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Selected novel approaches for the integrated pest management of *Aphelenchoides fragariae* in ornamental plants

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Declaration

I hereby declare that this thesis has been composed by me and that the work is my own, except as acknowledged by means of references. This thesis has not been submitted for any other degree of professional qualification except as specified.

Idowu Joseph Rotifa

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Table of Contents

List of Abbreviations

AHDB	Agriculture and Horticulture Development Board
ASM	Acibenzolar -S - methyl
DNA	Deoxyribonucleic acid
EAMU	Extension of Authorisation for Minor Use
EC	Emulsifiable concentrate
EPN	Entomopathogenic nematodes
GR	Granule
IPM	Integrated Pest Management
IPPC	International Plant Protection Convention
ISPM	International Standards for Phytosanitary Measures
ISR	Induced systemic resistance
LAD	Leaf area damage
LBN	Leaf and bud nematodes
MAPP	Ministerially Approved Pesticide Product
NIA	Nematode infested area
PIA	Percentage infested area
PCR	Polymerase chain reaction
PPN	Plant parasitic nematodes
ROC%	Reduction over control (in percentage)
SA	Salicylic acid
SAR	Systemic acquired resistance

SP	Soluble powder
SRUC	Scotland’s Rural College
TLA	Total leaf area
UK	United Kingdom
USA	United States of America

Output from PhD Project

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Lay summary

Leaf and bud nematodes (LBN) including *Aphelenchoides fragariae*, cause major economic damage to many ornamental and agricultural plants in nurseries and landscapes all over the world. LBN penetrate into the leaves through leaf stomata or wounds on the leaf, and feed on the internal cells, causing angular-shaped blotches on leaf veins, which can turn into necrotic lesions usually surrounded by large veins. They often cause serious economic loss to over a thousand nursery-grown ornamental plants worldwide. Common host plants include anemone, begonia, bergenia, chrysanthemums, dahlia, ferns, ficus, hibiscus, hosta, salvia and weigela among others. LBN problems have become more common due to the withdrawal and subsequent loss of effective pesticides due to regulatory issues and environmental concerns.

To gain new perspectives on LBN management using *A. fragariae* as a model species, this project examined in detail new management strategies which could be used as an integrated pest management (IPM) system along with improved hygiene to prevent the continuous infestation and spread of LBN. This project developed and adapted methods for direct inoculation of nematodes on leaves and soil to screen the efficacy of novel products for LBN management. To find suitable alternatives for the management of these important pests, this project investigated in the laboratory and glasshouse some currently available products used to control pests and diseases. Treatments investigated include Caliente Liquid mustard (isothiocyanates & capsicum), Cercobin (thiocyanate), Dynamec (abamectin), EcoGuard (garlic extract), Flocter (*Bacillus firmus)*, Jet 5 (peroxyacetic acid), Movento (spirotetramat), NeemAzal (azadirachtin), Nemanator (molasses & *Bacillus thuringiensis*), SC400 (fluopyram), Vydate 10G (oxamyl). The outcome led to field studies in commercial nurseries where the most effective products were evaluated under commercial field conditions as curative and preventative approaches to LBN management. Along with some commercially available insecticides, another treatment evaluated was an inducing/elicitor agent that triggers the defence mechanisms of plants during attack by pathogens – Acibenzolar -S - methyl (ASM).

Results showed that all the products evaluated in the field trials reduced the multiplication of *A. fragariae* on both naturally infested plants (curative approach) and the nematode inoculated plants (preventative approach) as individual treatment programmes and as programmes involving a combination of insecticides + ASM. This thesis demonstrates that the ASM (elicitor treatment) examined in this project has the potential to improve nematode management by enhancing pesticide efficacy.

LBN can migrate from infested soil/plant media or via infested leaf debris in pots to the leaves through water films on the stem with reported evidence of such transmission by past authors. Products applied to nematode infested soil media in this thesis limited the movement of LBN from the media into plants, and also reduced the inoculum of nematodes in the soil media.

Results obtained from this project also demonstrated a direct link between the visual symptoms of lesions found on the leaves and the nematode population within the leaf. The symptoms guide developed in this thesis assists in providing a quick identification of LBN symptoms upon emergence on leaves; lack of recognition of nematode symptoms is an important factor in the spread of infestation in the nursery. Growers found this guide helpful as it indicates infestation levels on leaves based on symptoms appearance, and thus it assists in providing an important signal for action or an indicator of assessing plant resistance (or tolerance), and evaluating the effectiveness of control treatments. The awareness created by the symptoms guide promotes immediate action as soon as symptoms are observed on leaves. Actions suggested include an immediate isolation of infested plants to prevent further spread in the nursery. Such plants could be treated or discarded based on a visual assessment of the symptoms as recommended in the thesis.

Commercially available products evaluated in this research have the potential to be combined with cultural control methods to effectively manage *A. fragariae* (and potentially other LBN species) in ornamental plants.

Abstract

Leaf and bud nematodes 'LBN' (*Aphelenchoides fragariae*), are microscopic widespread pests of the ornamental industry causing distortion and angular-shaped lesions / blotches on leaves of woody, perennial and herbaceous plants worldwide. They cause an annual loss of estimated millions of dollars on affected plants. These pests spread and infest aerial plant parts by various means including leaf touching from infested plants to healthy plants; movement of LBN in water films during rainfall, misting or irrigation from infested to clean plants; nematode presence in infested leaf debris found on the surface of soil / planting media, sand beds or ground / floor cover matting. These are just some of the infestation routes by which nematodes can be spread in the field. Growers can inadvertently transmit LBN via cut materials taken from infested mother stock, especially when the mother plants are asymptomatic. Symptoms become visible as the new plant grows and nematode numbers build up. Therefore, cleaning and sterilisation of implements / pots are important to reduce the chance of nematode spread to healthy plants. LBN can also be spread from the soil where they can overwinter as juveniles and adults (not as eggs) for some months, and sustenance can be maintained in the soil by feeding on saprophytic fungi in the absence of host plants. In addition, they can overwinter in the plant parts such as buds, rhizomes and bulbs, but not in the root. After sexual reproduction takes place, the life cycle from egg to adult is generally completed in 10-11 days at 18^{o}C. The nematode exhibits both ecto- and endoparasitic lifestyles. LBN moves up externally of the plant during spring to invade the new leaves through natural stomata or wounds. Symptoms include deformation of buds, leaves and flowers causing brown to black, or chlorotic, vein-delineated angular lesions that can become necrotic. If buds or young leaves are infested, they may not develop properly and may become deformed, and this would render such plants unmarketable. Since ornamental plants are sold for their aesthetic value, infestation leading to visual symptoms should be prevented in order to avoid economic loss. Management of *A. fragariae* has become challenging because of the revocation and subsequent loss of systemic pesticides, lack of approved bio-pesticides products, mis-diagnosis of symptoms, continuous movements of asymptomatic plants and increased production

of these vegetatively propagated plants. In the UK, *A. fragariae* and *A. ritzemabosi* are the two main LBN of economic importance. The last approved nematicide in the UK was Vydate 10G (oxamyl) with an Extension of Authorisation for Minor Use (EAMU) for use on protected ornamental plants, which expired at the end of December 2017. Therefore, there is a need for Vydate 10G's replacement to be identified and evaluated for the management of LBN, along with an improvement on the cultural control methods used, as an important component of integrated management of these pests.

To develop new approaches for the management of LBN using *A. fragariae* as a model species in ornamental plants, this project evaluated individually, and in combination, the efficacy of currently approved pesticides including Movento (spirotetramat) and Dynamec (abamectin), elicitor treatments 'acibenzolar-S-methyl' (ASM), known to induce resistance against pest in plants, and some bio-pesticide products derived from plant extracts such as azadirachtin, isothiocyanates and garlic extract. Experiments were conducted in bioassays for contact mortality to *A. fragariae*, inoculation methods were developed and adapted for nematode screening purposes, and used during glasshouse and commercial nurseries to investigate efficacy of these products, and subsequent foliar application of curative and preventative approaches on (naturally and artificially) infested plants, with these products in glasshouse and nursery conditions.

Results showed that isothiocyanates, garlic and abamectin had >75% contact mortality to *A. fragariae* in water bioassays. The elicitor ASM significantly reduced the population of *A. fragariae* by up to 60% compared with untreated Control in a curative approach after a 3x foliar application programme in ornamental plants. A curative approach method on 9 naturally infested plants (*Gunnera manicata*, *Anemone hupehensis*, *Cistus corbariensis*, *Buddleja davidii*, *Bergenia cordifolia*, *Astrantia major*, *Brunnera macrophylla*, *Astilboides tabularis*, *Dryopteris filix-mas*) indicated that all the treatments led to a >60% reduction of nematode population over the untreated Control (ROC%). The highest reduction was obtained with a combination of ASM + spirotetramat on most evaluated plants. A preventative approach with the use of azadirachtin, abamectin, spirotetramat and ASM on

artificially inoculated plants as single product programmes, and in combination with ASM led to low (73-609) mean nematode populations per 1g leaf, compared to the Control populations of 2454-5005 per 1g of leaf eight weeks after nematode inoculation on *B. davidii* and *A. hupehensis* plants. The lowest mean population (73) was obtained from the spirotetramat + ASM programme. As a preventative approach, ASM applied alone in a spray programme on *Anemone hupehensis* inoculated with 200 nematodes / leaf, had a mean nematode population of 255 compared with 1757 nematodes from untreated control 8 weeks after inoculation.

A glasshouse test was conducted on LBN infested soil media with 6 products to evaluate activity in preventing plant invasion from the soil. Oxamyl and treatments such as *Bacillus thuringiensis* (biological), fluopyram (fungicide), garlic extract, isothiocyanates & capsicum and *Bacillus firmus* (biological) limited nematode movement from infested soil media to the plant with reduced nematode multiplication within the leaf. The nematode symptom visual rating assessment guide developed in this thesis identified a correlation between nematode symptom severity (leaf lesions) and nematode population within the affected leaf. This guide will also help growers to improve on identification of LBN symptoms on leaves at the early stage. It will be useful in making decision for immediate action to prevent further spread of infestation by treating symptomatic plants with less than 15% leaf area damage (LAD) or dispose of plants with over 15% LAD in which such infested leaves may not likely respond to any treatment applied thereafter.

Results from field studies have demonstrated the potential of several novel products to manage LBN, and the potential for ASM combined with azadirachtin, abamectin and spirotetramat. As a preventative approach, a foliar spray programme of ASM should be considered to prime plants ahead of LBN symptoms, while a combination of ASM + spirotetramat or abamectin is suggested for a curative approach on plant exhibiting symptoms. Both spirotetramat and abamectin are currently registered in the UK for insect control in ornamental crop production, and the elicitor ASM approved for use on protected chrysanthemum. It is important to note that treatment will be most effective at the first sign of nematode symptoms when plants are actively growing. Considering various potential control methods of LBN, the

combination of elicitor with insecticides offered the best control methods in this study compared to results from pesticide or elicitor as a stand-alone treatment. The effectiveness of combined elicitor + insecticides programmes is likeley to have been due to the elicitor increasing plant resistance against further nematode multiplication, while the insecticides, known to reduce inoculum levels, have acted on the nematode population, either by systemic or contact action, thereby leading to a significant reduction of nematode levels compared with sole candidate treatments. However, the application of cultural control methods and a high level of hygiene, when incorporated with the above treatments in a practical IPM approach, will enhance LBN management on ornamental plants.

Key words: *Aphelenchoides* species, *Aphelenchoides fragariae*, *Bacillus*, biopesticides, curative and preventative approaches, elicitors, leaf and bud nematodes, nematode population, nematicide, ornamental plants, pesticides

Chapter 1

General introduction

1.1 Nematodes

Nematodes of the phylum Nematoda are classified as multicellular animals belonging to the group Ecdysozoa (animals with a cuticle that can shed) along with other organisms like insects, arachnids and crustaceans (Lambert & Bekal, 2002). They belong to classes of Chromadorea and Enoplea, and orders Rhabditida and Dorylaimida / Triplonchida (Fig. 1.1; Lambert & Bekal, 2002). Information about the evolution of nematodes is deduced from the comparative anatomy of existing nematodes, their habits (trophics) and from the comparison of deoxyribonucleic acid (DNA) sequences (Thomas *et al.*, 1997). Nematodes have probably developed several times their ability to parasitise animals and plants during their evolution (Blaxter *et al.*, 1998). Nematodes are second in diversity to insects because they are widely abundant animals. They are mostly free living and can feed on bacteria, fungi, protozoans and other nematodes while many are parasites of vertebrates, invertebrates and plants (Lambert & Bekal, 2002).

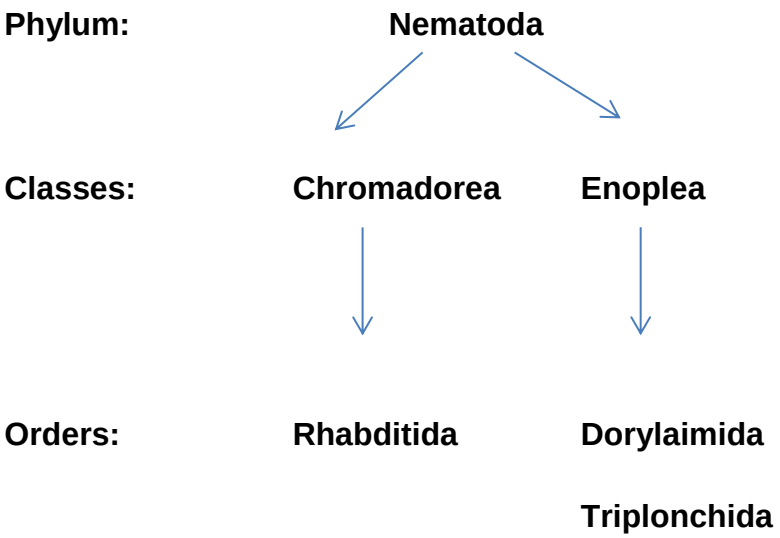

Figure 1.1. The classes and orders of nematodes (Lambert & Bekal, 2002)

The Guinea worm, *Drancunculus medinensis*, which is found in the human body, and the large roundworm *Ascaris lumbricoides* are among the important animal parasitic nematodes (Thorne, 1961). Some of these have been described in ancient Chinese scientific literature since 2700 B.C. (Maggenti, 1981). The first plant parasitic nematodes (PPN) were discovered in wheat seeds by Needham in 1743; root-knot nematodes were identified on cucumber by Berkeley in 1885, and sugar beet 'cyst nematodes' by Schacht in 1859 (Lambert & Bekal, 2002). Due to the simple anatomy and transparent bodies of nematodes, they have been used widely for biological research (Lambert & Bekal, 2002). An example is the bacterial feeding *Caenorhabditis elegans* which has been widely used to investigate animal functioning, behavioural study and DNA sequencing leading to discoveries with particular importance to modern medical research (Riddle *et al.*, 1997). Although, some species of nematodes can become swollen and rounded later in their life cycles, nematodes are mostly vermiform (worm shaped), with an outer cuticle secreted from an inner hypodermis which enables movement in a dorsal ventral direction, therefore, a simple way to describe body features is a 'tube within a tube' (Fig. 1.2; Lambert & Bekal, 2002). Nematodes have an alimentary canal, and the head of a PPN has a hollow mouth spear object (needle-like) called a stylet (Fig. 1.2), connected to the pharynx which contracts and expands during feeding through the ejection of secretions (Lambert & Bekal, 2002). Most of the common nematode genera can be identified by a standard microscope, while identification to species level may require morphological analysis, host-plant study and DNA analysis. Common features that are used to identify nematodes include the mouth cavity, shape/overlap of pharyngeal glands with the intestine, size and shape of the adult stage, tail, number and position of ovaries among others (Fig. 1.2; Mai & Mullin, 1996).

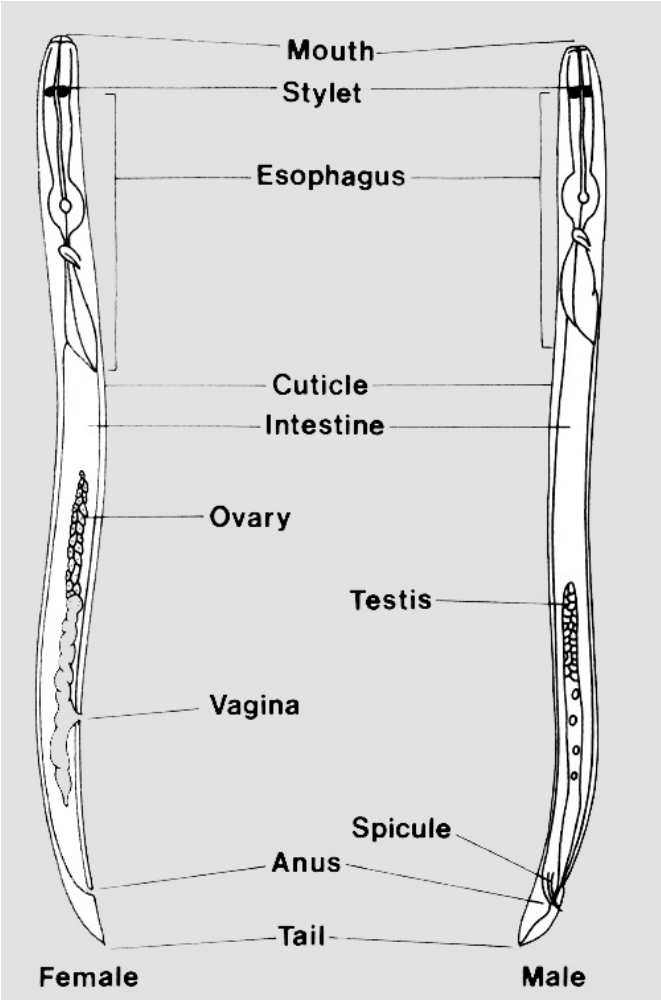

Figure 1.2. Typical nematode structures (Lambert & Bekal, 2002).

1.1.1 Survival strategies

Nematodes are found not only in soil but also in fresh & salt water (Thorne, 1961). In soil they are confronted with predators, unstable soil temperature, moisture and loss of host plants (Lambert & Bekal, 2002). Nematodes can escape predation by living in host plant tissues (Lambert & Bekal, 2002). PPN are mostly found in the soil, while others can be found inside plant living tissue, and tend to escape predation but can be terminated with disease or death of the host-plant, while some can move from host to host, with the possibility of encountering predators or pathogens (Lambert & Bekal, 2002). Nematode survival is affected by biotic and abiotic factors such as temperature and the presence of water. While winter or soil dryness can be hazardous, some nematodes can survive abiotic stress through cryptobiosis (a state of suspended metabolic activity) until favourable environmental conditions occur, while

others can survive on many plant species, therefore enjoying a cosmopolitan host range (Lambert & Bekal, 2002). The ability to survive harsh conditions is one of the factors responsible for their difficult eradication after infestation, leading to an area of nematology research focused on tackling nematode management in the field. Nematode movement may be limited in soil but more spread occurs through farm equipment, muddy footwear or infested soil to uninfested environments or plants. Nematode management involves detection, description and recognition of nematodes as the first step and for quarantine purposes in terms of the importance of nematode pathogenicity on crops or animal (Luc *et al.*, 2005). However, this thesis will focus on *Aphelenchoides* as they pose major concerns to horticultural / agricultural crops worldwide.

1.2 Plant Parasitic Nematodes

PPN form important groups of organisms found in the soil, roots and leaves of plants with a major effect on plant growth and production (Thorne, 1961; Decraemer & Hunt, 2013). They constitutes an important position among the problems confronting agricultural production and management worldwide (Singh *et al.*, 2013). Though PPN can be found in rounded and swollen shapes, the typical shape is long and slender - worm-like (Fig. 1.2), ranging from 250um to 12mm but often with an average length of 1mm, and a width of 15-35µm (Lambert & Bekal, 2002). Nematodes often look segmented due to body annulations, but they are not segmented. Nematodes like insects moult between juvenile stages, with three body layers (ectoderm, mesoderm and endoderm), and no skeleton but supported by the hypodermis (Lambert & Bekal, 2002). There have been around 4000 described species as PPN 'groups that can feed on plant tissue', usually characterised by the presence of a stylet (Nicol *et al.*, 2011; Decraemer & Hunt, 2013). New species are being described continually, and as the population increases, those that have been already described are now becoming pests due to environmental changes and crop patterns (Nicol *et al.*, 2011). Those that are of economic importance are grouped as causing direct damage and indirect damage as virus carriers, with less comprehensive details available on their specific economic impact, especially from the low resources parts of the world (Nicol *et al.*, 2011).

There have been estimated 14.6% of crop production losses caused by PPN in tropical and sub-tropical areas against 8.8% losses in the developed areas of the world (Nicol *et al.*, 2011). Despite this, Sasser & Freckman (1987) reported that a low proportion of the crop loss value (0.2%) is allocated to fund nematode management research.

1.2.1 Economic importance

PPN have been recognised as important pathogens responsible for crop losses throughout the world with an estimated annual loss of $100-157 billion (Sasser and Freckman, 1987; (Koenning *et al.*, 1999; Nicol *et al.*, 2011). Losses may be higher than estimated considering the areas where there are no active nematology research and lack of a data, which will affect estimated world losses of crop production caused by PPN (Singh *et al.*, 2013). Nematode impact is often difficult to assess especially when the effect is not obvious such as complete 'plant topple' (fall) in banana due to root infestation by migratory endoparasitic nematodes causing loss of unripe banana bunches (Gowen *et al.*, 2005). The degree of damage caused by nematodes can be influenced by environmental and climatic conditions apart from the age and host plant, hence increasing nematode density above damage thresholds (Nicol *et al.*, 2011). Significant increase in nematode population has been reported with increasing temperature from spring to summer seasons on infested Hosta and *Lantana camara* plants (Jagdale & Grewal, 2006; Kohl *et al.*, 2010).

1.2.2 Feeding

Nematodes feed in different ways on all parts of the plant such as roots, stems, leaves and buds, flowers and seed with the help of their stylet. Length and shape of the stylet can determine their mode of feeding and classification. During nematode feeding on plants, the contents of plant cells are withdrawn leaving the cells dead while lesions are formed in the plant tissue. Some nematodes 'trick' the plant cells to enlarge and form nutrient-rich feeding points which develop to giant cells/galls such as the root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Heterodera glycines*) (Fig. 1.3). Some nematodes reduce root ability to transport water and nutrients which leads to weak plants and subsequent invasion by other plant pathogens; other nematodes feed on the aerial parts of the plants causing lesions and

distortion leading to dead leaves, bulbs or flowers such as stem and bulb nematodes (*Ditylenchus* spp.), and leaf and bud nematodes - LBN (*Aphelenchoides* spp.) (De-Waele, 2002; Lambert & Bekal, 2002).

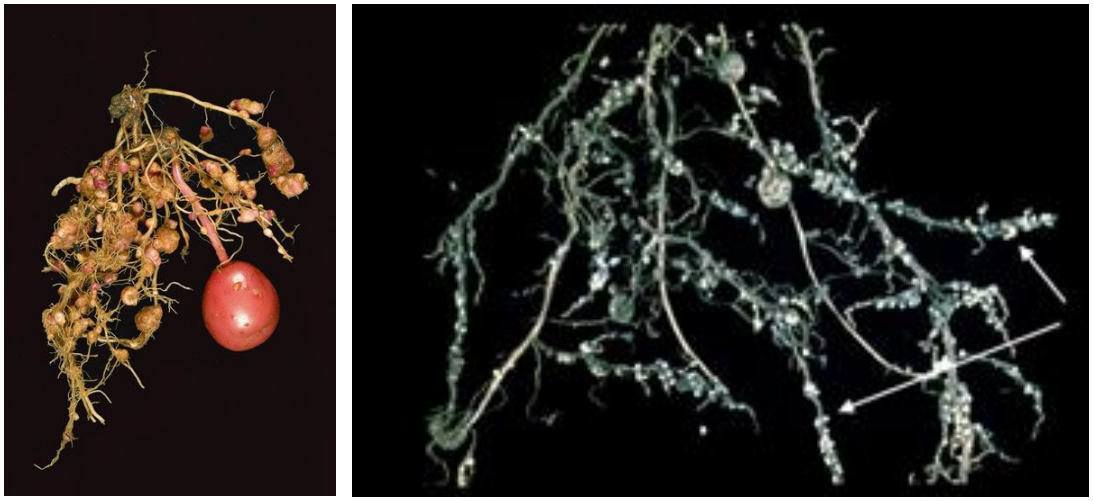

Figure 1.3. . (Left) False root-knot nematode, *Nacobbus bolivianus,* on potato roots. (Photo Courtesy: Rosa H Manzanilla-López, Rothamsted Research, UK; from (Manzanilla-Lopez, 2010). (Right) *Heterodera glycines* (soybean cyst nematode) Photo courtesy: R. A. Motsinger from Lambert & Bekal (2002).

1.2.3 Classification

Plant nematode interactions can be classified according to their mode of attack: ectoparasitic (nematodes feeding outside the plant); semi-endoparasitic (partially penetrate the plant forming permanent feeding cells); migratory endoparasitic (causing necrosis during migration and feeding on roots); sedentary endoparasitic (most damaging by forming giant cells – cyst and root knot nematodes). Others are stem and bulb nematodes (affects both lower and upper parts of plant), seed gall nematodes (migrate as ectoparasites at the tips of leaves causing distortion, and can penetrate to feed on developing seed), LBN (adult migrates in water films via stem to the leaves, enter through stomata and feed on leaf tissue forming chlorosis and necrosis). Plant parasitism occurs in the orders Trichodoridae (Thorne, 1961); Longidoridae (Thorne, 1935); Panagrolaimida (Hodda, 2007) and in the suborders Tylenchina (Chitwood, 1950) or Tylenchida (Thorne, 1949; Siddiqi, 1986). Classification of nematode feeding habits was proposed as an important factor to

consider in grouping and was therefore reflected in the classification of Tylenchida with a special feeding habit by (Siddiqi, 1986; Decraemer & Hunt, 2013)

1.3 Aphelenchoides

1.3.1 Sub-Classification

The superfamily *Aphelenchoidea* (Fuchs, 1937) are known to consist of 7 families including *Bursaphelenchus* (Fuchs, 1937) and *Aphelenchoides* (Allen, 1952); they are primarily fungal feeding species, insect parasites, predators and plant pathogens. While most of the *Aphelenchoides* are fungivores according to (Kanzaki *et al.*, 2012), thirteen species, namely; *A. arachidis*, *A. besseyi*, *A. bicaudatus*, *A. blastophthorus*, *A. dalianensis*, *A. ensete*, *A. fragariae*, *A. nechaleos*, *A. paranechaleos*, *A. ritzemabosi*, *A. saprophilus*, *A. sphaerocephalus* and *A. subtenuis* have been reported as plant parasitic (Fig. 1.4). Three of them are commonly referred to as the main plant parasitic *Aphelenchoides*; with their number of associated plant species as: *A. besseyi* (91), *A. ritzemabosi* (321) and *A. fragariae* (620) (Sánchez-Monge *et al.*, 2015). Thus, primary focus has been on these three species within the "foliar and bulb nematodes" namely *A. besseyi* Christie 1942, *A. fragariae* (Ritzema-Bos, 1891) Christie, 1932 and *A. ritzemabosi*, (Steiner, 1932), due to their economic importance and resultant yield losses.

The nematodes in the genus *Aphelenchoides* may represent a primitive type of nematode evolution, because of their ability to feed on both plants and fungi, with a very wide range of host compared to other plant pathogenic nematodes (Dropkin, 1969). Adult nematodes of the genus *Aphelenchoides* are vermiform and about 1mm in length (Shurtleff & Averre, 2000). The plant parasitic *Aphelenchoides* species can be distinguished from non-parasitic *Aphelenchoides* by the presence of a stylet with basal knobs (see Fig. 1.6). *Aphelenchoides* species can be separated from most other PPN taxa by the presence of a very large metacarpus and a finely annulated cuticle (Fig. 1.5), a lateral field which contains 2-4 lines, and a tapering, conical tail end that is either rounded or pointed (Siddiqi, 1975; Franklin, 1978; Hockland, 2001).

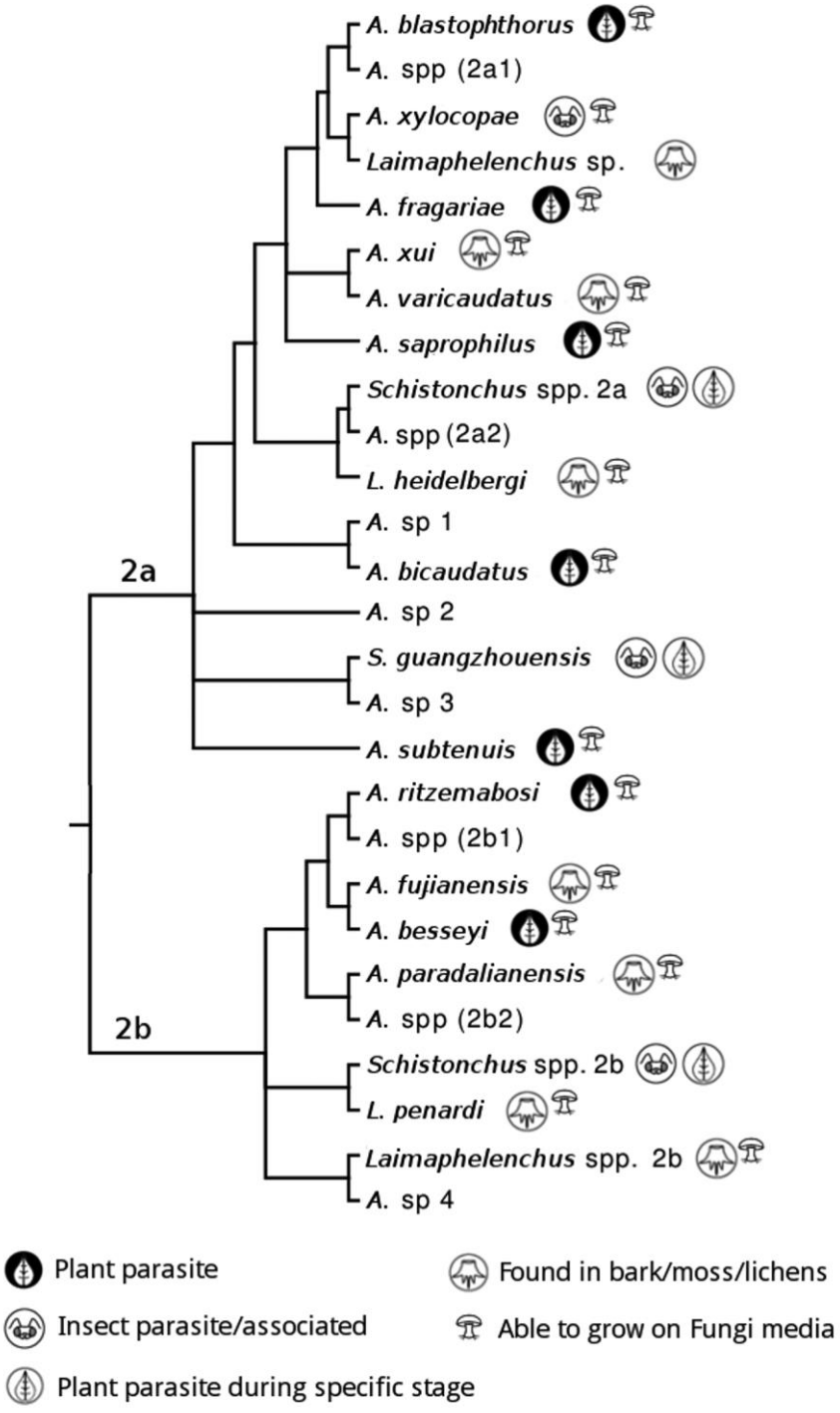

Figure 1.4. Phylogeny tree of *Aphelenchoides* and related taxa (*Laimaphelenchus* and *Schistonchus*) including their feeding behaviour. Tree reconstructed by (Sánchez-Monge *et al.*, 2015).

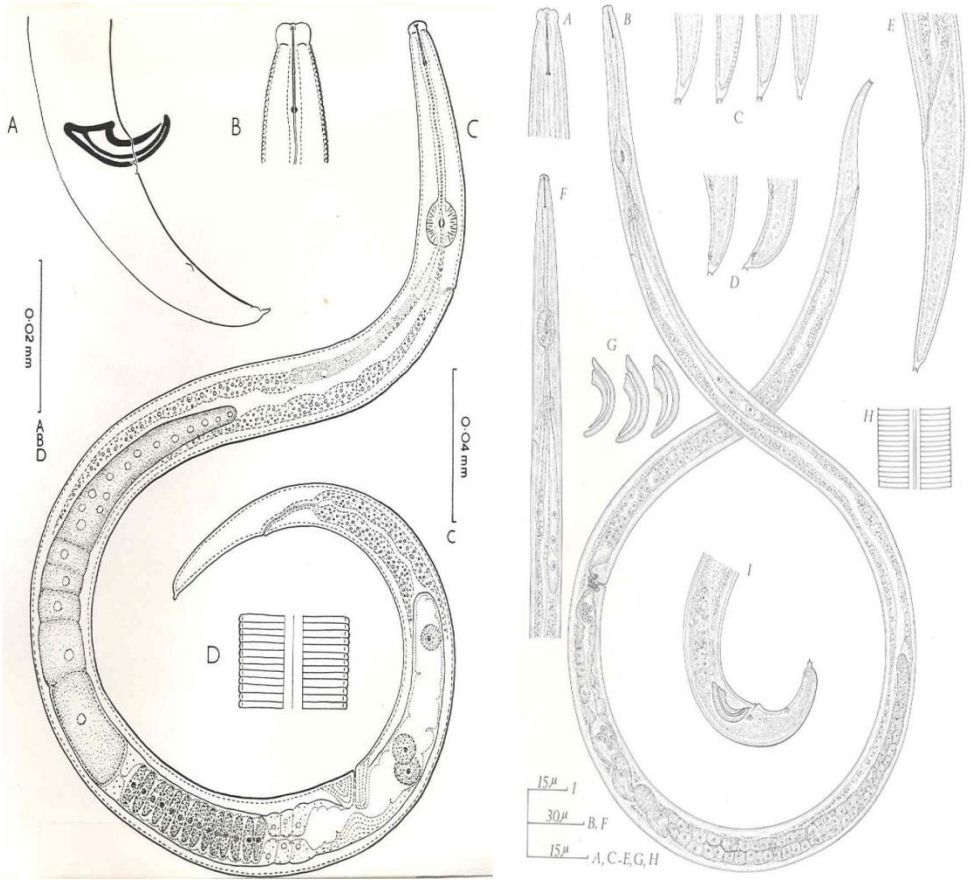

Figure 1.5. (left): *Aphelenchoides fragariae* showing A-male tail, B- head, C-matured female, D-Lateral field. (Right): *Aphelenchoides ritzemabosi*. A-female head, B-female, C-female tail ends, E- female tail, F- oesophageal region, G-spicules, H-lateral field, I- male tail region with spicule (Photo from - Siddiqi, 1975)

1.3.2. Economic Importance

Leaf and bud nematode (LBN) are endo and ectoparasites of various plants in the ornamental sector. They cause damage to a broad range of ornamental plants in greenhouses, nurseries and in landscape settings in many parts of the world including Asia, United States, Canada and European countries (Heinlein, 1982; LaMondia, 1999; Jagdale & Grewal, 2002). They often cause serious economic loss to a broad range of plants (over 1104 species) from 126 botanical families by thirteen plant parasitic species of *Aphelenchoides* (Sánchez-Monge *et al.*, 2015). When considering the wide host range and economic importance of *Aphelenchoides*, the 3 main plant

parasitic *Aphelenchoides* are most important - *A. besseyi, A. fragariae* and *A. ritzemabosi* (Sánchez-Monge *et al.*, 2015). These three species are reported to have economic impact and cause plant yield losses on ornamentals: *A. besseyi* was listed as one of the ten most damaging PPN, while *A. fragariae* and *A. ritzemabosi* are reported as the most commonly found nematodes in the aerial (leaf and bud) parts of ornamental plants (McCuiston *et al.*, 2007).

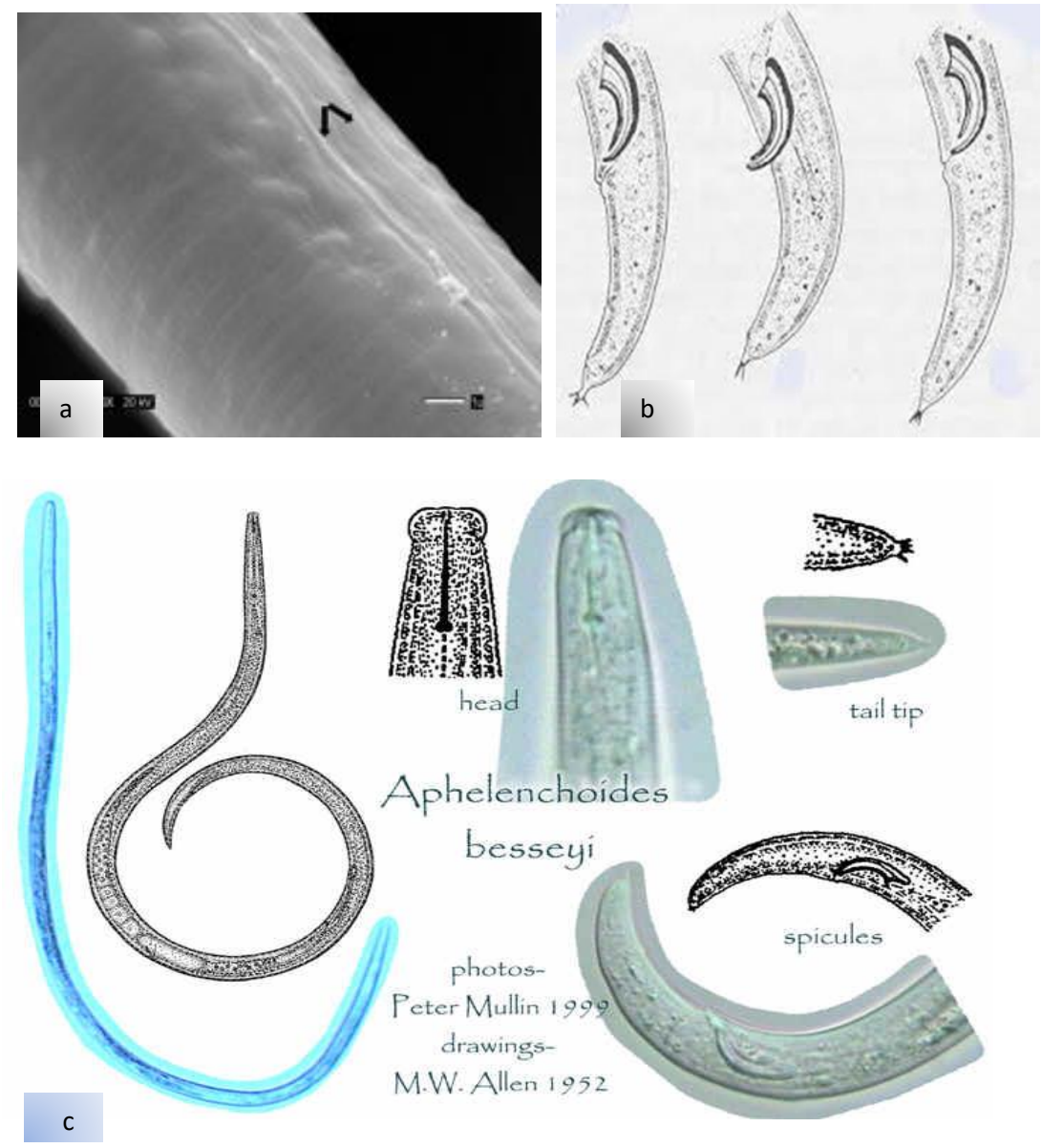

Figure 1.6. (a) Annulations and lateral field (with arrows) of *Aphelechoides fragariae* (Courtesy: Kohl, 2011); (b) Tail region of male *Aphelenchoides ritzemabosi* with 'mucro'- short pointed projections (www.ucdavis.edu/nemaplex) (c) *A. besseyi* showing matured male with body structures (courtesy- (Allen, 1952; Mai & Mullin, 1996)

1.3.2.1 *Aphelenchoides besseyi* described by Christie in 1942 (reported in Franklin, 1985; Franklin, 1978) is often associated with rice, where it causes "white tip" disease in Japan, and parts of southern part of the United States and parts of the European Union and also on Strawberry (Franklin & Siddiqi, 1972; Ward & Hockland, 1996; IPPC, 2016). It was listed as one of the quarantine PPN in the European Union (Ward & Hockland, 1996). Zhuo *et al.* (2010) described *A. besseyi* on pine wood from China (*Pinus massoniana*) but more studies are needed to confirm their relationship. *A. besseyi* has also been found on many ornamental plants (Goodey *et al.*, 1965; Seni de Jesus *et al.*, 2016). Morphological structures of *A. besseyi* are shown in Figure 1.6c. *A. besseyi* currently has 91 associated host plant species (Sánchez-Monge *et al.*, 2015).

