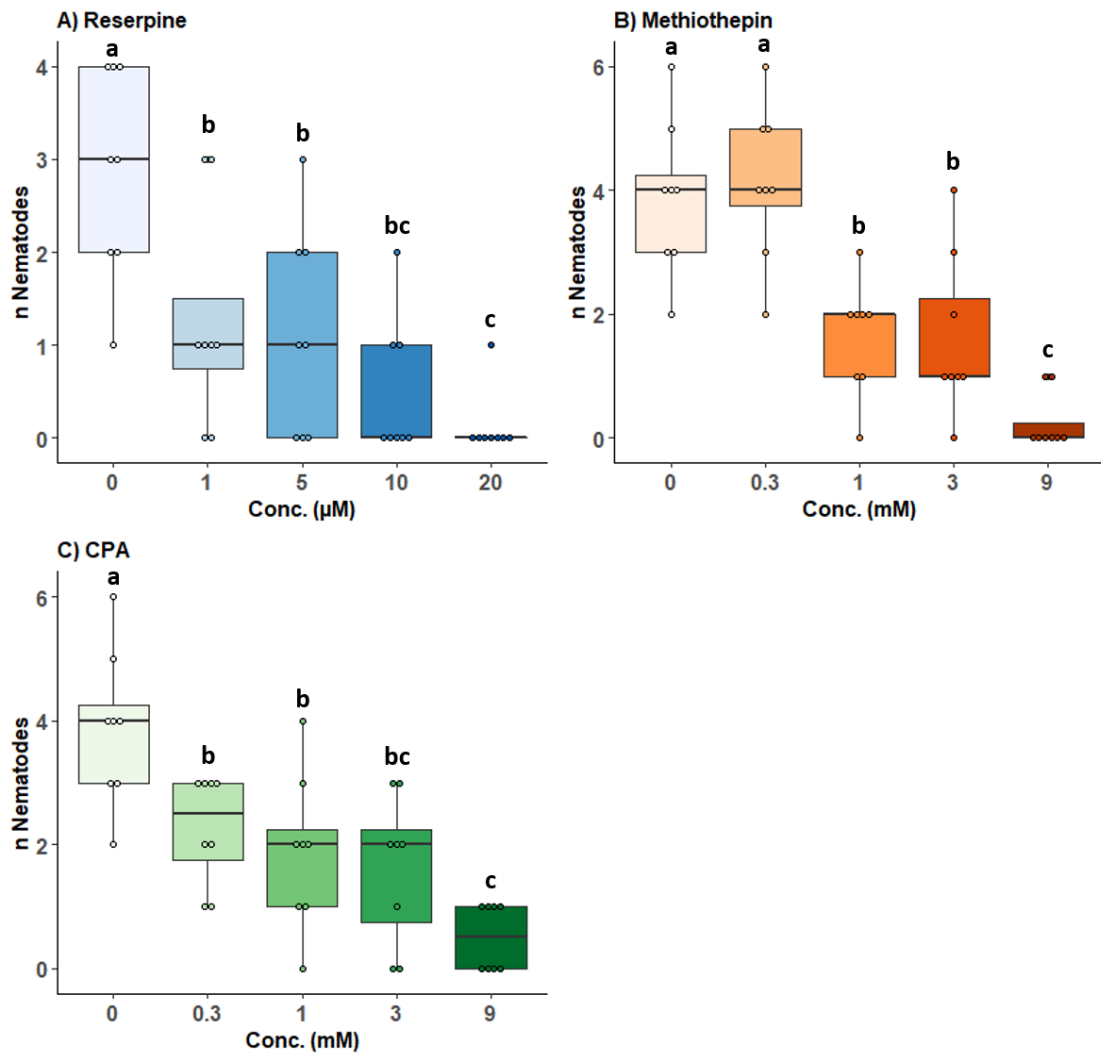


Figure 5. 6. Effect of reserpine, methiothepin and CPA exposure on the invasion ability of *M. incognita*.

Number of infective stage juvenile (J2) *M. incognita*, incubated for 24 hours in pharmacological blockers, counted within roots of aduki plants 24 hours post inoculation. Approximately 20 J2s were pipetted onto each root system following a 24-hour incubation in a) reserpine b) methiothepin and c) 4-chloro-DL-phenylalanine methyl ester hydrochloride (CPA), using sterile distilled water as a negative control (0 mM). Eight replicates were used per treatment, performed in two biological repeats of nematode cultures. Bold lines in boxes represent the median, with boxes representing the interquartile range, and whiskers extend to the smallest and largest value within 1.5x interquartile range. Letters above each boxplot represent groups which are significantly different in Wilcox post hoc tests ($p < 0.05$) following a Kruskal-Wallis analysis.

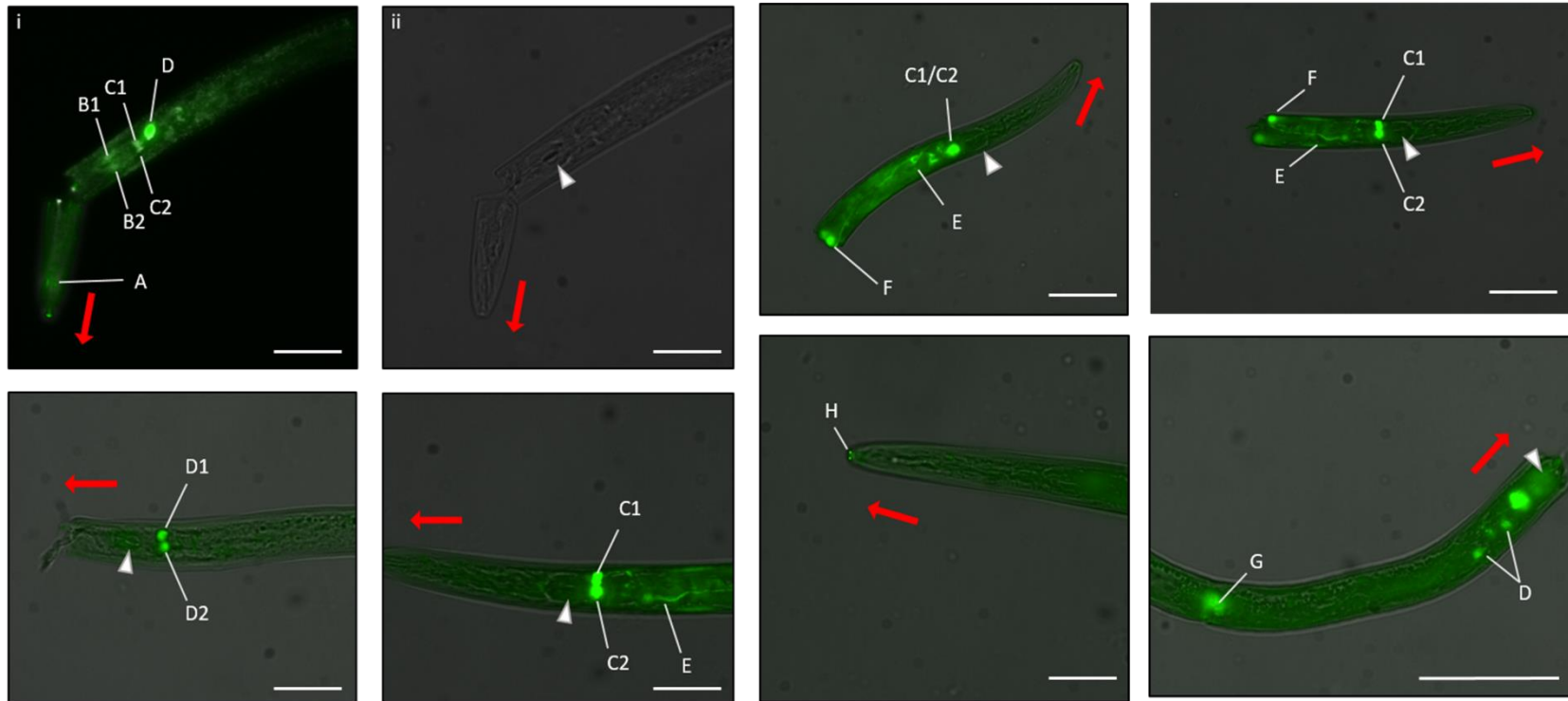


Figure 5. 7. Immunolocalisation of endogenous serotonin in *M. incognita* juveniles.