1.3.2.2 *Aphelenchoides fragariae* (Fig. 1.5) according to Goodey (1933) was first described when E.A. Ormerod sent infested strawberry plants to Ritzema Bos in England in 1890. The strawberry plants were found to be stunted and deformed with the crown and lateral branches resembling a cauliflower. This made Bos describe the plants as suffering from 'cauliflower disease', and Bos in 1891 later named the nematodes causing the disease as *Aphelenchoides fragariae* (Goodey, 1933). *A. fragariae* is currently the most reported species of *Aphelenchoides* with plant associations on 621 plant species (Sánchez-Monge *et al.*, 2015). While Escuer & Bello, (2000) and Zhen *et al.* (2012) quoted Siddiqi (1975) with a total of 250 plant species associated with *A. fragariae*, recent updated reports described at least 621 plant species and varieties from 287 genera presently associated with *A. fragariae*, with a large percentage (84%) within flowering plants (Sánchez-Monge *et al.*, 2015). The species widely reported on a number of ferns was *A. fragariae* (162) followed by *A. ritzemabosi* (7) and 4 with *A. besseyi* (Sánchez-Monge *et al.*, 2015).

1.3.2.3 *Aphelenchoides ritzemabosi* had been detected in chrysanthemums suffering from 'eelworm disease' as early as 1890, although the nematode was confused with other *Aphelenchoides* species due to the morphological similarities and resemblance, especially with *A. fragariae* (Fig. 1.5 & 1.6) until Schwartz identified it as *A. ritzemabosi* in 1911 (Goodey, 1933). *A. ritzemabosi* (Fig. 1.6) has a lateral field with four lines while *A. fragariae* has two lateral lines (Goodey *et al.*, 1965; Franklin &

Siddiqi, 1972; Franklin, 1978). The female tail of *A. fragariae* has elongate conoid with a single central spike (mucro) at the tail tip, while the conoid tail shape of *A. ritzemabosi* contain a terminal peg of two to four minute, but posteriorly pointed processes, appearing as a paintbrush (Franklin, 1978; IPPC, 2016). Though male spicules in both species have rose thorn-shaped, *A. fragariae* spicules are shorter in length (14-17µm) while *A. ritzemabosi* has a little longer spicules length of 20-22µm (Siddiqi, 1975; Hunt, 1993) *A. ritzemabosi* has the second highest number (321) of reported associated hosts after *A. fragariae*, which consist of 314 flowering plants and 7 ferns (Sánchez-Monge *et al.*, 2015). The principal host plant of *A. ritzemabosi* is Chrysanthemum (Juhl, 1978; De-Waele, 2002).

The use of molecular tools (PCR) is available to confirm identification of *Aphelenchoides* species from plant and soil material more accurately (McCuiston *et al.*, 2007; IPPC, 2016). Internal transcribed spacer (ITS)-1 PCR (polymerase chain reaction) was suggested as a diagnostic test for early detection and identification of *A. fragariae* in host plant material (McCuiston *et al.*, 2007). This was followed by the development of small subunit (SSU) ribosomal DNA based species-specific primers for 4 species of the genus *Aphelenchoides* (*A. besseyi*, *A. fragariae*, *A. ritzemabosi* and *A. subtenuis*), to rapidly identify any of these LBN species detected from soil and plant sample (Rybarczyk-Mydłowska *et al.*, 2012).

1.3.3 Host range, symptoms and identification

The list of Kohl, (2011) recently updated by Sánchez-Monge *et al.* (2015) reported a compilation of associated hosts of *A. fragariae*, *A. ritzemabosi* and *A. besseyi*. The compilation from around the world, (including Oceania, Asia, North America, South America and Europe) reveals how wide the LBN host range is. Among the plant families described as host plants are Asteraceae, Caprifoliaceae, Lamiaceae, Liliaceae, Primulaceae, Ranunculaceae and Scrophulariaceae, while there are also reports of LBN infesting members of such diverse plant families as Agavaceae, Cactaceae, Orchidaceae, Pinaceae and Poaceae (Kohl, 2011; Sánchez-Monge *et al.*, 2015). It is worth noting that while there are very few reports of *A. ritzemabosi* and *A. besseyi* infesting ferns, there are numerous reports of *A. fragariae* infesting ferns in the families Dryopteridaceae, Pteridaceae, and Aspleniaceae (Kohl, 2011).

Sánchez-Monge *et al.* (2015) reported further associated host plant species for *Aphelenchoides* spp.

Host plants of *Aphelenchoides* spp. include nursery grown herbaceous perennials, woody perennials and ferns such as anemone, begonia, bergenia, chrysanthemum, dahlia, dryopteris, ficus, hibiscus, hosta, salvia, weigela and many others (Fig. 1.7; Fig. 1.8; (LaMondia, 1999; Kohl *et al.*, 2010; Sánchez-Monge *et al.*, 2015). There have been increases in the number of associated host plants of *Aphelenchoides* in recent years as reported by Sánchez-Monge *et al.* (2015) based on the host numbers/feeding habits of *Aphelenchoides* following the description of (Koprivnikar & Randhawa, 2013).

Figure 1.7. LBN symptoms on infested ornamental plants (a) *Weigela florida* var. 'Bristol Ruby' (b) *Anemone hupehensis* var. japonica 'Prinz Heinrich' (c) *Astilboides tabularis;* and (d) *Dryopteris filix-mas*

Aphelenchoides fragariae is found in a broad range of plants, including ferns, bedding plants, herbaceous perennials and many other different crops across the United States and many parts of Europe (Crossman & Christie, 1936; McCuiston *et al.*, 2007). *Aphelenchoides ritzemabosi* attacks a range of ornamental plants but is rarely found on ferns, and is commonly found in Europe (Juhl, 1978).

Figure 1.8. (Left) Nematode infested *Buddleja davidii*; (Right) infested Bergenia 'Bressingham white'

It is important to note that neither of the species is limited to a particular continent. However the two species above are among the quarantine nematodes of economic importance as defined by the international plant pest convention (IPPC) and the quarantine list produced by Ravichandra, (2014). *Aphelenchoides besseyi* ranks the least common among the three species in ornamentals, but has been reported as an economically damaging pathogen of rice and a quarantine nematode species on rice (Daughtrey *et al.*, 1995; EPPO, 2004). *Aphelenchoides besseyi* is reported to prefer warmer climates while the two other species can be found in both tropical and temperate environments (IPPC, 2016). While all three species have been found to infest a wide variety of ornamental and crop plants, this thesis will pay attention mainly to *A. fragariae* as the model species used in this thesis. *A. fragariae* was the only LBN species found in the infested ornamental plants collected from grower's fields, cultured and used in all experimental tests conducted in this project. This thesis will now focus more on *A. fragariae* (model species); bearing it in mind that

the management strategies highlighted in this thesis may be applicable to other LBN species such as *A. ritzemabosi*, although this is subjected to further test. In addition, *A. fragariae* and *A. ritzemabosi* are the most commonly found species to infest ornamental plants in the UK.

The response of host plants to LBN infestation can vary considerably, but generally leaves, stems, flowers or buds often become distorted, dwarfed and dried off (Fig.1.7; Fig. 1.8; LaMondia, 1999). When the leaves of some woody plants such as *Lantana camara* are heavily infested, the plants become defoliated, while on other plants including *Heuchera sanguinea, Astilboides tabularis* and *Dryopteris filix-mas* (Fig. 1.7), the infested leaves eventually turn necrotic and die (Kohl, 2008; Kohl *et al.*, 2010). The affected leaf tissue of Hosta plants and *Helianthus* spp. can drop off leaving a shot-hole and tattered appearance after a serious infestation (Daughtrey *et al.*, 1995; Jagdale & Grewal, 2002).

However, additional infections by other pathogens could make diagnosis of LBN symptoms to be difficult on infested leaves (LaMondia, 1999). Such infections include the fungus, powdery mildew and also bacterial infections (Fig. 1.9). Great caution must be taken when dealing with diagnostic analysis of *Aphelenchoides* spp. as highlighted in diagnostic protocol on *A. besseyi*, *A. fragariae* and *A. ritzemabosi* (IPPC, 2016).

Figure 1.9. Downy Mildew (*Peronospora arborescens*) symptoms on Opium Poppy (*Papaver somniferum*) leaves (a); Grey mould caused by *Botrytis cinerea*, causing leaf dieback on rose in autumn (b) - Courtesy of Nigel Cattlin

Nowadays, there is a standard practice in nematology with the use of molecular methods for early detection and identification of *A. fragariae* in suspected host plant

tissues using the species-specific primers for an accurate identification (McCuiston *et al.*, 2007). After morphological identification (Siddiqi, 1975) of nematodes extracted from infested evergreen ferns (*Woodwardia fimbriata*) obtained from a commercial nursery, further confirmation was carried out. This was confirmed by the use of molecular techniques (polymerase chain reaction - PCR) via species-specific primer, which amplified DNA from samples containing *A. fragariae* at the James Hutton Institute Dundee. This work used the protocol described by Seni de Jesus *et al.* (2016) and was assisted by a molecular scientist at the Hutton. In support of the importance of molecular tools for early detection of asymptomatic plants, portable hand held tools should be developed for effective control and nematode management. The use of molecular techniques has a vital role to play through species identification and plant genes expression, which will assist in the screening for nematode resistance among plant cultivars**.** In view of the above statement, this study enabled me to undergo 'shadow' training on molecular techniques of nematodes species identification such as potato cyst nematodes (*Globodera* spp.), at Scotland's Rural College (SRUC) Edinburgh. This I hope will equip me for future work on nematode resistance screening. Because ornamental plants are being sold for their aesthetic value, visual symptoms on plants should be prevented due to fast rate of nematode multiplication; otherwise, plants become unsaleable (Fig. 1.7). Consequently damage by LBN can be very costly for horticultural growers if timely prevention/treatment on plants is not taken (Kohl *et al.*, 2010; Kohl, 2011).

1.4 Biology of leaf and bud nematodes

LBN including *A. fragariae* have a unique biological mode of parasitism as they infest aerial plant parts as against most of the other PPN which infest plants through the roots (Siddiqi, 1975). *A. fragariae* further differ from other PPN in their ability to survive and multiply on soil fungi in addition to their range of host plants (Richardson & Grewal, 1993; Zhen *et al.*, 2012). LBN are about 1 millimetre long (Franklin & Siddiqi, 1972). The life cycle is very similar for both *A. ritzemabosi* and *A. fragariae*. Fertile offspring require sexual reproduction, and fertilized females can still lay eggs after emerging from dormancy without re-fertilization, which makes it easier for fertilized females to continue reproduction for months without an

additional re-fertilization (De-Waele, 2002; Singh *et al.*, 2013). *Aphelenchoides fragariae* is an obligate parasite of above-ground plant parts of many ornamental and agricultural crops worldwide and may be ecto- or endoparasitic, especially of strawberry (De-Waele, 2002). LBN lay eggs usually within healthy, green sections of the ornamental plant leaf tissues (LaMondia, 1999; Lambert & Bekal, 2002). Populations of *A. fragariae* monitored in *Begonia lorraine* at 18^{o} C showed that females laid about 32 eggs which hatch between 3-4 days after the first moulting and subsequently the second stage hatches (Strümpel, 1967; Singh *et al.*, 2013). *A. fragariae* is a bisexual species which can produce new generation in 10-11 days at 18^{o}C (Hunt, 1993). According to Wallace (1960) and Franklin (1985), *A. ritzemabosi* deposited 25-30 eggs as a compact group, this takes between 3-4 days to hatch after the first moulting and subsequently the second stage hatches. Population can swell at a fast rate within leaf tissue with up to 15,000 nematodes per infested leaf (Fig. 1.10; Hunt, 1993; Lambert & Bekal, 2002). *A. fragariae* can survive in the soil up to 3 months and tolerate exposure to harsh temperature as low as -20^{o}C for up to 2 hr in infested Hosta leaf tissues (Jagdale & Grewal, 2006). Further investigation should be carried out on the long term exposure using a more realistic temperature obtainable in the UK such as freezing point (0^{o}C) which would reflect an ideal temperature for the growers under commercial conditions especially as against temperatures in the USA. During winter, *A. fragariae* can observe a dormant state in the soil, and plant parts such as buds, dormant crowns, dry leaves and rhizomes thereby becoming completely dehydrated with reduced metabolic activities (anhydrobiotic condition); normal activities resume when moisture is available (Jagdale & Grewal, 2006). Ability to survive desiccation helps this pathogen to persist infestation, and appear more difficult to eliminate in an infested nursery (Jagdale & Grewal, 2006; Zhen *et al.*, 2012). Jagdale & Grewal, (2006) found higher number of *A. fragariae* nematodes surviving in soil and dormant buds of Hosta plants collected from a polyhouse (a common Quonset-type of overwintering structure built from half-circle galvanized pipes, and covered with a white polythene sheet during winter) than plants from unprotected overwintering bare soil and under polythene cover (a structureless overwintering protective method in which containers are consolidated, covered with a white polythene sheet, and firmly secured around

the perimeter before the onset of winter. Authors therefore suggested that the higher number of survived nematodes was probably due to the protection from harsh temperatures, in addition to the favourable microclimate provided by the polyhouse.

The author observed that the level of sanitation by growers nowadays tend to increase in their nurseries compared with what was obtainable in the past. However, modern method of cultivation such as the use of polytunel and protected structure with little space between plants; along with irrigation methods have also supported the spread of nematode and increase of infestation in the nursery. The stoppage of *A. fragariae* migrating to leaves of healthy plant from infested pot media was investigated later in this thesis.

1.5 Infestation

Infestation of *A. fragariae* humidity is higher, and spreads up the stem of the plant (Fig. 1.11; Lambert & Bekal, 2002). Dew, rainfall and overhead irrigation provide the moisture conditions that allow *A. fragariae* to migrate up the stems in water films over plants surfaces to reach the leaves (Lehman & Miller, 1988). start from the base of the lower leaves of susceptible plants, where the Feeding primarily takes place endo-parasitically and occasionally ecto-parasitically on the plant tissue, depending on the host and prevailing environmental conditions (Singh *et al.*, 2013).

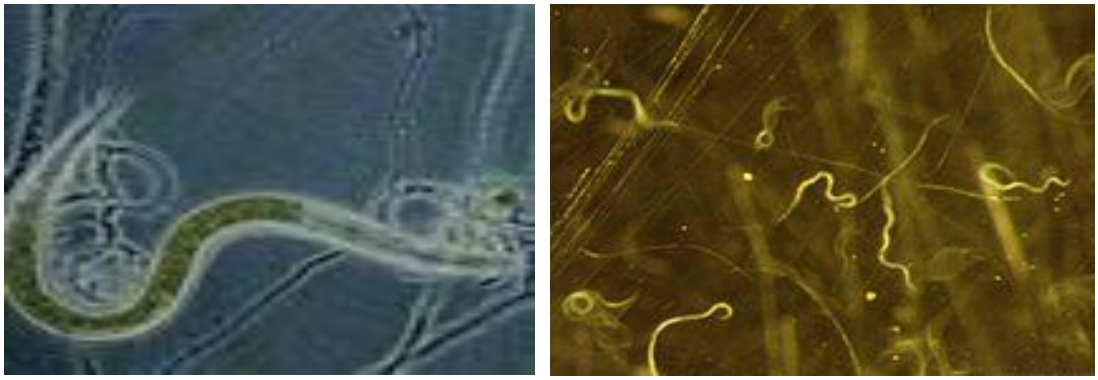

Figure 1.10 LBN: *Aphelenchoides ritzemabosi* feeds and reproduces in chrysanthemum leaf tissue (www.apsnet.org/publication)

There have been evidence of the nematodes feeding ecto-parasitically on buds and flower tissue, which indicates that the nematodes are able to parasitize plants without entering leaf tissue (Richardson & Grewal, 1993). A pilot study conducted on

infested ferns plants prior to the test of treatments (products) in this thesis indicated that nematodes are found ecto-parasitically on the leaf surfaces upon availability of moisture. This led to adoption of leaf wetness method in subsequent experimental chapters of this thesis for a successful efficacy of contact products.

LBN enters and exits leaf tissue through the stomata on the leaf surface; the pore openings in the leaf surface though which gas and water vapours exchange occurs, and move between the mesophyll cells, with no resistance from the guard cells of the stoma to invasion (Szczygiel & Hasior, 1972; Lambert & Bekal, 2002). Hesling & Wallace, (1961) observed adults of *A. ritzemabosi* entering plant tissue through open stomata with their head inserted into the opening while moving the rest of its body back and forth until it fully entered the stomata. Adult LBN usually enter through stomata on the underside of the leaf surface which has about forty times stomata more than found in the upper surface of the leaf (Lambert & Bekal, 2002; Jagdale & Grewal, 2006). *A. fragariae* and *A. ritzemabosi* feed (Fig. 1.10) on cell contents of healthy tissue by inserting their stylet into cells to withdraw contents, resulting in cell death and discolouration (Heinlein, 1982; Lambert & Bekal, 2002). Limitation of LBN migration by large veins within the leaf tissue usually results in the frequently observed angular leaf spots (Fig. 1.7; Sanwal, 1959; Kohl, 2011). On ferns and ornamentals, the endoparasitic life is assisted by the presence of a thin film of water on the leaf surface, through which nematodes enter the leaves via the natural stomata (Richardson & Grewal, 1993). Endoparasitic feeding in leaves can cause sections of the affected leaves to become brown to black or have vein-delimited angular lesions (Fig. 1.7; Jagdale & Grewal, 2002; Kohl *et al.*, 2010). Lesions later become chlorotic; the chlorotic part of the leaf can become necrotic usually surrounded by large veins (Lehman & Miller, 1988). The infested part of the leaves may shrivel (Fig. 1.7), and any parts of buds or young leaves affected may struggle with normal development, while flower development in plant may equally be affected (Jagdale & Grewal, 2002). *A.fragariae* exhibits ectoparasitic lifestyle as found in the folded crown and runner buds, while feeding takes place during the folded-bud stage in plant such as strawberries, causing malformations, twisting and puckering of leaves, discoloration and reduced flowers leading to the death of crown bud (LaMondia,

1999; De-Waele, 2002). Symptoms of LBN damage to leaves are shown in Figure 1.7 & 1.8.

1.6 Dispersal and spread

Movement and spread of LBN are usually restricted to high humidity environments, with the presence of four-water associated factors (Kohl, 2008). These include a water-film thicker than the diameter of the nematodes; thick water film with available high density of epidermal hairs; water-film thinner than nematode diameter usually called thin-films; and lastly water droplets (found during humid weather or after irrigation) (Wallace, 1959; Jagdale & Grewal, 2006).

Furthermore, mobility of LBN is highest when nematodes are moving between epidermal hairs in a thick film of water, which can occur on the upper surface of the stem and on the under surface of leaves during wet weather, which are likely to assist the maximum spread of nematodes on a plant (Lambert & Bekal, 2002; Kohl, 2011). Spread of *A. fragariae* between plants and leaves in nurseries is facilitated by the availability of a film of water as detailed above, and can occur through sprinkler irrigation rainfall and canopy touch (Fig. 1.11; Marlatt, 1970; Lehman & Miller, 1988; Kohl *et al.*, 2010). Lambert & Bekal, (2002) reported that movement of LBN on stems is achieved by the flow rate of water on the plant stem. Mobility of similar nematodes, such as juveniles of *Heterodera schachtii* is controlled by the thickness of the water film coupled with external resistance to movement such as size of soil particles (Singh *et al.*, 2013). Similarly the movement of LBN may be influenced by the distribution and availability of water on the plant surface along with the presence of epidermal hairs (Kohl, 2008). It is therefore important that for nematode spread to be achieved, there must be the presence of a film of water to assist locomotion and transmission from one part of the host to another (Lambert & Bekal, 2002; Kohl, 2008). The overwintering *A. fragariae* from infested leaves moves upward during the spring onto the surface of the fresh leaves, stems and petioles for eventual invasion of healthy leaves through the stomata or mechanical injured parts of leaves, and feed on the mesophyll cells of the infested leaves (Jagdale & Grewal, 2006). A diagram illustrating LBN pathway is shown in Figure 1.11.

Epidemiology studies on LBN (*A. fragariae*) investigated by Szczygiel (1966) reported that the population density of the nematodes increased from November to December and then decreased in January, remaining low throughout the spring season with the highest population density occurring in late autumn. LaMondia (1999) theorized that the LBN population does respond to changes in atmospheric temperature and humidity. Population densities of *A. fragariae* and *A. ritzemabosi* increased during early spring of 1967 and 1968 when the air temperature was low and humidity was high (Szczygiel & Hasior, 1972). Yamada & Takakura (1987) when examining populations of *A. fragariae* in lilies determined that the population density of nematodes in the leaves increased during the rainy season. Jagdale & Grewal (2006) suggested that high relative humidity supported the migration and survival of LBN on the outer surface of hosta leaves.

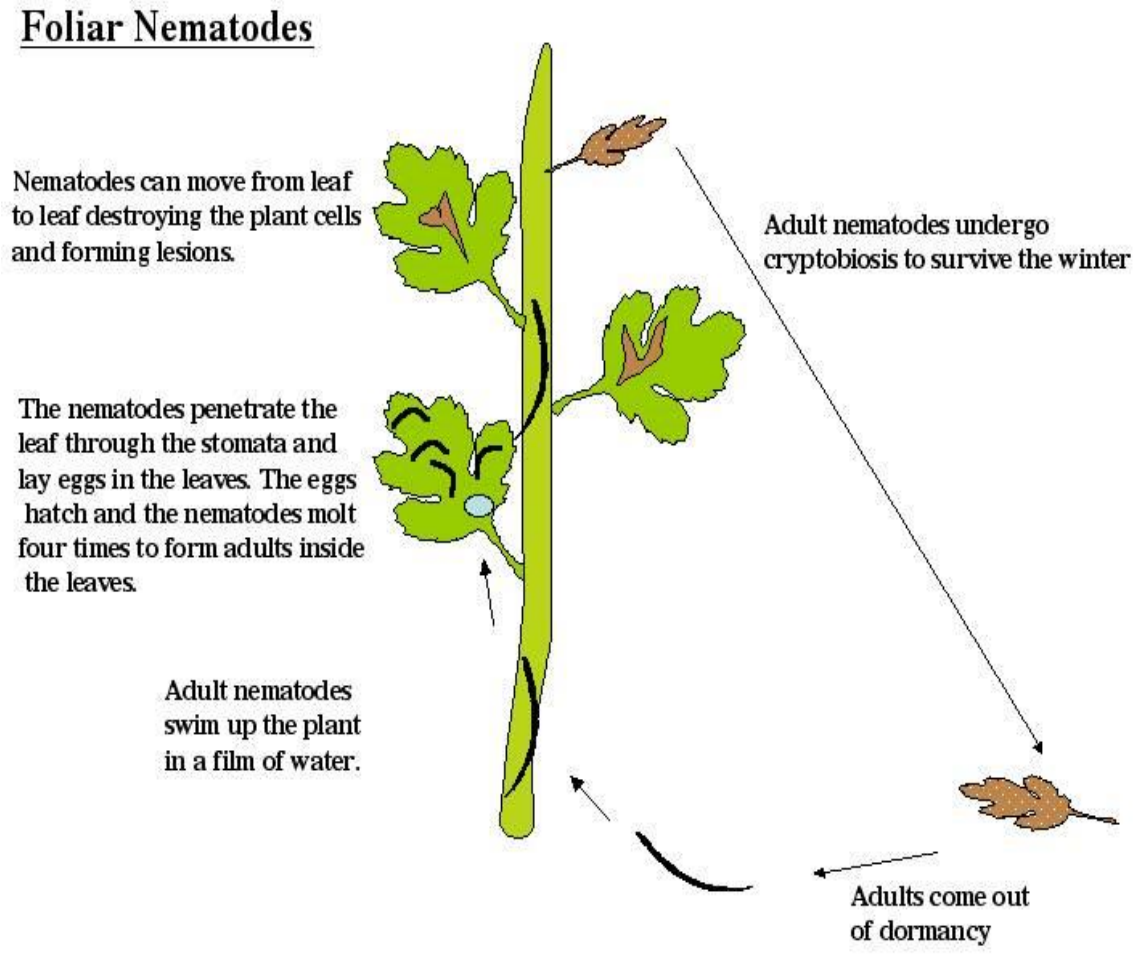

Figure 1.11. LBN plant invasion pathway (Lambert & Bekal, 2002).

LBN can be dispersed throughout greenhouses and nurseries by water splash (Lehman, 1996). Jagdale & Grewal, (2006) and Kohl *et al.(*2010) reported that overhead irrigation encourages *A. fragariae* to be dispersed in water droplets to neighbouring uninfested plants, while (Jagdale & Grewal, 2006) observed the spread of *A. fragariae* through direct contact between leaves of infested and uninfested healthy plant tissue. Likewise the canopy touch encouraged the spread of *A. fragariae* from infested plants of *Lantana camara* to nearby healthy plants (Kohl, 2008). Availability of moisture through sprinkler irrigation, rainfall or dew supports the survival and spread of LBN from leaves to leaves, and or plants to plants as observed in the nurseries during this project. As nematodes rely on moisture to move, care should be taken to avoid leaf wetness, hence use of drip or manual irrigation is preferred over sprinkler irrigation. Unfortunately, this is often very difficult for growers to achieve, especially when overhead irrigation systems are already in place for large sections of greenhouse space. At the commencement of this project, the initial observation of this author during sampling of plants in the nurseries include growers complaints of the potential inconveniencies in term of additional manpower and resources if the use of sprinkler irrigation (already in place) was to be done manually or replaced.

In addition, the author's view on the inadequate spacing between plants was observed as a common practice in the nurseries during this project (Fig. 1.12). Adequate plant spacing in the nursery is very important to avoid canopy touch, which can increase the chances of nematode spread between infested / asymptomatic and healthy plants; and can aid dispersal during overhead irrigation through water splash (Kohl *et al.*, 2010). Picture shown (Fig. 1.12) was part of the author's observations in one of the grower's nurseries in the UK. Changing the irrigation system alone was insufficient to prevent spread; consideration of canopy distance of not less than 30cm was recommended to discourage canopy touch and maintain good sanitation within the nursery. Previous work reported that healthy plants placed at 0cm-30cm near symptomatic plants developed continuous symptoms until 100% of the plants became symptomatic within 11 months (Kohl *et al.*, 2010).

My advice to the growers during this project was to employ use of adequate spacing (>30cm-100cm) between plants. Manual irrigation method via pot saucer for individual plant was used in this project with spacing of ≥50cm between plants.

Figure 1.12. Example of inadequate plant spacing in a commercial nursery

1.7 Management

The management of LBN focusses on reducing the impact of inoculum on the plant, which can be achieved in two ways. The first and most effective method is by isolating the host-plant from the nematode, and the second less effective approach is by introducing control measures once the nematode is present. LBN management can be very challenging because of the survival behaviours of the nematode, and a wide host range (De-Waele, 2002; Kohl, 2011).

A preventative method is very important in the management of LBN. Establishment of nursery with healthy plant material by pre-planting treatment with hot water, pesticides or elicitor product (inducing agent) will ensure a preventative approach in the nursery. Integration of above measures with various cultural controls including quarantine of new stock of plants to assess potential symptoms, adequate spacing between plants and avoiding the use of overhead irrigation will ensure nematodes free nursery. Above practices should be routinely carried out two or more times during growing season for adequate management of LBN. It is always very difficult to eradicate LBN once they are established in the nursery. Generally, the management systems of LBN include cultural control methods, which are an important component of an integrated pest management (IPM) programme which also includes other methods of management as mentioned above, such as chemical control, biological control, natural plant extracts/biopesticides, host plant resistance and induced resistance. As mentioned in 1.3.3, some infections caused by bacteria or other pathogens can be easily confused with symptoms of LBN (Fig. 1.9), therefore an accurate identification with subsequent confirmation by molecular tools is recommended.

1.7.1 Cultural control

One of the most important components for the management of LBN within IPM programmes is cultural control. The most effective of these is a programme of high crop hygiene in the nursery and glasshouse. *Aphelenchoides fragariae* can survive for several years in infested dried leaf debris (Jagdale & Grewal, 2006). Regular sanitation in nursery as part of hygiene practices are sometimes lacking especially when plants are tightly arranged with little space in between pots (Fig. 1.12), thus preventing regular crop-walk to remove infested abscised leaves in pots. When infested leaves abscise on planting media, and upon moisture availability, nematode can migrate from these leaves to infest healthy plants. Kohl *et al.* (2010) reported that availability of moisture can encourage emergence of nematodes from infested leaf debris, thereby infest newly growing plants. A similar observation was made by Jagdale & Grewal, (2006) who found that *A. fragariae* from overwintering soil, assisted by high relative humidity, successfully infested fresh leaves of Hosta plants

during spring. In addition, *A. fragariae* extracted from abscised leaves of nursery grown *Lantana camara* in pots had almost the population as the one obtained from symptomatic attached leaves (Kohl *et al.*, 2010). This suggest that debris or abscised leaves on the surface of substrates/media or overwintering soil can harbour *A. fragariae*, and serves as route for further infestation to emerging plants when condition becomes favourable. This issue has been gradually improved upon by growers during this project, but still serves as an important medium for nematode spread.

Generally, cultural management programmes should include the removal and destruction of infested plants and debris, abscised leaves in pots/ground should be disposed of accordingly, sterilisation of pots and equipment (trowel, pruning shears/pruning saw, scissors), avoid sprinkler irrigation and misting which can create ideal condition for nematode dispersal (LaMondia, 1996; Young, 2000; Zhen *et al.*, 2012). Also the use of certified nematode free planting materials can prevent spread of PPN, including *Aphelenchoides besseyi* on species' hosts (Ward & Hockland, 1996; Coyne *et al.*, 2013). It is advisable to monitor any potential symptom development in newly obtained plants by isolating them in a separate place in the garden or nursery for few weeks. Any plant that develops symptom should be isolated from the stock and treated or destroyed depending on the degree of symptoms observed.

When it comes to the issue of LBN control in the nursery, prevention is the best practice.

1.7.2 Hot water treatment

Management of LBN are mainly based on chemicals except for a few described methods involving hot-water treatment (LaMondia, 1999; Jagdale & Grewal, 2002, 2004; An *et al.*, 2017; Chałańska *et al.*, 2017). Hot-water treatment is a method to reduce or eliminate infestations, particularly with the aim to provide clean mother plants for propagation (Young, 2000; Coyne *et al.*, 2010; Hauser & Coyne, 2010). Various studies have recommended hot-water treatments of different plants which include immersing chrysanthemums in 43^{o}C hot water for 20 minutes against *A.*

ritzemabosi (Fallik, 2004), and treating bulbs of *Polianthes tuberosa* at 57^{o}C for 30 minutes to reduce the infestation of *A. besseyi* (Thi Thu Cuc & Pilon, 2007). Hot water treatments have also been used to manage other PPN by treating bulbs, bare-rooted plants, dormant crowns, suckers and runners of many economically important crops (Birchfield, 1954; Tsang *et al.*, 2001; Fallik, 2004; Coyne *et al.*, 2010). Successful hot water drenching at 70^{o}C, 90^{o}C and 100^{o}C have been carried out against overwintering *A. fragariae* in pots to prevent migration to the leaves of Hosta plants with no adverse effect on dormant crowns (Jagdale & Grewal, 2004). A pre-treatment temperature of 30^{o}C for 30 minutes followed by hot water treatment at 46^{o}C for 10 minutes have been recommended for strawberry plants against *A. besseyi* and *A. fragariae* (EPPO, 2012). However, despite the efficacy and recommendation of hot water treatment (Hauser & Coyne, 2010 ; EPPO, 2012), and from my previous experience of using hot water treatment to produce clean planting materials such as in *Musa* spp. (banana/plantain suckers) and *Dioscorea* spp. (yam seed/setts) according to Coyne *et al*. (2006) and Coyne *et al.* (2010), the required temperature and duration of individual plant species and cultivars varies. Therefore, significant resources would be needed to investigate requirements for most plants, in order to give effective control and to avoid phytotoxicity.

1.7.3 Chemical control

Chemical treatments such as aldicarb, diazinon, parathion and oxamyl have been used in the past for effective control of LBN (Jagdale & Grewal, 2002; An *et al.*, 2017). However most of these chemicals are no longer available due to government regulations and environmental concerns. Thus chemicals are now limited in availability and efficacy and this has significantly affected the nursery industry (LaMondia, 1999; Jagdale & Grewal, 2002). Depending on the plants being treated, modern chemical control methods may have variable results. Investigation with chemicals may produce successful mortality in an aqueous suspension, but prove ineffective when treating infested leaves (Jagdale & Grewal, 2002). Some insecticides have been demonstrated to be effective incidentally or under test conditions against LBN on some ornamentals (LaMondia, 1999; Young, 2000; An *et al.*, 2017), but several are not registered as approved in the UK.

In the UK, after the withdrawal of the effective chemical aldicarb (Temik), a HDC project HNS 131 evaluated a range of alternatives for the control and management of LBN (Bennison, 2007). The results suggested oxamyl, amongst others, as the most effective replacement for aldicarb, and reported abamectin as ineffective against leaf and bud nematodes (Bennison, 2007). Abamectin (18 g/l) is an emulsifiable concentrate containing (1.84% w/w). The product works by targeting the transmissions in the neuromuscular systems of insects. The contact of abamectin with invertebrates stimulates a neural transmitter which causes breakdown of nerve to nerve, and nerve to muscle, hence the term nerve poisons. The targeted insects become paralysed, stop feeding and die (Hague & Gowen, 1987). Abamectin has a translaminar movement and the mode of action results in mortality of approved pests. However it is harmful if swallowed, causes serious eye irritation and is very toxic to aquatic life (Cayrol *et al.*, 1993). Abamectin has previously been reported to show control activity against mites, insects, root knot nematodes and LBN (Cayrol *et al.*, 1993; LaMondia, 1996). Furthermore, LaMondia, (1999) and Young & Maher (2000) reported that abamectin demonstrated effective control against LBN on some ornamentals both i*n vitro* and *in vivo*; and thereby suggested abamectin as a potential treatment for short term suppression of LBN in hardy ornamentals (Young, 2000). The potential of abamectin for the management of LBN (*A. ritzemabosi*) on infected *Anemone hupehensis* was recently reported by Chałańska *et al.* (2017). Significant mortality at 24-72h was recorded when *A. fragariae* was exposed to an aqueous suspension of abamectin at a 2-fold dilution (An *et al.*, 2017). Various conflicting reports by past workers on abamectin in the literature posed a critical area of confusion on the efficacy of abamectin to manage LBN. While workers such as Young & Maher, (2000), Chałańska *et al.* (2017) and An *et al.* (2017) reported the potentials of abamectin in the management of LBN, Bennison (2007) found that abamectin at the same concentration of (18g litre $^{-1}$ 'active ingredients') as was used by the above authors was ineffective in the control of LBN. Abamectin is approved in the UK as a foliar treatment for the control of two-spotted mite and western flower thrips in protected and outdoor flower crops and other ornamentals. Despite not having a label recommendation for LBN, some growers observed incidental control of LBN symptoms when abamectin was used for the approved pests. Abamectin will

be fully evaluated in subsequent Chapters of this thesis in a laboratory water-bioassay, glasshouse and in commercial conditions, as an individual treatment and in combination with other products.

The results of two nematicides investigated by Jagdale & Grewal, (2002) showed that while both oxamyl and ethoprophos were effective in direct mortality when applied in the soil, oxamyl had the most consistent efficacy in reducing *A. fragariae* in both leaves and soil of Hosta. Oxamyl is grouped as a family of pesticides called carbamates. Its action is to block the normal function of cholinesterase, an essential nervous system enzyme of targeted insect pests (Anon, 1990)**.** Oxamyl works as an insecticide on a broad spectrum of insects, and as an acaricide for mites, ticks and as a nematicide against nematodes (Anon, 1990). Oxamyl action is both systemic and contact product. Oxamyl is classified as extremely poisonous to humans, fish, birds and other wildlife on prolonged or repeated exposure to the product (Cornell University Agricultural Extension, 1993). Oxamyl is applied directly and incorporated in soil, readily adsorb in soil with high organic matter and fairly slow in adsorb in sandy soil, and a decrease in adsorption at high temperature of 25 degrees and above (Anon, 1990 ; Arias-Estevez *et al.*, 2008). Oxamy 10% (as Vydate 10G) is approved in the UK for the suppression of nematodes in potatoes, carrots, sugar beet and parsnip. The efficacy of oxamyl as a nematicide against free living nematodes has long been established, and is known to work systemically against target pests(Wright *et al.*, 1980; Whitehead *et al.*, 1984; Osborn *et al.*, 2010). Oxamyl applied as a soil drench was reported by (Strider, 1973) to have significantly reduced number of *A. fragariae* in red begonia leaves within 20 days. Oxamyl was reported to cause over 70% reduction in LBN population (*A. fragariae*) in the leaves and soil around Hosta plants 45 days after treatment compared with the control (Jagdale & Grewal, 2002). There was an effective control of *A. ritzemabosi* in the leaves of infested *anemone hupehensis* by oxamyl during a 2-year field trial (Chałańska *et al.*, 2017).

During this study oxamyl had an Extension of Authorization for Minor Use (EAMU) for outdoor ornamental plant production until the end of 2017, targeting insect pests and stem and bulb nematode. However, some growers did not wish to use oxamyl as

it is not compatible with biological control agents, which are being used for other pests within IPM programmes, and is difficult to use as it is supplied in Surefill closed transfer packs which makes access to the product problematic. Consequently, growers of protected ornamentals and containers were not able to use oxamyl. In addition, the use of oxamyl also requires precautions for operator and environmental protection, along with a re-entry time to any treated glasshouses and a harvest interval. However, despite above restrictions and issues about oxamyl, Chapter 2 and 7 of this thesis included oxamyl as a standard nematicide among other products investigated in a laboratory water bioassay, and as soil treatment against *A. fragariae*.

Other pesticides include spirotetramat, a tetramic acid derivative insecticide, registered as Movento in the UK. Its mode of action involve inhibition of lipogenesis in treated insects, resulting in decreased lipid biosynthesis; inhibits growth of younger insects; and reduced the ability of adults insects to reproduce (Brück *et al.*, 2009). It is a foliar applied systemic insecticide that penetrates plant leaves when sprayed on. Spirotetramat is ambimobile, being transported both upwards and downwards through vascular bundles. It has moderate to low acute toxicity, irritates eyes and potentially skin sensitive (Vang *et al.*, 2016). Nauen *et al.* (2008) observed that the plants' phloem and xylem system enhances the absorption and distribution of spirotetramat throughout the entire plant; this according to the authors has allows spirotetramat to be an effective insecticide and potential nematicide. Smiley *et al.* (2011) also reported that spirotetramat works systemically within the plant, having both phloem and xylem mobility in different crop species. Spirotetramat has shown activity against *Pratylenchus vulnus*, the root feeding lesion nematodes in walnut orchards (DeBuse, 2011). Activity of spirotetramat against cereal cyst nematode (*Heterodera avenae*) was reported by Smiley *et al.* (2011), where 2 foliar applications at 2 week intervals reduced the postharvest egg density of *H. avenae* along with the juveniles by 35% compared to the untreated control. Spirotetramat was reported to cause a significant reduction from development to reproductive maturity of *Heterodera glycines* and *Meloidogyne incognita* when applied as a foliar spray on Soybean plants (Vang *et al.*, 2016). Spirotetramat may well have an effect on nematode reproduction and fecundity, and may not demonstrate any direct

activity against nematodes including *A. fragariae* if tested in water suspension due to its mode of action (Vang *et al.*, 2016). Spirotetratmat has an EAMU on outdoor and protected crops of ornamental plant production and forest nursery for the control of aphids, mealybugs and whiteflies (Salazar-López *et al.*, 2016). However, some growers observed incidental control of LBN symptoms when spirotetramat was used for the approved pests on some ornamental plants. Subsequent Chapters 2, 5 and 6 of this thesis will examine spirotetramat as a potential product against LBN, both in an aqueous suspension and as a preventative/curative treatment under commercial conditions.

Peroxyacetic acid is an organic compound with a colorless liquid and a characteristic odor of acetic acid (Cristofari-Marquand *et al.*, 2007). Peroxyacetic acid is an oxidizing agent, in which its exposure can cause irritation to the skin, eyes and respiratory system, while long term exposure can cause permanent damage (Cristofari-Marquand *et al.*, 2007). Peroxyacetic acid is an environmentally friendly fungicide/algicide, approved as a general disinfectant on protected horticultural crops. Its uses include cleaning floors and benches between crops for the control of disease pathogens. Peroxyacetic acid is marketed in the UK as Jet 5 and in the US as Zerotol. Peroxyacetic acid (as the product Zerotol) has been successfully investigated for activity against *Aphelenchoides* spp. both *in vitro* and *in vivo* (Jagdale & Grewal, 2002; An *et al.*, 2017). Results of studies on peroxyacetic acid showed significant mortality in water suspensions within 24h of exposure to *A. fragariae* and reduction of nematode numbers in leaves when used as a foliar spray 45 days after treatment of infested Hosta plants (Jagdale & Grewal, 2002; An *et al.*, 2017). In addition, 75% mortality was obtained *in vitro* when peroxyacetic acid was investigated against the stem nematode, *Ditylenchus dipsaci* in a laboratory bioassay, the authors therefore suggested that peroxyacetic acid should be tested on leaf and bud nematodes (Lole, 2001). Peroxyacetic acid therefore has potential to be used in the management of LBN. This product was assessed in this thesis for its contact mortality against *A. fragariae* in bioassays (Chapter 2).

Use of insecticidal soap (fatty acid products) was reported as effective as a foliar spray 48 days after treatment against *A. fragariae*, while it gave low efficacy in water

suspension (Jagdale & Grewal, 2002). The similar fatty acid product, Savona® is approved in the UK as a foliar spray against aphids, whiteflies and spider mites on both outdoor and protected ornamentals. However it was ineffective against LBN in an HDC project (HNS 131) (Bennison, 2007) and was not evaluated within this thesis. Savona® was not investigated within this thesis unlike other products which have had incidental control or controversial report on LBN or successful treatments of other nematode species. Future work can also investigate Savona® as a potential product.

While the use of chemical treatments has proved effective to manage LBN for many years, there is possibility of phytotoxic effect in plants; which is one of the disadvantages of chemical treatments. Moreover, plant species can react differently to the effect of chemical treatments. It is advisable to pre-test few plants with recommended products and observe any potential phytotoxic symptom such as death, distorted and dead areas on plants before treatment of the whole stock. However, chemical treatments are still the most effective way to manage LBN along with the integration of cultural control and other pest management strategies.

1.7.4 Biological control

The potential of natural enemies and extracts of plant species with microbial and nematicidal properties as alternatives to traditional pesticides is worthy of consideration. Efforts to reduce chemical usage have encouraged growers to seek pest management strategies which are environmentally friendly. Biological control was described according to DeBach & Rosen (1991) as the "study, importation, augmentation, and conservation of beneficial organisms to regulate population densities of other organisms". This aspect of IPM programs has played an important role for the manipulation of beneficial organisms against insect pests worldwide (Orr, 2009).

Biological control such as microorganisms antagonistic to nematodes and use of entomopathogenic nematodes (EPN) such as *Steinernema feltiae*, *S. glaseri* and *S. riobrave* have been investigated against some nematodes such as root-knot nematodes (*Meloidogyne incognita* and *M. javanica)* in the laboratory, glasshouse

and under field conditions (Bird & Bird, 1986; Grewal *et al.*, 1999; Shapiro-Ilan *et al.*, 2006; Kenney & Eleftherianos, 2016). Various species of entomopathogenic nematodes have been successfully used to effect suppression both in the field and greenhouse conditions (Richardson & Grewal, 1993; Lu *et al.*, 2016). In the use of EPN to manage LBN, Bennison (2007) found *Steinernema carpocapsae* ineffective in controlling leaf and bud nematodes with a 5-spray programme of 500 million/1000m^2. However, Jagdale & Grewal (2008) used 100 infested cadavers of *Galleria mellonella* mixed into soil medium to successfully suppress *A. fragariae* in the soil of infested Hosta 30 and 40 days after treatment as a curative and preventative approach. In addition, infested cadavers of *G. mellonella* applied to soil suppressed populations of *A. fragariae* in the infested Hosta plants, with reduced lesions compared to control (Jagdale & Grewal, 2008). There is a great potential to use EPN for management of LBN. However, the author did not have the opportunity to investigate further into EPN along with other management strategies that were used in this project due to time and other considerations, and as agreed by the industry representatives. Whatever the modification of agricultural production may be, the adoption of IPM with the use of biological control as a major tool will continually play a leading role (Orr, 2009; Kenney & Eleftherianos, 2016).

Other biological control agents such as *Bacillus subtilis* have demonstrated nematicidal activity against various nematodes species including *A. besseyi* during *in vitro* experiments (Xia *et al.*, 2011). *Bacillus firmus* in an aqueous suspension caused from 98-100% reduction in egg hatching on *Meloidogyne incognita* 24 days after treatment, while gall formation, nematode populations and number of eggs were reduced on tomato seedlings during glasshouse experiment (Terefe *et al.*, 2009).

Burkholderia cepacia, a non-parasitic rhizobacterium applied as foliar spray to nematode infested Hosta leaves caused a 50-85% reduction of *A. fragariae* in soil around the plant and in leaves; while mortality of *A. fragariae* when exposed to *B. cepacia* in water suspension was 34% compared to the control (Jagdale & Grewal, 2002). *B. cepacia* proved ineffective against *Meloidogyne incognita* in the soil and laboratory culture (Meyer *et al.*, 2000), which made Jagdale & Grewal, (2002) recommend more study on *B. cepacia* as the product could be nematode specific.

Bacillus firmus was investigated in this thesis as a soil treatment (Chapter 7) among other products against *A. fragariae*.

1.7.5 Plant extracts

There are commercially available products from plant extracts that have been previously tested against soil dwelling nematodes such as cyst nematodes, root-knot nematodes and other free living nematodes, although more work is needed to confirm product efficacy such as Nemagold (a liquid extract of marigold, *Tagetes erecta*, seaweed and 'organic matter') on LBN.

In a recent study, Azadirachtin (Neem tree extract) registered as AzaMax in the US, caused between 64-77% mortality in aqueous suspension when exposed to *A. fragariae*, while Neem oil demonstrated between 90-100% mortality at 24-72h of exposure to *A. fragariae* in aqueous suspension (An *et al.*, 2017). Azadirachtin acts as an anti-feedant, interferes with the moulting process, reduces fecundity, and disrupts respiration and oviposition in targeted insect pests (Howard *et al.*, 2009; Khalil, 2013). In support of Khalil's (2013) recommendation for azadirachtin as an important tool for the management of nematode pests, (An *et al.*, 2017) suggested azadirachtin as being toxic to *A. fragariae*. Efficacy of azadirachtin will be evaluated in this thesis in an aqueous suspension (Chapter 2), and further as a foliar treatment under a commercial field conditions (Chapter 6). Recently an emulsifiable neem concentrate formulation, registered as Azatin (azadirachtin 217g litre $^{-1}$ active ingredients) has been approved in the UK for use on protected ornamental plant production (MAPP 18301; Authorisation Number – 0360). Growers in the UK now have the option to use Azatin on ornamental plant production against insect pests, but there is currently no on-label approval for use against LBN.

The efficacy of Nemakill, a product containing Cinnamon (32%), Clove (8%) and Thyme (15%) oils as a nematicide was investigated in nematode infested leaves of Hosta (An *et al.*, 2017). Nemakill caused significant reduction of *A. fragariae* population in leaf-disc assays, while mortality of 100% was recorded in aqueous suspensions after 24h of exposure (An *et al*., 2017).

Chałańska *et al.* (2017) found that the application of soapbark tree extract, *Quillaja saponaria* was ineffective in reducing the population of *A. ritzemabosi* in Anemone leaves, which was in contradiction of the findings of previous authors (Roner *et al.*, 2007; Giannakou, 2011; Insunza *et al.*, 2001) who reported nematicidal activity of *Q. saponaria* extract and successful nematode reduction. Chalanska *et al.* (2013) had earlier reported the effectiveness of *Q. saponaria* for reducing the population of *A. ritzemabosi* on chrysanthemum leaves, though at higher concentrations (50% solution) compared to the reduced dose (10% solution) used by (Chałańska *et al.*, 2017).

Other plant products investigated as potential nematicides include extract of garlic (*Allium sativum* L.), though garlic extracts according to Bennison, (2007) proved to be ineffective against LBN. However, other authors have reported garlic extracts to have nematicidal activity both in the laboratory and greenhouse (Gupta & Sharmaj, 1993; El-Nagdi & Youssef, 2013). The major constituents of garlic oils are allium, diallyl disulphide and trisulphide, and these have demonstrated toxic effects against the pine wood nematode *Bursaphelenchus xylophilus* in laboratory bioassays (Park *et al.*, 2005). Iranshahi (2012) reported that hydrolysation of sulphur compounds found in *A. sativum*, *A. cepa* and *A. fistulosum* forms a variety of isothiocyanate compounds with nematicidal and phytotoxic effects on pathogens. The efficacy of nematicidal activity of garlic was reported to show toxicity against the slug-pathogenic nematode *Phasmarhabditis hermaphrodita*, with high mortality due to the presence of polysulfides (Anwar *et al.*, 2009; Anwar *et al.*, 2016). Garlic based products recommended as insecticides have now been registered in Denmark and Norway as ECOguard for cabbage root fly control, and ECOspray in the UK received regulatory approval for a product called 'Eagle Green Care', a liquid nematicide for pest control on elite sports turf (Ministerially Approved Pesticide Product 'MAPP' No.14989). Other UK approved garlic extract products include NEMguard granules (MAPP No. 15254) for carrot and parsnip, NEMguard PCN Granules (MAPP No.. 17377) approved for potato, NEMguard DE (MAPP No. 16749) to control root knot nematodes (*Meloidogyne* spp) and stem & bulb nematodes (*Ditylenchus dipsaci*) on outdoor bulb onion; and against free living nematodes on outdoor garlic, leek, fodder

beet and red beet. Garlic extract will be investigated against *A. fragariae* in water suspension (Chapter 2) and as a soil treatment (Chapter 7) in this thesis.

1.7.6 Biofumigants

Biofumigants comprising of isothiocyanates derived from mustard plants have been reported to have activity against soil-dwelling PPN (Brown & Morra, 1995; Ramirez *et al.*, 2009). These compounds act as natural biofumigants (Brown & Morra, 1995), and have been reported to have suppressed soil-borne pest and diseases due to the biocidal effect of isothiocyanates derived from glucosinolates (Kirkegaard *et al.*, 1996). Isothiocyanates/glucosinolates have demonstrated suppression of PPN (Ramirez *et al.*, 2009), weeds (Brown & Morra, 1995), and pathogenic fungi (Kirkegaard *et al.*, 1996). Various activities of biofumigants have been demonstrated against different species of PPN (*Meloidogyne javanica*, *Tylenchulus semipenetrans)* by mustard bio-fumigants (*Brassica juncea*) according to Zasada & Ferris, (2003). The incorporation of *Brassica juncea* (Indian mustard), *Eruca sativa* (Nemat) and *Raphanus sativus* reduced significantly the population of *G. pallida* on potato field trials (Ngala *et al.*, 2015). This thesis in subsequent Chapter 7 will investigate a commercial liquid extract of isothiocyanates & capsicum against *A. fragariae* in an aqueous suspension and as a potential treatment of nematode infested soil.

The prevention of nematode migration from infested soil /media to growing plants as a way to combine treatment of both soils and foliar treatment of leaves was requested for investigation by the project funding body. The author critically analysed the importance of this factor as a potential route to plant infestation of healthy plants especially the use of infested pots or planting media, and when abscised (infested) leaves are being found on surface of soil or pot. Past workers have reported transmission of nematodes from infested media to heathy plants (Jagdale & Grewal, 2006; Kohl *et al.*, 2010). The author agreed that if this study is investigated, it can be useful to growers of protected ornamentals and container plants. The combination of both soil and leaves treatment can increase the effectiveness of nematode management in the nursery.

1.7.7 Resistant cultivars

Host plant resistance is an important management tool against PPN. Some plants, including four cultivars of Hosta have been reported as resistant to LBN – *A. fragariae* (Jagdale & Grewal, 2006). Development of nematode resistance is desirable as part of an IPM programme. Wallace (1960) identified varieties of chrysanthemum which showed resistance to *A. ritzemabosi* and suggested it may be due to the plant lacking nutritional factors that prevented further infestation to be spread to other leaves in an infested plant. Although some varieties can be described as resistant they are not immune to nematode infestation which implies that the plant can be subjected to attack by adult nematodes but reproduction is highly reduced (Lambert & Bekal, 2002). Despite the huge benefits of resistant cultivars, the limitation in terms of availability to commercial growers is a key barrier, as is breeding the resistant genes into commercially acceptable cultivars (Arora & Sandhu, 2017). There is still work to be done to get available germplasms screened for nematode resistance (Starr *et al.*, 2007).

1.7.8 Induced resistance

The ability of a susceptible plant to develop resistance to further infection following an initial infection by a microbial pathogen is called induced resistance (Kuć, 1982; Hammerschmidt, 2014). Induced resistance can be described as two types: systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Walters & Heil, 2007; Hammerschmidt, 2014).

SAR involves the growth restriction of a pathogen and inability of symptoms to develop after a pathogen attack, when compared to plants with no induction of defence but attacked by the same pathogen (Walters *et al.*, 2014). SAR is both important for the plant to resist disease and also for recovery from disease infection. Infections from a wide range of pathogens can cause SAR to be induced in plants (both locally and systemically), most especially pathogens that cause necrosis upon infection (Walters & Fountaine, 2009). This action is coordinated by mass presence of endogenous salicylic acid (SA) at the area of infection; SA is a plant hormone which plays active roles in plant growth and development (Ryals *et al.*, 1996). Induction of SAR requires the presence of pathogen-induced SA which helps in plant

defence against pathogens through the activation of pathogenesis-related (PR) genes (PR-I in particular); which produces pathogenesis-related proteins which are antimicrobial, and attack molecules in the cell walls of fungi and bacteria (Pieterse & van Loon LC, 1999; Conrath *et al.*, 2002; Walters *et al.*, 2014). The resistance carried out after SAR induction is effective against many pathogens; therefore resistance by SAR is referred to as broad spectrum resistance (Pieterse & Van Loon, 2007; Walters *et al.*, 2014). Agents that can mimic the natural inducers of resistance during plant-pathogen interactions, and which have been reported to elicit SAR include acibenzolar-S-methyl (ASM) (Parkunan, 2008); the non protein amino acid β-aminobutyric acid (BABA) and cis-jasmone (Pieterse & Van Loon, 2007).

ISR involves the colonisation of plant roots by particular strains of plant growth-promoting rhizobacteria (PGPR) usually coordinated by sensitive pathways of jasmonic acid and ethylene (Fig. 1.13; Pieterse & van Loon LC, 1999; Conrath *et al.*, 2002). ISR also works in similar way to SAR as they both do not act particularly against pathogens (Pieterse & Van Loon, 2007; Yi *et al.*, 2013). However, ISR is reported to act independent of SA unlike SAR (Vallad & Goodman, 2004), and it is not associated with expression of PR genes but depends primarily on the production of ethylene and jasmonic acid (Fig. 1.13; Ryan *et al.*, 2008).

While the two systemic responses above are direct activation of defences, the resistance can also be linked to the ability to recall previous pathogenic infection, root colonisation or treatment by chemicals, and is referred to as 'Priming', and the response of the plant is rapid and more effective during subsequent pathogen invasion (Pieterse & van Loon LC, 1999; Goellner & Conrath, 2007). Priming alone does not usually indicate any changes in gene expression or level of resistance traits, hence may be as a result of a chemical elicitor like Acibenzolar -S- methyl (ASM) or a pathogen (Walters & Fountaine, 2009).

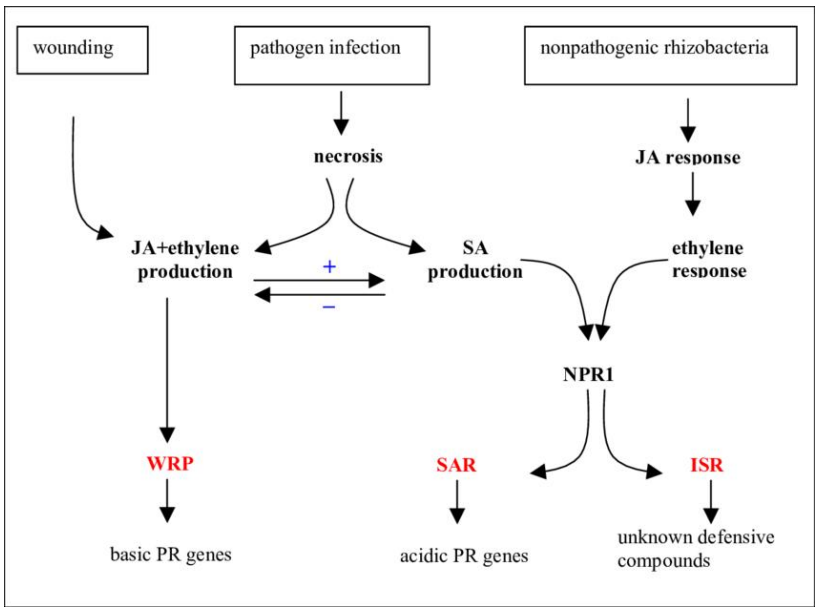

Figure 1.13 Signal pathways in response to induced resistance systemic caused by wounding, non-pathogenic rhizobacteria and pathogenic organisms in plant (Courtesy: Pieterse & van Loon, 1999).

It is important to note that agents that fully induce resistance at higher concentrations in plants are usually responsible for priming (Heil & Bostock, 2002; Walters & Heil, 2007). This is interpreted to mean that direct induced resistance and priming differs only quantitatively rather than qualitatively (Walters & Fountaine, 2009).

The mechanism of systemically induced plant defence which is best understood so far is SAR, which normally involves a broad-spectrum disease resistance mediated by SA (Kessmann *et al.*, 1994). When SA and compounds that can mimic the action of SA are applied to plants, they will chemically induce resistance to pathogens in such plants (Oostendorp *et al.*, 2001). SAR could be more active in restricting disease if it is activated before the arrival of the pathogen as previously demonstrated with various micro-organisms (Keller *et al.*, 1996; Ryals *et al.*, 1996).

A range of microbes and chemicals such as: β-Aminobutyric acid (BABA); polyacrylic acid, barium chloride, 2,6-dichloroisonicotinic acid (INA) and ASM among others have been reported to induce resistance to various pathogens upon application to plants (Ward *et al.*, 1991; Kessmann *et al.*, 1994; Malamy *et al.*, 1996). The above listed chemicals are not directly antimicrobial (Cole, 1999), therefore responses to the systemic resistance can be associated with direct activation of plant defences rather than any effect on the pathogen (Cole, 1999; Vallad &

Goodman, 2004). Walters & Fountaine (2009) outline some of the biotic and abiotic products developed after Probenazole, which was the first chemical resistance activator (elicitor) developed. Other chemical and microbial activators include ASM registered as Bion® (now Inssimo®) and Actigard® by Syngenta, Milsana® (extract of *Reynoutria sachalinensis*, KHH BioScience Inc., USA), Elexa (Chitosan SafeScience, USA), and Messenger (Harpin protein, Eden Bioscience, USA).

Reynoutria sachalinensis, an ethanoic extract from giant knotweed registered in the USA as Milsana®, and marketed as a plant activator on protected ornamental plants, and registered in Europe as Regalia®, has demonstrated control of fungal pathogens on crops such as cucumber (Daayf *et al.*, 1997; Fofana *et al.*, 2002), strawberry (Carlen *et al.*, 2004) and organic tomato crops (Dafermos *et al.*, 2012). Application of Milsana® at 7-10 day intervals gave a similar control of powdery mildew to those obtained when a commercial fungicide was used on tomato plants (Schmitt, 2002). Milsana® also demonstrated control of powdery mildew on grapes under field conditions by inducing phytoalexins which convey resistance by plants towards the pathogen (Konstantinidou-Doltsinis *et al.*, 2007). Milsana® therefore helps the plants to resist pathogen infection rather than act directly on the pathogen. Past molecular work on the mechanism of ASM in plant defences on tobacco and *Arabidopsis* showed that ASM activates the SAR pathway through mimicking the activity of SA (Friedrich *et al.*, 1996; Lawton *et al.*, 1996). ASM has been reported to induce resistance to pathogens when applied to various plants (Kessmann *et al.*, 1994). Activity of ASM on tobacco indicates a high level of disease control of *Pseudomonas syringae*, *Cercospora nicotianae* and *Alternaria alternata* by 99, 91 and 89% respectively (Cole, 1999; Perez *et al.*, 2003). Furthermore, previous reports on pre-treatment of oilseed rape with ASM against Phoma stem canker; *Leptosphaeria maculans* reduced lesions by 25-50% (Liu *et al.*, 2006). Infection caused by the leaf scald pathogen, *Rhynchosporium secalis* on barley was reduced by 45% (Paterson *et al.*, 2008).

Harpins are proteins rich in glycine and heat-stable secreted by type III secretion system from gram-negative plant pathogenic bacteria. They are directed to the extracellular space of the plant tissues as against inside the plant cells common with

other bacteria effector proteins (Choi *et al.*, 2013). Harpin, a hypersensitive response elicitor demonstrated induction of resistance to *Peronospora parasistica* and *Pseudomonas syringae* in Arabidopsis through the activation of SAR genes (Dong *et al.*, 1999). Foliar application of Harpin to soybean plants led to effective control of *Heterodera glycines* (soybean cyst nematode), and when used as a seed treatment significantly reduced the development of *Fusarium graminearum* in Soybeans (Navarro-Acevedo, 2016).

It is important to note that there is no guarantee that application of the elicitor alone can ensure complete eradication of pathogens (Walters *et al.*, 2005). Low control of powdery mildew and *Rhynchosporium commune* was witnessed on two barley cultivars (Optic and Cellar) after a field experimental treatment by ASM (Walters *et al.*, 2014). However, during the 3 years of experimental field trials, the ASM plus fungicide combination gave the most consistent disease control (Walters *et al.*, 2013). It was also reported that ASM controlled rust infection caused by *Uromyces pisi* on pea plants, but the control was incomplete (Barilli *et al.*, 2009). Similar reports from Ivors & Meadows, (2016) recommended combinations of ASM with fungicides and bactericides during tomato spray programs for increased plant resistance and reduction of early blight (*Alternaria solani*) inoculum levels in North Carolina, USA. The authors suggested that the use of elicitor + pesticide in combinations could be a valuable tool in reducing the total quantity of pesticide used, and delay pesticide resistance development thereby resulting in increased long term efficacy of pesticides (Ivors & Meadows, 2016).

While controlled environments can provide high levels of disease control by plant elicitors, effective performance under field conditions has not been consistent (Walters *et al.*, 2005; Walters & Fountaine, 2009). It has been suggested that under field conditions, expression of induced resistance by an elicitor can be influenced by the environment, genotype and crop nutrition level; consequently a better understanding of these interactions with the elicitor is needed to maximise the efficacy of induced resistance (Walters *et al.*, 2005). One of the major advantages associated with these synthetic elicitors include the absence of any direct antimicrobial activity when compared with normal traditional pesticides; a factor

which could assist the avoidance of pathogens developing resistance (Vallad & Goodman, 2004). The use of elicitors is deemed to be environmentally friendly compared with current pesticides (Vallad & Goodman, 2004). There have been no studies yet on the induction of resistance against LBN on ornamental plants. Most of the available investigation has focussed on root knot nematodes (*Meloidogyne incognita, M. javanica, M. chitwoodi*) in tomato plants (Oka *et al.*, 1999; Cooper *et al.*, 2005; Molinari & Baser, 2010) and a few studies on *M. chitwoodi* & *Pratylenchus* spp. of potato plants (Collins *et al.*, 2006; Dos-Santos *et al.*, 2013).

In view of the potential of elicitor treatments to induce resistance in plants against LBN as recorded on other pests/pathogens, this thesis will evaluate the application of products that act as elicitors of plant defences to determine whether they can confer a level of resistance to LBN in ornamental plants. The potential of ASM and *Reynoutria sachalinensis* as a single product, and in combination with other pesticides for their efficacy against multiplication of LBN (*A. fragariae*) in ornamentals will be investigated in greenhouse and in commercial field conditions. Having highlighted various ways to manage LBN in plants and especially on ornamental plants, success recorded so far on the management of LBN are mainly based on chemicals (LaMondia, 1999; Jagdale & Grewal, 2002, 2004; Chałańska *et al.*, 2017). Therefore, an investigation of elicitor treatments to induce resistance in plants with or without other pesticide products is necessary. However, the use of cultural control practices as an integral part of an IPM programme with a high level of hygiene is very important along with any choice of control method recommended in this thesis. This thesis will evaluate different methods as useful tools when combined in an IPM approach as a novel management strategy.

1.8 Conclusions

In this Chapter, some problems currently posed to both agricultural and horticultural crops by parasitic nematodes have been highlighted with the primary focus on *Aphelenchoides* species. Various ways by which LBN have been evaluated in the past were outlined, along with the potential for products that have been successfully applied to control other similar PPN, and with the potential efficacy of such products when tested on LBN.

There are some products that have been tested previously on LBN (Table 1.2), although some of them had controversial reports. Considering the current problems enumerated in this Chapter such as withdrawal of most chemicals previously used for the control of LBN due to regulatory and environmental concerns, lack of adequate cultural control (including regular sanitation, inadequate spacing between plants which can aid nematode spread, and irrigation methods which encourage water splash that helps in the spread of nematode from infested plant to healthy plants), lack of product efficacy due to lack of penetration on leaves, and inadequate information on symptoms identification and management. The factors above directly or indirectly contributed to the continued spread of nematodes and loss of ornamentals plants in the horticulture industry, and with no current efficient control practices nor approved products in place, therefore, the author's focus is to investigate different methods summarised below as useful tools when combined in an IPM approach as a novel management strategy.

- In response to the withdrawal of most chemicals previously used for the control of LBN, alternative products are needed as the potential alternatives to oxamyl, this project, in bioassays, will evaluate (against *A. fragariae*) some available chemicals and bio-pesticides products that are currently approved for other pests in the ornamental sector,.
- As part of cultural control strategies, this project aims to set up glasshouse bioassay which would investigate the prevention of *A. fragariae* from inoculated pot media (compost/soil) as a route to a successful infestation of subsequent healthy growing plants. This would serve as one of the preventative methods of managing *A. fragariae*.
- Due to the wide host range of *Aphelenchoides* spp., and the limitations associated with leaf penetration of pesticides during foliar application of products, this project will aim at utilising elicitor treatments which are known to induce resistance within the plant as systemic acquired resistance to evaluate whether plants can confer such resistance against LBN. This will be evaluated via laboratory bioassays, glasshouse trials and trials under commercial conditions, to ultimately produce new management strategies for LBN in ornamental plants. Most of my experimental studies for nematode

management will focus on chemicals and plant elicitors because chemical control have been reported as the most efficient method of management considering non tolerance to LBN symptoms (Section 1.7.8), complimenting cultural control with high level of hygiene in the nursery. In addition, plant elicitor works directly on the mechanism of plants to induce self resistance; elicitors are not antimicrobial, therefore the chances of developing resistance to elicitors like traditional pesticides are minimal. As previously highlighted, past workers have also recommended the combination of both chemical and elicitors suggesting that chemical effect will reduce / suppress inoculum levels while elicitor will act on the plant mechanism.

- This project will develop methods for artificial inoculation of nematodes on leaves and soil, a useful tool for screening resistance to LBN; and also investigate correlations between nematode population and infested leaves of ornamental plants. This evaluation will be a useful management aid that would provide a decision making approach on whether to treat infested plants or discard them based on severity of visual lesions (symptoms) on leaves.
- Novel products will be evaluated with an adopted management method such as manipulating time of product and adoption of leaf wetness strategy to optimise efficacy especially on products with controversial reports such as abamectin. This approach will involve both preventative and curative methods in the glasshouse and commercial conditions.
- Above studies will be narrowed down to *A. fragariae,* which is the only species extracted and identified from the infested (anemone, weigela and ferns) plants collected from the commercial nurseries during my pilot sampling. *A. fragariae* will be cultured in the leaves of healthy plant (to be generated from root stock) in the glasshouse, and use as model species in all experimental trials for this thesis. It is important to state that the findings in this study may be adopted for the treatment of *A. ritzemabosi,* although further test should be conducted to confirm this.
- Finally, there has been inadequate knowledge of symptoms recognition of LBN in the UK, and other part of the world including the USA. This has led to the wrong treatment being applied due to lack of proper diagnosis.

This project aims to enlighten growers in various forum including technical discussion group, seminar and farmer's group meetings by using the platform of AHDB. In addition, the project would produce a simple visual symptoms guide to aid nematode management by helping growers to better identify symptoms, create awareness for immediate treatment of infested plants or disposal as the case may be, and to assess the effectiveness of treatments for evaluation.

The overall aims and purpose of this thesis were to find effective novel approaches for the management of *A. fragariae*, using products that are currently approved for other pests in the ornamental sector; as preventative and curative management strategies in the leaves; and for prevention of nematode migration from infested soil / media to growing plants; and to develop a suitable method of nematode inoculation for use in glasshouse and field trials.

Chapter 2

Laboratory bioassay to investigate contact mortality of currently available chemical and bio-pesticide products on leaf and bud nematodes, *Aphelenchoides fragariae*

2.1 Introduction

LBN (*Aphelenchoides* spp.), are microscopic organisms found in the leaf tissue of ornamental plants with resultant serious damage to many ornamental plants grown in greenhouses, nurseries, and in the landscape settings throughout the United States, Canada, and Europe (Richardson & Grewal, 1993; LaMondia, 1999). They are a significant foliar pest of ornamental plants (over 1104 host species from 126 botanical families) whose feeding results in angular-shaped blotches on the leaves which are usually delimited by veins in most plants and often accompanied by leaf distortion (Fig. 2.1; Kohl, 2011; Sánchez-Monge *et al.*, 2015).

The affected part of the leaf may shrivel (Fig. 2.1). If any parts of buds or young leaves are infested, they may struggle with normal development and could become deformed; flower development may equally be affected (Jagdale & Grewal, 2002). As ornamentals are sold for their aesthetic value, these plants are often unsaleable (Fig. 2.1), making LBN damage very costly for ornamental growers which could result to estimated loss from £5,000 – £15,000 per annum depending on the size of the nursery as at 1996 (Young, 1996), and by current prices, annual loss to LBN's damage could probably be estimated up to £10,000 to £30,000 per nursery. In the UK, *Aphelenchoides fragariae* and *A. ritzemabosi* are the two main LBN species of economic importance on ornamental plants (Young, 1996, 2000).

LBN problems have become more common due to the revocation and subsequent loss of systemic nematicides, increased nursery production of these vegetatively - propagated plants and long distance movement of plants (Lehman, 1996; LaMondia, 1999; Jagdale & Grewal, 2006).

Figure 2.1. LBN infested nursery plants *Anemone hupehensis* var. japonica 'Prinz Heinrich' (top) *Astilboides tabularis (bottom left)* and *Dryopteris filix-mas* (bottom right)

Populations of *A. fragariae* (Fig. 2.2) can increase rapidly because they can be dispersed in detached or dried leaves, consequently infestation can rapidly become damaging and widely spread (LaMondia, 1999; Zhen *et al.*, 2012).

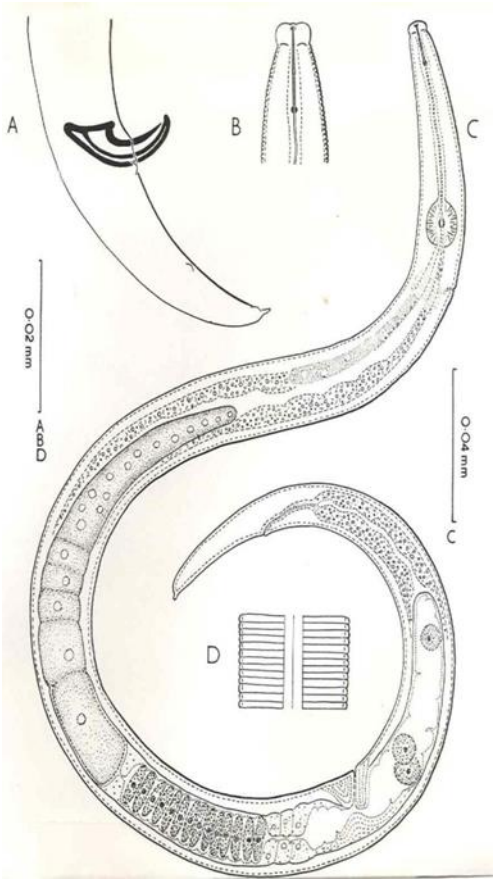

Figure 2.2 *Aphelenchoides fragariae* showing A-male tail, B- head, C- matured female, D-Lateral field.

Various chemical treatments such as diazinon, aldicarb, parathion and oxamyl have been used in the past for effective management of LBN (Young & Maher, 2000; An *et al.*, 2017). However, due to regulatory issues and environmental concerns, most of these chemicals are no longer available to growers today. A range of alternatives for the control and management of *A. fragariae* (Table 2.1) have been evaluated previously and some products such as diazinon EC (Emulsifiable concentrate), trichlorfon SP (Soluble powder), peroxyacetic acid, ethoprophos GR (Granule), oxamyl GR (Granule) were found to have significantly reduced nematode populations (*A. fragariae*) in a water bioassay, Hosta leaves and soil compared with the control (Jagdale & Grewal, 2002). The highest mortality (98.9%) of *A. fragariae* in a water bioassay was recorded with Zerotol (peroxyacetic acid) followed by 96.5%

mortality when nematodes were exposed to diazinon (Jagdale & Grewal, 2002). Over 70% population reduction of *A. fragariae* in Hosta leaves was observed after foliar treatment with five formulations (diazinon EC, trichlorfon SP, insecticidal soap, oxamyl and peroxyacetic acid) compared with the untreated control in two consecutive years of treatment (Jagdale & Grewal, 2002).

Table 2.1 Mortality of *Aphelenchoides fragariae* after direct exposure to dilutions of these products in water and leaf. Information obtained from previous published works as referenced.

Trade name	Chemical/scientific name	Contact mortality (*in vitro*)	Leaf mortality (*in vivo*)	Reference
Dynamec	Abamectin	Yes	Yes	1
Movento	Spirotetramat	Yes	N/A	1
Zerotol / Jet 5	Peroxyacetic acid	Yes	Yes	1,2,5
Vydate 10G	Oxamyl	Yes	Yes	1,2,3
EcoGuard	Garlic	Yes	N/A	4
NeemAzal	*Azadirachtin*	Yes	Yes	1,2

Ref: [1] (An *et al.*, 2017); [2] Jagdale & Grewal, 2002; [3] Young & Maher, 2000; [4] (Park *et al.*, 2005); [5] Bennison & Maulden, 2016.; N/A (Not available)

Apart from the fact that chemical controls are limited in availability and efficacy (LaMondia, 1999), modern chemical control methods have variable results, depending on the plant being treated (Young & Maher, 2000; Bennison, 2007). Table 2.1 summarises the previous studies of products used against *A. fragariae* as reviewed in the previous Chapter1, and with the plan to conduct further investigation on the listed products in this thesis. Some treatments by chemicals may be successful in contact mortality on nematodes in water, but may be ineffective when applied to infested leaves (Jagdale & Grewal, 2002; An *et al.*, 2017); thus, such chemical treatments are recommended for further research study to investigate their potential efficacy, as being carried out in this thesis.

Recent research in the USA has identified two US products Pylon (chlorfenapyr) and Nemakill (cinnamon-clove-thyme oil) with nematicidal activity against LBN in aqueous suspension, drench to soil and on a leaf assay (Hosta) at both 5% and 50% volume concentrations (An *et al.*, 2017). The study observed that at a 2-fold dilution of Ammonia, Oregano oil, Peroxyacetic acid, Potassium permanganate solution ($KMnO_4$), Sodium dichloroisocyanurate solution (NaDCC), Nemakill and Pylon, there was between 75% – 100% mortality to *A. fragariae* in aqueous suspension within 24h of exposure.

Nemakill, an oil mixture containing Cinnamon oil (32%), Clove oil (8%) and Thyme oil (32%) demonstrated a significant mortality of LBN in aqueous solution at concentrations of 2 and 20-fold dilutions (An *et al.*, 2017). These oils have demonstrated nematicidal activities against the pine wood nematode *Bursaphelenchus xylophilus* (Kong *et al.*, 2007), and the root-knot nematode, *Meloidogyne incognit*a (Meyer *et al.*, 2000). The authors therefore recommended further work on both Pylon and Nemakill for field evaluation for LBN management (An *et al.*, 2017). Information regarding Nemakill and Pylon only came to light in 2017, which was too late for their inclusion in this study.

Pesticides with translaminar activity may enter plant leaf after application, but the major medium for penetration is the leaf cuticle (Cloyd, 2016). Dynamec has translaminar activity whereby the active ingredients (abamectin) are distributed into plant tissues where the pests feed. Abamectin penetrates leaf tissues and forms a reservoir within the leaf, and can persist in an active state for some days (Cloyd, 2016). Abamectin was reported to have significant reduction *in vitro* and *in vivo* on *A. ritzemabosi* (Young & Maher, 2000; Chałańska *et al.*, 2017), while (Bennison, 2007) found abamectin to be ineffective against LBN, and proposed that oxamyl was the most effective available product. Abamectin has controversial reports on its management of LBN (Bennison, 2007; Young & Maher, 2000), hence the need for further investigation; while necessary strategies will be adopted in this thesis to maximize the efficacy of abamectin under commercial conditions.

There are no chemical nematicides currently registered for LBN in the UK. Although during this study, oxamyl had an Extension of Authorisation for Minor Use (EAMU)

on ornamentals for use against American serpentine leaf miner (*Liriomyza trifolli*), silverleaf whitefly (*Bemisia tabaci*), South American Leafminer (*Liriomyza huidobrensis*), and stem & bulb eelworm (*Ditylenchus dipsaci*), and many growers used this EAMU to try and manage LBN with oxamyl. EAMU of oxamyl expired at the end of December 2017. However, during this period, some growers did not wish to use oxamyl as it was not compatible with biological control agents, which are being used for other pests within IPM programmes, and is difficult to use as oxamyl is supplied in Surefill closed transfer packs which makes access to the product problematic. In addition, the last EAMU for oxamyl allowed only the use on outdoor ornamentals when applied by a mechanical granule applicator, hence it cannot be safely carried out by hand-held equipment, and should be followed by soil incorporation; therefore growers of protected ornamentals and containers were unable to use oxamyl as specified by the EAMU. Moreover, the use of oxamyl also requires precautions for operator and environmental protection, along with a re-entry time to any treated glasshouses and a harvest interval. However, oxamyl was included in this study as a standard nematicide despite the highlighted issues concerning its use.