A) Weak immunolocalisation of stylet protractor muscles. B1 and B2) Weak immunolocalisation of neurone cell bodies within the metacarpus. C1 and C2) Strong immunolocalisation of neurone cell bodies directly behind the metacarpus and around the nerve ring position. D1 and D2) Moderate to strong immunolocalisation of neurone cell bodies posterior to metacarpus and nerve ring. E) Serotonin transmission or activity originating from behind the nerve ring and following the excretory duct. F and G) Strong immunolocalisation of possible neuronal cell bodies around the juvenile mid-section. H) Strong immunolocalisation at the amphidial openings. White arrows indicate the position of the metacarpus within the nematode. Red arrows show orientation of the nematode, facing the anterior end. All images are composites of bright-field and GFP fluorescent photographs, with the exception of i) GFP only, and ii) Bright-field only. Scale bars (white bar, lower right on each image) represent 50 µm.

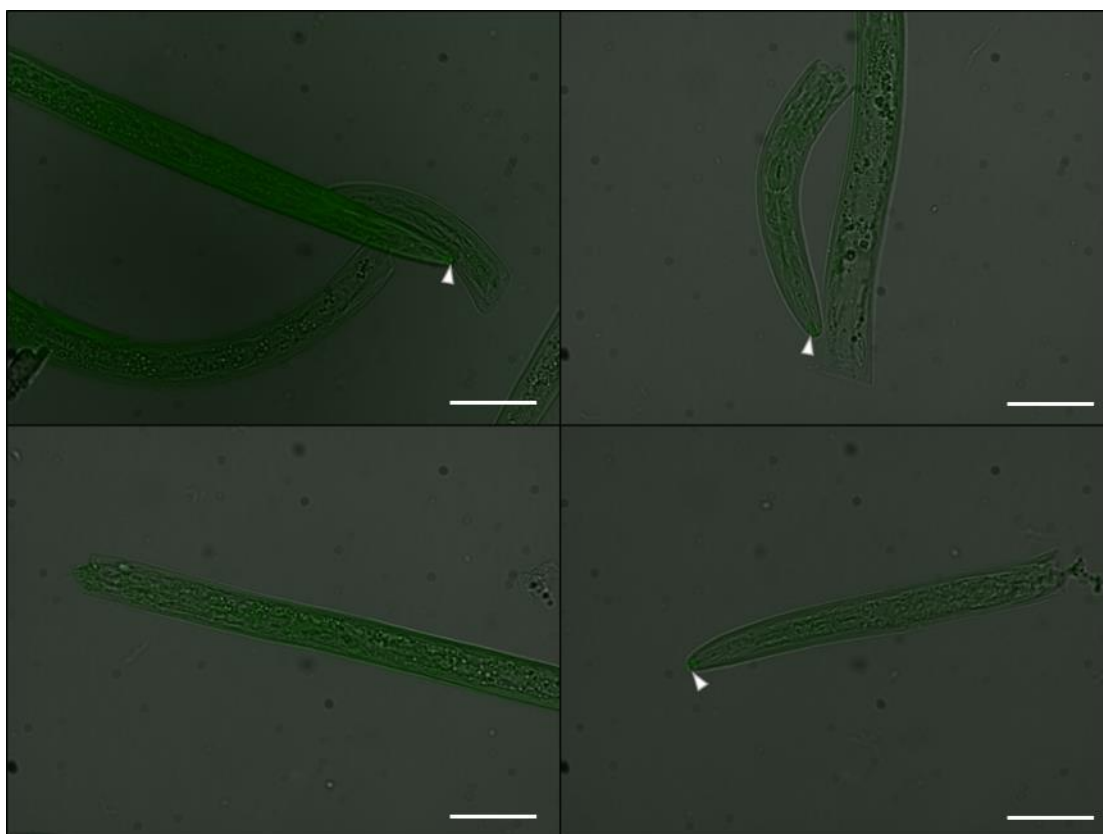


Figure 5. 8. Negative controls for the immunolocalisation of endogenous serotonin in *M. incognita* juveniles.

M. incognita juveniles treated as negative controls, incubated with only the secondary anti-rabbit FITC-labelled antibody and not the primary antibody, showed no strong immunoreaction under GFP excitement, with the exception of some moderate immunolocalisation at the stylet (White arrow). Scale bars (white bar, lower right on each image) represent 50 μm .

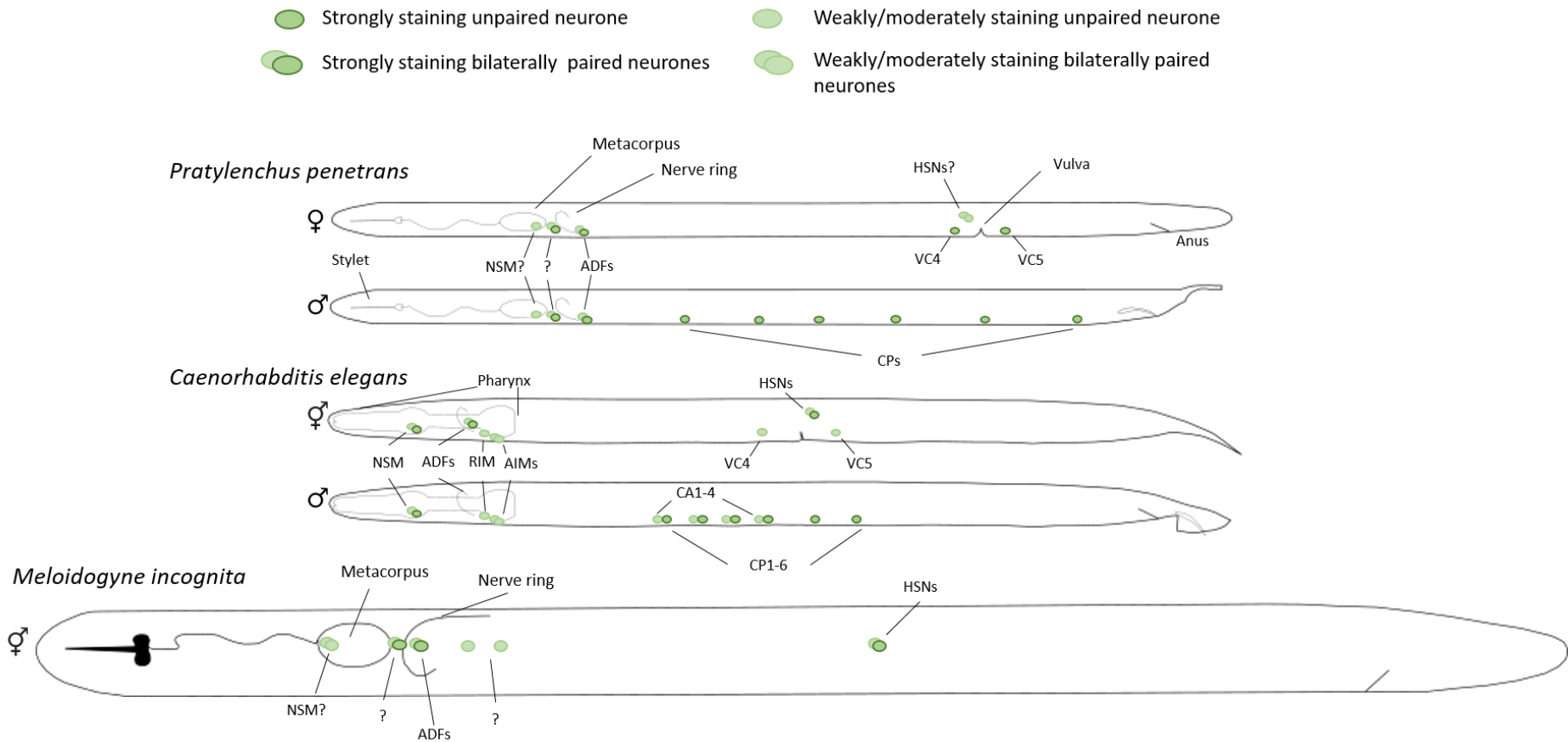


Figure 5. 9. Schematic diagram of serotonergic neurones in *M. incognita* juveniles based on anti-serotonin immunoreactivity.