Although some insecticides have been demonstrated to be effective against LBN on some ornamentals (LaMondia, 1999; Young, 2000), they are not registered as nematicides in the UK. Abamectin for example has a UK approval for use in ornamental plant production against spider mites, while spirotetramat is approved for use on aphids and whitefly in the UK, with an EAMU approval against insect pests in ornamental plant production. However, growers observed an incidental control of LBN symptoms on infested ornamental plants when the two products (spirotetramat and abamectin) were used for their approved pest's management. Spirotetramat was further investigated in this study to confirm its suspected potential for LBN control. It had previously been reported to have efficacy on other nematode species such as *Heterodera glycines* and *Meloidogyne* species (Vang *et al.*, 2016).

Other potential products (insecticides, fungicides or algicides) considered for investigation in this study were based on their previous potential reports against LBN or other PPN (LaMondia, 1999; Jagdale & Grewal, 2002; Vang *et al.*, 2016; An *et al.*, 2017; Chałańska *et al.*, 2017). In addition, bio-fumigant and bio-pesticide (plant

extract) products such as Isothiocyanates & capsicum and garlic extract had previously indicated control on *A. fragariae* (*in vitro*) and other nematode species (An *et al.*, 2017). Bio-fumigants suppress soil borne pests and diseases with naturally occurring gases, and are highly efficient as contact products (Ramirez *et al.*, 2009). Isothiocyanates/glucosinolates released during the incorporation of Indian mustard plants (*Brassica juncea*) in the soil suppressed PPN such as *Meloidogyne javanica, Tylenchulus semipenetrans* and *G. pallida* among others (Zasada & Ferris, 2003; Ngala *et al.*, 2015). Garlic has demonstrated contact effect on the eggs of pests such as Cabbage root fly, root knot nematodes (*Meloidogyne* spp.) and stem & bulb nematodes (*Ditylenchus dipsaci*) on outdoor bulb onion (Anwar, 2009; Anwar *et al.*, 2016). Garlic as 'Eagle Green Care' product was approved in the UK as a liquid nematicide for pest control on elite sports turf (MAPP No.14989). The presence of polysulphides in garlic confers toxicity against the slug-pathogenic nematode *Phasmarhabditis hermaphrodit*a (Anwar *et al.*, 2016). Azadirachtin, a systemic insecticide was selected in this study due to its effect as a slow anti-feedant, with the ability to reduce fecundity and breeding ability on various insect pests (Khalil, 2013); while its potential management have been reported on *A. fragariae* in both *in vitro* and *in vivo* studies (An *et al.*, 2017). Peroxyacetic acid has been successfully used against *A. fragariae* (Jagdale & Grewal, 2002), and approved in UK as a general disinfectant in ornamental production with surfactant activity to achieve penetrating action on pests.

In the search for an alternative to oxamyl, which is the main purpose of this study, laboratory bioassays were conducted to evaluate contact mortality of *A. fragariae* in water using a range of biological and chemical products including abamectin – insecticide; spirotetramat - insecticide, peroxyacetic acid - fungicide and algicide; oxamyl - nematicide; thiocyanate – fungicide; azadirachtin A. – biopesticide; allyl-isothiocyanate & capsicum – biofumigant, and garlic – biopesticide. Products such as peroxyacetic acid, abamectin, oxamyl and spirotetramat have been approved in the UK for use on ornamental plant production.

This study therefore evaluated the efficacy of abamectin, spirotetramat, peroxyacetic acid, oxamyl, thiocyanate, azadirachtin, allyl-isothiocyanate & capsicum and garlic extract against *A. fragariae* in a laboratory contact bioassay.

2.2 Materials and methods

2.2.1 Materials

2.2.1.1 Nematodes

The nematodes (*A. fragariae*) were sourced and extracted from infested evergreen fern (*Woodwardia fimbriata*) which was obtained from a commercial nursery. *A. fragariae* was identified based on morphological and morphometric features (Siddiqi, 1975; Hockland, 2001; Seni de Jesus *et al.*, 2016).

2.2.1.2 Pesticides

A range of biological and chemical pesticides were sourced from Agrochemical companies in the UK (see Table 2.2 & 2.3). There was no available recommendation specific to *Aphelenchoides* spp. for these pesticides (Table 2.2); therefore the product doses were prepared based on the recommendations made by the pesticide companies for the management of insects, mites and other pests.

2.2.2 Methods

Laboratory bioassay test was carried out to investigate contact mortality effect of the selected chemical and biological products against *A. fragariae* (Fig. 2.2) in water. Nematode extraction from infested leaf tissue was carried out based on a method adapted according to the techniques described by Jagdale & Grewal, (2002) and (Zhen *et al.*, 2012). Leaves were cut into 1cm² pieces, incubated in a plastic beaker containing distilled water to stimulate LBN emergence from leaves, and kept at a room temperature of 22° ± 2°C for 48h. After 48h, the leaf pieces and extraction water were passed through large nested 20-mesh sieve (850-µm openings) made by Endecotts Limited, London SW19 3TZ, England, to first remove leaf debris, and then followed by a 500-mesh sieve (25-µm openings) to collect extracted nematodes in a beaker. Nematodes that were extracted were washed from the 500-mesh sieve in 10ml distilled water.

The nematode suspensions were counted in a 3 x 5cm counting dish under an inverted microscope at x40. In view of the importance of molecular confirmation of nematode species as expected in nematology practice nowadays, samples were taken to laboratory of nematology, James Hutton Institute, Dundee. Assisted by a

molecular biologist, *A. fragariae* was confirmed through the use of molecular techniques (PCR) using species-specific primer, which amplified DNA from samples containing *A. fragariae,* according to the protocol described in (Seni de Jesus *et al.*, 2016).The suspensions were refrigerated at 4^{o} C and used within 48 hours of extraction period for laboratory bioassays.

Three different dose rates (levels) of the chemical and biological products were considered with level 1 being manufacturer's recommended dose rate while 2 and 3 were reduced by about half and one-third of the recommended dose rates respectively. Levels 2 and 3 were formulated to evaluate contact mortality in water at a reduced dose rates. The experiment was conducted in 108 (5cm) plastic petri dishes (Table 2.2; 2.3) on *A. fragariae* at room temperature of 22° ± 2°C. The bioassay evaluated 8 products at 3 dose rates: full recommended dose (Level 1) and two lower dose rates (Level 2 and Level 3), with each level replicated 4 times. Water was used as the control with the same number of replicates (n=4). Based on the adapted method of Jagdale & Grewal, (2002), this study consisted of nine treatments including water (Control), with four dishes used as replicates for each treatment. Having prepared a double strength concentration for each product according to the manufacturer's recommended dose rates, a 4ml aliquot solution of each treatment (in distilled water) was transferred into each dish, and 4ml of a suspension containing about 800 mixed stages nematodes was added to each Petri dish to obtain the desired active ingredient level for each product (Table 2.3). The control treatment was set up with a nematode water suspension used as above while ordinary distilled water was used instead of concentrations of chemical and bio-pesticide products. Observations were recorded on percentage nematode mortality at 24, 48 and 72h after exposure.

During sampling time (carried out at 24, 48 and 72h), a thoroughly mixed 2ml sub-sample from each Petri dish was added into a 5-cm diameter dish containing 10ml of distilled water. This was held at room temperature for a maximum period of 72h for nematodes to potentially recover from the effect and shock caused by the treatments. During each observation at 24, 48 & 72h, nematode suspensions were reduced to 3ml (concentrated), followed by counting of live and dead nematodes under an inverted microscope. Nematodes were classed as 'dead' based on the definition adopted by

Jagdale & Grewal (2002) and An *et al.* (2017) as a complete lack of movement even after prodding with a fine needle.

Data analysis: Data was analysed using the same approach as that used by Jagdale & Grewal (2002) and An *et al*. (2017). Data of arcsine-transformed values of corrected percentage mean mortalities were subjected to analysis of variance using One-way ANOVA at each observation. Data from overtime observations were analysed using repeated measures ANOVA with time and treatment as within-subject factors. Significant differences between treatments were determined with Tukey's mean comparison at $P<0.05$. All data analyses were performed in Minitab (V.16).

Table 2.2 Description of products investigated in laboratory bioassays against *Aphelenchoides fragariae* in this study

Trade names	**Chemical name**	**Description**
EcoGuard	Garlic (4%)	A garlic based bio-pesticide/insecticide product. The major constituents of garlic oils are allium, diallyl disulphide and trisulphide. The sulphur compounds present in garlic have potential as nematicides against nematodes of carrot and parsnip in the UK, and root knot nematodes of carrot & tomato.
Caliente Liquid Mustard	Allyl-isothiocyanate and capsicum	A bio-fumigant product, comprising isothiocyanates derived from mustard, and capsicums derived from chilli pepper. The bio-fumigant suppresses various soil-borne pests and diseases by releasing naturally occurring compounds. Suppression of a range of soil-borne diseases including verticillium wilt, rhizoctonia, sclerotinia, pythium, fusarium, and a range of harmful nematode species.
Cercobin WG	Thiocyanate 70%	Fungicide; a water dispersible granule formulation containing 700g/kg thiophanate-methyl for use as a protectant fungicide on a range of horticultural and agricultural crops; Use for the reduction of fusarium and mycotoxins in wheat and triticale.
Dynamec	Abamectin 18g/l	Insecticide; abamectin affects the nervous system of invertebrates, and is approved in the UK as a foliar treatment for the control of two-spotted mite and western flower thrips in protected and outdoor flower crops and other ornamentals.

Trade names	Chemical name	Description
Jet 5	Peroxyacetic acid 5%	Peroxyacetic acid is an environmentally friendly fungicide/algicide; approved in the UK as a disinfectant on protected horticultural crops. Its use includes cleaning floors and benches between crops for the control of disease pathogens.
Movento	Spirotetramat 150g/l	Spirotetramat is a systemic insecticide registered in the UK as Movento. It has a two-way (ambimobile) mode of action for controlling many sucking insect pests with an EAMU in the UK on aphids, mealybugs and whiteflies for ornamental plant production.
NeemAzal	Azadirachtin 1%	Bio-pesticide; a systemic insecticide with effect as an anti-feedant, with the ability to reduce fecundity and breeding ability on various insect pests. Azadirachtin has recently been approved as Azatin in the UK for protected ornamental plant production to control western flower thrips and onion thrips only.
Vydate 10G	Oxamyl 10%	Soil applied nematicide approved in the UK. Oxamyl works systemically for the suppression of nematodes in potatoes, carrots, sugar beet and parsnip. Oxamyl had an EAMU for outdoor ornamental plant production against American serpentine leaf miner, silver leaf whitefly, South American leaf miner, and stem & bulb eelworm. The EAMU ended December 31st, 2017.

Table 2.3 Trade and chemical names, formulations and sources of pesticides used in this experiment

Product name	*Active ingredients*	*Manufacturers*	Concentration level		
			1*	2	3
Movento	*Spirotetramat150g/l*	Bayer Cropscience	0.5L/600L/ha	0.4L/600L/ha	0.3L/600L/ha
Caliente L mustard	*Isothiocyanate*	HealthCare UK	50L/500L/ha	25L/500L/ha	12L/500L/ha
Jet 5	*Peroxyacetic acid 5%*	Certis	800ml/100L	650ml/100L	570ml/100L
NeemAzal	*Azadirachtin %*	NeemCo Ltd	743mg/1L	550mg/1L	350mg/1L
Vydate10G	*Oxamyl 10%*	DuPont	55kg/ha	40kg/ha	10kg/ha
EcoGuard	*Garlic 4%*	ECOspray	4ml:96ml	4ml:120ml	4ml:144ml
Cercobin	*Thiocyanate 70%*	Certis	1.1kg/500L/ha	900g/500L/ha	750g/500L/ha
Dynamec	*Abamectin 18g/l*	Syngenta	50ml/100L	25ml/100L	10ml/100L

*Note that level 1 is the manufacturers recommended dosage for use against nematodes / other pests while level 2 & 3 are reduced dose

2.3 Results

After 24h of exposure, several of the tested products caused significant mortality of *A. fragariae* compared with the Water control (Table 2.4). The highest nematode mortality at 24h was observed when the nematodes were exposed to Garlic, Abamectin and Isothiocyanate (Table 2.4).

Table 2.4. Effect of biological and chemical pesticides at 3 different levels (doses) of concentrations on *Aphelenchoides fragariae* in water after 24 hours exposure

	Mortality (%) at 24h for 3 levels of concentration		
Treatments	Dose 1	Dose 2	Dose 3
Spirotetramat	5.6 de (±0.98)	6.1 d (±1.23)	5.8 d (±0.76)
Isothiocyanate	51.4 b (±4.49)	37.6 b (±3.99)	29.4 b (±2.72)
Azadirachtin	12.9 de (±1.91)	10.1 cd (±1.79)	5.9 d (±1.19)
Peroxyacetic	14.9 cd (±2.09)	8.3 cd (±0.76)	8.1 cd (±0.72)
Abamectin	77.9 a (±3.01)	63.8 a (±1.12)	51.4 a (±3.30)
Thiocyanate	7.8 de (±1.42)	5.5 d (±1.22)	4.3 d (±0.60)
Oxamyl	24.4 c (±0.98)	20.3 c (±2.02)	15 c (±1.78)
Garlic	75.4 a (±3.16)	64 a (±7.25)	56 a (±2.48)
Water	4.1 e (±0.86)	3.6 d (±0.43)	3 d (±0.62)

Data are mean (±SE) percentage mortality of four replicates, and values in the same column followed by the same letter are not significantly different (Tukey's multiple range test $P<0.05$)

There was an increase in nematode mortality as the exposure period increases in all the treatments (except the control) from 48h and up to 72h at the 3 dose rates (Table 2.5 and Table 2.6). Increase in nematode mortality was observed in Peroxyacetic (40.5%) at 72h of exposure along with Garlic-86%, Abamectin-94% and Isothiocyanate-95% (Table 2.6). The less effective products were Spirotetramat, Thiocyanate, Oxamyl and Azadirachtin, although they all still caused mortality of 8.8-33.8% of *A. fragariae* after 72h of exposure (Table 2.6) at the highest dose (Dose 1).

Table 2.5. Effect of biological and chemical pesticides at 3 different levels (doses) of concentrations on *Aphelenchoides fragariae* in water after 48 hours exposure

	Mortality (%) at 48h for 3 levels of concentration		
Treatments	Dose 1	Dose 2	Dose 3
Spirotetramat	9.4 c (±1.16)	8.5 cd (±0.53)	8.9 cde (±1.04)
Isothiocyanate	89.3 a (±4.19)	70.3 a (±3.87)	27.4 b (±5.04)
Azadirachtin	31.4 b (±2.95)	16.3 bc (±2.84)	8.1 de (±0.95)
Peroxyacetic	32 b (±4.99)	27.3 b (±4.81)	15.9 cd (±3.50)
Abamectin	88.4 a (±2.85)	72.3 a (±3.34)	65.6 a (±1.64)
Thiocyanate	11.4 c (±1.80)	7.8 cd (±0.90)	6.1 e (±0.86)
Oxamyl	31.9 b (±3.05)	24 b (±0.75)	18.3 bc (±2.39)
Garlic	79.3 a (±3.41)	67.8 a (±2.77)	65.6 a (±1.01)
Water	4.8 c (±0.37)	4.8 d (±0.37)	3.9 e (±0.43)

Data are mean (±SE) percentage mortality of four replicates, and values in the same column followed by the same letter are not significantly different (Tukey's multiple range test $P<0.05$)

Table 2.6. Effect of biological and chemical pesticides at 3 different levels (doses) of concentrations on *Aphelenchoides fragariae* in water after 72 hours exposure

	Mortality (%) at 72h for 3 levels of concentration		
Treatments	Dose 1	Dose 2	Dose 3
Spirotetramat	8.8d (±1.49)	8.3de (±0.83)	7.4d (±1.01)
Isothiocyanate	95.8a (±2.07)	83a (±3.94)	28.3b (±5.46)
Azadirachtin	33.8c (±2.96)	17.6cd (±1.80)	13.1cd (±1.66)
Peroxyacetic	40.5c (±3.59)	32.6b (±3.94)	11.4cd (±1.95)
Abamectin	94.4ab (±0.49)	78.8a (±5.87)	59.8a (±2.73)
Thiocyanate	10d (±1.22)	6.9de (±1.11)	7.1d (±0.98)
Oxamyl	32.6c (±3.05)	29.8bc (±1.91)	20.6bc (±1.38)
Garlic	86.4b (±2.41)	74.1a (±3.79)	68a (±2.33)
Water	4d (±0.43)	4.5e (±0.47)	3.9d (±0.49)

Data are mean (±SE) percentage mortality of four replicates, and values in the same column followed by the same letter are not significantly different (Tukey's multiple range test $P<0.05$)

Table 2.7. Mortality grouping of 3 different levels (doses) of concentrations in water on *Aphelenchoides fragariae* 72 hours after exposure

	Low mortality (<15%)		Medium mortality (15-50%)		High mortality (>50%)	
	Products	*means*	*Products*	*Means*	*Products*	*means*
Dose 1	Spirotetramat	8.8d	Azadirachtin	33.8c	Isothiocyanate	95.8a
	Thiocyanate	10d	Peroxyacetic A	40.5c	Abamectin	94.4ab
	Control(Wt)	4d	Oxamyl	32.6c	Garlic	86.4b
Dose 2	Thiocyanate	6.9de	Azadirachtin	17.6cd	Isothiocyanate	83a
	Spirotetramat	8.3de	Peroxyacetic A	32.6b	Abamectin	78.8a
	Control(Wt)	4.5e	Oxamyl	29.8bc	Garlic	74.1a
Dose 3	Spirotetramat	7.4d	Oxamyl	20.6bc	Abamectin	59.8a
	Thiocyanate	7.1d	Isothiocyanate	28.3b	Garlic	68a
	Peroxyacetic.A	11.4cd				
	Azadirachtin	13.1cd				
	Control(Wt)	3.9d				

Data are percentage mortality means of four replicates, and values in the same dose category (dose 1, 2 or 3) followed by the same letter are not significantly different (Tukey's multiple range test, $P<0.05$)

Overall, the highest mortality (95.8%) was observed at 72h of nematode exposure to Isothiocyanate at Dose 1 (Table 2.6) followed by both Abamectin (94.4%) and Garlic (86.4%) at the manufacturers recommended dose.

The lowest percentage mortality (<15%) was recorded from exposure to Thiocyanate, Spirotetramat and Water (Table 2.7). Other products such as Peroxyacetic acid, Azadirachtin and Oxamyl had moderate contact mortality of 15-50% (Table 2.7).

2.4 Discussion

The bioassay results provided an indication of products which warrant further investigation as potential nematicides for *Aphelenchoides* management. From the results, some products which are currently used as disinfectants, bio-fumigants and insecticides demonstrated considerable contact mortality against LBN. Three promising products have been identified from this bioassay study: - isothiocyanate & capsicum, abamectin and garlic extract demonstrated the highest nematicidal activity against LBN at recommended rates for insect control, as well as at reduced dose rates of ±50% manufacturer's recommended dose rate (Table 2.7).

The results of 94% mortality with abamectin in this study are similar to the results of Young & Maher, (2000) who observed significant mortality (90%) of *A. ritzemabosi* (LBN) *in vitro* by abamectin with the same manufacturer's recommended rate (0.05% volume concentration) as was used in this study after 24 hours of exposure in a laboratory assay. Abamectin was suggested by Young & Maher, (2000) as a potential treatment for short term suppression of LBN in hardy ornamentals, and could be incorporated within an integrated pest management programme. Mortality of 71-75% of *A. fragariae* at 24h of exposure to a 2-fold dilution of abamectin, and with subsequent mortality increase up till 72h was observed by An *et al.* (2017). The results as reported by LaMondia (1999) on foliar application of abamectin to infested *Lamium maculatum* and Chałańska *et al.* (2017) to infested Japanese anemone confirms that abamectin has the potential to control LBN. Chałańska *et al.* (2017) demonstrated that oxamyl and abamectin mixed with an aqueous extract of

garlic (*A. sativum*) were effective at managing *A. ritzemabosi* in Japanese anemone (cv. *Hupehensis*).

In this current study, abamectin at the manufacturer's recommended rate against spider mites demonstrated high mortality of 77%, 88% and 94% on *A. fragariae* at 24h, 48h and 72h of exposure respectively. Abamectin is approved in the UK as a foliar treatment for the control of two-spotted mite and western flower thrips in protected and outdoor flower crops and other ornamentals. Despite not having a label recommendation for LBN, some growers have observed incidental control by abamectin on LBN infested ornamental plants (personal communication).

Peroxyacetic acid also demonstrated nematicidal activity with 40.5% mortality at 72h of exposure. Peroxyacetic acid is widely used in the UK as a disinfectant on protected horticulture. Its use includes cleaning floors and benches between crops are planting for the control of disease pathogens. Peroxyacetic acid (as the product Zerotol) has been tested previously for activity against *Aphelenchoides* spp. (Jagdale & Grewal, 2002; An *et al.*, 2017). They used a higher concentration of peroxyacetic acid (Zerotol) than used in this study at 270g litre $^{-1}$ (active ingredients) - aqueous solution, compared to the current study with a lower concentration of 40g litre $^{-1}$ (active ingredients) - aqueous solution. Their results demonstrated 100% mortality in water against *A. fragariae* during 24h exposure (Jagdale & Grewal, 2002) and 100% mortality of *A. fragariae* at 72h after being exposed to a 2-fold dilution of the same high concentration (An *et al.*, 2017). However, the use of peroxyacetic acid at a lower concentration (40g litre $^{-1}$ active ingredients) in this study, which is the UK standard recommended rate, caused 40.5% mortality rate of *A. fragariae* at 72h of exposure. Use of peroxyacetic acid in the UK has a role in 'good management practice' for reducing the risk of leaf nematode problems along with other pathogens, but at the UK recommended rate is not as effective as the rates used in the USA.

An *et al.* (2017) used a product called AzaMax (Azadirachtin) at both 2 & 20-fold dilutions (6g & 0.6g/l) treatment. A 2–fold dilution caused 60-77% mortality while 20-fold dilution demonstrated 33-51% mortality of *A. fragariae* in an aqueous suspension after 24-72h exposure. This current study recorded 12-33% mortality with the recommended rate of Azadirachtin-0.25g/l when exposed to *A. fragariae* after 24-72h, which is a much lower azadirachtin concentration than An *et al.* (2017).

Contact mortality may not occur with azadirachtin at this concentration as it affects the endocrine system of target pests after ingestion (Howard *et al.*, 2009). Azadirachtin acts as an anti-feedant, interferes with the moulting process, reduces fecundity, and disrupts respiration and oviposition in targeted insect pests (Howard *et al.*, 2009; Khalil, 2013). This could explain why the mortality is not immediate. Results of this study confirm the recent report of An *et al.(*2017) which suggested azadirachtin as being toxic to *A. fragariae*. This study agrees with the recommendations of Khalil (2013) on azadirachtin as an important tool for the management of nematode pests. An emulsifiable neem concentrate formulation, registered as Azatin (azadirachtin 217g litre $^{-1}$ active ingredients) has been approved in the UK for use on protected ornamental plant production (MAPP 18301; Authorisation Number – 0360). Growers now have the option to use Azatin on ornamental plant production against insect pests, although there is currently no on-label approval for use against LBN.

Spirotetramat had low contact mortality (6-9%) against *A. fragariae*. However, spirotetramat has been shown to cause a significant reduction in development to reproductive maturity of *Heterodera glycine* and *Meloidogyne incognita* when applied as a foliar spray on soybean plants in a greenhouse study (Vang *et al.*, 2016). Consequently, spirotetramat may well have an effect on nematode reproduction and fecundity rather than demonstrate any direct mortality against *A. fragariae* in water as observed in this study. Nauen *et al.* (2008) reported that upon the application of spirotetramat to foliage, it is hydrolysed to an -enol form (the main active form) known to inhibit acetyl-CoA carboxylase (ACC) in insects, the enzyme essential for fatty acid biosynthesis. The phloem and xylem access through the physicochemical properties of spirotetramat-enol enables the active ingredients to be taken and distributed throughout the entire plants, hence serving as an effective insecticide and potential nematicide product (Nauen *et al.*, 2008; Salazar-López *et al.*, 2016). This was very effective in subsequent trials in glasshouse and commercial conditions. Spirotetramat is registered in the UK as Movento for controlling many sucking insect pests in ornamental plant production, with EAMU in the UK on aphids, mealybugs and whiteflies. Having observed incidental control of LBN symptoms on infested plants, some growers are considering using spirotetramat for LBN management on ornamental crops in the UK.

Previous studies on leaf extracts of *Brassica juncea* (cv. Indian mustard) demonstrated that glucosinolates which break down into isothiocyanates, inhibit the motility of *Globodera pallida* infective juvenile nematodes in an *in-vitro* bioassay, leading to significant mortality and suppression of 89% of juveniles at 48h when compared with water alone (Lord *et al.*, 2011). The nematicidal effects of glucosinolate derived isothiocyanates have been demonstrated through the treatment of infected soil and confirmed toxicity to several nematodes species: *Caenorhabditis elegans, Heterodera schachtii, Xiphinema americanum* and *Globodera rostochiensis* (Lazzeri *et al.*, 1993; Donkin *et al.*, 1995). 2-phenylethyl isothiocyanate, found in *Sinapis alba* roots, and allyl isothiocyanate, in a bioassay inhibited egg hatching of potato cyst nematodes (*G. rostochiensis*) at 50ug/ml, with similar potential of allyl isothiocyanate reported in a field trial on potatoes (Ellenby, 1951). Isothiocyanates involved in the activity of Rapeseed (*Brassica napus*) as a biofumigant to control nematodes has made it popular as green manure or rotational crop (Halbrendt, 1996). *Capsicum annuum* was reported to possess nematicidal activity against *Meloidogyne incognita,* the root-knot nematodes on tomato cv '*Lycopersicon esculentum*' (Bawa *et al.*, 2014). The product Caliente Liquid Mustard contains allyl isothiocyanate and capsicum, and in this study recorded 95% nematode mortality after 72h of exposure. While isothiocyanate demonstrated high contact mortality in water, the use of isothiocyanate if not well incorporated into the soil, may cause phytotoxic effect to plants (as observed during soil treatment study of this thesis - Chapter 7). Therefore the effect of fumigants gases should be allowed to properly wear out before plants are introduced to the treated soil as recommended by the manufacturer. This suggests that the nematicidal properties of *Capsicum*, allyl- isothiocyanate and other isothiocyanates may have a role to play in the management of The bioassay results provided an indication of products which warrant further investigation as potential nematicides for *Aphelenchoides* management. From the results, some products which are currently used as disinfectants, bio-fumigants and insecticides demonstrated considerable contact mortality against LBN. Three promising products have been identified from this bioassay study: - isothiocyanate & capsicum, abamectin and garlic extract demonstrated the highest nematicidal activity against LBN at recommended rates for insect control, as well as at reduced dose rates of ±50% manufacturer's recommended dose rate (Table 2.7) in the soil stage of the nematode life cycle, and was demonstrated as a soil treatment in Chapter 7.

Garlic extract demonstrated high levels of contact mortality to *A. fragariae* in this study. The major constituents of garlic oils are allium, diallyl disulphide and trisulphide, and these have demonstrated toxic effects against pine wood nematode *Bursaphelenchus xylophilus* in laboratory bioassays (Park *et al.*, 2005). Garlic oils had 100% mortality at low concentrations of 62.5µl/litre on both adult and juveniles of *B. xylophilus* at 4h of exposure. It has been reported that hydrolysation of sulphur compounds found in *Allium sativum*, *A. cepa* and *A. fistulosum* form a variety of isothiocyanate compounds with nematicidal and phytotoxic effects on pathogens (Choi *et al.*, 2007). The efficacy of nematicidal activity of garlic was reported to show toxicity against nematode *Phasmarhabditis hermaphrodita* with high mortality due to the presence of polysulfides (Anwar, 2009). Concentrations of raw garlic straw '*Allium sativum*' at 1%, 2% and 4% (w/v) increased the mortality of *Meloidogyne incognita* from 9.8% to 36.6%, and at the same time reduced hatching activity (Gong *et al.*, 2013). In this study, garlic at a reduced dose rate of 3.3% and 2.8% percent volume concentrations demonstrated high mortality of 68% and 74% on *A. fragariae* respectively at 72h of exposure, while mortality of 86% was obtained at the manufacturers recommended rate (4%) after 72h of exposure. Garlic based insecticides have been approved in Denmark and Norway as ECOguard for cabbage root fly control, ECOspray in the UK got regulatory approval for product called 'Eagle Green Care', the liquid nematicide for pest control on elite sports turf (MAPP No.14989). Other approved garlic extract products include NEMguard granules (MAPP No. 15254) for carrot and parsnip, NEMguard PCN Granules (MAPP No.. 17377) approved for potato, NEMguard DE (MAPP No. 16749) to control root knot nematodes (*Meloidogyne* spp.) and stem & bulb nematodes (*Ditylenchus dipsaci*) on outdoor bulb onion; and against free living nematodes on outdoor garlic, leek, fodder beet and red beet. Easy penetration of leaf cuticle with attendant phytotoxic and nematicidal effect on pathogen contributes to the efficacy of garlic on pests (Choi *et al.*, 2007), and thereby assisted in the contact mortality of this study. Garlic was further investigated as a soil treatment in Chapter 7.

The lowest percentage mortality (<15%) of nematodes in this current study was recorded from exposure to thiophanate-methyl, spirotetramat and Water (Table 2.7). Other products such as peroxyacetic acid, azadirachtin and oxamyl had moderate contact mortality of 15-50% (Table 2.7).

Oxamyl is a soil applied nematicide widely used for the suppression of various nematodes in potatoes, sugar beet, carrots and parsnips. Oxamyl is known to work systemically against target pests hence may have less effect as a contact product (Wright *et al.*, 1980; Whitehead *et al.*, 1984; Osborn *et al.*, 2010). Spirotetramat, azadirachtin and oxamyl are known to systemically move within plants; therefore mortality could be gradual rather than immediate (Table 2.7). These compounds may exert an effect against *A. fragariae* by interfering with nematode reproduction and fecundity. Vang *et al.* (2016) suggested that the mode of action of systemic pesticides including spirotetramat may be due to having an effect on nematode reproduction and fecundity rather than demonstrating direct mortality against nematodes. There are no reports yet of LBN developing resistance to oxamyl or any of the historically used products before their withdrawal.

To compliment the efficacy of chemical and other products, cultural control serves as an important component within integrated pest management (IPM) programmes on LBN control. The adoption level of cultural methods as observed in the nurseries was not up to expectations. Growers were encouraged during this thesis to improve sanitation in the nurseries. They should avoid ways that could create ideal conditions for nematode infestation and spread as detailed in Chapter 1, and Kohl *et al.* (2010). Test was conducted in the following Chapter 3 to evaluate the possibility of nematode infestation to the plant using the route of infested soil, as a step towards soil treatment to ensure healthy plant.

Further research is described in subsequent Chapters of this thesis where some of these products are used as single treatments and in combination with other products to manage LBN under glasshouse and field conditions.

Chapter 3

Methods for artificial inoculation of *Aphelenchoides fragariae* on the leaves of *Anemone hupehensis* and *Weigela florida*, to soil for subsequent plant infestation, and the correlation of nematode populations with infestation symptoms

3.1 Introduction

Aphelenchoides species including *Aphelenchoides fragariae* (Aphelen-chida: Aphelenchidae) - LBN, cause major economic damage to many ornamental and agricultural plants in nurseries and landscapes all over the World (Johnson & Gill, 1975; Richardson & Grewal, 1993; Jagdale & Grewal, 2006). They are plant parasitic organisms found in the leaf tissue of over 1104 associated host (Kohl, 2008; Sánchez-Monge *et al.*, 2015).

LBN penetrate into the leaves through leaf stomata or wounds on the leaf, and feed on the mesophyll cells, thereby causing angular shaped blotches on leaf veins, which can become lightly chlorotic (Jagdale & Grewal, 2004; Kohl, 2011), and later turn into necrotic lesions which are usually surrounded by large veins (Sanwal, 1959). Management of LBN is challenging due to the survival behaviour of the pest in dried leaves/plant debris, dormant crowns and soil (De-Waele, 2002).

A serious setback on the control strategies of LBN is the revocation and restriction of use of some chemical pesticides such as diazinon, aldicarb, parathion and oxamyl due to their broad presumed toxicity to humans, plants and environmental concerns, despite their efficacy (LaMondia, 1996; Young & Maher, 2000; Jagdale & Grewal, 2002; An *et al.*, 2017).

Due to the wide host range of *A. fragariae* and its life cycle inside the leaf tissues of host plants and in soil (De-Waele, 2002), it is important to have suitable tools for screening plant resistance to nematodes, therefore reliable methods of nematode inoculation are important for research, and evaluating control and management strategies (De-Schutter *et al.*, 2001; Zhen *et al.*, 2012). A single leaf inoculation method termed ‘wet tissue-paper inoculation technique’ was used by Jagdale &

Grewal (2006), and supportive material such as black plastic bags to provide a moisture retaining medium after inoculation while nematodes undergo stomata penetration as demonstrated during resistance screening of Hosta plants to *A. fragariae* by Zhen *et al.*, (2012). Moisture and humidity are considered essential factors for successful nematode (*Aphelenchoides fragariae*) infestation on leaves (Jagdale & Grewal, 2006; Kohl *et al.*, 2010). Moisture retention is necessary to avoid high loss of nematode inoculum as reported by Plowright *et al.* (2002) who demonstrated that over 75% inoculum loss of *Ditylenchus angustus* was recorded after inoculation during a rice resistance screening study due to lack of moisture retention.

The use of a soil medium for nematode inoculation as a tool for nematode resistance screening (although on root-infesting nematode species) was previously reported in a banana/plantain pathogenicity pot study (Coyne *et al.*, 2013). Sterilised soil with clean sucker was inoculated with aqueous suspension of 1000 nematodes of five species (*Radopholus similis*, *Pratylenchus coffeae*, *Hoplolaimus pararobustus*, *Helicotylenchus multicinctus* and *Meloidogyne* spp.), and with resultant symptoms of lesion/necrosis on roots of the susceptible cultivars 8 weeks after artificial soil inoculation (De-Schutter *et al.*, 2001; Coyne *et al.*, 2013).

Cultural control is an important component of integrated pest management practices. Soil serves as an important infestation route for nematode migration from infested leaf debris or overwintering soil to new plants, when moisture is made available (Lambert & Bekal, 2002; Jagdale & Grewal, 2006; Kohl, 2008). A soil treatment bioassay is therefore necessary to evaluate potential products that may prevent nematode migration from the soil/plant media to new plants, thereby maintaining clean soil/plant media for healthy plant growth.

The pattern of visual symptoms exhibited by different host plant species as shown on weigela, anemone, hostas, astilboides, dryopteris, bergenia and buddleja could differ slightly, but the overall LBN description given in this thesis will help growers to distinguish symptoms of LBN from visual symptoms caused by bacteria infection on leaves as previously highlighted (Chapter 1- Section 1.3.3). Because the aesthetic appearance of ornamental plants dictates the grower's assessment for identifying nematode free plants suitable for sale, it is very important to evaluate symptom

severity from time to time by assessing the presence of newly infested leaves (Fig. 3.1). In view of this, leaf rating assessment was carried out to investigate the relationship between visual symptoms on leaf and corresponding nematode population as an essential tool for assessing plant resistance, and in addition to evaluate the effectiveness of control treatments.

There can also be issues with asymptomatic nematode infestation of leaves which will lead to the continuous reproduction of LBN in plants (McCuiston *et al.*, 2007), and spread throughout the nursery. Consequently the assessment of plant resistance can not overlook the importance of asymptomatic nematode reproduction within the leaf. An easily adaptable method of nematode inoculation of leaves with subsequent assessment of nematode populations within the leaf will be of assistance for resistance screening to LBN. Quality resistance screening procedures plays an important role that can be beneficial to breeders and horticultural managers (De-Waele, 2002).

Figure 3.1. Symptoms of LBN lesions on (red-tag) leaves of *Anemone hupehensis* var. japonica 'Prinz Heinrich' (left) and *Weigela florida* var. 'Bristol Ruby' (right) undergoing leaf inoculation test

This study has evaluated the efficacy of direct nematode inoculation methods to soil and subsequent plant infestation, inoculation of leaves as a suitable tool for resistance screening and as a research tool to evaluate control options, and in addition, investigated the relationship between LBN reproduction and leaf symptom severity ratings of the lesions caused by *A. fragariae* on two plant species.

3.2 Experiment (1). Methods for artificial inoculation of *Aphelenchoides fragariae* to soil and subsequent plant infestation of leaves of *Anemone hupehensis* var. japonica 'Prinz Heinrich' and *Weigela florida* var. 'Bristol Ruby'

3.2.1 Materials and methods

3.2.2 Materials

3.2.2.1 Materials (Soil Inoculation)

Nematodes: The nematode species (*A. fragariae*) used was from the same source as reported in Chapter 2, Section 2.2.1.1. LBN were isolated from infested evergreen fern (*Woodwardia fimbrata*) and multiplied on the leaves of clean *Anemone hupehensis* plants. Nematodes were extracted as described in Chapter 2 - Section 2.2.2.

Plants: Certified nematode-free 'plug' plants of *Anemone hupehensis* var. japonica 'Prinz Heinrich' (henceforth referred to as *Anemone hupehensis*) were obtained as rootstock from a commercial nursery, Jackdaws' Field Nursery, Horsham, West Sussex UK.

3.2.2.2 Materials (Leaf Inoculation)

Nematodes: The nematode species (*A. fragariae*) used were the same as described above in Section 3.2.2.1.

Plants: Certified nematode-free of *Anemone hupehensis* as above and *Weigela florida* var. 'Bristol Ruby' (henceforth referred to as *Weigela florida*) were obtained from a commercial nursery, Jackdaws' Field Nursery, Horsham, West Sussex UK.

3.2.3. Methods

3.2.3.1 (Method - Soil inoculation)

This test was developed to assess the efficacy of infestation by LBN (*A. fragariae*) on *Anemone hupehensis* via the soil route. Plants were grown in the glasshouse until they had a minimum of 6-8 leaves (Fig. 3.2). Leaf samples were taken at random for nematode extraction from these plants as described in Chapter 2 to confirm that they

were nematode free. Plants were kept in a glasshouse facility and isolated from other plants at conditions of 25 ± 2°C (Fig. 3.2).

Plants were laid out in a randomised block design (Fig. 3.2). The study had 4 treatments and 3 replicates. Treatments used were distilled water suspensions containing 500, 1000, 1500 and 2000 nematodes per plant, and water was used for the Control. Treatments containing mixed stage nematodes in an aliquot suspension of 3ml were dispensed using a glass pipette around the surface of the sterilised soil/plant media in each pot containing plants (approximately 4cm away from the stem of the plant), while the control treatment had a 3ml water treatment applied in the same way.

Figure 3.2. Showing a block of layout during soil inoculation test on *Anemone hupehensis*

The plants were left in the glasshouse for 8 weeks at 25 ± 2°C. At 8 weeks after inoculation, three leaves were randomly selected per plant from the base, middle and growing point of the plant for nematode extraction. Nematode extraction was carried out from leaves as previously described in Chapter 2, Section 2.2.1.1, based on a method adapted from techniques described by (Kohl *et al.*, 2010; Zhen *et al.*, 2012).