Schematics of *Pratylenchus penetrans* and *Caenorhabditis elegans* were adapted from Loer & Rivard (2007) and Han *et al.* (2017). Neurone identities that are stained in *M. incognita* are suggested based on strength and positioning of cell bodies in comparison to *P. penetrans* and *C. elegans* neurones.

Other features of *M. incognita* were consistently highlighted apart from neuronal cell bodies. Two small and strong fluorescent spots could be seen at the mouth opening. Whilst some immunoreactivity can be seen in the stylet of the negative controls (Figure 5. 8), the two distinct points at the mouth opening are more strongly fluorescent and separate from the stylet. These specifically could be at the amphidial openings of the *M. incognita* juveniles. Two areas were weakly highlighted surrounding the stylet, which could be the stylet protractor muscles, indicating serotonin transmission that mediates muscle activity for stylet thrusting. Finally, the excretory duct located posterior to the nerve ring was strongly and consistently highlighted.

5.3.6 Presence of serotonergic genes in plant parasitic nematodes

BLASTP and tBLASTn homology searches were performed using the amino acid sequences of known *C. elegans* serotonin receptors, as well as synthesis and metabolism genes, in order to identify the presence of orthologous genes in *Meloidogyne* species (Table 5. 1). Four species of *Meloidogyne* were chosen, two of which are known to infect coffee (*M. incognita* and *M. hapla*), and two others which are of scientific and economic importance (*M. enterolobii* and *M. graminicola*). The BLASTP search was also expanded to two migratory endoparasitic nematodes, *Radopholus similis* and *Pratylenchus coffeae*, for a larger representation of other important plant parasitic nematodes of coffee. Orthologues identified would be considered as essential regulators of essential parasitic behaviours, such as chemo-sensation and chemotaxis, locomotion and invasion and feeding within host roots using the stylet.

Genes encoding the G-protein coupled serotonin receptor SER-5 and the serotonin-gated chloride channel MOD-1 were identified in the genomes of *M. incognita*, *M. graminicola*, *M. enterolobii* and *M. hapla*. However, there were no clear orthologues for the gene encoding G-protein coupled serotonin receptor SER-7 in *M. enterolobii*, or for those encoding SER-1 and SER-4 in any of the four root-knot nematode genomes. Orthologues of all *C. elegans* G-protein coupled serotonin receptors were present in the genomes of the root lesion nematodes *Radopholus similis* and *Pratylenchus coffeae*, as well as the chloride channel MOD-1. Most of the synthesis and metabolism genes in the serotonin pathway were also present across the four *Meloidogyne* spp., with the exception of the vesicular monoamine transporter *cat-1*, which had no orthologue match in *M. graminicola*, and the monoamine oxidases *amx-1* and *amx-2*, which had no hits in any *Meloidogyne* spp. The two root lesion nematodes also did not have orthologues for *C. elegans amx-1* or *amx-2*, but no orthologue for *amx-3* was found in *R. similis* either.

Table 5. 1. Presence of molecular components of the serotonin pathway in root knot and root lesion nematodes

Presence/absence of orthologues of *Caenorhabditis elegans* serotonin biosynthesis pathway genes in the genomes of *Meloidogyne* species, *Radopholus similis* and *Pratylenchus coffeae* as identified by BLASTP and tBLASTn searches. Similarity (% ID) for each putative orthologue is shown if present.

Gene	Root knot nematodes				Root lesion nematodes	
	<i>M. incognita</i>	<i>M. graminicola</i>	<i>M. enterolobii</i>	<i>M. hapla</i>	<i>R. similis</i>	<i>P. coffeae</i>
ser-1 G-protein coupled receptor	x	x	x	x	57	68
ser-4 G-protein coupled receptor	x	x	x	x	59	61
ser-5 G-protein coupled receptor	77	71	78	75	51	59
ser-7 G-protein coupled receptor	76	71	x	71	74	64
mod-1 Serotonin gated chloride channel	80	87	80	91	47	75
tph-1 Tryptophan hydroxylase	81	84	86	85	68	51
cat-4 GTP-cyclohydrolase	82	80	83	81	75	79
bas-1 Aromatic AA decarboxylase	62	62	62	63	40	53
cat-1 Vesicular monoamine transporter	80	x	97	94	58	62
mod-5 Serotonin reuptake transporter	83	76	94	86	62	67
amx-1 Monoamine oxidase	x	x	x	x	x	x
amx-2 Monoamine oxidase	x	x	x	x	x	x
amx-3 Monoamine oxidase	54	65	77	58	x	51

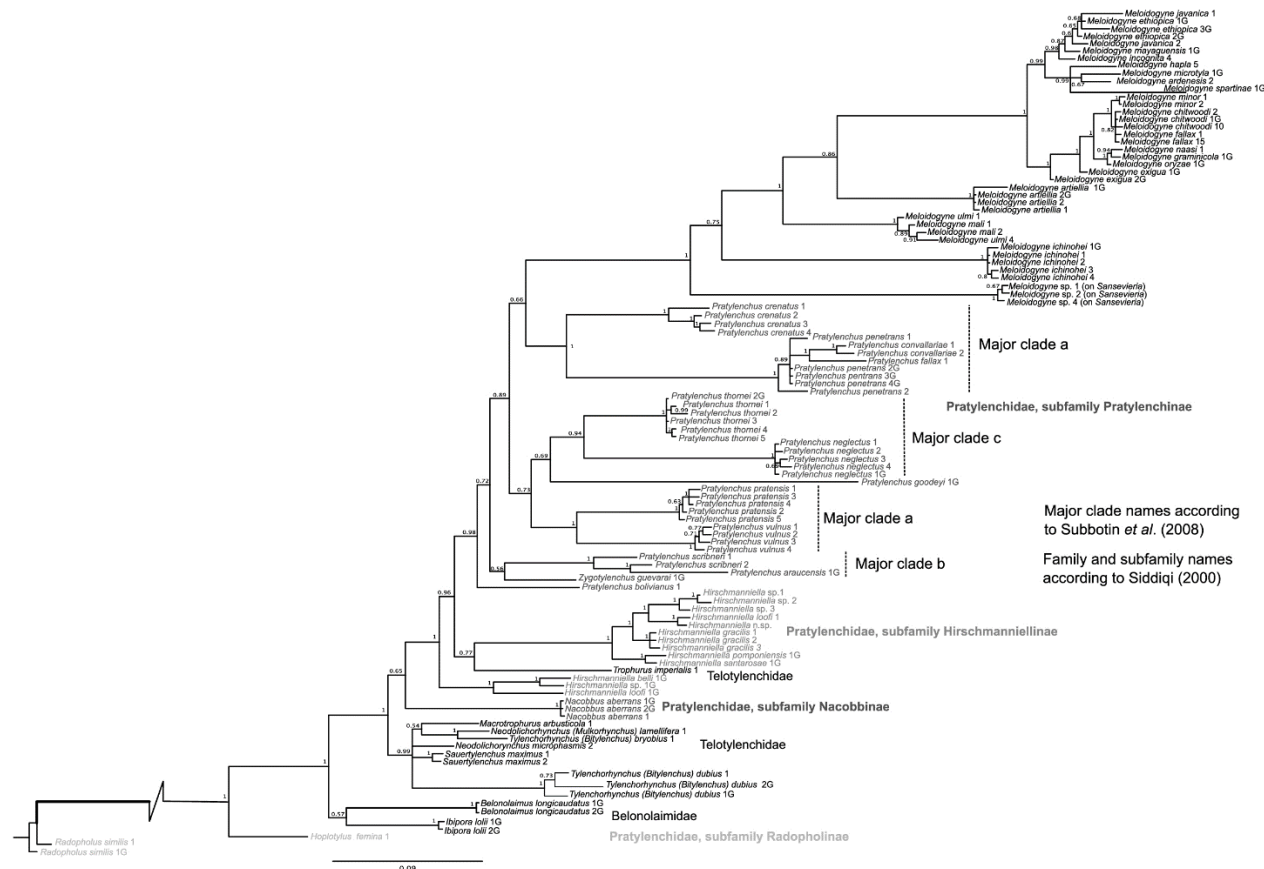


Figure 5.10. A Bayesian inference of a *Pratylenchidae* and *Meloidogynidae* tree based on partial sequences of the largest subunit of the RNA polymerase II gene (*rpb1*).

A Bayesian inference of a *Pratylenchidae* and *Meloidogynidae* tree based on (nearly) full length SSU rDNA sequences. The numbers next to nodes are posterior probabilities (pp). pp values > 0.95 are considered to be robust. For the nomenclature of taxa we adhered to Siddiqi (2000). For grouping of *Pratylenchus* species, major clade identifiers as proposed by Subbotin et al. (2008) are used. Figure taken from and analysis performed by Rybarczyk-Mydlowska et al (2014).

Genes involved in the serotonin pathway of plant parasitic nematodes here that are found across all species and genera (i.e. *ser-5*, *mod-1*, *tph-1*, *cat-4*, *bas-1*, and *mod-5*) offer ideal targets for plant parasitic control. Drugs, or nematicides, that are developed which target these genes will be effective against plant parasitic nematodes regardless of species or lifestyle, providing broad-spectrum protection.

5.4 Discussion

5.4.1 Role of serotonin in *M. incognita* parasitic behaviours

Several distinct behaviours are essential in the lifecycle of plant parasitic nematodes and the successful parasitism of host plants. Firstly, plant parasitic nematodes need to locate and move to host roots using compounds exuded from roots providing a chemical gradient. Once the nematodes are in close proximity to their hosts, they need to perform stylet thrusts to initially penetrate through cell walls and invade into root cells, and later to extract nutrients from cells for development. In the potato cyst nematode *G. pallida*, these behaviours have all been shown to be regulated, at least in part, by the serotonergic pathway, with multiple neurobiological components characterised (Crisford *et al.*, 2020). Molecular characterisation of the serotonin-signalling pathway, however, has not been performed for other plant parasitic nematodes, despite the availability of sequenced genomes. This characterisation of serotonin genes, which are required for key parasitic behaviours of root knot and root lesion nematodes, would unveil molecular targets for inhibition of infection and effective control for plant parasitic nematodes that infect coffee.

The serotonin immunoreactivities of *C. elegans* and *P. penetrans* were used as comparisons to locate and identify serotonergic neurones in *M. incognita*. In *C. elegans*, both NSM and ADF show the strongest immunoreactivity to serotonin, but only ADF was strongly fluorescent in *P. penetrans* (Loer & Rivard, 2007; Han *et al.*, 2017). The NSM class is situated within the metacarpus of the oesophagus (Albertson & Thomson, 1976). Interestingly, there is only weak immunoreactivity to serotonin in the metacarpus of both *P. penetrans* and in *M. incognita*, where this class of neurones is expected. The ADF neurones, however, are situated outside of the oesophagus and are strongly fluorescent in all three nematodes (Albertson & Thomson, 1976; Han *et al.*, 2017). Research in *C. elegans* has highlighted the role of ADF neurones in chemosensory and feeding behaviours, directly involving the serotonin receptor SER-7 and the tryptophan hydroxylase TPH-1 (Cumminham *et al.*, 2012; Song *et al.* 2013). Serotonin released from ADF neurones alone was also sufficient to mediate these behaviours. It is feasible, therefore, that the ADF-produced serotonin has a greater influence in the parasitic behaviours

of plant parasitic nematodes, such as stylet thrusting. The HSNs, VC4 and VC5 neurones are localised toward the posterior end of hermaphrodite *C. elegans* and female *P. penetrans* (Loer & Rivard, 2007; Han *et al.*, 2017). For juvenile *M. incognita*, only putative HSNs could be observed to be immunoreactive. The lack of VC4 and VC5 may be due to lower immunoreactivity, meaning they were not visible in this instance, but they could also indicate differences in neurobiology between nematodes of different lifestyles. The immunolocalisation of serotonin in male *M. incognita* could also be considered to confirm the presence of putative CP and CA neurones in this plant parasitic species. Further imaging and analysis of serotonergic neurones is recommended, utilising confocal microscopy, to confirm their identity in plant parasitic nematodes, especially since this study relies only on comparisons of immunoreactivity between nematode species based on positioning and the strength of staining. Regardless, the immunoreactive staining of *M. incognita* demonstrates the presence and activity of the neurotransmitter serotonin, particularly in the anterior region of the parasitic juvenile stage.

Three pharmacological blockers, which target components of the serotonin biosynthesis and signalling pathway, were effective in disrupting chemotaxis and stylet thrusting in *M. incognita*. A concentration-dependent reduction in chemotaxis toward salicylic acid was caused by overnight incubation in reserpine, methiothepin and CPA, supporting previous suggestions that chemotaxis and food-foraging behaviours are serotonergic-signalling dependent (Fleming *et al.*, 2017; Han *et al.*, 2017). CPA irreversibly inactivates the enzyme tryptophan hydroxylase, while methiothepin irreversibly blocks serotonin receptors (Jéquier *et al.*, 1967; Hobson *et al.*, 2003). The disruption of chemotaxis and stylet thrusting of *M. incognita* caused by these chemicals therefore would be long-lasting, if not permanent, and severe. The effect of reserpine on chemotaxis and stylet thrusting after 24-hour exposure was similar to both CPA and methiothepin, though more observations post 24 hours would be needed to confirm the long-lasting effects of the chemical to suggest its mechanism. Compounds developed to control plant parasitic nematodes that involve disruption of parasitic behaviours by targeting serotonin signalling should also function irreversibly to ensure their effectiveness. Though irreversible inhibitors are commonly less selective so could increase the risk of affecting off-target organisms.

The low doses of these chemicals required to inhibit chemotaxis reflects the action of other nematicides on parasitic nematodes. Aldicarb is a potent acetylcholinesterase inhibitor (Opperman & Chang, 1992). Acetylcholinesterase is essential in the nervous system of nematodes as it terminates neurotransmission at synapses by inactivating acetylcholine. Without its termination, the accumulation of acetylcholine would cause the constant

stimulation of nerves. Aldicarb uptake in nematodes can lead to tremors, paralysis and death (Opperman & Chang, 1992). At low concentrations however, Aldicarb, as well as a synthetic acetylcholinesterase inhibitor peptide, disrupts *G. pallida* chemosensation (Winter *et al.*, 2002; Wang *et al.*, 2011). Levamisole is also a widely used anthelmintic, as an agonist to nicotinic acetylcholine receptors (Fanelli *et al.*, 2005), to control livestock and human nematode parasites. At low concentrations, it also causes disruption of chemosensation, with symptoms at higher concentrations including spastic paralysis of body wall muscles, protraction of male spicules, stimulation of egg laying and ultimately death (Kim *et al.*, 2001; Winter, 2002).

The stylet thrust response of *M. incognita* following serotonin and fluoxetine exposure was impaired by incubation with all three pharmacological blockers. Methiothepin is an antagonist to both SER-7 and MOD-1 in *G. pallida*, which leads to the inhibition of the stylet thrust behaviour (Crisford *et al.*, 2020). The incubation of *M. incognita* in methiothepin blocked the stylet thrust suggesting that it was antagonistic to G-coupled serotonin receptors or a serotonin gated chloride channel in this plant parasitic species too. Reserpine, which is a naturally occurring plant alkaloid from *Rauwolfia serpentine* used medically for its tranquilising action, inhibits the mammalian vesicular monoamine transporter and the *C. elegans* and *G. pallida* orthologue CAT-1 (Erickson *et al.*, 1992; Crisford *et al.*, 2020). As reserpine blocked stylet thrusting in response to fluoxetine but not serotonin, it must also inhibit serotonin signalling pre-synaptically since the exogenous serotonin could still stimulate postsynaptic serotonin receptors. It is likely, then, that reserpine is also targeting a vesicular monoamine transporter and disrupting function. CPA causes the irreversible inactivation of mammalian and nematode tryptophan hydroxylases (Jéquier *et al.*, 1967; Crisford *et al.*, 2020), and appears to act pre-synaptically to inhibit stylet thrusting in *M. incognita*, as only fluoxetine and not exogenous serotonin-stimulated stylet thrusting was inhibited by the chemical. Together, the disruptive effect of these chemicals on stylet thrusting strongly supports the presence of an orthologous serotonin-signalling pathway in *M. incognita* to that of *C. elegans* and *G. pallida* that mediates stylet function.