Mean nematode population were expressed as number of LBN per 1g of leaf. The method of drenching soil with live nematodes (*A. fragariae*) in the pot of healthy plant was developed to be used for further identification of novel products as a soil treatment (Chapter 7), and a similar method was used by (An *et al.*, 2017).

3.2.3.2 (Method - Leaf Inoculation)

This test investigated the efficacy of direct inoculation of LBN (*A. fragariae*) on leaves of *Weigela florida* and *Anemone hupehensis* plants. Plants were grown in the glasshouse as described above (Section 3.2.2.1) until they had a minimum of 6-8 leaves (Fig. 3.5). Plants were kept in a glasshouse facility and isolated from other plants with conditions of 25 ± 2°C (Fig. 3.5). The study had seven plants of each species. Six plants were inoculated with LBN while the control plants were inoculated with distilled water only. Three leaves were randomly selected for inoculation per plant (Fig. 3.3; 3.4). The methods described by Jagdale & Grewal, (2006); Zhen *et al*, (2012) were adapted to allow direct inoculation of 200 mixed stage LBN (*A. fragariae*) to the leaf. Leaves were injured by making 10 perforations on the leaf surface with a sharp needle scattered between veins at the upper side of the leaf (Fig. 3.3). This approach was adapted to mimic the unavoidable mechanical damage to leaves in the nursery through handling and transportation of plants or injury caused by insects. Leaves were wrapped with wet tissue paper called Kimwipes tissue (11cm x 21cm - Box size code number KC34155 by Kimberly-Clark Professional; Kimtech Science, West Malling, Kent, ME19 4HA, United Kingdom; Fig. 3.4). An aliquot (3ml) suspension containing 200 mixed stage nematodes was dispensed via a glass pipette onto the wet tissue paper. The plants were covered with black plastic bags after inoculation in order to maintain moist conditions. The bags and tissue paper were removed after 72 hours. All plants were randomised and kept in glasshouse conditions of 25 ± 2°C for 8 weeks (Fig. 3.5).

LBN multiplication was assessed at 3, 5 and 8 weeks after inoculation by leaf extraction using the adapted extraction method based on techniques described by (Jagdale & Grewal, 2002; Kohl *et al.*, 2010; Zhen *et al.*, 2012); as previously outlined in Chapter 2, Section 2.2.2. Nematode populations from leaves were expressed as nematode number per 1g leaf.

Figure 3.3. Mechanical injuries on leaf of *Anemone hupehensis* by perforations during direct nematode inoculation

Figure 3.4. Leaves of *Anemone hupehensis* covered in wet tissues during direct nematode inoculation on leaf

Figure 3.5. Showing layout of leaf inoculation test on selected (red-tagged) leaves of *Weigela florida* plants

3.3 Experiment (2). To investigate the relationship between the visual symptoms of nematode infestation on leaves and corresponding nematode population on *Weigela florida* and *Anemone hupehensis* plants by *Aphelenchoides fragariae*

3.3.1 Materials and methods

3.3.2 Materials

Plants (*Weigela florida* and *Anemone hupehensis*) and nematodes (*A. fragariae*) were from the same source as described above (Section 3.2.1).

3.3.3 Methods

This study had 4 treatments and 3 replicates for each plant species. All plants were randomised and kept in a glasshouse for 10 weeks at 25 ± 2°C. Direct nematode inoculation on leaves was carried out according to the adapted method described by Zhen *et al*. (2012), and previously used in Section 3.2.3.2. Three plants each were inoculated with 50, 100 and 200 nematodes per leaf while the remaining 3 plants

(Control) were inoculated with distilled water only. Three leaves were randomly selected per plant for nematode inoculation.

Plants were left for nematodes to multiply in leaf tissue, and to produce symptoms on leaves. Leaf sampling for nematode numbers was undertaken from 3 weeks after inoculation when the symptoms were first noticeable (Fig. 3.7; 3.9; 3.11), and subsequently at 5 and 8 weeks after inoculation.

Different degrees of nematode symptoms were observed on the leaves of both plant species. Leaves showing lesions symptoms of various degrees of nematode infestation were carefully selected and categorised into 6 groups according to their different levels of visual (lesions) symptom caused by nematode infestation per total leaf area (TLA). The 6 categories of leaves are as shown in Table 3.1, with 'category 1' as clean leaf (non-infested), and 'category 6' indicates symptomatic (highly infested) leaf.

Table 3.1 Category of (%) visual symptoms on leaf

Leaf category	Percentage of infestation
1	0%
2	1-10%
3	10-15%
4	25-50%
5	50-75%
6	75–100%

Five leaves were randomly selected from each visual symptom category for nematode extraction and other necessary parameters. Leaf samples for the above 6 categories were used for image analysis by Computer Software called 'ImageJ' (1997) developed by Rasband, W. S. of National Institutes of Health, USA. The parameters recorded include: total leaf area (TLA), nematode infested area (NIA), and percentage infested area (PIA%).

Percentage infested area (PIA) per leaf was calculated as NIA/TLA*100, and expressed as a percentage of the total leaf area. Nematode extraction was carried out on all 5 selected leaves per category. This was to assess nematode population in

relation to the degree of visual symptoms. Nematode extraction was carried out according to the technique described in Chapter 2 - Section 2.2.2 by Zhen *et al.* (2012), while nematode count was also achieved using the method described in Chapter 2 - Section 2.2.2.

Data analysis: Analysis of data collected was carried out as described by Zhen *et al.*, (2012). Data from the mean nematode population from this study were subjected to analysis of variance using One-way ANOVA at each observation. Data from overtime observations (3, 5 and 8 weeks) were analysed using repeated measures ANOVA with treatments and time as subject factors. Significant differences between treatments were determined with Fishers multiple range test at P <0.05. All the data analyses were performed in Minitab (Vs.16). Other parameters considered include the percentage of nematode infested area (PIA) which is calculated as:

$$PIA\% = \left(\frac{nematode\ \ infested\ area\ (NIA)}{total\ leaf\ area\ (TLA)} \times 100\right)$$

3.4 Results

3.4.1 Experiment (1). Methods for artificial inoculation of *Aphelenchoides fragariae* to soil and subsequent plant infestation of leaves of *Anemone hupehensis* and *Weigela florida*

3.4.1.1 Results – Soil inoculation

Table 3.2 indicates the mean nematode population 8 weeks after inoculation in relation to the nematode inoculum applied per treatment. Apart from the non-inoculated (Control) plants, the lowest nematode population (1191.6 / 1g leaf) was obtained from leaves inoculated with 500 nematodes / pot (Table 3.2), whereas a mean of 4699.9 nematodes (the highest population) came from soil inoculated with 2000 nematodes/pot 8 weeks after inoculation. Although treatments with 500 and 1000 nematodes/pot had different mean nematode populations, there was no significant difference between them at P <0.05 (Table 3.2). Similarly, treatments inoculated with 1500 and 2000 nematodes/pot had no significant difference at (P <0.05) between them. In this study, inoculation of soil with nematodes demonstrated

a level of invasion in *Anemone hupehensis* leaves as shown above. Nematode infestation was recorded on leaves lower and further up the plant stem, although infestation was first observed at the lower leaves nearer the soil surface (Fig. 3.7). Nematode infestation of healthy (upper) leaves may have been aided through touching of infested leaves near the soil surface.

Table 3.2. Nematode (*A. fragariae*) infestation route via soil by artificial inoculation method on *Anemone hupehensis* in a glasshouse study. Data are mean values of nematode population of 3 replicates per treatment. Values with same letter are not significantly different (Tukey's multiple range test, P <0.05).

	Nematode reproduction / 1g leaf at 8 weeks after inoculation	
No. of nematodes Applied	Mean	
500	1191.6	b
1000	1650.5	b
1500	3484.7	a
2000	4699.9	a

In general, nematode infestation symptoms were observed on leaves irrespective of their position, although leaves closer to the soil surface were observed to first express lesion symptoms before the leaves higher up the stem (Fig. 3.6). Nematode mean population increased in line with the amount of inoculum applied to the soil (Table 3.2).

3.4.1.2 Results – Leaf inoculation

Table 3.3 shows significant differences in nematode population in relation to time of assessment for both plant species.

Mean nematode populations rose significantly from 3 weeks to 8 weeks after inoculation. *Weigela florida* had the lowest mean nematode population of 373.8/g leaf at week 3 (Table 3.3), but with an increase to 802.3 at week 5 and 1495.8 nematodes/g of leaf at 8 week (Table 3.3). Significant differences (P <0.05) were observed between dates after inoculation (3, 5 & 8 weeks) in terms of the nematode

population (Table 3.3). A similar trend of a gradual increase over time was observed in nematode population after inoculation in *Anemone hupehensis* leaves (Table 3.3).

Table 3.3. Nematode (*A. fragariae*) artificial inoculation test on *Anemone hupehensis* and *Weigela florida* in a glasshouse study. Data are mean values of **Table 3.3**. Nematode (*A. fragariae*) artificial inoculation test on *Anemone hupehensis* and *Weigela florida* in a glasshouse study. Data are mean values of nematode population of 6 replicates per plant species when inoculated with 200 nematodes / leaf. Values with same letter are not significantly different (Tukey's multiple range test, P <0.05).

	Mean nematode population/1g leaf at 3, 5 & 8 weeks after inoculation	
Weeks after inoculation	Weigela	Anemone
3	373.8c	607c
5	802.3b	1358.3b
8	1495.8a	2068.5a

However, mean nematode populations were higher in *Anemone hupehensis* compared with Weigela (Table 3.3). The lowest nematode population of 607 nematodes/g of leaf were recorded at week 3 while week 5 had a value of 1358, and the highest population of 2068.5/g of leaf was obtained in week 8 (Table 3.3). There were significant differences in the mean nematode populations between week 3, 5 and 8 (P <0.05; Table 3.3). Nematode symptoms were obvious on the leaves directly inoculated with nematodes on both *Anemone hupehensis* and *Weigela florida* (Fig. 3.7, 3.8).

Figure 3.6. Nematode lesion symptoms (arrowed) on leaf of *Anemone hupehensis* during soil inoculation test

Figure 3.7. Picture showing LBN lesion symptoms on direct inoculated leaf of *Weigela florida*

Figure 3.8. LBN lesions on (red-tag) *Anemone hupehensis* leaf inoculated with *A*. *fragariae* 8 weeks after inoculation

3.4.2 Experiment (2). To investigate the relationship between the visual symptoms of nematode infestation on leaves and corresponding nematode population of *Weigela florida* and *Anemone hupehensis* by *Aphelenchoides fragariae*

The results of the mean nematode population obtained per leaf category against the percentage of infested leaf area (PIA) on both plant species indicate that the higher the lesion percentage score, the higher the nematode population (Fig. 3.10 & 3.12).

Anemone hupehensis:

The results of clean leaf 'category 1' with '0'% lesion indicate no nematode, while nematode populations increased in response to increasing lesion severity (Fig. 3.9, 3.10; Table 3.4). The higher the lesion severity symptom found on the *Anemone hupehensis* leaf, the higher the nematode population (Fig. 3.10; Table 3.4).

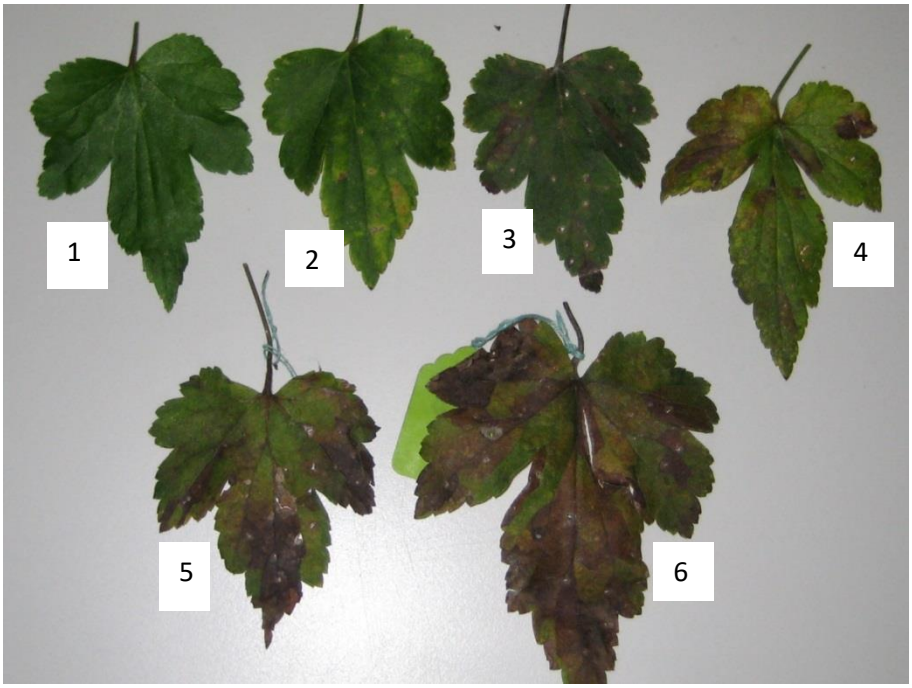

Figure 3.9. *Anemone hupehensis* leaves showing severity of lesion symptoms caused by *Aphelenchoides fragariae* at 3-10 weeks after inoculation with 50, 100 and 200 nematodes in the glasshouse. Ascending degree of lesion from uninfested 'leaf 1' (clean) to 'leaf 6' (highly infested).

Table 3.4. Parameters showing relationship between degree of lesions symptoms caused by *A. fragariae* and nematode population in leaves of *Anemone hupehensis* inoculated with 50, 100 and 200 nematodes/ leaf. Nematode population are mean values per 5g of leaf from 5 replicates. Means that do not share a letter are significantly different (Fisher's multiple range test P <0.05).

Leaf	TLA	NIA	PIA (%)	Lesion%	Mean nema population/5g of leaf	Nema(±SE)
1	412542	332	0.080477	0	0	0
2	378218	21005	5.553675	1-10	867d	±44.37
3	351491	51365	14.61346	10-15	1832cd	±160.99
4	396862	108018	27.21803	25-50	3955bc	±88.37
5	466899	277782	59.49509	50-75	6053b	±638.23
6	818326	689305	84.23355	75-100	10,712a	±789.418

TLA = total leaf area; NIA = nematode infested area; PIA = percentage infested area

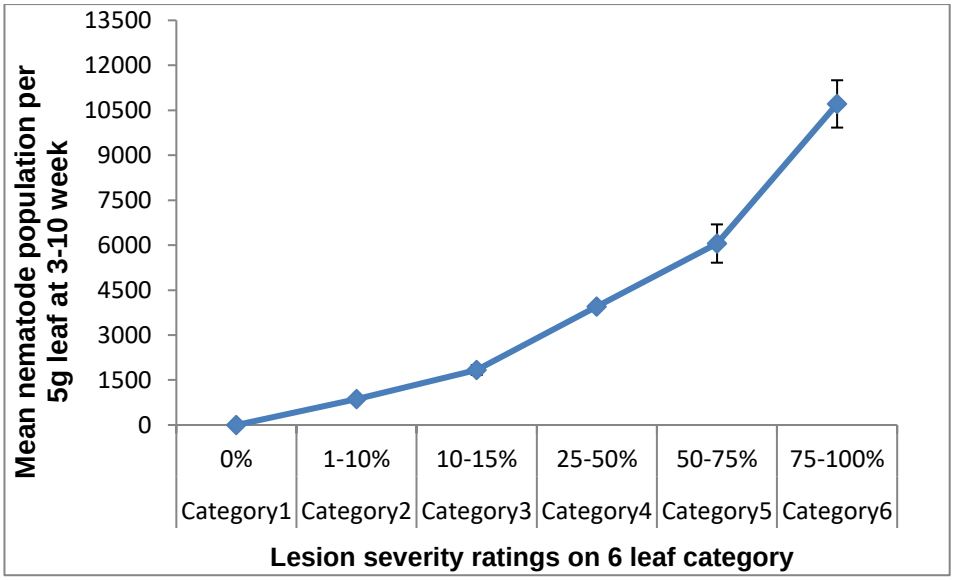

Figure 3.10. Relationship between symptom severity in leaves of *Anemone hupehensis* and mean nematode population. Values are mean of nematode population per 5g of leaf including error bars.

Weigela florida:

Figure 3.11. *Weigela florida* leaves showing severity of lesion infection caused by *Aphelenchoides fragariae* 3 -10 weeks after inoculation. Ascending degree of lesion from uninfested 'leaf 1' (clean) to 'leaf 6' (highly infested).

Table 3.5. Parameters showing relationship between degree of lesions symptoms caused by *A. fragariae* and nematode population in leaves of *Weigela florida* inoculated with 50, 100 and 200 nematodes / leaf. Nematode population are mean values per 5g of leaf from 5 replicates. Means that do not share a letter are significantly different (Fisher's multiple range test P <0.05).

Leaf	TLA	NIA	PIA (%)	Lesion%	Mean nema population/5g of leaf	Nema(±SE)
1	405988	1605	0.395332	0	0	0
2	350015	21121	6.034313	1-10	728d	±37.83
3	434957	52793	12.13752	10-15	1396d	±105.68
4	309870	106264	34.29309	25-50	3548c	±222.47
5	344995	216077	62.63192	50-75	4375b	±255.41
6	269831	250415	92.80438	75-100	8582a	±447.41

TLA = total leaf area; NIA = nematode infested area; PIA = percentage infested area
Nema = nematodes

The results of clean leaf 'category 1' with '0'% lesion indicate no nematode, while nematode population from category 2 increased in response to increasing lesion severity (Fig. 3.11, 3.12; Table 3.5).

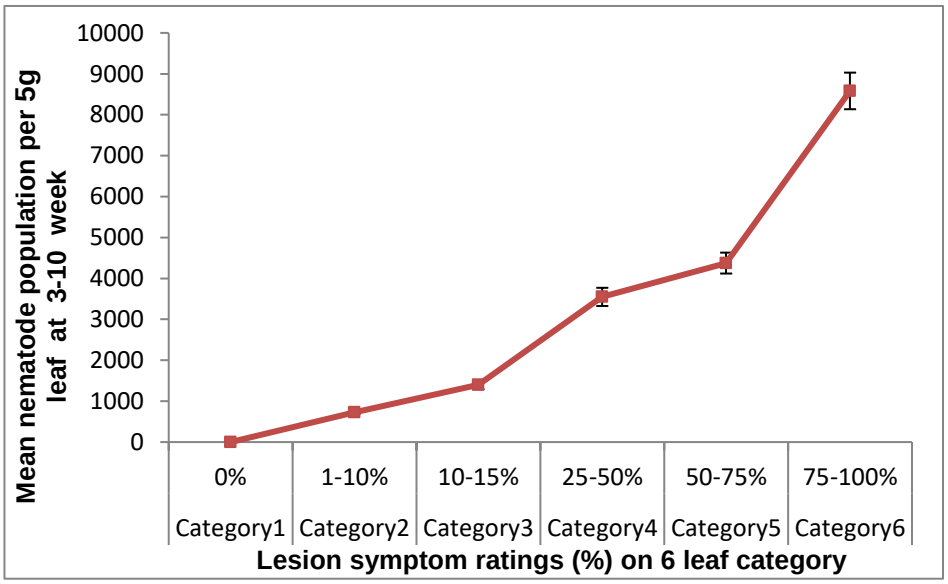

Figure 3.12. Relationship between symptom severity in leaves of *Weigela florida* and mean nematode population per 5g of leaf including error bars.

The higher the lesion severity symptom found on the *Weigela florida* leaf, the higher the nematode population (Fig. 3.12; Table 3.5).

3.5 Discussion

The key outcomes from this study are the identification of suitable methods of LBN inoculation of leaves, a method for infesting plants via nematode infested soil, and the development of a leaf symptom scoring system that can be related to the nematode population within the infested leaf. Results on leaf inoculation and soil inoculation from both plant species demonstrated that characterisation of *A. fragariae* infestation and multiplication is possible through artificial inoculation. In addition, findings from *A. fragariae* used as a model species in this study could be applicable to other LBN such as *A. ritzemabosi,* although subjected to further test. This approach was utilised for subsequent evaluation of *A. fragariae* management treatments (Chapters 4, 6 and 7).

Zhen *et al,* (2012) applied a similar approach for inoculating *A. fragariae* on Hosta plants. The results were similar in LBN multiplication with this study, although the authors used 5,000 as inoculum number while this study used 200 nematodes / leaf. It was suggested that single leaf inoculation is a reliable tool for resistance screening of LBN (*Aphelenchoides* species) on other ornamentals (Zhen *et al.*, 2012). The efficacy of direct nematode inoculation on roots was reported by De-Schutter *et al.* (2001) with suspensions of live *Radopholus similis* inoculated directly on banana roots to detect symptoms of necrosis/lesions on susceptible cultivars 8 weeks after inoculation. De-Schutter *et al.* (2001) observed 6-14 fold population increase of the initial inoculum with direct nematode inoculation on roots, while 7-10 fold population increase was obtained with this study. Both studies demonstrated significant visual (lesions) symptoms, at the same duration of eight weeks after inoculation. Also, a similar approach of single leaf inoculation ensured a successful production of typical symptoms of LBN infestation in leaves during a pathogenicity test of *A. fragariae* on seven cultivars of Hosta plants (Jagdale & Grewal, 2006). De-Schutter *et al.* (2001) concluded that a single inoculation method was effective as a fast resistance screening approach in banana nematodes.

Results from artificial soil inoculation confirm that soil is a route through which LBN can infest plants. The technique of drenching soil with 800 live LBN (*A.*

fragariae) per pot of healthy Hosta plant was used to identify potential products as soil treatment by (An *et al.*, 2017), although the method outlined in this Chapter was developed independently of this. Knowledge regarding the soil route of infestation is important because LBN can migrate up the stem of healthy plants either from soil infested through water splash during irrigation; or from infested leaf debris in the soil to the healthy leaves up the plant stem when wet-weather conditions lead to moisture or thin films of water on the stem (Jagdale & Grewal, 2006; Kohl *et al.*, 2010). The developed test reported in this thesis could also be used to evaluate soil applied management approaches to control LBN infestation resulting from diseased leaf debris in the soil. Although this study investigated using sterile soil artificially inoculated with LBN, in order to optimise control products during soil treatment, growers should avoid the presence of leaf debris which could serve as a potential infestation route. Soil treatments should be done as a preventative approach for younger plants, and may require additional curative treatment during the growing season. This method was successfully applied to evaluate soil treatments of several products against *A. fragariae* (Chapter 7).

Investigation of leaf lesion severity symptoms and the corresponding LBN population in leaves of Weigela and *Anemone hupehensis* demonstrated a correlation between the two variables. Results with both plant species were similar in terms of a corresponding increase in severity of leaf symptoms with LBN population. The lower the percentage symptom severity, the lower was the population of *A. fragariae* in the leaves of both species. There is ongoing work by AHDB Horticulture to develop other quick ways (PCR method and portable hand-held test kit) for early detection of LBN infestation and to quantify LBN infestation in plants.

This relationship can be adopted as a useful management guide for growers to determine the extent of damage caused to the plant, and to help in taking the decision of whether it is worth treating plants to make them viable for sale. This is very useful especially when the infestation level on leaves is getting to ≥15% leaf area damage as described in the guide, then such plants should be discarded immediately (Bennison *et al.*, 2018). Treatments will be most effective at the first signs of symptoms when the plant is actively growing in the field. Importantly, assessment of plant resistance can be enhanced using this symptom key, and in evaluating the effectiveness of control treatments against LBN in the nursery.

Zhen *et al.* (2012) observed a similar relationship between symptom severity and nematode population density of three Hosta cultivars (Aureo marginata, Patriot and Guacamole) and results was more consistent from mechanically injured leaves. The degree of symptom severity increased directly with LBN population, although this varied by cultivar (Zhen *et al.*, 2012), as nematode symptoms may not appear exactly the same on different host plants. Successful LBN penetration, typical lesion production and corresponding nematode population were more successful on mechanically injured leaves during a pathogenicity study on seven Hosta cultivars by Jagdale & Grewal (2006). Although growers strive to maintain good appearance of plants in the nursery, the author's observation during this thesis showed that it is still a challenge for growers to avoid damage to leaves during handling and transportation of plants, especially in a fairly big nursery. Mechanical damage to leaves is an important factor which can aid the spread of LBN in the nursery; hence growers are advised to improve plant handling to avoid damage. Mechanical damage to leaves of Hosta plants was suggested to have possibly increased chances of LBN infestation (Jagdale & Grewal, 2006; Zhen *et al.*, 2012). Plant leaves used for this study were carefully injured with a sharp needle to mimic the natural occurrence in the nurseries. While using needles to cause injury on the leaf in order to mimic mechanical damage of plants is an acceptable method, leaf injury should be minimised during direct inoculation of plants with succulent leaves, especially if using a scalpel. Zhen *et al.* (2012) reported that leaf injury is unavoidable in a commercial setting either during transportation or by insect damage; consequently, leaf injury is still advisable during screening for optimum LBN penetration.

The results from both species in term of corresponding LBN population from the leaf categories 2–6 indicate that nematode populations are likely to increase as the symptom severity increases. For the symptoms to be evident in the leaf, LBN populations must have attained 10 nematodes/g of leaf or more according to Kohl *et al.* (2010) who reported that symptomatic leaves always had a minimum of 10 LBN or more per 1g fresh leaf weight to develop symptoms. Therefore, population increase is likely to occur if plants are not treated upon first detection of symptoms, and this will not be ideal for marketability as no level of infestation is acceptable based on commercial plant aesthetic assessments.

The outcome of this study on the categories of LBN symptoms related to nematode population would be a useful guide for growers to allow them to make a decision on whether further management of infested plants is worthwhile.

Because of the importance of a successful integrated nematode management programme using nematode resistant and tolerant cultivars, a successful screening approach to *Aphelenchoides* or other nematodes species is essential. The techniques developed to reliably inoculate leaves through applying LBN inoculum to the plant media provide a sound method for evaluation of a range of approaches in subsequent Chapters of this thesis.

Chapter 4

Induction of plant defences against leaf and bud nematodes (*Aphelenchoides fragariae*) in *Anemone hupehensis* and *Weigela florida* by the elicitor products Regalia (*Reynoutria sachalinensis*) and ASM (acibenzolar-S-methyl)

4.1 Introduction

LBN, *Aphelenchoides fragariae* (Ritzema-Bos, 1891) (Christie, 1932) (Aphelenchida: Aphelenchidae) are economically important pests in the ornamental industry worldwide causing damage to herbaceous and woody perennials (Winslow, 1960; LaMondia, 1999 ; Jagdale & Grewal, 2006). The number of host plants of *Aphelenchoides* spp. has increased over time compared to other plant parasitic nematode species. They often cause serious economic loss to a broad range of plants (over 1100 species) from 126 botanical families by thirteen plant parasitic species of *Aphelenchoides* (Kohl, 2011; Sánchez-Monge *et al.*, 2015). They are a significant foliar pest of ornamental plants and feed endo-parasitically on the mesophyll and parenchyma leaf tissues causing shaped blotches on the leaves which are delineated by the veins and often accompanied by leaf distortion (Sanwal, 1959; LaMondia, 1999; Jagdale & Grewal, 2002). They and can also can be found ecto-parasitically in the folded crown/runner buds of plant such as strawberries with feeding observed in their folded bud stage (De-Waele, 2002; Kohl *et al.*, 2010). When leaves are infested by *A. fragariae*, they become chlorotic and subsequently turn necrotic; abnormal plant growth with stunting, deformation of buds, leaves flowers are observed. Such plants often become unsaleable and can lead to significant economic loss to growers (LaMondia, 1999; Kohl *et al.*, 2010). Current popular management strategies include among others, avoiding the use of nematode infested materials for planting, sterilisation of equipment and minimal use of overhead irrigation as water splash can aid movement of nematodes from infested leaves to non-infested leaves.

There has been an acceptance that plants can actively defend themselves or have resistance induced against potential pathogens (Pieterse & Van Loon, 2007; Walters & Heil, 2007). Past work has demonstrated that following infection of plants by a

microbial pathogen, an enhanced resistance can thereafter be developed by plants to avert further infection (Kuć, 1982; Hammerschmidt, 2014). Application of certain compounds or plant extracts (inducing agents or elicitors) to plants can trigger resistance to subsequent attack from pathogens both locally and systemically (Walters *et al.*, 2013, 2014). When an inducing agent is applied to a plant, the defence mechanism may be triggered directly or may be triggered only when a pathogen challenge occurs; this plant response is termed induced resistance and can be described as systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Cole, 1999; Walters *et al.*, 2005).

Systemic acquired resistance (SAR) involves the growth restriction of a pathogen and inability of symptoms to develop after a pathogen attack, when compared to plants with no induction but attacked by the same pathogen (Molinari & Baser, 2010; Walters *et al.*, 2014). This action is coordinated by mass coordination of the plant hormone salicylic acid (SA), a plant hormone which plays active roles in plant growth and development (Ryals *et al.*, 1996), at the area of infection. Induction of SAR requires the presence of SA which helps in plant defence against pathogens through the activation of pathogenesis-related (PR) genes (PR-1in particular); which produces PR proteins which are antimicrobial, but attack molecules in the cell walls of fungi and bacteria, and are capable of breaking them down (Cole, 1999; Pieterse & Van Loon, 2007; Spoel & Dong, 2012; Walters *et al.*, 2014). PR proteins may transmit the information regarding pathogen invasion to nearby cells, or upon pathogen/insect attack, local barricades may occur through deposition of lignin between cells thereby slowing the spread of infection to other parts of the plant (Lyon, 2007).

Agents that can mimic the natural inducers of resistance during plant- pathogen interaction, and which have been reported to elicit SAR include acibenzolar-S-methyl - ASM (Leadbeater & Staub, 2014); the non-protein amino acid β-aminobutyric acid (BABA) and cis-jasmone (Jakab *et al.*, 2001; Walters & Heil, 2007).

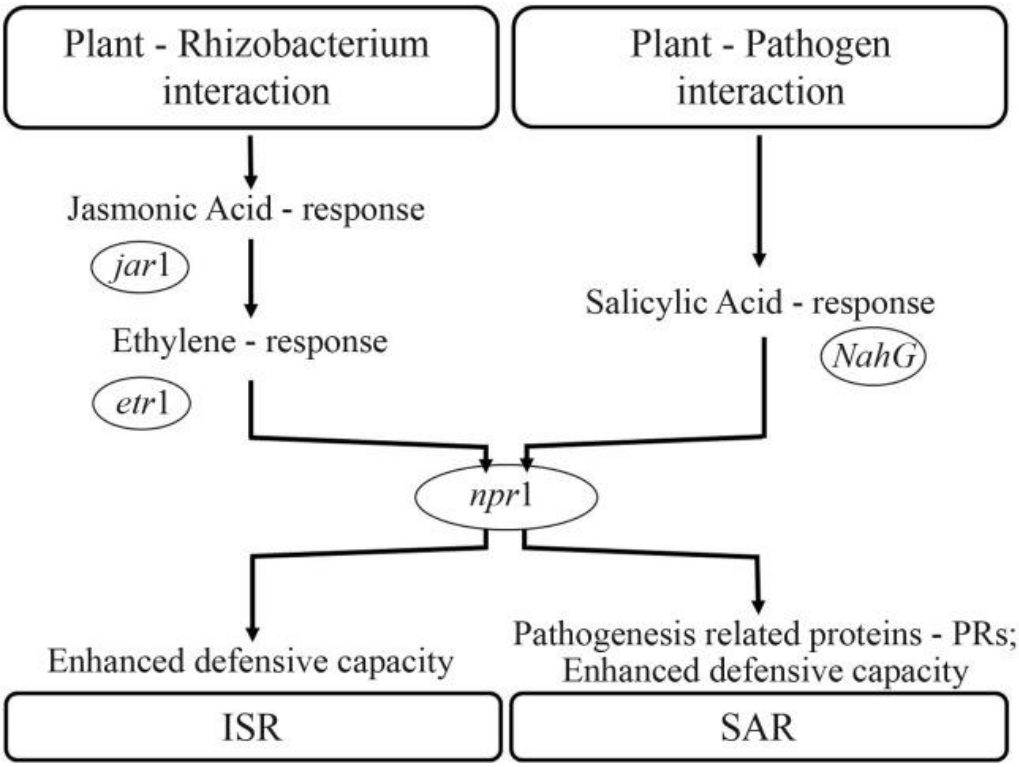

Figure 4.1. Response of induced systemic (signal) pathways caused by non-pathogenic rhizobacteria and pathogenic organisms in plant - Courtesy: (Pieterse & Van Loon, 1999).

Induced systemic resistance (ISR) involves the colonisation of plant roots by particular strains of plant growth-promoting rhizobacteria (PGPR) usually mediated by sensitive pathways of jasmonic acid and ethylene (Fig. 4.1; Van-Loon *et al.*, 1998; Pieterse & Van Loon, 2007). ISR also works in similar way to SAR as they both do not act particularly against pathogens but directly on plant mechanism (Zehnder *et al.*, 2001; Pieterse & Van Loon, 2007). ISR differs from SAR as ISR is reported to be independent of SA, and primarily depending on the production of ethylene and jasmonic acid (Fig. 4.1; Van-Loon *et al.*, 1998; Spoel & Dong, 2012).

The mechanism of systemically induced plant defence which is best understood so far is systemic acquired resistance (SAR), which normally involves a broad-spectrum disease resistance mediated by SA (Kessmann *et al.*, 1994). When SA and compounds that can mimic the action of SA are applied to plants, it will chemically induce resistance to pathogens in such plants (Oostendorp *et al.*, 2001). There are developed chemicals such as polyacrylic acid, barium chloride, 2,6-dichloroisonicotinic acid (INA) and ASM that can induce resistance to various pathogens upon application to plants (Ward *et al.*, 1991; Kessmann *et al.*, 1994;

Malamy *et al.*, 1996). The above chemicals are not directly antimicrobial (Cole, 1999). Walters & Fountaine, (2009) outlined several products that have been developed after Probenazole; the first chemical resistance activator (elicitor) was developed in Japan. Developed chemical and microbial activators include ASM registered in Europe as Bion®, (later re-registered as Inssimo) and Actigard by Syngenta, Milsana® (extract of *Reynoutria sachalinensis* - KHH BioScience Inc., USA), Elexa (Chitosan SafeScience, USA), and Messenger (harpin protein, Eden Bioscience, USA) (Walters & Fountaine, 2009). In addition, it is important to note that SAR could be more active on restricting disease if it is activated before the arrival of the pathogen as previously demonstrated with various micro-organisms (Keller *et al.*, 1996; Ryals *et al.*, 1996).

A major advantage of synthetic elicitors apart from being environmentally friendly are their non-antimicrobial activity, which means that systemic resistance is a direct activation of plant defences which can help to avoid developing disease resistance by pathogens unlike traditional pesticides (Vallad & Goodman, 2004). Furthermore, a response related to a plants ability to recall previous infection, root colonisation and treatment of infection by chemicals is referred to as 'priming', and it usually results in a rapid and effective response during a subsequent pathogen attack on the plant (Goellner & Conrath, 2007). ASM has been reported to induce resistance to pathogens when applied to various plants (Kessmann *et al.*, 1994). Use of ASM on tobacco led to a high level of disease control of *Pseudomonas syringae*, *Cercospora nicotianae* and *Alternaria alternata* by 99, 91 and 89% respectively (Cole, 1999; Perez *et al.*, 2003).

Other elicitors worth mentioning include Chitosan/Elexa which is a common polymer in the shells of crustaceans, cell walls of fungi and exoskeleton of insects (Obanor *et al.*, 2013). The main active compound is chitosan, and has been reported to reduce incidence of grape downy mildew (GDM) on grapevine leaves between 56% and 95%, when combined with laminarin and low copper, with multiple foliar applications in a 2-year trial compared with the untreated control (Romanazzi *et al.*, 2016).

There are several microbial protein based products such as Harpin obtained from *Erwinia amylovora* with products such as Messenger which activates SAR in the

host plant against attack from pathogens (Chang & Nick, 2012). There was successful control with Harpin of blue mould on apples (Capdeville *et al.*, 2008). Foliar applications of harpin and ASM at 4x rates both reduced the numbers of lesion nematodes (*Pratylenchus* spp.) by potato harvest, while high dose rates of Harpin (4x) and ASM both reduced nematode (*Meloidogyne incognita)* infection index compared with the untreated Control (Collins *et al.*, 2006). Navarro-Acevedo (2016) reported that the root exudates from Harpin (foliarly) treated soybean plants reduced hatching rates of the eggs of soybean cyst nematode (*Heterodera glycines*) in greenhouse experiments compared to the root exudates of the untreated Control. Other elicitors include those derived from plants such as *Reynoutria sachalinensis*, also known as Reysa, an extract of giant knotweed (registered as Milsana® in the USA and Regalia® in Europe). Reysa is marketed as a plant defence activator for glasshouse ornamental and vegetable crops, and has recorded success as a fungal control product on crops such as strawberry (Carlen *et al.*, 2004) and cucumber (Daayf *et al.*, 1997; Fofana *et al.*, 2002). Schmitt (2002) reported control of powdery mildew and bunch rot of grape berries equivalent to a commercial fungicide when applied at 7-10 day intervals. Reysa has been shown to increase the amount of phenolic substances which act as phytoalexins capable of preventing the attack of powdery mildew in ornamental plants, vegetable and fruit crops (Copping & Duke, 2007). There are however, some reports where induced resistance agents gave no significant disease control on crops (Walters *et al.*, 2005). An example of such work is the study by Zhang *et al.*, (2001). ASM was used to control the infection of *Cercosporidium personatum* (leaf spot) pathogen on peanut. The elicitor increased the disease levels on treated plants by 52% compared to the untreated plants. It has been suggested that under field conditions, expression of induced resistance by an elicitor could be influenced by the environment, genotype and crop nutrition level; consequently a better understanding of these interactions with the elicitor is needed to maximise the efficacy of induced resistance (Walters & Heil, 2007).

The choice of ASM for this study was based on its previous reports under glasshouse and field conditions; and has reduced pathogen infections on various crops such as barley, tobacco, tomato, Arabidopsis. This product is available as plant defence activator in Europe, and registered as Inssimo. Also, Reysa is a plant extract product marketed as plant defence activator for glasshouse ornamental and vegetable crops. It

has been successfully used for fungal control on crops such as cucumber and strawberry. The elicitor choice was supported by project supervisors, industry representatives and the project sponsor (AHDB).

Although there are no previous reports on the use of inducing agents (elicitors) to manage *Aphelenchoides* spp. on ornamental plants, both products have been reported to reduce disease levels on various crops, hence the justification for their use in this study (Cole, 1999; Molinari & Baser, 2010; Walters *et al.*, 2014. There is need to reduce reliance on traditional pesticides which often results in resistance development overtime by pathogens. The fact that elicitors are not directly antimicrobial, but can induce resistance via activating plant defence mechanisms, can lead to the risk of fungicide development resistance by pathogens to be reduced or eliminated (Cole, 1999; Vallad & Goodman, 2004). Moreover, reports on the successful management of various pathogens including root knot nematodes by inducing agents, either as stand alone or in combination with other pesticides motivated an investigation of elicitors to manage *A. fragariae* on ornamental plants. This current study has evaluated the application of products that act as elicitors of plant defences to determine whether they can confer a level of resistance to LBN in ornamental plants.

This Chapter reports on results obtained from the foliar application of two elicitor products, Reysa (*Reynoutria sachalinensis)* and ASM (acibenzolar-S-methyl) on two ornamental plants – *Weigela florida* and *Anemone hupehensis*, after inoculation with *A. fragariae*.

In addition, a further study comparing single and multiple treatments of ASM to *Anemone hupehensis* inoculated with *A. fragariae* was undertaken.

4.2 Experiment (1). Induction of plant defences against LBN (*Aphelenchoides fragariae*) in *Anemone hupehensis* and *Weigela florida* by the elicitor products Reysa (*Reynoutria sachalinensis*) and ASM (acibenzolar-S-methyl)

4.2.1 Materials and methods

4.2.2 Materials

Nematodes: The nematode species (*A. fragariae*) used in this study was from the same source and extracted as described in Chapter 2 - Section 2.2.2.