5.4.2 Serotonin signalling pathway genes conserved in plant parasitic nematodes

The development of neurobiological control agents can be aided by improved knowledge of the precise molecular characteristics of the serotonin-signalling pathway. In *G. pallida*; *cat-1*, *tph-1*, *ser-7* and *mod-1* were all functionally characterised using *C. elegans* as a heterologous model (Crisford *et al.*, 2020). Using the same principles, we identified putative orthologues of the same genes in root knot and migratory endoparasitic nematodes, as well as other serotonergic related genes. Whilst most genes appear to be conserved across the two superfamilies of nematode,

there are instances where no orthologues of genes were found in plant parasitic species. Putative orthologues to genes encoding G-protein coupled receptors SER-1 and SER-4 were present in the genomes of *R. similis* and *P. coffeae*, but no matches were found in the genomes of any root knot nematode. SER-4 was identified previously in *G. pallida*, but this cyst nematode had no clear SER-1 orthologue (Crisford *et al.*, 2020). The lack of some of these G-protein coupled receptors begins to highlight phylogenetic differences between plant parasitic nematodes of different families. NemChR-DB, a recently developed database containing information on chemosensory G-protein-coupled receptors in nematodes, predicts the presence of many of these receptors in root knot nematodes (Langeland *et al.*, 2021). Though root knot nematodes appear to lack direct orthologues of characterised receptors, the large expanse of nematode chemosensory G-protein coupled receptors means that other genes performing the same function are still important to study to understand and potentially disrupt host-seeking behaviours (Bargmann, 2006; Krishnan *et al.*, 2014).

Root-knot and root-lesion nematodes are closely related genera of plant parasitic nematodes, based on both shared morphological characteristics and Ribosomal DNA (rDNA) sequence analysis (Figure 5. 10). Root-knot nematodes are also recognised to have arisen from a common migratory Pratylenchidae ancestor (Rybarczyk-Mydłowska *et al.*, 2014), though the species has yet to have been unequivocally identified. However, the migratory nematode *Radopholus similis* within the Pratylenchidae family is evolutionary closer to endoparasitic cyst nematodes (Heteroderidae) than it is to both *Meloidogyne* and *Pratylenchus* spp., based on small subunit ribosomal DNA (Mathew & Opperman, 2019). As some serotonergic genes appear in the genomes of *P. coffeae*, but not root-knot nematodes, particularly those encoding G-protein coupled receptors (*ser-1* and *ser-4*), it is most likely that they have been lost in the evolution of *Meloidogyne* species, as they are present in the closely related and ancestral *R. similis* genome. In contrast, the presence of monoamine *amx-3* may be a gene gain within these families, as it is the only gene which is not present in *R. similis*.

The presence of genes in the amine oxidase family was explored in plant parasitic nematodes due to their expression and role in chemosensory neurones, e.g. AMX-2 is the primary monoamine oxidase that metabolises serotonin, and therefore are potential drug targets (Wang *et al.*, 2017). However, only putative orthologues for *amx-3* were found across all species considered, with the exception of *R. similis* that had no obvious orthologue. The absence of *amx-1* and *amx-2* in all root knot and the root lesion and burrowing nematodes highlights the distinct neurobiology of plant parasitic nematodes compared to other nematodes such as the model, free-living *C. elegans*.

In some cases, instead of whole groups or families of genes absent between different nematodes, individual species may lack one gene involved in serotonin signalling. The guava root-knot nematode, *M. enterolobii*, was the only *Meloidogyne* spp. that had no obvious orthologue to *ser-7*. This species has been classed in the same clade as *M. incognita*, and so would be expected to be genetically similar, but has also been described as clearly distinguished from other species in that clade by mitochondrial and ribosomal RNA sequence comparisons (Janssen *et al.* 2016). *M. graminicola* was also the only species not to have an obvious orthologue for the *cat-1* gene. The discrepancies in which only singular species appear have singular gene losses may highlight an issue in the completeness of available genomes. Compared to the model free-living nematode *C. elegans*, and more recently sequenced genomes of nematodes, the sequencing and assemblies of less studied plant parasitic nematodes may be at a lower standard. The gaps in these assemblies may explain the unusual apparent gene losses in singular species.

The absence of *ser-7* and *cat-1* in individual species of nematode emphasises the importance to select a drug target that is conserved across all plant parasitic nematodes. A crop such as coffee will be under threat from multiple species at any one time and so any control agent developed will need broad-spectrum coverage against multiple species; a drug that targets all nematode CAT-1, for example, would not protect coffee against *M. graminicola* if the genome information is accurate, and so yields would still be affected. In contrast, a highly conserved gene across nematode superfamilies will likely also be highly conserved across all animals. This would lead to difficulties in developing a control method that would be considered environmentally safe due to effects on off-target organisms.

The serotonin gated chloride channel MOD-1 offers an ideal target for plant parasitic nematode control. The gene encoding the channel is not only conserved but also selectively found in invertebrate phyla (Jones & Satelle, 2008). This means that any novel drug that targets the channel would not only be effective against all plant parasitic nematodes that infects a crop, but also be selective enough to reduce damage to off-target organisms, a concern for the development and approval for anthelmintics (Araujo *et al.*, 2022). The feasibility of any approach that targets MOD-1 of course would need to be assessed. Many anthelmintic drugs that target serotonin-gated chloride channels, however, have been largely successful in both veterinary and human treatment of nematode and arthropod parasites. Albendazole, for example, is used for the treatment of lymphatic filariasis in humans, caused by filarial worm infections of *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (Molyneux *et al.*, 2003). Other anthelmintics that target serotonin-gated chloride channels include mebendazole and praziquantel. These drugs are generally well-tolerated and have a low risk of side effects, although they may not be

effective against all types of helminths (Dayan, 2003). The delivery and mechanism to inhibit MOD-1 in plant parasitic nematodes would also need to be established, though, in a selective manner not to disrupt the serotonin signalling of off-target organisms.

5.4.3 Serotonin signalling as a target for plant parasitic control

The efficacy of targeting serotonin signalling as a potential nematode control for crops was demonstrated by the reduction in nematodes able to infect aduki plant roots following exposure to each chemical. The potential of neurobiological affecting nematicides has also been investigated and demonstrated against several plant parasitic nematodes. Khalil (2014) summarises the nematicidal effectiveness of avermectins, which stimulate the release and binding of gamma-amino butyric acid (GABA) at nerve endings leading to neuromuscular paralysis, on *Meloidogyne*, *Radopholus* and *Ditylenchus spp.* Several nematode neuropeptides were also shown to disrupt chemosensation, host-finding and invasion behaviours in *M. incognita* and *G. pallida* when applied exogenously to the nematodes, whilst also having no effect on off-target organisms (*C. elegans* and *Steinernema carpocapsae*) (Warnock *et al.*, 2017). The low doses required to reduce the infective capability, using the pharmacological blockers in this study, are also comparable to other potential chemicals currently investigated to be used as nematicides; fluopyram at low toxicity reduced the infection of *M. incognita* on tomato and *Heterodera schachtii* on *Arabidopsis thaliana* (Faske and Hark, 2015; Schleker *et al.*, 2022). Similarly low concentrations of aldicarb and abamectin, previously used as nematicides, have also been used as chemical control against *M. incognita* and *Rotylenchus reniformis* (Haydock *et al.*, 2013).

An alternative to chemical controls discussed so far would be to silence or disrupt plant parasitic specific serotonin-related genes with RNAi (Lilley *et al.*, 2012). If crops are successfully engineered to express dsRNA molecules that disrupt serotonergic signalling, which plant parasitic nematodes will ingest through feeding on host cells, it would provide a control with less environmental risk than using polluting chemicals with off-target organism toxicity (Dutta *et al.*, 2015). The efficacy of this control has been demonstrated *in planta*. Root knot nematodes experienced defective phenotypes (e.g. paralysis and irregular movement) upon ingestion of siRNA/dsRNA, targeting either genes in siRNA and microRNA (miRNA) pathways, led to the reduction of infectivity *Arabidopsis thaliana* (Iqbal *et al.*, 2022). Soybean roots were transformed to incorporate siRNAs that disrupted the function of genes involved in reproduction, fitness and development, and led to decreased infectivity of the soybean cyst nematode, *Heterodera glycines*, and *M. incognita* on the transformed soybean upon ingestion (Li *et al.*, 2010; Ibrahim *et al.*, 2011). Attempts to use RNAi to inhibit *ser-7* revealed non-specific toxic effects of dsRNA

on the stylet system, demonstrating caution required when characterising genes based on stylet thrust behaviour in plant parasitic nematodes (Crisford *et al.*, 2015). Introducing RNAi through soaking nematodes in dsRNA has led to the selective disruption of other neuropeptide genes, however. Nematode FMRFamide-like peptides are a diverse family of neuropeptides that regulate both sensory and motor functions. Kimber *et al* (2007) were able to use RNAi, by soaking, to disrupt *G. pallida* FMRFamide-like peptide genes. The limitation from this finding is that the uptake of dsRNA was most likely via the nematode amphids and so might not be possible in an *in-planta* RNAi system where dsRNA would need to be ingested.

There are more considerations on the use of host induced silencing of serotonergic signalling genes for plant parasitic nematode control. Firstly, neuronal cells of *C. elegans* have shown to be resistant to RNAi interference (Suvi *et al.*, 2005). GABAergic and dopaminergic neurones showed the greatest resistance, but cholinergic and glutamatergic neurones were more sensitive. When considering RNAi for any plant parasitic gene, not just neuronal genes, the efficacy of gene silencing can be variable. While RNAi can lead to large differences in plant parasitism reduction, demonstrating the effects of phenotype are of a direct result of RNAi can be difficult (reviewed by Lilley *et al.*, 2012). Joshi *et al* (2022), whilst summarising the potential of RNAi technologies for control, also highlights the success of targeting nematode effectors (pathogen proteins and small molecules that alter host-cell structure and function), which may be a more ideal target for control rather than genes involved the nematode nervous system. Finally, only locomotion and feeding behaviours have been explored here. RNAi, and other control methods, could also be used to inhibit egg laying and reproductive behaviours to disrupt the lifecycle of plant parasitic nematodes. The hatching of *Meloidogyne artiella* juveniles, for example, was delayed by soaking the eggs in dsRNA targeting against a chitin synthase gene involved in the synthesis of the chitin layer in eggshells (Fanelli *et al.* 2005). Targeting other stages of the lifecycle, therefore, may prove a better mechanism for nematode parasitism disruption and should be considered and compared with findings here.

The use of RNAi and other genetic engineering technologies has been efficiently demonstrated in coffee for many years (Ogita *et al.*, 2003; Simon-Gruita *et al.*, 2019) and should be considered in the development of new coffee varieties, which are protected against plant parasitic nematode damage.

5.5. Key Findings

Immunolocalisation of serotonin confirms its neurotransmission in *M. incognita* and the presence of serotonergic neurones. Plant parasitic behaviours, such as locomotion, chemotaxis and stylet function were shown to be mediated by serotonin in *M. incognita*. The inhibition of the serotonin biosynthesis pathway in *M. incognita* reduces the parasitic effectiveness of infective stage juveniles to invade into a host plant, and therefore, genes that regulate serotonin signalling in root knot nematodes are promising novel targets for control.

Chapter 6

General Discussion

The interaction of plant parasitic nematodes with a host plant can be viewed in two paradigms: 1) the ability of the nematode to find its host, invade into root tissue and set up feeding sites to develop, and 2) the ability of the host plant to respond to invasion, migration and development of the nematodes within its roots. Defining the molecular mechanisms that drive aspects of these two paradigms helps the general understanding of how these organisms interact with each other, from which we can then develop sophisticated controls against plant parasitic nematodes and protect the host plant from parasitic damage. This study has aimed to characterise the molecular mechanisms that drive interactions of plant parasitic nematodes and Robusta coffee with a view to the longer-term development of improved nematode control strategies and technologies.

6.1 Genetic resources of root-knot nematode resistance and tolerance in coffee

The natural genetic variation found within different varieties of a crop is a vital resource both for the understanding of a trait, and as a tool for introducing desired traits into a crop through breeding. Coffee genebanks are a rich resource for genes of interest. Over 100 species have been identified in the genus *Coffea* that exhibit variations in morphology and ecology but from a genetic perspective are still capable of being readily hybridised to produce fertile interspecific hybrids (Davis *et al.*, 2006; Bertrand & Anthony, 2008). Whilst these resources have been utilised to identify some species and varieties that show resistance to plant parasitic nematodes, reports on genetic resources of Robusta coffee that are resistant to root-knot nematodes are few (Bertrand & Anthony, 2008). Examples include accessions resistant to *M. incognita* race 1 (Goncalves *et al.*, 1996), accessions resistant to Guatemala isolates of *M. paranaensis* (Bertrand *et al.*, 2000) and several accession of Robusta resistant to *M. exigua* (Bertrand & Anthony, 2008). The genes that mediate the resistances in these resources, if mapped and identified, are key in the development of new resistant coffee varieties. Many resistance genes identified and used in crop breeding programmes are effective against only a limited number of nematode species (Discussed in Chapter 1). Multiple genes will therefore have to be “pyramided” to provide adequate broad-spectrum protection if bred into new varieties (Bertrand & Anthony, 2008). This is also important, as reliance on one resistance gene will lead to a selection for nematode virulence over time, leading ultimately to the breakdown of resistance (Verdejo-Lucas *et al.*, 2009; Giné & Sorribas, 2017).

In Chapter 3, the resistance and tolerance of five Robusta varieties to root-knot nematodes were assessed as a screen for potential genetic sources of tolerance or resistance. In comparison to reports on the resistance of coffee varieties to root-knot nematodes, we observed lower than expected numbers of nematodes that were able to develop across all varieties tested. This could be indicative of generally high levels of resistance found in these varieties. Reasons, other than natural resistance, that could lead to low infection rates include High initial inoculation densities, short infection periods and the lack of varieties with defined susceptibilities. These factors, discussed in Chapter 3, highlight the limitations of translating laboratory and greenhouse studies on coffee to the interaction of plant parasitic nematodes and coffee in the field. Carraro-Lemes *et al* (2021) further detail factors required for ideal infection of *M. paranaensis* on coffee to assess resistance. For example, if plants are too young, the volume of root will introduce competition, too old and roots become too lignified which can prevent successful invasion. In the field, however, coffee plants can be grown for cultivation over 20 years, during which time the population dynamics of plant parasitic nematodes infecting coffee can change drastically (Ziska *et al.*, 2018; Siebert *et al.*, 2020). It is obviously not feasible to reflect such conditions in a smaller scale experiment. While samples can be taken from established coffee fields to screen for tolerance or resistance against plant parasitic nematodes identified in surrounding soils, fluctuating and uncontrollable conditions outside of experimental control mean that determining molecular mechanisms of either resistance or tolerance cannot be performed.

Root-knot nematodes showed differential behavioural responses to the root exudate of different coffee varieties (Chapter 3.3.4). The relationship between chemo-attractiveness of a plant parasitic nematode toward root exudate of a host and the suitability of the host has already been established in other systems (Yang *et al.*, 2016; Kirwa *et al.*, 2018). If this correlation is confirmed for coffee and plant parasitic nematodes, then the root exudate of coffee could be considered a measurable phenotype as an indicator for nematode susceptibility. Assessing the chemotaxis of nematodes to the root exudates of a coffee variety would be a high-throughput screening method for susceptibility in comparison to infection assays that require weeks to months for results. The composition of an attractive or repulsive exudate would also be useful knowledge for the development of new coffee varieties that show a level of resistance towards plant parasitic nematodes. It has been proposed that Robusta roots contain and exude high levels of phenolic compounds that contribute to their general resistance against plant parasitic nematodes, compared with Arabica (Villain *et al.*, 2004). This is supported by evidence that phenolic compounds, which can be present in root exudates, are repellent to some root-knot and root lesion nematodes (Wuyts *et al.*, 2006). Genes that are involved in the synthesis of

phenolic compounds could then be of interest for incorporation into commercial varieties of coffee to help protect against plant parasitic nematode damage.