Elicitor: ASM and Reysa were supplied by Syngenta Agrochemical Company, UK.

Plants: Certified nematode-free *Anemone hupehensis* var. japonica 'Prinz Heinrich' and *Weigela florida* var. 'Bristol Ruby' (henceforth referred to as *Anemone hupehensis* and *Weigela florida* respectively) were obtained from a commercial nursery, Jackdaws' Field Nursery, Horsham, West Sussex UK. The test was carried out using 2 litre pots containing *Weigela florida* and *Anemone hupehensis* plants.

4.2.3 Method

The three treatments used were: (i) ASM + inoculated nematodes (ii) Reysa + inoculated nematodes, and (iii) Inoculated nematodes only (Control). All treatments were arranged in a randomised design with five replicates per treatment. Plants were grown in 2 litre pots containing sterilised compost (Fisons Levington, UK) until they had a minimum of 6-8 leaves, and maintained in a glasshouse at conditions of 25° ± 2°C. Leaf samples were taken from plants at random for extraction to confirm that they were nematode-free. Plants were kept in a glasshouse and isolated from other plants. Fifteen plants were used for each species.

The two elicitor products were separately dissolved in litre $^{-1}$ of water based on the manufacturer's instructions and applied 3 days prior to nematode inoculation of the plants. ASM was applied at 0.175g litre $^{-1}$ of water (equivalent to 35g/ha/200L water), while Reysa was applied at 5ml / litre $^{-1}$ of water (equivalent to 2.5l/ha/300L water). An adjuvant (Tween 20) was added at the rate of 100µl per litre $^{-1}$ of water (0.01%) to all treatments including the water Control. A hand-held pressurised sprayer supplied by Scientific Laboratory Supplies (SLS) UK, was used to spray products as a foliar application on each plant until all parts of the plant was well covered (run off). Control treatments had only water + Tween 20 sprayed on the plants.

Plants were left for 72h before three randomly selected leaves per plant were inoculated with nematodes using methods described by Jagdale & Grewal, (2006); Zhen *et al.*, (2012), and as previously described in Chapter 3, Section 3.1.3.2.

200 live mixed stage nematodes in an aliquot suspension of 3ml were inoculated per leaf. Plants were randomised and left in glasshouse conditions of 25° ± 2°C for 8 weeks. Leaf sampling (from the inoculated leaves) were used for extraction to assess nematode infestation at 3, 5 and 8 weeks after inoculation using the extraction method outlined in Chapter 2 - Section 2.2.1.1. Nematode numbers were expressed as numbers per 1g of leaf.

4.3 Experiment (2). Evaluation of the elicitor ASM with different treatment programmes to manage LBN (*A. fragariae*) on *Anemone hupehensis*

4.3.1 Materials and methods

4.3.2 Materials

Plants (*Anemone hupehensis*) and nematodes (*A. fragariae*) were from the same source as described in the above experiment (Section 4.2.1).

4.3.3 Method

This study consisted of 5 treatments, with each treatment programme replicated 5 times. Plants were arranged in a randomised design. The main factor considered in this study was nematode multiplication despite the treatment by elicitor-ASM.

Treatments details were;

Treatment 1 = ASM + nematode at week 1 (x1 application)

Treatment 2 = ASM + nematode at week 1 & 3 (x2 applications)

Treatment 3 = ASM + nematode at week 1 & 5 (x2 applications)

Treatment 4 = ASM + nematode at week 1, 3 & 5 (x3 applications)

Treatment 5 = Nematode only – 'Control'

All treatments had Tween 20 added at a dose of 100µl / litre $^{-1}$ of water. All the treatments were applied as a foliar spray on plants until total cover of all plants surfaces (run-off). Three days after the 'Treatments 1' were applied (week 1), 200 mixed stages of live nematodes were directly inoculated on three randomly selected leaves per plant (using the method described in Chapter 3, Section 3.1.3.2).

Leaf sampling for nematodes from the inoculated leaves was undertaken at 3, 5 and 8 weeks after inoculation. Nematode numbers were expressed as numbers per 1g of leaf. Plant maintenance in the glasshouse and sampling of leaves to assess nematode multiplication were the same as described in Chapter 2 - Section 2.1.1.

Data analysis: Analysis of data collected was carried out as described by An *et al.*, (2017). Data from the mean nematode population from this study were subjected to analysis of variance using One-way ANOVA at each observation. Nematode number data at 3, 5 and 8 weeks were analysed using repeated measures ANOVA with treatments (nematode, elicitors and time) as subject factors. Significant differences between treatments were determined with Tukey's mean comparison at P <0.05. All the data analyses were performed in Minitab (Vs.17). Other parameters considered include the percentage of nematode increase which is calculated as:

$$Percentage\ nematode\ increase = \left(\frac{Mean\ final\ population}{number\ of\ initial\ inoculum} \times 100\right)$$

4.4 Results

4.4.1 Experiment (1). Induction of plant defences against LBN (*Aphelenchoides fragariae*) in *Anemone hupehensis* and *Weigela florida* by the elicitor products Reysa (*Reynoutria sachalinensis*) and 'ASM' acibenzolar-S-methyl

The results obtained from the untreated control (N) in *Anemone hupehensis* demonstrated the highest mean nematode number of 2523.8 per 1g of leaf during the study at week 8 (Table 4.1). Generally, the control treatment had more nematode multiplication than the other 2 treatments (Table 4.1). Treatment with Reysa led to the highest mean nematode number (1224.5) obtained at week 3. However nematode population was later reduced at week 5 to 334.2, with an increase to 876.1 at week 8. ASM had a gradual but low decrease in mean nematode number when compared with the other 2 treatments on *Anemone hupehensis* plants (Table 4.1). The lowest nematode reproduction (109.5) of ASM and in the study was observed at week 8.

The results obtained from untreated control (N) in *Weigela florida* demonstrated a gradual and steady nematode increase from week 3 to week 8 (Table 4.1). The highest nematode multiplication was obtained from control during the study with

mean nematode number of 1781.1. Generally, control had more nematode multiplication than the other 2 treatments (Table 4.1).

Treatment with Reysa had the lowest nematode reproduction at week 3 (198) but increased to the highest mean nematode number obtained during week 5 (1268.6), and later reduced to 862.2 at week 8. ASM had a gradual increase in nematode reproduction from week 3 to week 5 (416.1 to 692.5) and declined at week 8 to 477.7 (Table 4.1).

Overall, treatment with ASM demonstrated the lowest nematode reproduction in the study (Table 4.1).

Table 4.1. Test of Reysa and ASM on the reproduction of *A. fragariae* in a glasshouse when inoculated with 200 nematodes / leaf on *Weigela florida* and *Anemone hupehensis*. Data are percentage (+SE) mean values of nematode reproduction of five replicates when inoculated with 200 nematodes / leaf (N = nematodes). Values with same letter are not significantly different (Tukey's multiple range test, P <0.05).

		Mean nematode reproduction at 3, 5 & 8 week after inoculation	
Duration	Treatment	Weigela	Anemone
3 week	Reysa +N	198g (±16.43)	1224.5bc (±66.3)
	ASM +N	416.1fg (±40.43)	438d (±30.14)
	N	584.1ef (±41.51)	1112.2c (±51.9)
5 week	Reysa +N	1268.6b (±94.73)	334.2d (±34.71)
	ASM +N	692.5de (±51.1)	219d (±15.07)
	N	994.1c (±60.3)	1638.7b (±59.9)
8 week	Reysa +N	862.2cd (±31.5)	876.1c (±60.29)
	ASM +N	477.7ef (±41.22)	109.5d (±7.53)
	N	1781.1a (±49.08)	2523.8a (±93.9)

4.4.2 Experiment (2). Evaluation of the elicitor ASM with different treatment programmes to manage LBN (*A. fragariae*) on *Anemone hupehensis*

Values obtained as mean nematode population in Figure 4.2 indicate that 3x application doses of ASM significantly reduced (P <0.05) nematode populations in all the treatments when compared with the untreated Control 't5' (Fig. 4.2).

Nematode multiplication during week 3 from all the treatments (excluding the untreated Control) indicates that nematode reproduction was similar with no significant differences between any of the treatments (Fig. 4.3). During week 5, treatments t2 (ASM + nematode at weeks 1 & 3) and t4 (ASM + nematode at weeks 1, 3 & 5) had lower nematode populations than the other treatments. A significant difference (P <0.05) between treatments was only obtained with the t4 programme (ASM applied at week 1, 3 and 5) against the other treatments (Fig. 4.3).

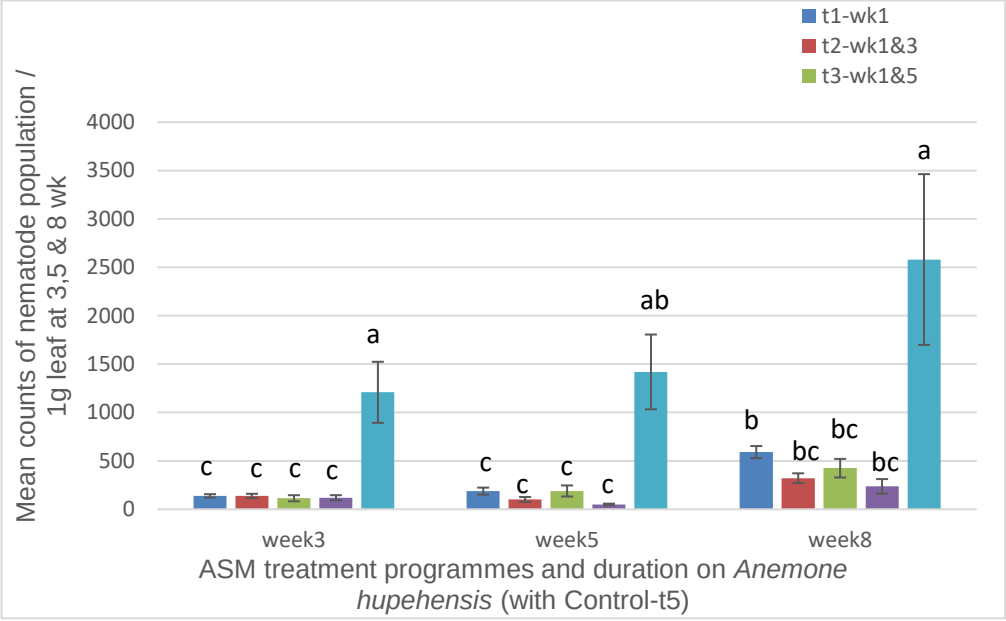

Figure 4.2. Mean counts of *A. fragariae* after an initial inoculation of 200 nematodes per leaf, with differing ASM application programmes: (t1 = ASM + nematode at wk 1; t2 = ASM +nematode at wk 1 & 3; t3 = ASM +nematode at wk 1 & 5; t4 = ASM +nematode at wk 1, 3 & 5; t5 = Nematode only). Each bar represents a mean (±SE) of five replicates per treatment. Bars with same letter are not significantly different (Tukey's multiple range test, P <0.05).

The t1 (ASM application at week 1) was the least effective treatment and demonstrated the highest nematode population at week 8 compared to the other three

ASM treatment programmes (Fig. 4.3). In general, all treatments had significantly lower nematode multiplication than the untreated Control (Fig. 4.2).

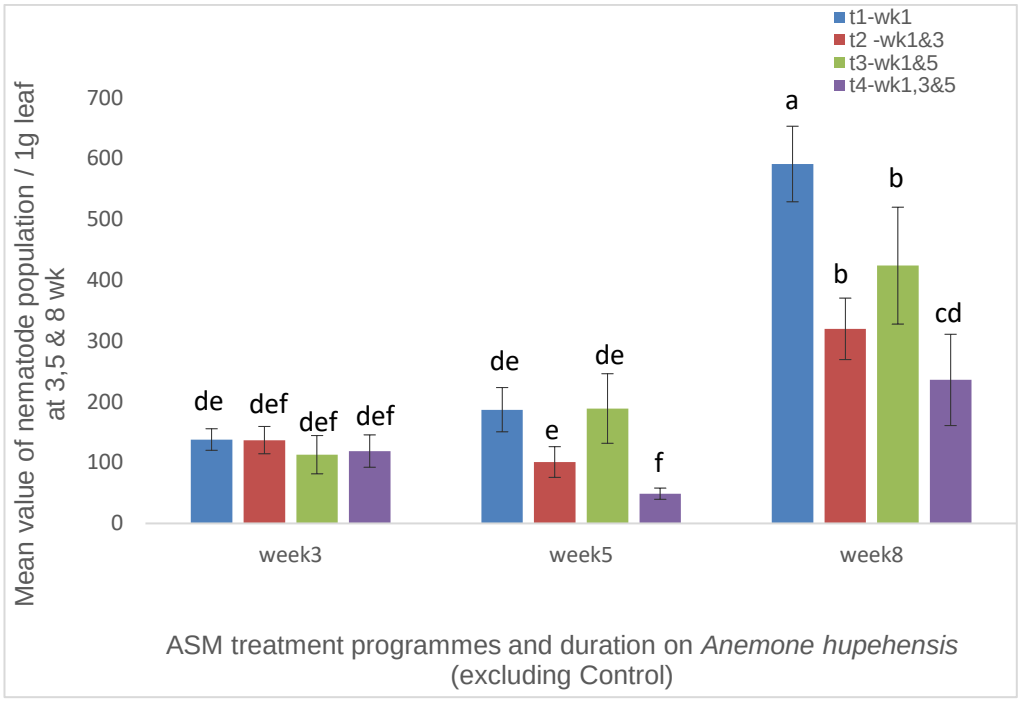

Figure 4.3. Mean counts of *Aphelenchoides fragariae* between treatments, after an initial inoculation of 200 nematodes / leaf at 3, 5 & 8 weeks with differing ASM programmes: (t1 = ASM +nematode at week 1; t2 = ASM +nematode at week 1 & 3; t3 = ASM +nematode at week 1 & 5; t4 = ASM +nematode at week 1, 3 & 5). Each bar represents a mean (±SE) of five replicates per treatment. Bars with same letter are not significantly different (Tukey's multiple range test, P <0.05).

4.5 Discussion

The glasshouse results on the two elicitors gave an indication of their nematicidal potential, and as promising products to manage *A. fragariae* on ornamental plants. Despite the inconsistent performance by Reysa as against what was observed in ASM, both elicitors led to lower nematode population increase by reducing nematode multiplication compared to the untreated control on both plant species. ASM gave better reduction of nematode population in plants when compared with untreated Control than Reysa.

Reysa, an ethanoic extract from giant knotweed registered in the USA as Milsana and registered in Europe as Regalia has demonstrated control of fungal pathogens on crops such as cucumber (Daayf *et al.*, 1997; Fofana *et al.*, 2002), strawberry (Carlen

et al., 2004) and organic tomato crops (Dafermos *et al.*, 2012). Application of Milsana at 7-10 day intervals gave a similar control of powdery mildew to those obtained when a commercial fungicide was used on tomato plants (Schmitt, 2002). Milsana also demonstrated control of powdery mildew on grapes under field conditions by inducing phytoalexins which convey resistance by plants towards the pathogen (Konstantinidou-Doltsinis *et al.*, 2007). Milsana therefore helps the plants to resist pathogen infection rather than act directly on the pathogen. Reysa exhibited potential as an induced defence agent against nematodes by reducing *A. fragariae* multiplication compared with the untreated control in both plant species during this study.

Likewise, ASM demonstrated a reduction of nematode multiplication on both plant species in this study. Past molecular work on the mechanism of ASM in plant defences on tobacco and *Arabidopsis* showed that ASM activates the systemic acquired resistance (SAR) pathway through mimicking the activity of salicylic acid (SA) (Lawton *et al.*, 1996; Friedrich *et al.*, 1996).

McGrann *et al.* (2017) demonstrated effective control of light leaf spot (LLS), caused by *Pyrenopeziza brassicae,* on winter oilseed rape by foliar application (3 spray programmes) of ASM (Bion-0.175g litre $^{-1}$) at the same rate as was used in this study. It was also reported that ASM lowered light leaf spot (LLS) infection on inoculated Brussel sprouts during field trials. Reysa –(5%) as used in this study was not as effective as ASM against LLS of Brussel sprouts except when it was combined with other elicitors. McGrann *et al*. (2017) concluded that levels of LLS on early maturing Brussel sprout were consistently reduced when ASM was used alone as a single product compared to other elicitors such as Reysa-5%, Softguard® (chitinosan 2.6%), SiTKO-SA (Salicylic acid-4% and Silica-5%) applied singly.

Application of the elicitor alone will not ensure complete eradication of pathogens (Walters *et al.*, 2005), which is also the case with chemical pesticides. Low control of powdery mildew and *Rhynchosporium commune* was witnessed on two barley cultivars (Optic and Cellar) after a field experimental treatment by ASM (Walters *et al.*, 2013). However, during the 3 years of experimental field trials, the ASM plus fungicides combination gave the most consistent disease control (Walters *et al.*, 2013). Similar reports from Ivors & Louws, (2007); Ivors & Meadows, (2016)

recommended combinations of ASM with fungicides and bactericides during tomato spray programs for increased plant resistance and reduction of early blight (*Alternaria solani*) inoculum levels in North Carolina, USA. The authors suggested that the use of elicitor + pesticide in combination could be a valuable tool in reducing the total quantity of pesticide used, and delay pesticide-resistance development thereby resulting in increased long term efficacy of pesticides (Ivors & Louws, 2007). Considering the findings from above authors, and the results from this study, the author suggested that optimum management can only be attained by a combination of treatments in an IPM approach suitable for practical application for the growers.

In this regard, this thesis in subsequent Chapters examined along with ASM treatment programmes, the combination of elicitor with fungicides to improve the efficacy of ASM in controlling nematode infestation. This current study did not repeat microscopic work on infestation levels on leaves since previous work (described in Chapter 3) has demonstrated a positive correlation between nematode populations and infestation levels on both plant species (*Weigela florida Anemone hupehensis)* used in this study. Therefore this investigation focussed on visual symptoms and nematode population on the two plant species in order to evaluate the efficacy of these elicitors.

A programme of 3x applications of ASM at 2-week intervals proved to be the most effective programme in all the treatments investigated for reducing *A. fragariae* reproduction. It had previously been suggested that application of elicitors earlier in the season before disease onset might reduce disease inoculum levels, and may lead to reduced use of fungicide (Walters *et al.*, 2013). However, this suggestion did not work in favour of spring barley treated by elicitors (Walters *et al.*, 2014). Here, the authors recommended that protection of later stages of crop growth is very important to maintain/sustain the grain yield. This study supports the requirement for an application programme rather than a single dose treatment of ASM to manage *A. fragariae* on ornamentals. Tween 20 was added to all the treatments in this study including untreated control (as an adjuvant to facilitate spray coverage), however, Tween 20 had been previously investigated for nematode control (Katiki *et al.*,

2011). Future work should therefore consider the use of Tween 20 as a separate treatment in order to investigate its potential on *A. fragariae* or other LBN.

Results in this study have demonstrated potential management of *A. fragariae* on ornamental plants by ASM with the 3x foliar application programme proving more effective than either a single or 2x application programme. This study is in agreement with other reports of successful control of various diseases on crops by ASM including root-knot nematodes, and hereby suggests a potential approach for LBN management on ornamental plants.

One of the major advantages associated with these synthetic elicitors include the absence of any direct antimicrobial activity when compared with normal traditional pesticides; a factor which could assist the avoidance to developing resistance by pathogens (Cole, 1999; Vallad & Goodman, 2004). The use of elicitors seems to be environmentally friendly compared with current pesticides (Vallad & Goodman, 2004). There were no observed differences in the growth of both plant species treated with an inducing agent and the untreated control plants during this study.

The successful management of *A. fragariae* by ASM in this study which demonstrated a significant reduction of nematode population compared with untreated Control signifies its potential use against *A. fragariae* infestation.

In summary, despite the significant reduction of LBN populations by the ASM compared with untreated control in this study, the author suggests that ASM should be applied as a preventative measure before the onset of LBN symptoms (priming), rather than as a control treatment. However, in the presence of LBN mild symptoms, when the infestation level on leaves is below 15% leaf area damage as described in the guide (Chapter 3), ASM should be combined with other treatments in an IPM approach most suitable for practical application to manage the pest. Optimum LBN management is achieved by a combination of treatments.

The outcome of this study led to a further subsequent use of ASM in field trials with ASM alone and in combination with other pesticides previously identified from the contact mortality bioassays of Chapter 2.

Chapter 5

Curative efficacy of novel products to manage leaf and bud nematodes (*Aphelenchoides fragariae*) on naturally infested ornamental plants in two commercial nurseries in the UK

5.1 Introduction

Aphelenchoides spp., commonly called leaf and bud nematodes, are non-segmented organisms. They are large, compared to other plant parasitic species, being 0.5 to 1.2mm in length. LBN are important pest known to cause economic damage in the ornamental industry on a range of woody perennials and nursery grown herbaceous plants (LaMondia, 1999; Jagdale & Grewal, 2006). The range of symptoms exhibited by LBN varies considerably on flowering ornamentals depending on host plant. The action of nematode feeding on host plant may cause large sections of the leaf to become chlorotic, turning to necrotic lesions usually surrounded by large veins (Kohl, 2011). LBN may cause lesions in crops such as Hosta and Salvia, or bronzing and discoloration in crops such as begonia, buddleja, weigela and anemone (Jagdale & Grewal, 2002). See Figure 5.1.

Aphelenchoides fragariae, which is the most common LBN species, can attack at least 621 plant species and varieties (from 287 genera) of which about 84% of them are flowering ornamentals plants (Kohl, 2008; Sánchez-Monge *et al.*, 2015).

LBN populations can increase rapidly, due to the medium of dispersal in detached or infested dried leaves, thereby resulting in widespread infestation within a nursery (LaMondia, 1999). Spread to new leaves by LBN within the same plant, and from plant to plant, is aided primarily by swimming up stems or along the surface of wet plant parts through water films (Lambert & Bekal, 2002; Kohl *et al.*, 2010).

They prefer moist conditions and moderate temperatures. *Aphelenchoides fragariae* enter the plant either through the stomata or by direct penetration and reproduce within the leaves. Once inside new leaves, reproduction occurs rapidly as they feed on mesophyll cells (De-Waele, 2002; Jagdale & Grewal, 2002). *Aphelenchoides fragariae* can also exhibit ectoparasitic lifestyle. This means it is often found in the

folded crown and runner buds of plants such as strawberries, while feeding occurs in the folded bud stage (De-Waele, 2002).

(Hosta '*Abba Dabba Do*')

(*Weigela florida* var. 'Bristol Ruby')

(Dryopteris affinis)

(*Buddleja davidii)*

(*Heuchera macrorhiza*)

Figure 5.1 Foliar symptoms of ornamental plants infested by *Aphelenchoides* spp.

Because *A. fragariae* are able to be dispersed in detached or infested dried leaves, infestation on new plants can rapidly become widespread (Kohl *et al.*, 2010). LaMondia, (1999) suggested that propagation often carried out on perennial flowering ornamental plants aimed at achieving true cultivar can also increase spread of LBN.

In general, the most likely but undiagnosed source of infestation is through infested but asymptomatic plant material (Kohl *et al.*, 2010). *A. fragariae* not only destroys the aesthetic value of flowering plants, but also results in a financial loss to growers; therefore infestation should be strictly monitored. Once established, the control of LBN is challenging and difficult due to its transmission medium and ability to

survive for several years in infested dried leaf material. Nematode symptoms can be often misdiagnosed (as previously mentioned) due to possible additional infection by other pathogens such as *Botrytis cinerea* (LaMondia, 1999).

As highlighted in Chapter 1- Section 1.6 of this thesis, there are still challenges in most nurseries visited during this project regarding adequate spacing between plants. This can encourage easy nematode spread via canopy touch or water splash during overhead irrigation already in place in most nurseries. Therefore, good sanitation practice and irrigation management may help to slow the spread of nematodes, since there are no currently registered chemical nematicides for LBN in the UK. Oxamyl previously had an EAMU for outdoor ornamental plant production until the end of 2017, targeting insect pests and stem and bulb nematode. The lack of effective management options for LBN poses a significant challenge among many ornamental growers in the UK and other countries.

Preliminary research has indicated that some insecticides including abamectin have some nematicidal activity and may be useful for management of *A. fragariae* (LaMondia, 1996). In addition, Young & Maher (2000) reported the activity of abamectin against LBN (*Aphelenchoides ritzemabosi*) *in vitro* and *in vivo*. Abamectin is approved in the UK for the control of two-spotted spider mite and western flower thrips in protected and outdoor flower crops and other ornamentals.

Spirotetramat is a systemic insecticide with a two-way mode of action that controls sucking pests such as aphids and whitefly, and has an EAMU for use as an insecticide in ornamental plant production against insect pests. There is no literature on the potential of spirotetramat to control LBN, whereas abamectin has demonstrated some potential as reported above.

Within this research study, alternatives to synthetic chemical nematicides were considered, which could be environmentally friendly and acceptable for use against *A. fragariae* in particular and LBN in general. Compounds which induce plant defense mechanisms - systemic acquired resistance (SAR) were considered. Resistance to pathogens can be chemically induced in plants (Ward *et al.*, 1991; Kessmann *et al.*, 1994) by applying to foliage compounds which can mimic the action of salicylic acid (SA), such as acibenzolar-S-methyl ‘ASM’ (Malamy *et al.*, 1996). ASM has been shown to aid in the control of crop diseases caused by fungi,

bacteria and viruses in field trials (Cole, 1999; Vallad & Goodman, 2004; Walters *et al.,* 2014). ASM was the first synthetic chemical elicitor developed as a SAR activator and is marketed in Europe as Bion® and Inssimo®, and in the USA as Actigard® (Walters *et al.*, 2005). Although resistance induced by these agents is broad spectrum and long lasting, it rarely provides complete control of infection (Walters *et al.*, 2005). Ivors & Louws (2007) suggest a combination of elicitor and fungicides or other pesticides for the effective control of pest and diseases on crops. The rationale here was that the elicitor would increase plant resistance, while the fungicides and bactericides could reduce inoculum levels of pathogen. Walters *et al.*, (2014) suggested that the use of the elicitor in combination with fungicides, even at half rate, can provide levels of disease control and yield increases that are as good as fungicide treatment.

Over 3 years of field trials, the elicitor plus fungicide combinations provided the most consistent control of *Rhynchosporium commune* on spring barley (Walters *et al.,* 2014). This prompted the use of ASM in this study as individual treatments and in combination with other insecticides for LBN management. Major advantages in using these synthetic elicitors is the lack of any direct antimicrobial activity, which is common of traditional pesticides, hence could avoid direct selective pressures on pathogen populations, and, further, they appear to be environmentally friendly compared to current pesticides (Vallad & Goodman, 2004).

In order to improve the efficacy of contact products on *A. fragariae*, a pilot study was earlier conducted to investigate the presence of nematodes at the leaf surfaces, via water moisture as provided by dew, rainfall, and irrigation. Nematodes, if found on the leaf surface, could increase the efficacy of contact product such as abamectin on LBN. Infested plants of *Weigela Florida* and ferns (*Woodwardia fimbriata*) were placed outside overnight to attract nematodes to the leaf surface by either via dew or overnight rainfall. Early following morning (≈6am), four leaves were randomly collected per plant variety, and surface rinsed in glass beaker of 100ml distil water. The supernatant solution was observed under a light microscope to check for the presence of nematodes. The results indicated the presence of *A. fragariae* ranging between 250-380 nematodes from four leaves/plant variety. Presence of nematodes on the leaf surface was in support of Jagdale & Grewal, (2006) who observed *A.*

fragariae nematodes on the leaf surface of infested Hosta plants in the growth chambers at the relative humidity of 90% and 100%. The above investigation confirmed that nematodes can be found ectoparasitically on the leaf surface, and are attracted to leaf surface by the presence of water moisture. Results of this pilot study and other previous reports on the potential of abamectin as a contact product against LBN led to my adoption of leaf wetness prior to treatment application on plants in two commercial nurseries.

Preliminary laboratory and glasshouse bioassays study (Chapter 2 & 4) of this thesis has indicated some potential products including inducing agents, for the management of *A. fragariae*. These products have showed potential to reduce multiplication of *A. fragariae* either through contact / systemic action or induction of plant self defences. Such products have been investigated as a curative approach in this study on naturally infested plants under commercial conditions.

In the search for alternatives to oxamyl, two field trials in commercial nurseries were conducted to investigate the efficacy of novel products that could prevent spread of nematodes, and limit nematode multiplication, consequently preventing an increase in symptoms or infestation on already infested plants in both outdoor and controlled environments. Nurseries were picked based on disease pressure as reported by the growers, although the author could not replicate conditions in nursery to nursery.

The objectives of this field study were to determine the curative efficacy of selected foliar insecticides and a synthetic chemical elicitor, as individual treatments, and in combination for post-infestation management of *A. fragariae* on naturally infested ornamental plants.

5.2. Field study 1 – (Commercial nursery 1 in Oxfordshire, UK)

5.2.1 Materials and methods

5.2.2 Materials

Plants: Seven naturally infested plants species - *Astrantia major*, *Gunnera mannicata*, *Bergenia cordifolia*, *Brunnera macrophylla*, *Astilboides tabularis*, *Dryopteris filix-mas* and *Anemone hupehensis* were isolated at a commercial ornamental nursery in Oxfordshire, United Kingdom.

Pesticides: Products used were Spirotetramat - 1.67ml/ litre^{-1} - a systemic insecticide; Abamectin - 18g litre^{-1} – a contact and translaminar insecticide; and ASM 500g Kg−1 (acibenzolar-S-methyl) - an elicitor of induced resistance in plants, 'Tween 20' (0.01%) - an adjuvant commonly used to enhance spread of product and facilitate action of the principal product was used in all treatments as well as the water Control treatment. The above were used as individual treatments and also in combination with ASM.

5.2.3 Methods

The field trial was conducted to evaluate the efficacy of different products on LBN infested plants as individual treatments and in combination with ASM. These infested plants were isolated based on visual symptoms of nematode infestation shown on the leaves. The symptoms observed on the leaves included angular leaf spots, blotches, discolouration and brown to dark necrotic lesions characteristically contained in the patterns of the leaf veins. Leaf samples randomly taken from a selection of plants confirmed infestation by *A. fragariae*. Plants were kept in an open, uncovered and quarantine area isolated from other 'clean' plants. Average temperatures of 10.2^{o}C (night) and 22.6^{o}C (day) were observed during the duration of the trial. Daily manual irrigation was carried out (early in the morning) on individual plant to avoid water splash.

Plants in both 2 litre and 4 litre pots were arranged in a randomised block design containing six treatments and four replicates per treatment (Fig. 5.2). All plants were adequately spaced (±50cm) to avoid canopy touch.

The six treatments included (i) Spirotetramat, (ii) Abamectin, (iii) ASM (elicitor), (iv) Spirotetramat + ASM, (v) Abamectin + ASM, and (vi) Control (Water). Based on manufacturer's instructions, all products were prepared in 1 litre of water as shown below:

- Spirotetramat - 1.67ml/ litre^{-1} of water (equivalent to 0.5L/ha/300L water);
- Abamectin - 500µl litre^{-1} of water (equivalent to 50ml/ha/100L water)
- ASM 50% (500g Kg−1) - 0.175g litre^{-1} water (equivalent to 35g/ha/200L water).

- Adjuvant (0.01% Tween 20) was added at the rate of 100µl litre^{-1} of water to all treatments including the water Control.

Figure 5.2 Layout of the experimental trial with randomised block design at Nursery 01, Oxfordshire, UK

A hand-held pressurised sprayer (1 Litre) supplied by Scientific Laboratory Supplies (SLS) UK, was used to spray products as a foliar application on each plant until all parts of the plant was well covered (run off). Early morning application of treatments before sunshine was carried out on plants that had been watered (to wet the leaves) 1 hour before product application. Jagdale & Grewal (2006) found a significant number of nematodes on the leaf surface at 100% relative humidity, and they suggested that free moisture is required to aid movement and survival of LBN. This was also supported by earlier findings by Wallace (1959) who observed that wet-weather conditions are an important factor for the movement of *A. ritzemabosi* to the leaves of chrysanthemum. Control treatments had only water + Tween 20 sprayed on the plants.

Spirotetramat and abamectin were applied 2 times while ASM had 3 applications. All the treatments were applied together on the same day at the start of the trial.

Spirotetramat and ASM treatments had 14 day interval between each application, while abamectin had 7 days interval between applications (Table 5.1).

Table 5.1 Treatments and timing of applications

Treatments	**1st (*Day 1*)**	**2nd (*Day 7*)**	**3rd (*Day14*)**	**4th (*Day 28*)**
Spirotetramat	√		√	
Abamectin	√	√		
ASM	√		√	√
Spirotetramat + ASM	√		√	√ (ASM only)
Abamectin + ASM	√		√	√ (ASM only)
Control (water)	√	√	√	√

Plants were left in the nursery under natural outdoor conditions throughout the duration of study. Plants were watered daily, and assessed for nematode population by random leaf sampling before the first treatment application and at eight weeks after the final treatment application (12 weeks after the first application). No phytotoxicity effect was observed on any plants after the treatments of the products. Leaf sampling was carried out on plants to determine their initial (referred to as p_i) and final (referred to as p_f) nematode population. Leaf samples (including both symptomatic and asymptomatic) were randomly taken per infested plant during both (p_i and p_f) sampling periods. Asymptomatic leaves were considered during sampling even though they were scored as 'clean' - Category 1' with no symptom. Leaves were cut into 1cm² sections, and 5g fresh weight of leaf sample was used for nematode extraction. Nematodes were extracted from leaves of plants using the method outlined in Chapter 2 - Section 2.2.2. based on a technique described by Kohl *et al.*, (2010) and Zhen *et al.*, (2012).

Using the lesion rating key developed in Chapter 3 of this thesis, visual scoring of nematode symptoms on leaves was carried out on each plant at the beginning and end of the trial, with a value of '1' - 0% (clean - no symptom) and maximum value of '6' for 75% - 100% (highly infested) per leaf (Fig. 5.3). Plants were left in the field trial for eight weeks after the final ASM application.

Figure 5.3 Examples of infested *Dryopteris* filix-mas with visual symptoms scores: (left – scored as 2 of 6) and (right – scored as 4 of 6) at Nursery 1 – Oxfordshire, UK

Eight weeks after the application of the final treatment, leaf sampling was carried out for nematode extraction as outlined above to obtain the final nematode population (p_f).

Leaf samples were randomly taken from the base, middle and top of each plant for nematode extraction. The nematode extracted samples were refrigerated at 4 ^{0}C until counted and quantified. Numbers of live and dead nematodes were counted in a 7.5cm counting dish under an inverted microscope at x40. The total number of *A. fragariae* in the dish was corrected for 5g fresh leaf weight per plant. As leaf samples were sometimes less than 1g, counts were extrapolated (Kohl *et al.*, 2010). Saprophytic and parasitic nematodes were recovered from the leaf samples, however, the only pathogenic nematode species found during each sampling period was *A. fragariae* identified using morphological and morphometric features (Siddiqi, 1975).

5.3. Field study 2 – Commercial nursery 2 in Herefordshire, UK

5.3.1 Materials and methods

5.3.2 Materials

Plants: Two naturally infested plants species – *Buddleja davidii* and *Cistus corbariensis* were isolated based on visual symptoms as explained in Field study 1 above. Plants were kept in a covered glasshouse facility isolated from other 'clean' plants.

Pesticides: The same (products) treatments as used in Field study 1 and as shown below (Table 5.2)

Table 5.2 Treatments and timing of applications

Treatments	**1st (*Day 1*)**	**2nd (*Day 7*)**	**3rd (*Day14*)**	**4th (*Day 28*)**
Spirotetramat	√		√	
Abamectin	√	√		
ASM	√		√	√
Spirotetramat + ASM	√		√	√ (ASM only)
Abamectin + ASM	√		√	√ (ASM only)
Control (water)	√	√	√	√

5.3.3 Methods

The study had the same six treatments as used in Field Study 1, but with four replicates for *Buddleja davidii* (Fig. 5.4) and ten replicates for Cistus (Fig. 5.5). Other methods used were the same as in Field Study1 (Section 5.2.3). Plants were watered manually via saucer everyday.

Figure 5.4 Layout of Buddleja experimental plants at Field Study 2

Figure 5.5 Layout of Cistus experimental plants at Field Study 2

Leaf sampling was carried out at the beginning and eight weeks after the final product treatment to determine both p_i (initial) and p_f (final) nematode population per plant. Leaves were cut into 1cm² sections, and 5g fresh weight of leaf sample was used for nematode extraction. Nematodes were extracted from leaves of plants using the method outlined in Chapter 2, Section 2.2.2 based on a technique described by Kohl *et al.*, (2010) and Zhen *et al.*, (2012). No phytotoxicity was observed due to products applied on both plant species after the treatments.

Data analysis: Data of nematode population and leaf symptom score at pre-treatment (p_i) and post -treatment (p_f) were analysed by analysis of variance (ANOVA) using a

General Linear Models Procedure (Minitab 15). Variables include treatment, plant species, leaf symptom score and nematode population count (initial & final). Significant differences between treatments were determined with Fisher's multiple range test at P <0.05 (Jagdale & Grewal, 2002)

The other parameter considered was Reproduction Factor (RF) which is calculated as:

$$RF = \frac{p_f}{p_i}$$

Where p_f is the 'mean final population' after the treatment and p_i is 'mean initial population' before the treatment, 'Reduction of population over Control' (ROC) was also determined for each treatment (Jagdale & Grewal, 2002) which is calculated as:

$$ROC = \left(\frac{mean\ of\ treatment}{mean\ of\ Control} \times 100\right) - 100$$

5.4 Results

Results from both nurseries demonstrated the differences between the treatments and the Control for each plant species, and the difference within treatments with and without the untreated Control. Since the initial nematode population (p_i) of individual plant species were different, the key values considered were the reproduction factor (RF), and reduction of nematode population over control (ROC %) as explained above Jagdale & Grewal, 2002).

5.4.1 Field Study 1 - Commercial nursery 1 in Oxfordshire, UK

5.4.1.1 *Gunnera manicata*

There was a significant (P < 0.05) reduction in reproduction factor (RF) between all the treatments and the Control (Table 5.3).

Values obtained as the reproduction factor (RF) range between 0.20 and 7.16. (Table 5.3) spirotetramat + ASM had the lowest RF (0.20) while the untreated Control gave the highest RF of 7.16 (Table 5.3). Significant (P <0.05) reduction of the RF of nematodes was observed in all the treatments compared to the Control (Fig. 5.6a).

The RF results excluding the untreated Control (Fig. 5.6b) indicate that spirotetramat, abamectin, ASM and abamectin + ASM treatments were not significantly different (P >0.05) between each other.

Table 5.3 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Gunnera manicata* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Nematode per 5g leaf			
	Gunnera manicata			
Trmts	***pi***	***pf***	***RF***	***ROC%***
Spirotetramat	740.1	557.5	0.78b (±0.28)	89.1
Abamectin	1656.5	922.6	0.53bc (±0.04)	92.6
ASM	923.9	575.7	0.59bc (±0.11)	91.8
Spirotetramat+ASM	828.9	140.9	0.20c (±0.03)	97.2
Abamectin+ASM	540.5	331.9	0.60bc (±0.23)	91.7
Control	449.1	3254.2	7.16a (±0.12)	

Similarly, there was no significant difference between the four treatments: spirotetramat + ASM, abamectin + ASM, abamectin and ASM (P >0.05). However, there was a significant difference in RF between the spirotetramat (RF of 0.78) and the spirotetramat + ASM (RF of 0.20) treatments (P <0.05; Fig. 5.6b).

Reduction of nematode population over the Control (ROC) expressed as a percentage (Jagdale & Grewal, 2002), had the highest value with spirotetramat + ASM (97.2%) followed by 92.6% with abamectin. The lowest value (89.1%) was obtained with spirotetramat (Table 5.3).