A novel method to screen for either susceptibility or tolerance to plant parasitic nematodes would be to measure the effect on coffee leaves, specifically chlorophyll content and fluorescence. Multiple Robusta varieties were shown to be intolerant to the infection of root-knot nematodes, and suffered a decrease in photosynthetic efficiency, which could be measured in the leaves. The more tolerant Robusta variety FRT101 also showed comparatively few transcriptomic changes in leaf tissue following root-knot nematode infection. These results together suggest that measurements of leaves could indicate the presence of plant parasitic nematodes and could therefore be used as screen for levels of tolerance in coffee. The ability to sample the leaves of coffee has the major advantage of being a quick method that preserves the plant for further use, as the root system does not need to be collected, washed etc. for the detection of nematode presence.

6.2 The constitutive and induced defences against plant parasitic nematodes in plants

In Chapter 4, root-knot nematode infection was shown to have a large effect on the transcriptomic regulation of Robusta coffee. These transcriptional changes demonstrate the significant reprogramming that is required for successful plant parasitic nematode infection, as well as the diversity of responses from the plant host to defend against parasitism. The experiments revealed genes involved in plant defence and immunity that could be ideal markers of resistance or susceptibility. These genes would also be candidates for crossing within a coffee breeding programme to introduce nematode protection as a trait.

A role for cell-wall-regulation was highlighted as a means to protect against root-knot nematode infection in coffee. The cell wall represents a physical barrier for the migration of plant parasitic nematodes through roots and can therefore be viewed as a constitutive defence. The natural variation in cell wall composition that occurs between plant hosts could be a major driver for a plant parasitic nematode to determine the suitability of a plant for parasitism. *Pratylenchus coffeae*, for example, regulates the expression of cell wall degrading enzyme genes based on the concentrations of xylan and cellulose in root exudate to which it is exposed (Bell *et al.*, 2019). Results in Chapter 3 also show enhanced chemo-attraction of plant parasitic nematodes to the root exudate of more susceptible hosts. The composition of the cell wall detected via root exudates pre-invasion is a key indicator to plant parasitic nematodes of the suitability of the plant as a host, and so it may be an initial deterrent, preventing any invasion and pathogenicity occurring.

The strength of the cell wall, as well as its elasticity and digestibility, is conferred by the cross linking of phenolic compounds by the formation of covalent bonds and the exclusion of water. Root-knot nematode infection has been shown to cause the accumulation of the cell wall polymer lignin for cell wall reinforcement or repair (Veronico *et al.*, 2019; Sato *et al.*, 2021). Ferulate cross-links, involving the hydroxycinnamic acid derivatives ferulic acid and p-coumaric acid, play an important part in cell wall extensibility and biodegradability. Several negative correlations have been established between the content of ferulate acids and disease severity or vulnerability to herbivory, although so far, a possible relationship has not been investigated with below ground pathogens (Bily *et al.*, 2003; Buanafina & Fescemyer *et al.*, 2012). Finally, extensin peroxidases can also harden the cell wall as a defence response against multiple plant parasitic nematodes (Rashid 2016). The observation that Robusta var. Nemaya has a higher content of phenolic compounds within its roots in comparison to other coffee species (Villain & Sarah, 2004), and is highly resistant to plant parasitic nematodes may not be a coincidence. The high concentration of phenolic compounds in Nemaya may lead to increased phenol cross-links that mediate effective induced defences against soil-borne pathogens.

A key component of the plant immune system is the induction of pathogenesis-related (PR) proteins and antimicrobial peptides (AMPs). These proteins and peptides are widespread throughout the plant domain, present in a wide range of plant organs, and are induced by a broad-spectrum of pathogens (van Loon *et al.*, 1994; Ali *et al.*, 2018a). Pathogenesis-related proteins and antimicrobial peptides are usually induced following the activation of defence signalling pathways; via salicylic acid for biotrophic pathogens, and via jasmonic acid for necrotrophic pathogens. Their induction can be both locally at the site of pathogen challenge, and systemically at a distance from the pathogen, leading to systemic acquired resistance. Furthermore, the induction of PRs and AMPs following pathogenicity can also lead to increased stress tolerance. The antifungal PR proteins (e.g. PR2 and PR3) protect cell damage due to cold stress, and cold stress induces the expression of PR12 and PR13 AMPs in wheat plants (Gaudet *et al.*, 2003; Janská *et al.*, 2010). Moreover, the upregulation of PR gene PR10 has been reported in response to multiple abiotic stresses in maize (Jake *et al.*, 2010). PR genes are also induced in potato and tomato following root-knot nematode infection (Bar-Or *et al.*, 2005; Shukla *et al.*, 2018). The multiple protection that PR proteins and AMPs provide, against both biotic and abiotic factors, makes them ideal targets for coffee improvement through genetic engineering.

6.3 Developing genetic controls for plant parasitic nematodes of coffee

Genetic engineering and gene editing provide tools for safe, efficient and effective means to remodel crops and introduce desirable traits. Technologies include zinc-finger nucleases and

transcription-activator-like effector nucleases, but more commonly used now is the CRISPR/Cas-9 system (Ye *et al.*, 2022). Rather than the random insertion of a target gene, the major advantage of gene editing over previous transgenic technologies is the ability to target the editing within the genome.

The use of genetic engineering paves the way for effective plant parasitic nematode controls, as previously discussed in Chapter 5. Genetic traits that mediate resistance or tolerance could be introduced into a commercial coffee variety to provide plant parasitic nematode protection, without spending the time and resources on crossing the genomic trait into a variety through breeding. Gene editing can also be used to knockout or knockdown a gene that contributes to the susceptibility to plant parasitic nematodes. Leibrok *et al* (2022) demonstrate the biological feasibility of using CRISPR/Cas-9 to knock out 7-methylxanthosine methyltransferase and 3,7-dimethylxanthine methyltransferase in coffee, producing a plant which does not produce caffeine. The same principles, therefore, could be applied to resistance and susceptibility genes of plant parasitic nematodes. Examples of engineered crops that perturb susceptibility to plant pathogens include the knockout of *MLO* (Mildew Locus O) in potato to increase resistance to the oomycete pathogen *Phytophthora infestans*, and disruption of amino acid metabolism genes in wheat resulted in reduced susceptibility to wheat yellow rust, *Puccinia striiformis* f. sp. *tritici* (Corredor-Moreno *et al.*, 2021; Kieu *et al.*, 2021).

The technology could also be used for host-induced gene silencing, in which crops are engineered to express dsRNA or ssRNA that disrupt the function of essential nematode genes upon digestion, as discussed in Chapter 5. The use of gene-edited crops is still strictly regulated in many regions, which would impede the use of the technology for plant parasitic nematode control. For example, the EU still regulates and prohibits gene-edited crops as if they were genetically modified (Gelinsky & Hilbeck, 2018). Many regions, however, do not restrict gene edited crops and food, regulating them as conventional plants, including Brazil, the United Kingdom and the United States (Gene Literacy Project, 2022).

'Omics' knowledge on the interaction of plant parasitic nematodes and host plants can still be utilised to develop control methods without the genomic engineering of a crop. Chapter 5 describes the investigation of the neurobiology that mediates root-knot nematode parasitic behaviours, focussing on the neurotransmitter serotonin. This could lead to the development of anthelmintics that can be applied at sites of infected coffee to reduce nematode burden. A better understanding of the plant immune response to plant parasitic nematodes, and specifically the mechanism of systemic acquired resistance, has also led to novel control methods. Root-knot nematode infection leads to induction of PR genes through salicylic acid

signalling which can result in the development of a broad-spectrum, systemic resistance (Durrant & Dong, 2004; van Loon *et al.*, 2006). When applied exogenously onto tomato, through soil drenches or root dips, salicylic acid or its synthetic analogues can inhibit root-knot nematode development and reduce root galling, whilst not negatively affecting plant growth (Molinari, 2006). Molinari and Leonetti (2019) were then later able to prime systemic acquired resistance in tomato using applications of beneficial microorganisms, including Bio-control fungi, arbuscular mycorrhizal fungi, and plant growth promoting rhizobacteria. The pre-treatment by these beneficial microorganisms also induced the expression of PR genes (PR-1, PR-3, PR-5, ACO). The priming of systemic acquired resistance in crops such as coffee, using the application of chemicals or bio-control agents, is therefore a potential strategy to reduce root-knot nematode damage from infection. Further investigation would be needed however, due to the complex interactions beneficial microorganisms may have with the existing soil microbiome of coffee crops, or the environmental impact of any systemic acquired resistance-inducing chemical.