In general, there were significant differences in RF (P <0.05) between all treatments and the Control, and all the treatments caused a >80 % reduction in nematode population in leaves compared with the Control in this plant species (Table 5.3).

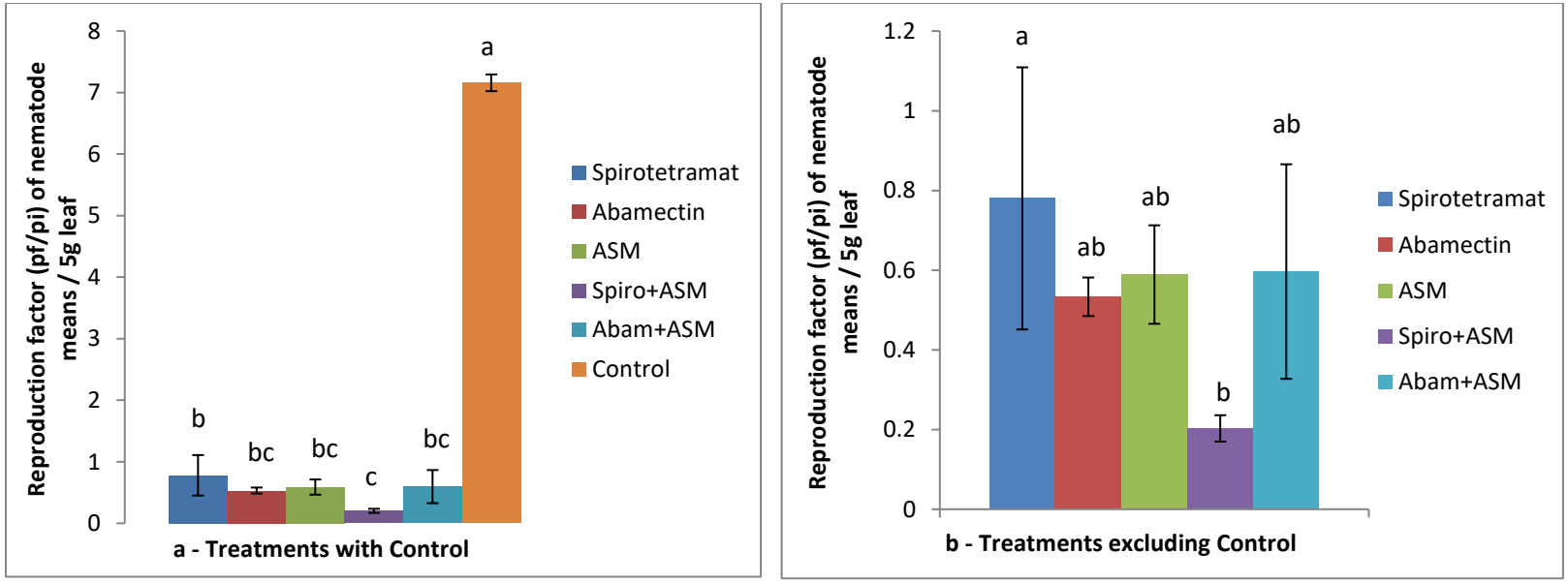

Figure 5.6 Mean (± SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Gunnera manicata* plants with the untreated 'Control' (a) and excluding the 'Control' treatment (b). Columns followed by the same letter are not significantly different (Fisher's multiple range test, $P<0.05$).

5.4.1.2 *Bergenia cordifolia*

There was a significant (P <0.05) reduction in RF in all the treatments compared with the Control (Table 5.4).

Table 5.4 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Bergenia cordifolia* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Nematode per 5g leaf			
	Bergenia cordifolia			
Trmts	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	2529.4	900.6	0.36c (±0.012)	74.5
Abamectin	1529.9	994.2	0.56b (±0.06)	61.2
ASM	2080.9	632.2	0.30c (±0.008)	79.1
Spirotetramat+ASM	1100.8	383.7	0.32c (±0.037)	77.6
Abamectin+ASM	1689.0	512.1	0.34c (±0.029)	76.0
Control	1127.7	1595.5	1.43a (±0.061)	

Values obtained as the reproduction factor (RF) range between 0.30 with ASM and 1.43 in the Control (Table 5.4). The four treatments (ASM, spirotetramat, abamectin + ASM and spirotetramat + ASM) had no difference (P >0.05) between them (Table 5.4). Significant (P <0.05) reduction of the RF of nematodes was observed in the leaves in all the treatments compared to the Control (Fig. 5.7a).

The RF results obtained excluding the untreated Control (Fig. 5.7b) indicate that abamectin was the only treatment significantly different (P <0.05) from the other four treatments (Fig. 5.7b), having a significantly higher RF value. The other treatments (spirotetramat, ASM, abamectin + ASM and spirotetramat + ASM) had no difference (P >0.05) in RF between them (Fig. 5.7b).

Reduction of nematode population over Control (ROC) expressed as a percentage show that ASM obtained the highest value of 79.1% followed by 77.6% in spirotetramat + ASM. The lowest value (61.2 %) was obtained with abamectin (Table 5.4).

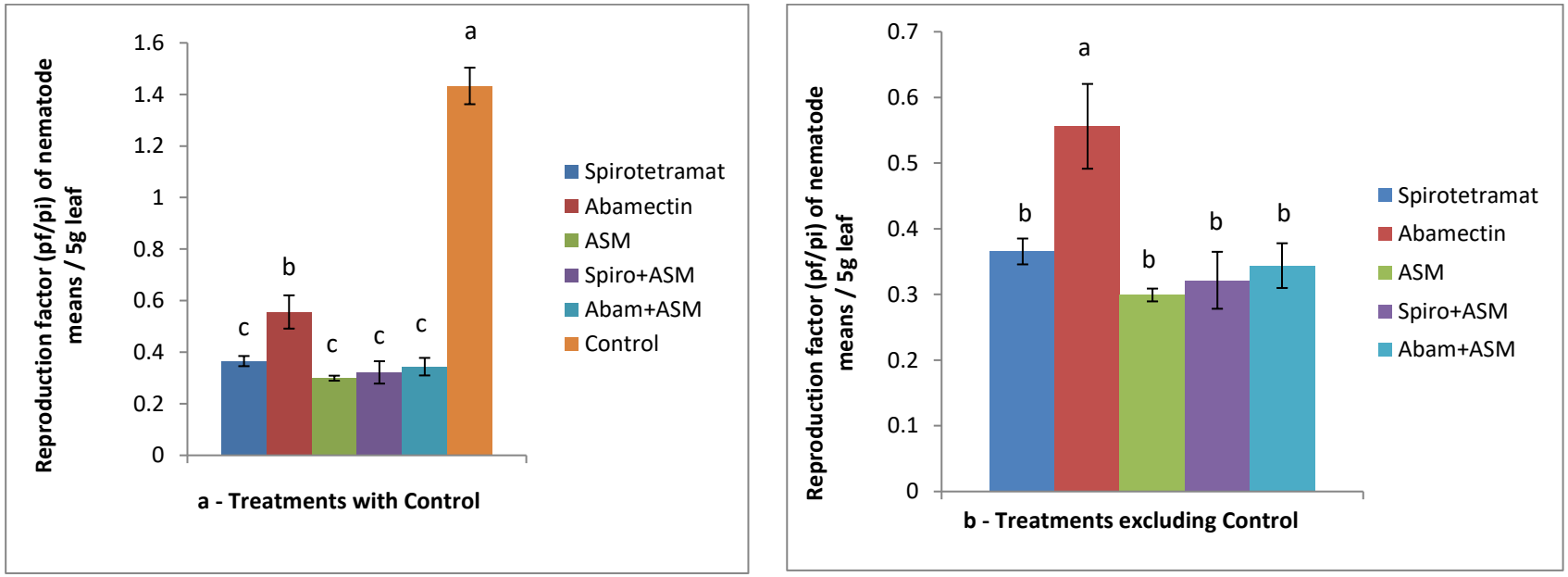

Figure 5.7 Mean (± SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Bergenia cordifolia* plants with the untreated 'Control' (a) and excluding the 'Control' treatment (b). Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

Generally, there was only a significant difference in RF results (P <0.05) between abamectin and the other treatments excluding Control (Fig. 5.7b), while all the treatments caused a >60% reduction in nematode population in leaves compared with the Control (Table 5.4).

5.4.1.3 *Dryopteris filix-mas*

There was a significant (P <0.05) reduction in reproduction factor (RF) between all the treatments and the untreated Control (Table 5.5).

Table 5.5 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Dryopteris filix-mas* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Nematode per 5g leaf			
	Dryopteris filix-mas			
Trmts	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	2334.5	354.7	0.16b (±0.013)	92.04
Abamectin	3931.5	503.4	0.14b (±0.013)	93.33
ASM	4456.6	562.6	0.12b (±0.013)	94.04
Spirotetramat+ASM	2890.4	234.2	0.09b (±0.005)	95.84
Abamectin+ASM	5138.8	427.7	0.10b (±0.013)	95.36
Control	5581.1	14785.2	2.07a (±0.59)	

Values obtained as the reproduction factor (RF) range between 0.09 and 2.07 (Table 5.5). Spirotetramat + ASM had the lowest RF (0.09) while the untreated Control gave the highest RF value of 2.07 (Table 5.5). Significant reduction (P <0.05) of the RF of nematodes was observed in the leaves in all the treatments compared to the untreated Control (Fig. 5.8a).

The RF results obtained excluding the untreated Control (Fig. 5.8b) indicate that spirotetramat, abamectin and ASM were not significantly different (P >0.05) between each other. Spirotetramat had a significantly higher RF than ASM, abamectin + ASM and spirotetramat + ASM (P <0.05).

However, spirotetramat + ASM exhibited a significantly lower (P <0.05) RF compared to the other treatments without untreated Control (Fig. 5.8b).

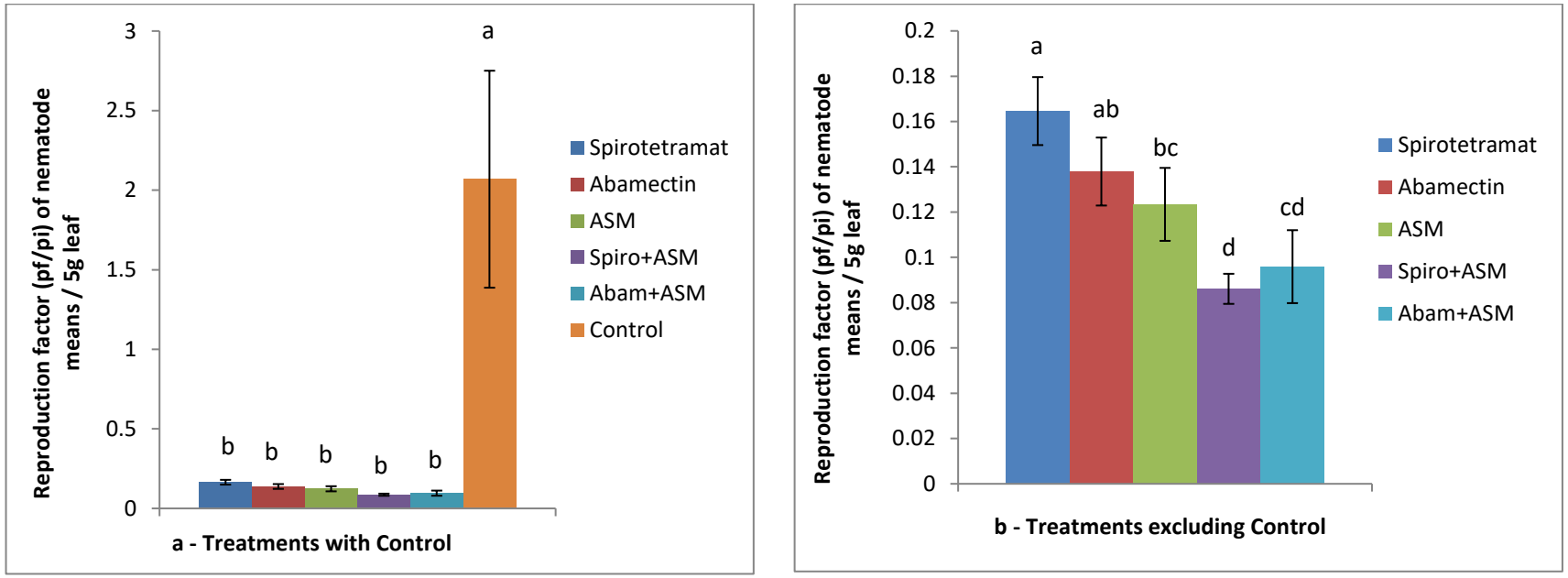

Figure 5.8 Mean (±SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Dryopteris filix-mas* plants with the untreated 'Control' (a) and excluding the 'Control' treatment (b). Columns followed by the same letter are not significantly different (Fisher's multiple range test, $P<0.05$).

Figure 5.9 Pictures showing untreated 'Control' plant (left) of *Dryopteris filix-mas* before the application of treatment and treated plant - foliar applied spirotetramat + ASM - elicitor (right) after eight weeks of treatment application in the grower's nursery.

Reduction of nematode population over the untreated Control (ROC) expressed as a percentage (%) indicate a range of values between 92.04 - 95.84% (Table 5.5). Spiroteramat + ASM had the highest (ROC) value (95.84%) closely followed by 95.36 % with abamectin + ASM. The lowest value (92.04%) was obtained with spirotetramat (Table 5.5).

In general, there were significant differences in RF (P <0.05) between all treatments and the Control, and all the treatments caused a >90% reduction in nematode population in leaves compared with the Control in this plant species (Fig. 5.9 - left & right; Table 5.5).

5.4.1.4 *Astrantia major*

There was a significant reduction (P <0.05) in reproduction factor (RF) between all the treatments and the untreated Control (Table 5.6).

Table 5.6 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Astrantia major* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Nematode per 5g leaf			
	Astrantia major			
Trmts	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	3988.9	1217.8	0.32c (±0.01)	83.7
Abamectin	3656.9	1510.4	0.38b (±0.020)	80.5
ASM	4972.8	1121.6	0.23d (±0.010)	88.3
Spirotetramat+ASM	3989.2	853.0	0.21d (±0.005)	89.4
Abamectin+ASM	4599.4	1382.8	0.29c (±0.022)	85.0
Control	3654.2	7242.1	1.96a (±0.024)	

The reproduction factor (RF) results range between 0.21 – 1.96 (Table 5.6). The untreated Control had the highest RF value of 1.96 while spirotetramat + ASM had the lowest RF of 0.21 (Table 5.6). A significant (P <0.05) reduction of the RF of nematodes was observed in the leaves of all the treatments compared to the Control (Fig. 5.10a).

The RF results excluding the untreated Control (Fig. 5.10b) indicate that spirotetramat + ASM (RF of 0.21) and ASM (RF of 0.23) were not significantly different (P >0.05) between each other, and the same was applicable between spirotetramat (RF of 0.32) and abamectin + ASM (RF of 0.29) with no difference (P >0.05) between the two treatments. However, there was a significant (P <0.05) difference between abamectin (RF of 0.38) and all the other treatments (Fig. 5.10b).

Reduction of nematode population over the Control (ROC) expressed as a percentage had the highest value with spirotetramat + ASM (89.4%) followed by 88.3% with ASM. The lowest value (80.5%) was obtained with abamectin (Table 5.6).

In general, there were significant differences in RF (P <0.05) between all treatments and the Control, and all the treatments caused a >80% reduction in nematode population in leaves compared with the Control in this plant species (Table 5.6).

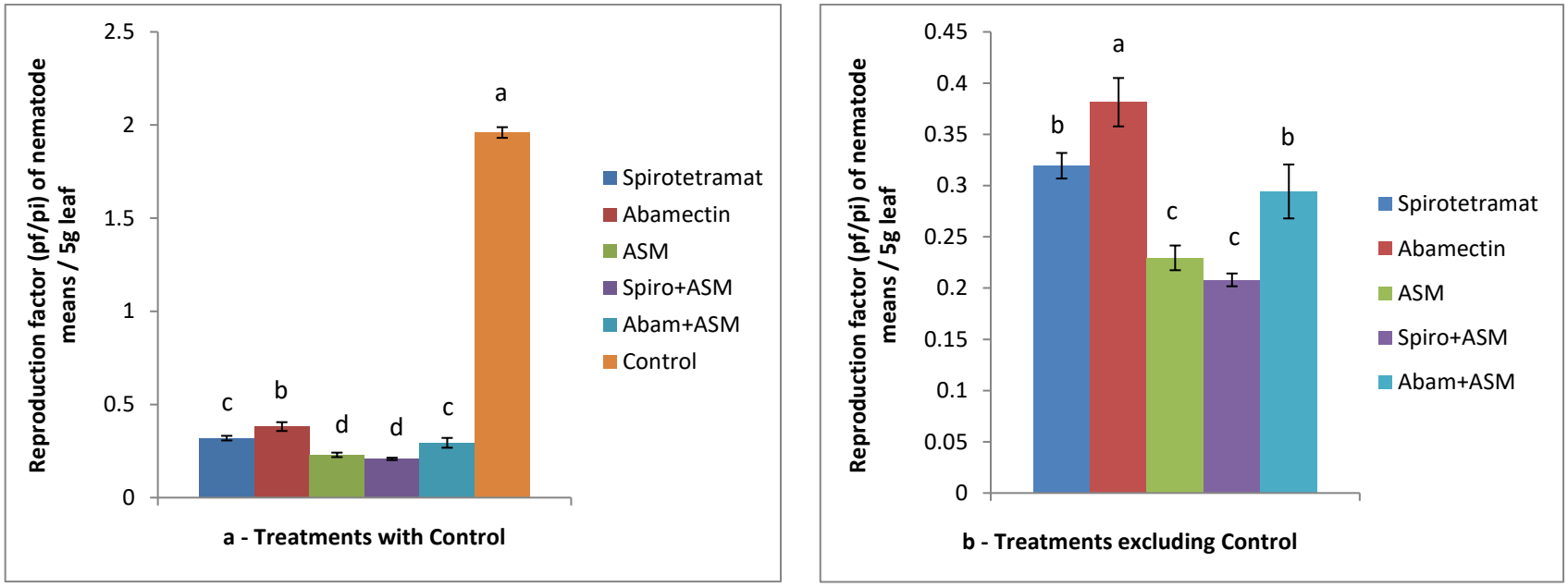

Figure 5.10 Mean (± SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Astrantia major* plants with the (a) untreated 'Control' and (b) excluding the 'Control' treatment. Columns followed by the same letter are not significantly different (Fisher's multiple range test, $P<0.05$).

5.4.1.5 *Brunnera macrophylla*

There was a significant reduction ($P < 0.05$) in reproduction factor between all the five treatments compared with the untreated Control (Table 5.7).

Table 5.7 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Brunnera macrophylla* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, $P < 0.05$).

	Nematode per 5g leaf			
	Brunnera macrophylla			
Trmts	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	8753.1	1131.8	0.26b (±0.084)	87.2
Abamectin	2629.3	1519.3	0.44b (±0.087)	78.5
ASM	6051.6	1077.1	0.24b (±0.048)	88.2
Spirotetramat+ASM	4221.0	821.8	0.24b (±0.047)	88.2
Abamectin+ASM	2892.3	484.8	0.15b (±0.014)	92.5
Control	2454.0	3537.2	2.04a (±0.36)	

Values obtained as the reproduction factor (RF) range between 0.15 and 2.04 (Table 5.7). The Control treatment had the highest RF value (2.04) with the lowest RF value (0.15) obtained with abamectin + ASM (Table 5.7). A significant ($P < 0.05$) reduction of the RF of nematodes was observed in the leaves in all the treatments compared to the Control (Fig. 5.11a).

The reproduction factor (RF) results obtained from all treatments excluding the untreated Control (Fig. 5.11b) indicate that abamectin and spirotetramat had no difference ($P > 0.05$) between each other. Similarly, there was no significant difference between the three treatments - spirotetramat + ASM, abamectin + ASM, and ASM ($P > 0.05$). However, there was a significantly higher RF with abamectin and the three other treatments - spirotetramat + ASM, abamectin + ASM and ASM ($P < 0.05$; Fig. 5.11b).

Reduction of nematode population over Control (ROC) expressed as a percentage had the highest value of 92.5% with abamectin + ASM (Table 5.7). The lowest value (78.5%) was obtained with abamectin.

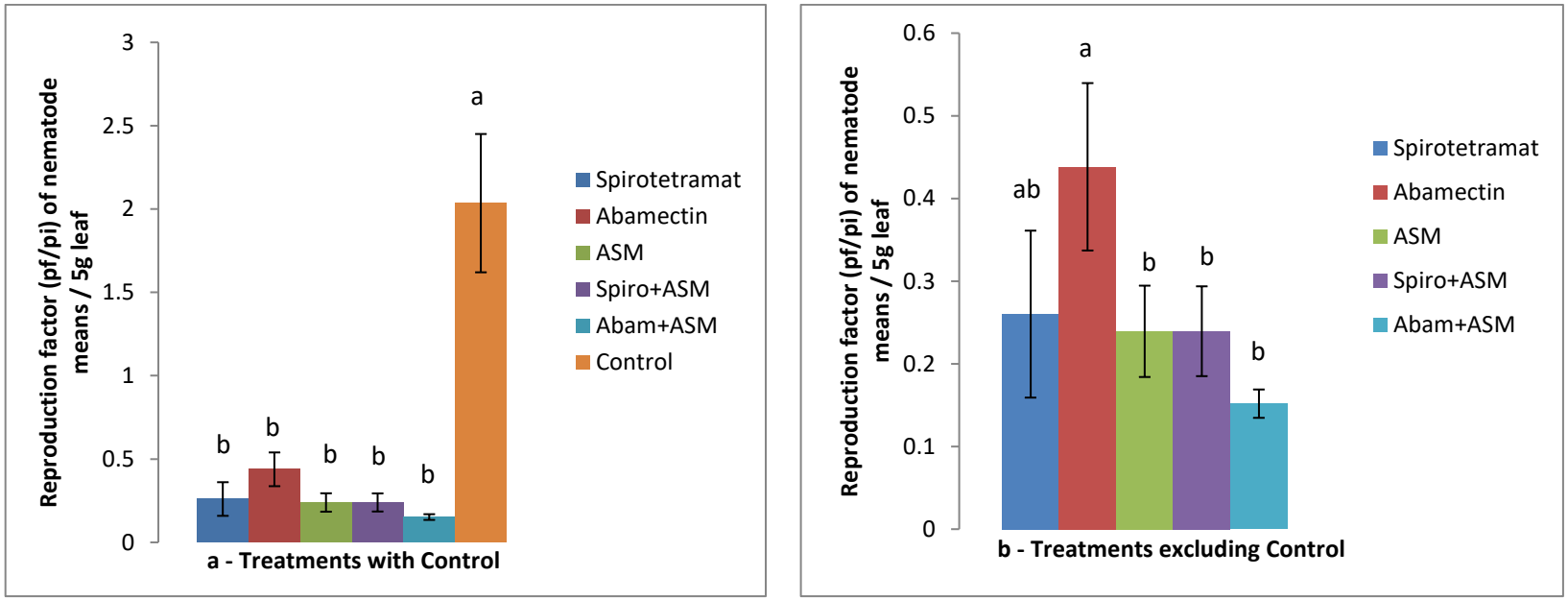

Figure 5.11 Mean (± SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Brunnera macrophylla* plants with the (a) untreated 'Control' and (b) excluding the 'Control' treatment. Columns followed by the same letter are not significantly different (Fisher's multiple range test, $P<0.05$).

In general, there were significant differences in RF (P <0.05) between all the treatments and the Control (Fig. 5.11a), and all the treatments caused a >75% reduction in nematode population in leaves compared with the Control in this plant species.

5.4.1.6 *Astilboides tabularis*

There was a significant (P < 0.05) reduction in reproduction factor (RF) between all the treatments and the Control (Table 5.8).

Table 5.8 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Astilboides tabularis* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Nematode per 5g leaf			
	Astilboides tabularis			
Treatments	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	8122.19	2363.15	0.29b (±0.014)	89.95623
Abamectin	9566.86	4720.26	0.48b (±0.030)	83.74598
ASM	1606.25	1045.15	0.49b (±0.11)	83.19198
Spirotetramat+ASM	5798.77	628.76	0.17b (±0.038)	94.06333
Abamectin+ASM	7731.62	1129.32	0.25b (±0.091)	91.44723
Control	9046.45	33198.62	2.92a (±0.54)	

The values obtained as the reproduction factor (RF) range between 0.17 and 2.92 (Table 5.8). Spirotetramat + ASM had the lowest RF value (0.17) while the untreated Control gave the highest RF of 2.92 (Table 5.8). There was a significant (P < 0.05) reduction of the RF of nematodes observed in all the treatments compared with the untreated Control (Fig. 5.12a).

The RF results excluding the untreated Control (Fig. 5.12b) indicate that spirotetramat (RF of 89.96) had no difference (P >0.05) with the other four treatments – abamectin, ASM, spirotetramat + ASM and abamectin + ASM (Fig. 5.12b). Similarly, there was no significant difference between abamectin (RF of 0.48) and ASM (RF of 0.49), likewise between spirotetramat + ASM (RF of 0.17) and abamectin + ASM (RF of 0.25).

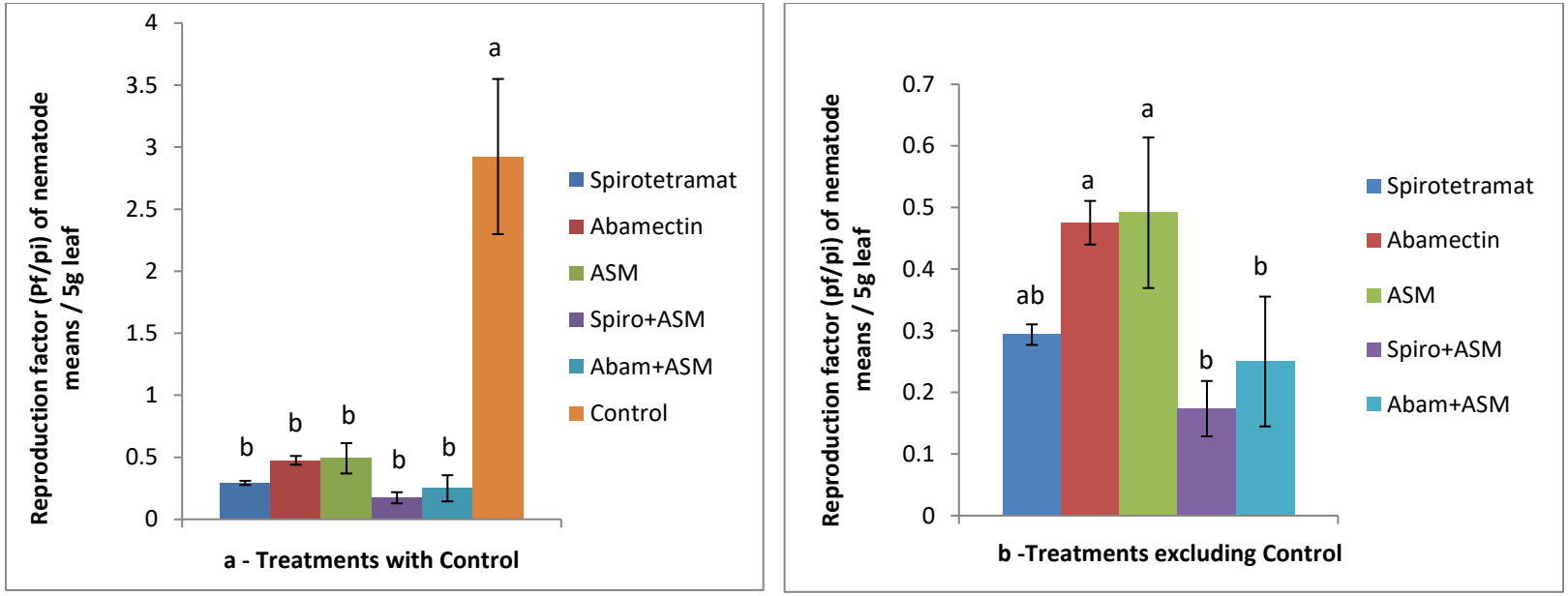

Figure 5.12. Mean (±SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Astilboides tabularis* plants with the untreated 'Control' (a) and excluding the 'Control' treatment (b). Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

However, there were significantly lower RF values when abamectin and ASM are individually compared with spirotetramat + ASM and abamectin + ASM ($P < 0.05$; Fig. 5.12b).

Reduction of nematode population over the Control (ROC) had 94.06% as the highest value with spirotetramat + ASM followed by 91.45% with abamectin + ASM. The lowest value (83.19%) was obtained with ASM (Table 5.8).

In general, there were significant differences in RF (P <0.05) between all the treatments and the Control. In addition, all the treatments caused a >80% reduction in nematode population in leaves of *A. tabularis* compared with the Control (Table 5.8).

5.4.1.7 *Anemone hupehensis*

There was a significant ($P < 0.05$) reduction in reproduction factor (RF) between all the treatments and the untreated Control (Table 5.9).

Table 5.9 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Anemone hupehensis* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Nematode per 5g leaf			
	Anemone hupehensis			
Trmts	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	2732.536	1218.37	0.45b (±0.039)	79.63672
Abamectin	6636.642	2293.78	0.35bc (±0.014)	84.4143
ASM	2877.411	697.78	0.25bc (±0.029)	88.92595
Spirotetramat+ASM	6812.223	291.87	0.04c (±0.005)	98.04841
Abamectin+ASM	4292.742	445.18	0.11c (±0.016)	95.23595
Control	4573.576	10086.45	2.22a (±0.17)	

Values obtained as the reproduction factor (RF) range between 0.04 and 2.22 (Table 5.9). Spirotetramat + ASM had the lowest RF (0.04) while the untreated Control gave the highest RF of 2.22 (Table 5.9). Significant ($P < 0.05$) reduction of the RF of nematodes was observed in the leaves of all the treatments compared to the Control (Fig. 5.13a).

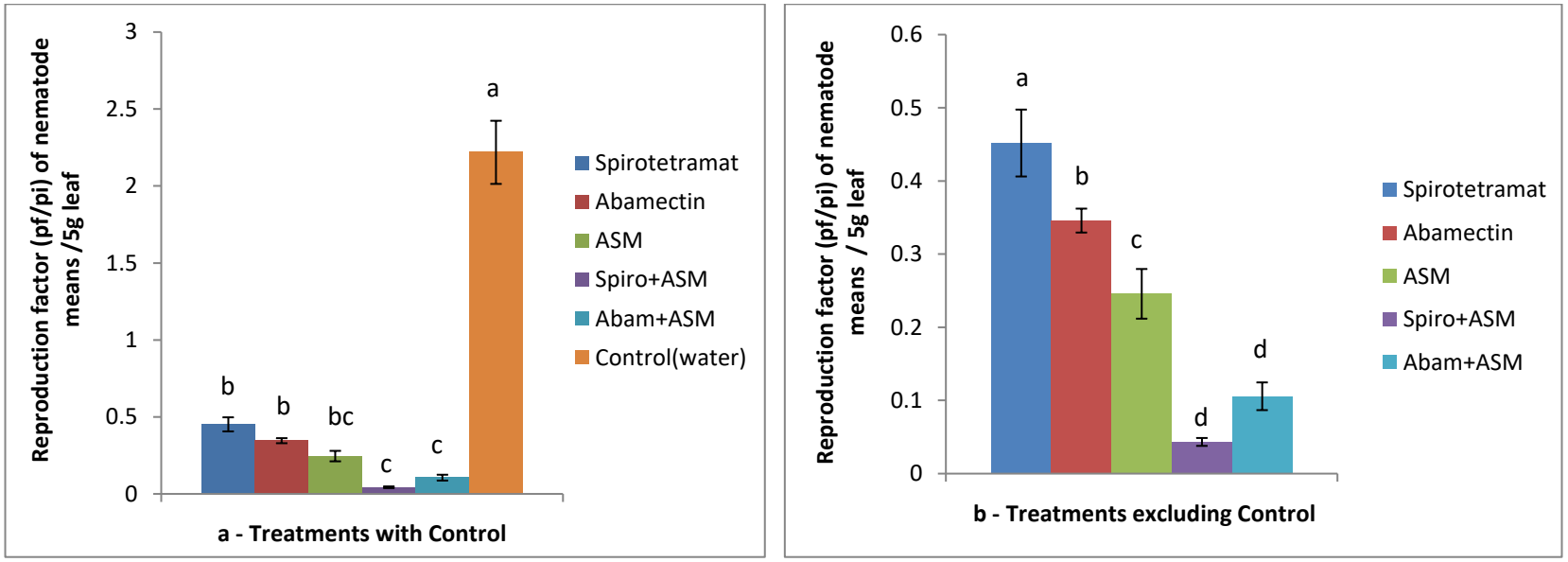

Figure 5.13 Mean (±SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Anemone hupehensis* plants with the (a) untreated 'Control' and (b) excluding the 'Control' treatment. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

The RF results excluding the untreated Control (Fig. 5.13b), indicated that spirotetramat + ASM and abamectin + ASM were not significantly different (P >0.05) between each other. However spirotetramat + ASM and abamectin + ASM had significantly lower RF values compared to the other three treatments. There were also significant differences (P <0.05) in RF between spirotetramat, abamectin and ASM (Fig. 5.13b).

Reduction of nematode population over the Control (ROC) expressed as a percentage had the highest value with spirotetramat + ASM (98.04%) followed by 95.23% with abamectin + ASM. The lowest value (79.63%) was obtained with spirotetramat (Table 5.9).

In general, there were significant differences in RF (P <0.05) between all treatments and the Control, and all the treatments caused a >75 % reduction in nematode population in the leaves of anemone compared with the Control (Table 5.9).

5.4.1.8 Summary of the efficacy of ASM ± pesticides for the management of A. *fragariae* on ornamental flowering plants at Field Study 1.

Comparison was made between treatments on each of the plant species to investigate the efficacy of products as a result of the addition of the elicitor ASM.

Gunnera manicata: There was a reduction (P <0.05) in reproduction factor (RF) when ASM was applied with spirotetramat (RF of 0.20) compared to spirotetramat alone (RF of 0.78) - (Table 5.10). There was no significant difference between abamectin + ASM and abamectin alone (P >0.05; Table 5.10).

Bergenia cordifolia: There was no difference (P >0.05) between reproduction factor (RF) values of 0.32 in spirotetramat + ASM and 0.37 with spirotetramat (Table 5.10). However, addition of ASM to abamectin led to a significant difference (P <0.05) with a reduced RF of 0.34 by abamectin + ASM compared with 0.57 with abamectin alone (Table 5.10).

Dryopteris filix-mas: There was no difference (P >0.05) in reproduction factor (RF) between abamectin + ASM (0.09) and abamectin (0.13). However, addition of ASM gave a significant reduction in RF (P <0.05) with spirotetramat + ASM (0.09) and spirotetramat alone (0.16) – (Table 5.10).

Astrantia major: There was a significant difference (P <0.05) in reproduction factor (RF) due to the addition of ASM with a reduced RF value of 0.21 in spirotetramat + ASM, compared with 0.32 obtained with spirotetramat (Table 5.10). Similarly, abamectin + ASM had a significant (P <0.05) reduced RF value of 0.29 compared with 0.38 with abamectin alone (Table 5.10).

Anemone hupehensis: Results of reproduction factor (RF) indicate a significant difference between spirotetramat + ASM (0.04) and spirotetramat alone (0.45) at P <0.05 (Table 5.10). Similarly, addition of ASM gave a significant reduction in RF (0.11) with abamectin + ASM compared to an RF of 0.35 with abamectin alone (P <0.05; Table 5.10).

Astilboildes tabularis: There was a significant (P <0.05) reduction of reproduction factor (RF) with spirotetramat + ASM (0.17) compared to spirotetramat alone (0.29; Table 5.10). However, addition of ASM gave no difference (P >0.05) between abamectin + ASM (0.25) and abamectin alone (0.47), Table 5.10).

Brunnera macrophylla: Results of the reproduction factor (RF) obtained with spirotetramat + ASM (0.23) and spirotetramat alone (0.26) gave no significant (P >0.05) difference (Table 5.10). In contrast, there was a significant reduction in RF with abamectin + ASM (0.15) compared to abamectin alone (0.43) (P <0.05; Table 5.10).

Table 5.10 Reproduction factor (RF) values with spirotetramat and abamectin ± ASM in plants at Field Study 1

Plants	Trmt	RF		Plants	Trmt	RF	
Gunnera manicata	Spirotetramat vs Spiro+ASM	0.7801 0.2029	a b	***Astrantia major***	Spirotetramat vs Spiro+ASM	0.31943 0.20798	a b
Gunnera manicata	Abamectin vs Abam+ASM	0.5965 0.5332	a a	***Astrantia major***	Abamectin vs Abam+ASM	0.38136 0.29427	a b
Bergenia cordifolia	Spirotetramat vs Spiro+ASM	0.36553 0.3216	a a				
Bergenia cordifolia	Abamectin vs Abam+ASM	0.55601 0.34395	a b				

Data are mean values of four replicates, and values not sharing a common letter indicate a significant difference (Fisher's multiple range test, P <0.05). RF – reproduction factor ($p_f \div p_i$)

Table 5.11. Reproduction factor (RF) values with spirotetramat and abamectin ± ASM in plants at Field Study 1

Plants	Trmt	RF	Plants	Trmt	RF
Anemone hupehensis	Spirotetramat vs Spiro+ASM	0.45184 a 0.04332 b	***Astilboides tabularis***	Spirotetramat vs Spiro+ASM	0.29366 a 0.17365 b
Anemone hupehensis	Abamectin vs Abam+ASM	0.34579 a 0.10571 b	***Astilboides tabularis***	Abamectin vs Abam+ASM	0.4753 a 0.2501 a
Dryopteris filix-mas	Spirotetramat vs Spiro+ASM	0.16455 a 0.08614 b	***Brunnera macrophylla***	Spirotetramat vs Spiro+ASM	0.2601 a 0.2394 a
Dryopteris filix-mas	Abamectin vs Abam+ASM	0.13794 a 0.09587 a	**Brunnera *macrophylla***	Abamectin vs Abam+ASM	0.4383 a 0.1518 b

Data are mean values of four replicates, and values in the same column followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05). RF – reproduction factor ($p_f \div p_i$)

5.4.2 Field Study 2 - Commercial nursery 2 in Herefordshire, UK

5.4.2.1 *Buddleja davidii*

There was a significant ($P < 0.05$) reduction in reproduction factor (RF) between all the treatments and Control (Table 5.12).

Table 5.12 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Buddleja davidii* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, $P < 0.05$).

	Nematodes per 5g leaf			
	Buddleja davidii			
Treaments	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	2092.00	356.00	0.17b (±0.017)	94.1
Abamectin	2901.90	499.00	0.17b (±0.009)	94.1
ASM	2476.00	446.40	0.18b (±0.010)	93.8
Spirotetramat+ASM	2700.50	178.20	0.07b (±0.010)	97.7
Abamectin+ASM	1765.00	283.00	0.17b (±0.029)	94.1
Control	1841.00	5065.00	2.91a (±0.37)	

Values obtained as the reproduction factor (RF) range between 0.07 and 2.91 (Table 5.12). Spirotetramat + ASM had the lowest RF (0.07) while the untreated Control had the highest RF value of 2.91 (Table 5.12). A significant ($P < 0.05$) reduction of the RF of nematodes was observed in the leaves in all the treatments compared to the Control (Table 5.12).