6.4 Integrated management strategies for plant parasitic nematodes of coffee

A successful control strategy against plant parasitic nematodes needs to fulfil four key requirements (McSorely & Phillips, 1993; Nyczepir & Thomas, 2009). 1) Controls need to be available and attainable to a grower. If a control measure is limited to a few growers in only some regions, the development of such control may not be justifiable. 2) Knowledge of current plant parasitic nematode infestations. This includes understanding the species composition of nematodes within a field and the precise location of infestations within the field so that correct applications of controls can be made. 3) Control measures need to be cost effective. They need to be affordable for a grower concerned about plant parasitic nematodes, and result in an improvement of yields and profit to justify any initial costs. 4) Knowledge of how nematodes will respond to control measures. If nematode populations are only reduced transiently at the point of intervention, multiple applications of the control measure will be required. These factors together exemplify the difficulties in controlling pathogens within agriculture.

Integrated pest management is an agricultural practice that utilises both chemical and biological controls for pathogens whilst also adopting improved cultural practices, to reduce the frequency and quantity of any one control and reduce environmental damage associated with traditional crop yield improvements (Radcliff *et al.*, 2009). The integrated pest management strategies for other coffee pathogens could be used as models to inform best practices for plant parasitic nematode control. The coffee berry borer, *Hypothenemus hampei*, is a primary arthropod pest of coffee plantations worldwide (Vega *et al.*, 2015). The pest develops on and damages coffee

berries, directly affecting the yield quality of coffee production. The persistence of the pest within coffee fields in Latin America has led to development of multiple strategies to control it and prevent losses due to reduced quality of coffee yields (Reviewed by Aristizábal *et al.*, 2016). Alcohol traps are used to monitor the seasonal flight activity of the berry borer in Central and South America and estimate periods of dispersal. When more regular and efficient harvesting was taught in Columbia, the presence of coffee berry borers reduced massively. The entomopathogenic fungus *Beauveria bassiana* has also been used as an environmentally friendly bioinsecticide in Colombia, though its effectiveness can depend on several factors including the strain, concentration, virulence, weather conditions, and application efficiency (Vega *et al.*, 2012). Finally, sanitation practices have been improved in Colombia to prevent re-infestation of coffee berry borer post-harvest. The knowledge of coffee berry borer biology, ecology and behaviour in relationship to coffee has led to the successful development of biological and cultural control strategies. The application of these multiple strategies means the pest can be managed with little to no reduction to yields and demonstrates the effectiveness of an integrated pathogen management strategy.

Integrated pest management has also been shown to be beneficial to smallholder Arabica coffee farmers in Uganda (Isoto *et al.*, 2014). Coffee farms implementing this agricultural strategy, although not in response to plant parasitic nematodes, had an increased demand for commodities and increases in rural income. If implemented correctly, integrated pest management could therefore be an effective and productive control of plant parasitic nematodes of coffee.

A major issue in the development and application of pathogen control measures is the difficulties for growers to adopt new control measures. Implementing multiple controls requires intricate knowledge of each measure to ensure their effectiveness, as well as their effect on other plants and soil borne organisms. For former chemical controls, such as the application of methyl bromides, the knowledge requirement and successful application needed only information on an accompanying label (Nyczepir & Thomas, 2009). Methods that are more sophisticated may have a higher barrier of knowledge for successful implementation, especially if used in combination. Parsa *et al.* (2014) highlight other obstacles to the adoption of integrated pest management in developing countries. The main obstacle highlighted within their survey of practitioners from 96 countries was “insufficient training and technical support to farmers”. The poor support offered was due to weaknesses or a complete lack of research, outreach and adoption incentives from stakeholders. It is clear then, that even if effective control measures

for plant parasitic nematodes are developed, there is still a need to improve the ability for coffee growers to adopt and efficiently use such measures.

The assessment and development of control measures for coffee is also difficult due to its perennial nature. Damage from diseases and pathogens can be delayed over several years. This means crop loss assessment, necessary for both the diagnosis of pathogenicity and the effectiveness of any control measures implemented in response, is a major challenge to sustainable management (Avelino *et al.*, 2018). Growers may not have the skill or means to identify plant parasitic nematode damage; especially as major symptoms are below ground or non-specific. This lack of skill will also hamper accurate evaluation of control measures, leading to errors in further or on-going applications.

6.5 Future research for the control of root-knot nematodes

The fundamental biology of root-knot nematode and coffee interaction studies here can be used to inform future integrated pest management strategies. Two effective control strategies to implement would be the development of i) nematode-resistant and tolerant crops, and ii) nematicides that specifically targets serotonergic neurobiological components of plant parasitic nematodes.

On the first point, findings in Chapter Three show methods on phenotyping of Robusta varieties that show traits of either resistance/susceptibility or tolerance/intolerance. The practical and rapid identification of variety phenotype is essential for crop breeding programmes when developing any new crop. Chapter Four also highlighted molecular components that drive these phenotypes, including defence and pathogen response genes as well as cell wall regulatory genes. Not only should these genes and gene pathways be considered in crop breeding programmes but should also be targeted in gene editing for more expedient development of nematode-resistant and tolerant varieties. While some functional confirmation was performed using *Arabidopsis* as a homologous system, further research would be needed to validate the contribution of these genes have more precisely in the coffee crop itself, and how effective gene editing would be in coffee in reducing nematode parasitism.

Further functional confirmation is also required regarding the second strategy, development of serotonergic-targeting nematicides. Chapter Five highlighted *M. incognita* candidate genes in serotonin signalling pathway which are integral for nematode parasitism, based on homology to the model organism *C. elegans*. However, these still need to be functionally confirmed within the root-knot nematode species. This could be achieved by observing if RNAi knockdown, with siRNA or dsRNA which targets these genes within *M. incognita*, results in lower infection of a

susceptible host. Another approach would be to clone candidate *M. incognita* genes and attempt to rescue their functional phenotype in a transformed analogous *C. elegans* mutant. Once functionally confirmed, chemicals which target and disrupt such serotonergic signalling would need to be identified, followed by assessments on its the efficacy to reduce nematode populations in soils, and its specificity on these parasites and not to off-target organisms.

Finally, results discussed so far only consider root-knot nematodes. As multiple genera of plant parasitic nematode often infect coffee at any time, the strength of these control strategies will also depend on the effects of other nematode species, most notably root-lesion nematodes, and would be critical to assess. Regardless, these two approaches offer two new strategies in controlling plant parasitic nematodes that infect Robusta coffee and should be considered and included in wider integrated pest management strategies.

6.6 Concluding Remarks

The impact of plant parasitic nematodes on the yields of coffee is not negligible. Plant parasitic nematodes that infect coffee need to be managed to ensure growers, particularly in developing countries, do not suffer major losses in yields. This is especially important when growers face additional production constraints from other coffee pests and diseases, and the effects of climate change. The interaction of the root-knot nematodes and coffee studied here has revealed the potential for novel genomic-based controls that would be advantageous over previous methods. Firstly, using gene editing to introduce genes which confer resistance to plant parasitic nematode could provide better protection in new coffee varieties. Specifically improving cell wall regulation as a constitutive response to pathogen challenge will also offer broad-spectrum defences and tolerance against soil-borne pathogens. Whilst these engineered crops are in development, and for regions where gene editing is more heavily regulated, the use of nematicides should be considered. If developed to target only plant parasitic nematodes and no off-target organisms, can provide immediate protection in coffee fields with high nematode burdens. Implementing these two methods in an integrated management strategy could prevent global losses in coffee yields caused by plant parasitic nematodes.

Chapter 7

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