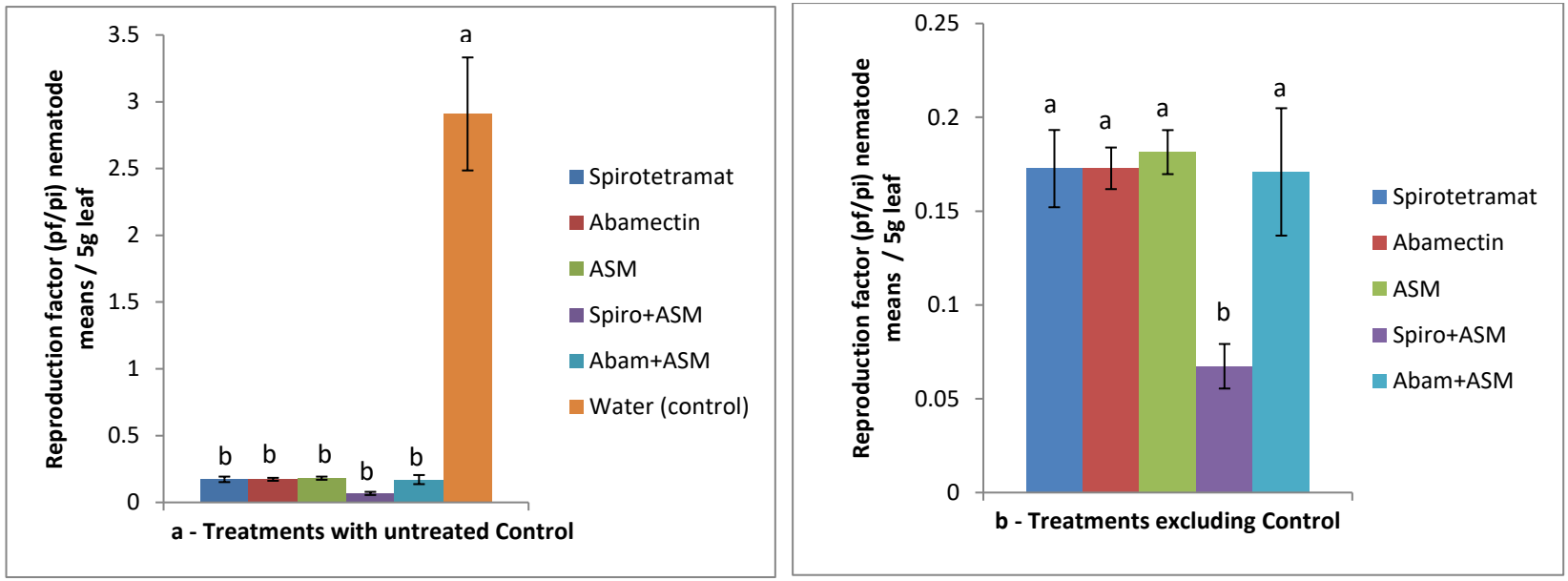

Figure 5.14 Mean (±SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Buddleja davidii* plants with the untreated 'Control' (a) and excluding the 'Control' treatment (b). Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

The RF results excluding the untreated Control (Fig. 5.14b) indicate that spirotetramat, abamectin, ASM and abamectin + ASM treatments were not significantly different (P >0.05) between each other. However, there was a significant reduction in RF between the spirotetramat + ASM (0.07) compared to the other four treatments – spirotetramat, abamectin, ASM and abamectin + ASM (P <0.05; Fig. 5.14b).

Reduction of nematode population over the control (ROC) expressed as a percentage had the highest value with spirotetramat + ASM (97.7%), while the lowest value (93.8%) was obtained with ASM (Table 5.12). In general, there were significant differences in RF (P <0.05) between all treatments and the Control, and all the treatments caused a >90% reduction in nematode population in leaves compared with the untreated Control in this (*Buddleja davidii)* plant species (Table 5.12).

5.4.2.2 *Cistus corbariensis*

There was a significant (P < 0.05) reduction in reproduction factor (RF) between all the treatments and the Control (Table 5.13).

Table 5.13 Reduction over control (%) and mean value (±SE) of nematode reproduction factor from 5g of leaf for *Aphelenchoides* infested *Cistus corbariensis* plants. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Nematodes per 5g leaf			
	Cistus corbariensis			
Treatments	***pi***	***pf***	***RF***	***ROC(%)***
Spirotetramat	3130.0	1019.7	0.35b (±0.037)	78.0
Abamectin	2330.7	629.0	0.30bc (±0.05)	80.8
ASM	2717.0	771.0	0.28bc (±0.04)	82.0
Spirotetramat+ASM	3283.0	408.9	0.13c (±0.021)	91.5
Abamectin+ASM	2331.0	329.9	0.14c (±0.017)	90.9
Control	2544.0	3802.0	1.58a (±0.146)	

The reproduction factor (RF) values obtained from Cistus range between 0.13 and 1.58 (Table 5.13). Spirotetramat + ASM had the lowest RF (0.13) closely followed

by abamectin + ASM (0.14) while the untreated Control gave the highest RF of 1.58 (Table 5.13).

There was a significant reduction of the RF of nematodes as observed in all the treatments compared to the untreated Control (Fig. 5.15a).

The RF results excluding the untreated Control (Fig. 5.15b) indicate that spirotetramat, abamectin and ASM treatments were not significantly different (P >0.05) from each other. Similarly, there was no significant difference between two treatments - spirotetramat + ASM (RF of 0.13) and abamectin + ASM (P >0.05). However, there was a significantly lower RF with spirotetramat + ASM and abamectin + ASM compared to the three other treatments – spirotetramat, abamectin and ASM (P <0.05; Fig. 5.15b). Reduction of nematode population over the control (ROC) expressed as a percentage, had the highest value with spirotetramat + ASM (91.5) followed by 90.9% with abamectin + ASM. The lowest value (78%) was obtained with spirotetramat (Table 5.13).

In general, there were significant differences in RF (P <0.05) between all treatments and the Control, and all the treatments caused a >75% reduction in nematode population in leaves compared with the Control in this Cistus plant species (Table 5.13).

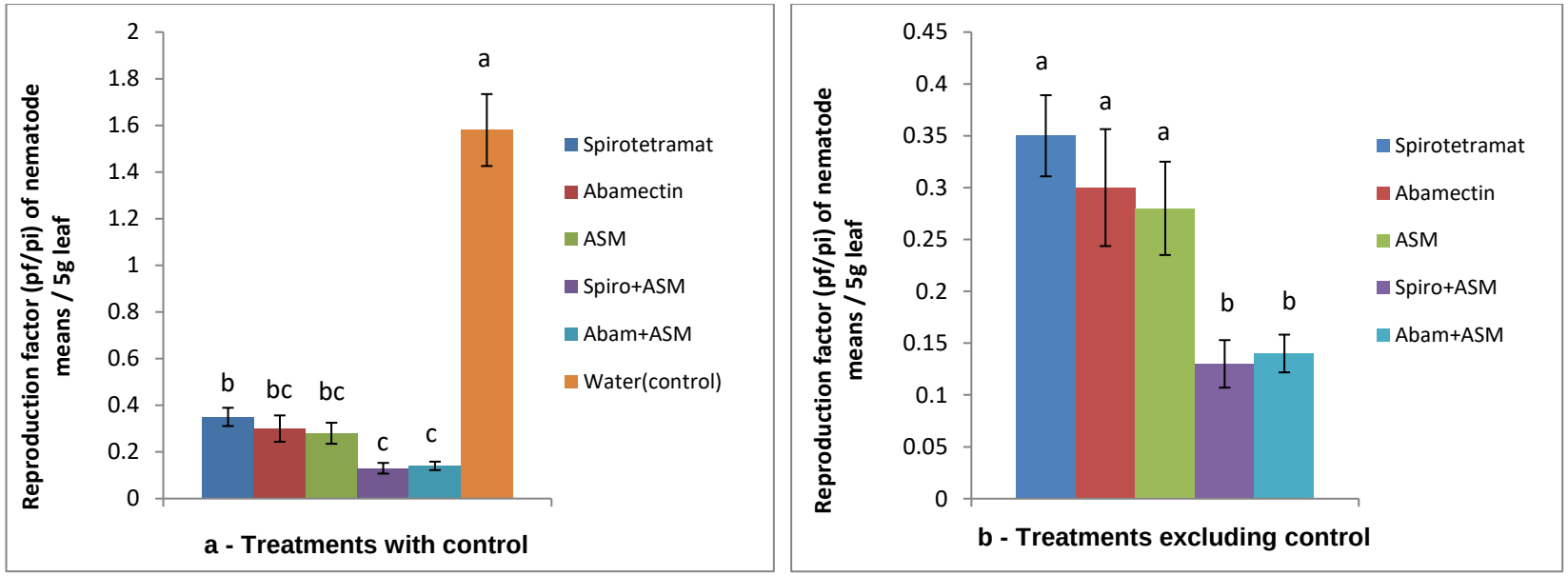

Figure 5.15 Mean (±SE) Reproduction Factor (RF – p_i/p_f) from 5g of leaf for *Aphelenchoides* infested *Cistus corbariensis* plants with the untreated 'Control' (a) and excluding the 'Control' treatment (b). Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

5.4.2.3 Summary of the efficacy of ASM ± pesticides for the management of *Aphelenchoides* on ornamental flowering plants at Field Study 2

Comparison was made between treatments on each of the plant species to investigate the efficacy of products with the addition of ASM.

Buddleja davidii: There was a significant reduction (P <0.05) in reproduction factor (RF) with spirotetramat + ASM (0.07) compared to spirotetramat alone (RF of 0.17, Table 5.14). Addition of ASM had no significant difference in RF of nematode population with abamectin + ASM (RF of 0.17) and abamectin alone (RF of 0.17, Table 5.14).

Table 5.14 Reproduction Factor (RF) values with spirotetramat and abamectin ± ASM in plants at Field Study 02

Plants	Trmt	RF	Plants	Trmt	RF
Buddleja	Spirotetramat vs Spirot+ASM	0.1726 a 0.0673 b	**Cistus**	Spirotetramat vs Spirot+ASM	0.34749 a 0.13485 b
Buddleja	Abamectin vs Abam+ASM	0.1728 a 0.1709 a	**Cistus**	Abamectin vs Abam+ASM	0.3035 a 0.1437 b

Data are means of four replicates (*Buddleja*) and ten replicates (Cistus) plants. Values not sharing a common letter indicate a significant difference (P <0.05) according to Fisher individual test. RF - Reproduction factor (p_f / p_i) per 5g leaf

Cistus corbariensis: Addition of ASM with spirotetramat had a significant (P <0.05) reduction in reproduction factor (RF) (0.13) compared with spirotetramat alone (0.35, Table 5.14). Similarly there was a significant reduction (P <0.05) with reduced RF values in abamectin + ASM (0.14) when compared with abamectin alone (RF of 0.30, Table 5.14).

5.5 Discussion

This study at both nurseries identified potential products (pesticides and elicitors) for the effective management of LBN. Although nematode infestation has not been

completely eliminated, the identified products reduced infestation to economically viable levels, and when used in combination with other management practices, as part of an IPM approach, could serve as replacements for oxamyl. Application of these pesticides with or without the elicitor 'ASM' significantly reduced the continuous reproductive capacity of *Aphelenchoides fragariae* on these naturally infested plants compared to untreated plants ($P < 0.05$). This study has demonstrated that ASM (acibenzolar-S-methyl), spirotetramat and abamectin can significantly reduce the population of LBN on all the plants species tested by >60 % when compared with the untreated Control.

The results of 94% mortality with abamectin in this study are similar to the results of Young & Maher (2000) who observed significant mortality (90%) of *A. ritzemabosi* (LBN) *in vitro* by abamectin with the same manufacturer's recommended rate (0.05% volume concentration) as was used in this study after 24 hours of exposure in a laboratory assay. The results as reported by LaMondia (1999) on foliar application of abamectin to infested *Lamium maculatum* and Chałańska *et al.* (2017) to infested *Anemone hupehensis* confirms that abamectin has the potential to control LBN. An *et al.* (2017) demonstrated between 71-75% mortality of *A. fragariae* by abamectin during laboratory bioassays test. However, both spirotetramat and abamectin have not been fully evaluated for their capacities to suppress A. *fragariae* in ornamental plants, and this study appears to be the first investigation on the management of LBN by the elicitor ASM.

Spirotetramat is a registered product for controlling many sucking insect pests including aphids, with an EAMU approval for use. Foliar application of spirotetramat at the manufacturers recommended rate for use against insect pests to LBN infested plants led to a significant reduction in the reproduction factor (RF) of the nematode population. This suggests that multiplication of nematodes in the leaves was altered through the foliar application of spirotetramat. This effect was similar to the report of a reduced reproduction of cereal cyst nematode (*Heterodera avenae)* when spirotetramat was applied to the foliage of spring wheat on two nematode infested fields in the United States (Smiley *et al.*, 2011). Spirotetramat is reported to have both phloem and xylem mobility (ambimobile) in several plant species (Nauen *et al.*,

2008), so when used in this study the effects of spirotetramat on LBN could be expressed throughout the whole plant.

The reduction in nematode population over control (ROC%) by spirotetramat during both field trials in this study range between 74% (*Bergenia*) to 94% (*Buddleja).* This demonstrates that spirotetramat has significant potential for LBN control on ornamental plants. There was no observed phytotoxicity on any plant species from spirotetramat in this study.

Abamectin has demonstrated activity against a number of mites, insects, and root-knot nematodes (Cayrol *et al.*, 1993; LaMondia, 1999). Abamectin was reported to have potential to control LBN on hardy nursery stock in the UK (Young & Maher, 2000). Abamectin is registered in the UK for the control of spider mites and leaf miners on ornamentals. Past research work has reported that repeated applications of abamectin - 0.011 litre^{-1} (as Avid 0.6 ml litre^{-1}) reduced both *A. fragariae* and *Ditylenchus dipsaci* populations in *Lamium maculatum* in the USA (LaMondia, 1999).

In this study, application of abamectin at the rate recommended for insect pests on ornamentals led to a significant reduction in the multiplication of nematodes when compared with the untreated Control on plants tested at both nurseries. There was a significant (P <0.05) reduction in the nematode reproduction factor (RF) on all the plant species tested when compared with the untreated Control. The reduction of nematode multiplication over the control (ROC%) ranged between 61% in *Bergenia* to 94% in *Buddleja*. These results confirmed the results of LaMondia (1999) who reported significant reduction of nematode populations (*A. fragariae*) extracted from infested foliage of *Lamium maculatum* 5 weeks after foliar application (at 0.011g litre^{-1}) with abamectin in a greenhouse experiment, although the author used 0.6 ml/l Avid – (0.011g litre^{-1} abamectin), a little bit higher than 0.5 ml litre^{-1} – (0.009g litre^{-1} abamectin) that was used in this study. The activity of abamectin could be influenced by the use of adjuvants and manipulation of the timing of application.

During this study, early morning application of treatments was carried out one hour after plants were irrigated (to wet the leaves). This is in accordance with the report of Jagdale & Grewal (2006) that at 100% relative humidity, a significant number of nematodes were recorded on the leaf surface, while an earlier report (Kohl *et al.*,

2010) observed that wet-weather conditions are an important factor for the movement of *A. ritzemabosi* on to the leaves of chrysanthemum.

The results of this study are at odds with the report of Bennison, (2007), who evaluated a range of alternatives for the control and management of *Aphelenchoides*, and found abamectin to be ineffective. However, unlike this study, that research work did not involve the use of an adjuvant, wetting of plant leaves before product application or early morning timing of application. The adjuvant 'Tween 20' used in this study had a good compatibility with abamectin, and other products including ASM and spirotetramat. While past works have recorded successful combination of Tween 20 as surfactant with ASM in the past, this study did not investigate Tween 20 in detail as the author in agreement with the project supervisors decided to prioritise products / treatments with focus on chemical and bio-pesticides along with elicitor agent (considering time scale for the project). Leaf characteristics of different plant species could differ on the absorption of abamectin by leaves and its translaminar activity. The cuticle of the leaf may serve as a barrier to abamectin absorption and activity (Price, 1982). For example, during this study, it was observed that leaves of *Gunnera manicata* and *Astilboides tabularis* were of thick textures while *Anemone hupehensis* and *Astrantia major* have soft and tender leaves, hence leaf cuticle could influence product penetration and foliar uptake of pesticides in the leaves.

While symptom on infested leaves of cistus may not visibly appear at the initial level of infestation, symptom on *Buddleja davidii* appears glaring / visible as soon as infestation occurs on the leaf. Also, the spread of symptom on infested leaves of *Anemone hupehensis* was rapidly noticed than it was observed on the leaves of *Gunnera manicata* during this study. Generally, there was no observed phytotoxicity on the leaves of any plant species investigated due to the product (treatment) in this study.

Further work may be necessary on the addition of adjuvants to determine whether they are necessary for effective LBN control, and whether some adjuvants are more effective than others. Generally, adjuvants may influence surface properties of product, prepare leaf cuticle to the transportation of solute, thereby enhancing product activity (Kirkwood, 1999).

Moreover, further study could examine if Tween 20 (used in this study) contributed additional effect as a potential agent for the management of LBN, other than improving products efficacy. Tween 20 has been investigated as treatment for disease and pest control. Pehlevan & Kovanci (2016) conducted laboratory evaluations of Tween 20 against the pear psylla [*Cacopsylla pyri* L. (Hemiptera: Psyllidae)] at different concentrations. Tween 20 showed significant toxic effects against psyllid nymphs especially at concentrations of 20ml and 30ml / litre $^{-1}$ while less toxicity effect was observed against psyllid eggs (Pehlevan & Kovanci, 2016).

The elicitor ASM (50% w/w) 'acibenzolar-S-methyl' when used individually and in combination with spirotetramat and abamectin demonstrated a significant (P <0.05) reduction in nematode multiplication on infested plants in both nurseries. Similar results in reduced pathogen multiplication were observed by past workers on ASM to control pests and diseases on plants including root knot nematodes (Molinari & Baser, 2010; Walters *et al.*, 2014; McGrann *et al.*, 2017). ASM has been reported as a novel plant protection product that mimics the pathogen-host interaction and thereby results in (SAR) systemic acquired resistance in plants (Cole, 1999).

Furthermore, ASM has been reported to induce resistance to pathogens when applied to plants (Kessmann *et al.*, 1994). ASM is not directly antimicrobial as it works directly on plant mechanisms rather than pathogen itself, hence reducing the probability of pathogens developing resistance, therefore a great advantage over conventional pesticides (Vallad & Goodman, 2004). The product ASM applied 3x as a foliar application at the manufacturer recommended dose rate for barley and protected chrysanthemums against fungal disease (0.175g litre $^{-1}$ water–equivalent to a spray volume of 35g/ha/200L water) significantly reduced nematode multiplication (P <0.05), with less lesion symptoms on leaves when compared with the untreated Control eight weeks after the final ASM treatment. ASM is registered in the UK as a plant protection product, and approved as Bion® (now Inssimo®) for use on protected Chrysanthemum against fungal diseases. Foliar application of ASM 3x at an interval of 14 days during this study significantly reduced the reproduction factor (RF) of nematodes when compared with the untreated Control plants. Addition of ASM caused a significant (P <0.05) reduction of nematode population over the Control (ROC%) which ranged between 79% (*Bergenia*) to 91% (*Gunnera manicata)*.

This confirmed the previous results of Chapter 4 that 3x foliar application of ASM on *Anemone hupehensis* plants infested with LBN significantly reduced the population of *A. fragariae* in a glasshouse study, and with no observed phytotoxic effect on the plants (see Chapter 4). The results from this study are similar to those seen with foliar fungal pathogens and other nematodes, where ASM protected tobacco plants from angular leaf spot compared with controls in Zimbabwe (Cole, 1999), reduction of 74% egg masses of root knot nematode (*Meloidogyne incognita*) when ASM was soil-drenched on tomato in a glasshouse study (Molinari & Baser, 2010), and a 70% reduction in *Rhynchosporium commune* infection on spring barley in a glasshouse experiment (Walters *et al.*, 2014).

There was similarity between results obtained from the 2 nurseries in term of efficacy due to the addition of ASM to spirotetramat and abamectin in most of the plant species tested. Results also confirmed that addition of ASM to spirotetramat and abamectin had significantly (P <0.05) lower reproduction factor (RF) values, and a suppressive effect on nematode multiplication compared to the insecticides alone. Spirotetramat + ASM gave a consistent reduction in nematode multiplication in most of the plant species investigated. Similar observations were noticed in field experiments where ASM plus fungicide combinations provided most consistent disease control of *R. commune* on barley crops (Walters *et al.*, 2014), and a consistently reduced infection of light leaf spot disease (*Pyrenopeziza brassicae*) on winter oilseed rape by foliar application of ASM during the field trials (McGrann *et al.*, 2017). Other authors recommended the use of ASM (sold as Actigard®) in combinations with fungicides and bactericides in tomato spray programs in North Carolina, USA for increased plant resistance and reduction of fungus (inoculum levels) such as early blight (*Alternaria solani*) (Ivors & Louws, 2007).

While previous research work has showed significant reductions (up to 74%) in root-knot nematode (*M. incognita*) reproduction in tomato due to ASM application (Molinari & Baser 2010), the combination of ASM with other pesticides in this study increases the reduction of nematode multiplication on plants. However, more work is needed on the efficacy and economics of large scale application of elicitors such as ASM under commercial field conditions as the compounds related to defence when induced by ASM treatment could be significantly lower where the supply of nitrogen

is limited to plants (Walters & Fountaine, 2009). Further work is also suggested to consider various dose rates of the pesticide and elicitor products to determine the cost effectiveness of combining elicitors with other pesticides. For example, a reduced dose rate if effective would reduce overall cost accrued from the use of combined products. The use of elicitor / pesticide combinations could be valuable in reducing overall pesticide use, and this could delay resistance to pesticide arising and increased longevity in their use. In this study, the combination of elicitor with insecticides offered the best control methods than results obtained from pesticide or elicitor as a sole treatment. However, the application of cultural control methods when incorporated with above in an IPM approach most practical for growers to apply will in doubt enhance LBN management.

Moreover the developed visual symptom key in Chapter 3 is a good tool to assess resistance and its level, and in addition, to evaluate the effectiveness of treatment administered.

It is important to note that nematode extraction method could influence final nematode population from samples, while the method used in this study has been widely used by past workers, various extraction methods could be adopted in future study to compare optimum nematode extraction for *A. fragariae*.

Reaction to treatment by plants could differ, as nematode populations can vary significantly among host plants and over time. In light of this, management of very high nematode populations by insecticide application would be very difficult, especially when the reproductive potential of *Aphelenchoides* species is considered (LaMondia, 1999; Kohl *et al.*, 2010), and as indicated by the correlation between lesions symptom vs nematode population on leaf (Chapter 3). A high level of hygiene is recommended to reduce the risk of nematode infestation, while the use of a pesticide-based approach (with or without elicitors) should be adopted in high-risk situations (e.g. previous history of nematode infestation or nematode presence in soil), at the earliest signs of nematode infestation and or as a preventative approach.

In conclusion, having tested various curative treatments, with resultant reduction in LBN population compared with untreated control. The best management practices, as observed in this study, that could give optimum control is to combine these chemical

treatments (including ASM) along with cultural control methods and a high level of hygiene in an IPM approach.

Chapter 6

Preventative management of leaf and bud nematodes (*Aphelenchoides fragariae*) using novel products on ornamental plants (*Anemone hupehensis* and *Buddleja davidii)* in outdoor and glasshouse conditions in the UK

6.1 Introduction

Aphelenchoides fragariae are microscopic nematodes that live in leaf and tissue and cause significant injury to ornamental plants worldwide (Kohl, 2008). Some of the host species include strawberries, alfalfa, and numerous ornamental plants such as ferns, weigela, begonia and anemone among others (Fig. 6.1; Kohl *et al.*, 2010; Sánchez-Monge *et al.*, 2015).

Chemical treatments such as diazinon, aldicarb, parathion and oxamyl have been used in the past for effective management of LBN (Young & Maher, 2000). However, because of issue related to regulations and environmental concerns, the majority of these chemicals are no longer available to growers. Current chemical control methods have variable efficacy according to the plant species being treated (Bennison, 2007). Treatments by chemicals may be successful via direct contact mortality on nematodes in water, but may be less effective when applied to infected leaves (Jagdale & Grewal, 2002, 2004; An *et al.*, 2017). There are no chemical nematicides currently registered for LBN in the UK, although oxamyl (Vydate 10G) during this study had an Extension of Authorization for Minor Use (EAMU) for outdoor ornamental plant production, the EAMU expired at the end of December 2017.

It has been known that plants are able to actively defend themselves or induce self resistance towards virulent pathogens such as nematodes and fungi (Walters *et al.*, 2014). The treatment of plants against pathogens with various agents including synthetic chemicals, may lead to induced resistance to the attack by pathogens both locally and systemically (Walters *et al.*, 2005). However, Kessmann *et al.*, (1994) observed that 'systemic acquired resistance' (SAR) is the best known defence mechanism for induce resistance, and is identified by the broad spectrum disease

resistance usually mediated by plant hormone 'salicylic acid' (SA). The first synthetic chemical produced as SAR activator is ASM, and has been commercially available in Europe as Inssimo® for use against white rust (*Puccinia horiana*) on chrysanthemums. ASM has demonstrated potential to control LBN in previous glasshouse and field studies (Chapters 4 and 5).

Past research work has indicated that some current insecticides such as abamectin have nematicidal potential, and may be useful for the management of *A. fragariae* (LaMondia, 1999), while Young & Maher (2000) reported the activity of abamectin against leaf and bud nematodes (*A. ritzemabosi*) in both *in vitro* and *in vivo* studies.

Abamectin is currently approved in the UK for the management of two-spotted spider mite and western flower thrips in protected and outdoor flower crops and some ornamentals. Spirotetramat is a systemic insecticide with a two-way mode of action that controls sucking pests such as aphids and whitefly, and has an EAMU for use in ornamental plant production.

Both spirotetramat and abamectin were evaluated against nematode infested nursery plants as a curative treatment (Chapter 5). The results of these trials confirmed the potential of both spirotetramat and abamectin to control LBN by significantly reducing nematode multiplication in the leaves. Both abamectin and spirotetramat were chosen for evaluation against *A. fragariae* as a preventative treatment in field trials due to positive results from the curative trials reported in Chapter 5.

Azadirachtin A. is an important active ingredient of Neem (Khalil, 2013), a product derived from the neem tree (*Azadirachta indica*) known to originate from India and now widely grown in tropical and subtropical areas. Various components derived from neem tree attracted attention for their insecticidal, fungicidal, bactericidal and nematicidal properties (Gajalakshmi & Abbasi, 2004). NeemAzal with the main active ingredient azadirachtin has demonstrated potential as an insecticide in which upon ingestion of azadirachtin by insect larvae, based on multi-action pathways affects insect growth regulation, inhibition of fecundity, toxicity, anti-feedant properties, damage to the larval cuticle and harmful effects on the endocrine system which could prevent moulting activity (Mulla & Su, 1999; Howard *et al.*, 2009; Lynn *et al.*, 2010). Neem based formulations have demonstrated suppression of populations of root knot nematodes, *Meloidogyne incognita* (Lynn *et al.*, 2010), and

potato cyst nematode (*Globodera rostochiensis)* and free living nematodes on potato (Akhtar & Alam, 1991). To date, there has been little research on azadirachtin against LBN under field conditions, although recent bioassay studies showed that azadirachtin (in the form of Neem oil) exhibited between 36-90% mortality in a 20 and 2-fold dilution respectively on *A. fragariae* within 24-72h of exposure (An *et al.*, 2017). Previous results in Chapter 2 in this study also confirmed direct contact mortality of *A. fragariae* with an azadirachtin product. Although azadirachtin was not investigated in the previous curative field trial (Chapter 5), but with the foreseen possibility to approve azadirachtin product in the UK as suggested by the industry representative, this plant extract was consequently chosen for evaluation against *A. fragariae* in a preventative field study. Recently, an emulsifiable neem formulated product, registered as Azatin (azadirachtin 217g litre $^{-1}$) has been approved in the UK for use on protected ornamental plant production (MAPP 18301; Authorisation Number – 0360). Growers in the UK now have the option to use Azatin on ornamental plant, although its approval relates to the control of western flower thrips and onion thrips only.

In addition, as a result of the positive results demonstrated by abamectin, spirotetramat and ASM during the preliminary (bioassays) investigation in Chapters 2 & 4, and when investigated as a curative approach in a field study (Chapter 5), a preventative approach to nematode (*A. fragariae*) infestation was undertaken using these products, and azadirachtin under commercial conditions.

In the search for effective alternatives to oxamyl, two field trials in commercial nurseries and one glasshouse study were conducted to investigate the efficacy of novel products that could limit multiplication of nematodes on nematode-free plants that were inoculated with nematodes post-first treatment, to determine whether plants remain symptom-free and have limited nematode multiplication within the leaves.

Figure 6.1 Foliar symptoms of ornamental plants infested by *Aphelenchoides* spp.: (left) – *Anemone hupehensis*; (middle) - *Dryopteris affinis* (right) - *Buddleja davidii*

The objectives of this study were to determine the efficacy of selected foliar insecticides (spirotetramat and abamectin), Neem plant extract (azadirachtin) and a synthetic chemical elicitor (ASM), as individual treatments, and in combination, for the preventative management of *A. fragariae* on nematode-inoculated ornamental plants.

6.2. Field Study 1 - Commercial nursery 1 – Oxfordshire, UK

6.2.1 Materials and methods

6.2.2 Materials

Plants: Certified nematode-free *Anemone x hybrida 'Honorine Jobert'* commonly cultivated in the United Kingdom was obtained from a commercial ornamental nursery in Oxfordshire, United Kingdom.

Pesticides: Products used in this study were spirotetramat - a systemic insecticide; Abamectin – a contact and translaminar insecticide; Azadirachtin - a plant extract with antifeedant and repellent activity; and ASM (acibenzolar-S-methyl) - an elicitor of induced resistance in plants. Spirotetramat, abamectin and azadirachtin were used as individual treatments and also in combination with ASM.

Nematodes: The nematodes (*Aphelenchoides fragariae*) were sourced and extracted from infested anemone plants. *A. fragariae* was identified based on morphological and morphometric features (Siddiqi, 1975).

6.2.3 Methods

Nematodes were extracted from the infested anemone leaves using the method outlined in Chapter 2, Section 2.2.2 based on a technique described by Kohl *et al.*, (2010); Zhen *et al.*, (2012). The nematode solutions were counted in a 3 x 5cm counting dish under an inverted microscope at x40 to the required concentration of mixed stage individuals per ml. The nematodes were used within 2-3 days after extraction.

Experimental plants were certified to be nematode free using the methods outlined in a previous Chapter 2 - Section 2.2.2. Plants were kept in an isolated area from other nursery plants to avoid any potential nematode infestation, with prevailing average temperature of 11.9^{o}C (night) and 23.5^{o}C (day) as observed during the duration of the trial. Manual irrigation was carried out daily (early in the morning) using water hose for individual plant. This study had eight treatments with five replicates.

Figure 6.2 Layout (left) of the LBN (preventative) experimental trial and treatment application (right) on *Anemone x hybrida 'Honorine Jobert'* plants, Oxfordshire, UK

Plants in 2 litre pots were arranged in a randomised block design (Fig. 6.2). The eight treatments were (i) Spirotetramat (ii) Abamectin, (iii) ASM (elicitor), (iv) Azadirachtin, (v) Spirotetramat + ASM, (vi) Abamectin + ASM, (vii) Azadirachtin + ASM and (viii) Control (Water).

Based on the manufacturer's instructions, all products were prepared in 1 litre of water as shown below:

- Spirotetramat- 1.67ml litre^{-1} water (equivalent to 0.5L/ha/300L water).
- Abamectin 18g/l - 500µl litre^{-1} water (equivalent to 50ml/ha/100L water).
- Azadirachtin A. - 2.5ml litre^{-1} water (equivalent to 3L/ha/1200L water).
- ASM - 0.05g litre^{-1} water (equivalent to 50g/ha/1000L water).
- Adjuvant (0.01% Tween 20) was added to all treatments at the rate of 100µl per litre^{-1} of water including the Control (water).

A hand-held pressurised sprayer (1 Litre) supplied by Scientific Laboratory Supplies (SLS) UK, was used to spray products as a foliar application on each plant until all parts of the plant was well covered (run off). Early morning application of treatments was carried out on pre-irrigated plants (for leaf wetting) about 1 hour after irrigation as previously reported in Chapter 5. Jagdale & Grewal, (2006) found a significant number of nematodes on the leaf surface at 100% relative humidity, in which they suggested that free moisture is required to aid movement and survival of LBN. This report was also supported by findings of Kohl *et al.*(2010) who observed that wet-weather conditions are an important factor for the movement of *A. fragariae* to the leaves of host plants. Control treatments had only water + Tween 20 sprayed on the plants.

All the treatments were applied together on the same day as a foliar application as described above. Nematode inoculation was carried out 4 days after the first treatment on the plants as suggested by Cole (1999). Nematode inoculation was conducted on three randomly selected leaves per plant. 200 mixed stage individuals per ml was applied directly on the leaves based on a technique described by Zhen *et al.*, (2012) and described in detail in Chapter 3, Section 3.2.3.2. This method has been successfully used in previous glasshouse studies (Chapters 3 & 4).

For each treatment programme, spirotetramat and abamectin were applied a total of 2 times while azadirachtin and ASM had 3 applications. Spirotetramat and ASM treatments had a 14 day interval between each application while abamectin and azadirachtin had a 7 day interval between their applications (Table 6.1). Control treatment (water) + Tween 20 were applied at every other treatment applications.

Table 6.1 Treatments and timing of applications

Treatments	**1st (*Day 1*)**	**2nd (*Day 7*)**	**3rd (*Day14*)**	**4th (*Day 28*)**
Spirotetramat	√		√	
Abamectin	√	√		
Azadirachtin	√	√	√	
ASM	√		√	√
Spirotetramat+ASM	√		√	√ (ASM only)
Abamectin+ASM	√		√	√ (ASM only)
Azadirachtin+ASM	√		√	√
Control (water)	√	√	√	√

Plants were observed in the nursery and glasshouse under protected and natural outdoor conditions for eight weeks after nematode inoculation. Daily watering was manually carried out on plants individually along with other plants in the nursery. Random leaf sampling was carried out on plants before the leaf inoculation to confirm the plants were uninfested and again at eight weeks after nematode inoculation to determine the nematode population in the three randomly-selected inoculated leaves. Nematode extraction was carried out as previously described (Chapter 2, Section 2.2.2), according to the technique outlined by Kohl *et al.*, (2010); Zhen *et al.*, (2012).

The nematode extracted samples were refrigerated at 4 °C until counted. Numbers of live and dead nematodes were counted in a 7.5cm counting dish under an inverted microscope at x40.

The total number of *A. fragariae* in the dish was corrected for 5g fresh weight per plant. As leaf samples were sometimes less than 1g, counts were extrapolated according to Kohl *et al.*, (2010).

Visual scoring of nematode symptoms on the 3 inoculated leaves was carried out on each plant at the end of the trial (8 weeks post nematode inoculation). A value of '1-0%' was used as a score for clean (no symptoms) and '6' (75-100%) as maximum lesion symptoms score per inoculated leaf (Fig. 6.3).

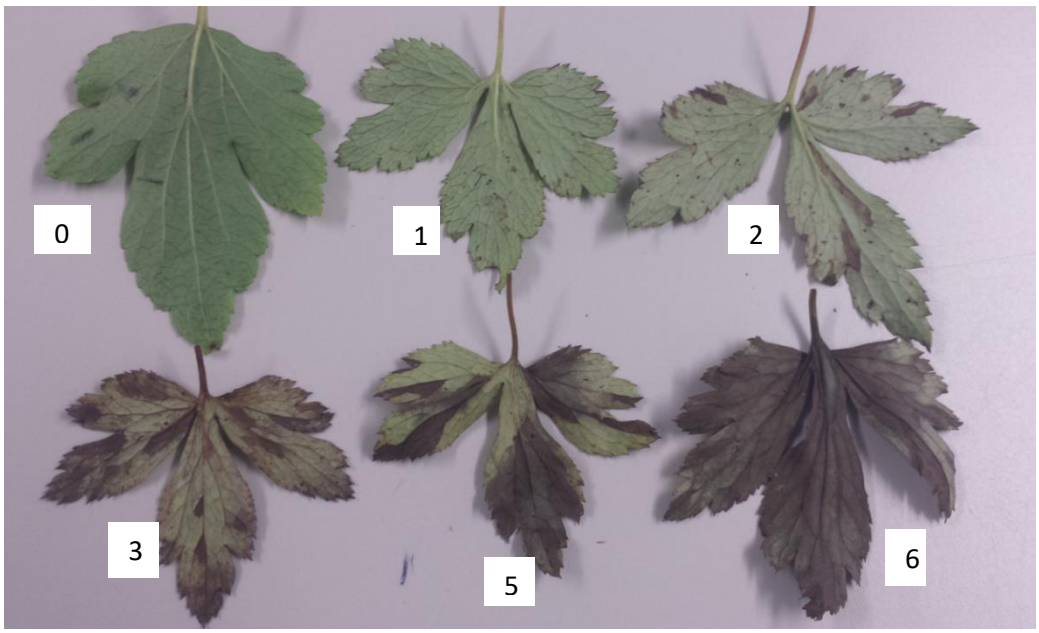

Figure 6.3 Examples of the visual scoring of nematode lesion symptoms on treated and untreated anemone leaves (From top left - Clean '0 %') to (bottom right – '90 %')

6.3. Field Study 2 – Commercial nursery 2 – Herefordshire, UK

6.3.1 Materials and methods

6.3.2 Materials

Plants: Certified nematode-free *Buddleja davidii* 'White Profusion' plants were obtained from Wyevale nurseries, Hereford UK.

Pesticides: The same (products) treatments as used in Field study 1 Section 6.2.3

Nematodes: The nematodes (*A. fragariae*) were sourced from infested *Anemone hupehensis* maintained at the SRUC Plant Growth Unit. The nematode extraction was as described in Chapter 2, Section 2.2.2. *A. fragariae* was identified based on morphological and morphometric features (Siddiqi, 1975).

6.3.3 Methods

Leaf samples taken at random for extraction from these plants confirmed that they were nematode free. Plants were kept in a covered glasshouse facility isolated from other plants (Fig 6.4 & 6.5), with conditions of 16 ± 2°C to 25 ± 2°C.The study had the same eight treatments and five replicates (Fig. 6.4), as used in Field Study 1. Other methods used were the same as in Section 6.2.3.

Daily watering was carried out manually on individual plants along with other plants in the nursery. Eight weeks after nematode inoculation on the leaves, (4 weeks post final product application), sampling of the inoculated leaves for nematode extraction was undertaken to obtain the final nematode population per treatment using the method described above for nematode extraction in Section 6.2.2.

Figure 6.4 Layout of *Buddleja davidii* 'White Profusion' experimental plants at Field Study 2

Figure 6.5 *Buddleja davidii* 'White Profusion' leaves (+yellow tag) wrapped with tissues after direct inoculation at Field Study 2

Visual scoring of nematode symptoms on the 3 inoculated 'tagged' leaves (Fig. 6.5 & 6.6) was carried out on each plant at the end of the trial. A value of '1' - 0% (clean - no symptom) and maximum value of '6' for 75% - 100% (highly infested) per leaf (Fig. 6.6) was used for leaf symptoms.

Figure 6.6 Nematode symptoms on *Buddleja davidii* 'White Profusion' leaves (blue arrowed) with visual scores: left – '10% 'and right – '30%' in Field Study 2.

6.4. Experimental Study 3 – Scotland Rural College (SRUC) – Plant Growth Unit, Edinburgh, Scotland, UK

6.4.1 Materials and methods

6.4.2 Materials

Plants: Certified nematode-free 'plug' plants of *Anemone hupehensis* var. japonica 'Prinz Heinrich' (henceforth called *Anemone hupehensis*) were obtained as rootstock from a commercial nursery, Jackdaws' Field Nursery, Horsham, West Sussex UK.

Pesticides: The same products were used as in Section 6.2.3.

Nematode: The nematodes (*A. fragariae*) were sourced and extracted from infested anemone plants (*Anemone hupehensis*) maintained in the SRUC glasshouse. The nematode extraction was as previously described in Chapter 2, Section 2.2.2. *A. fragariae* was identified based on morphological and morphometric features (Siddiqi, 1975).

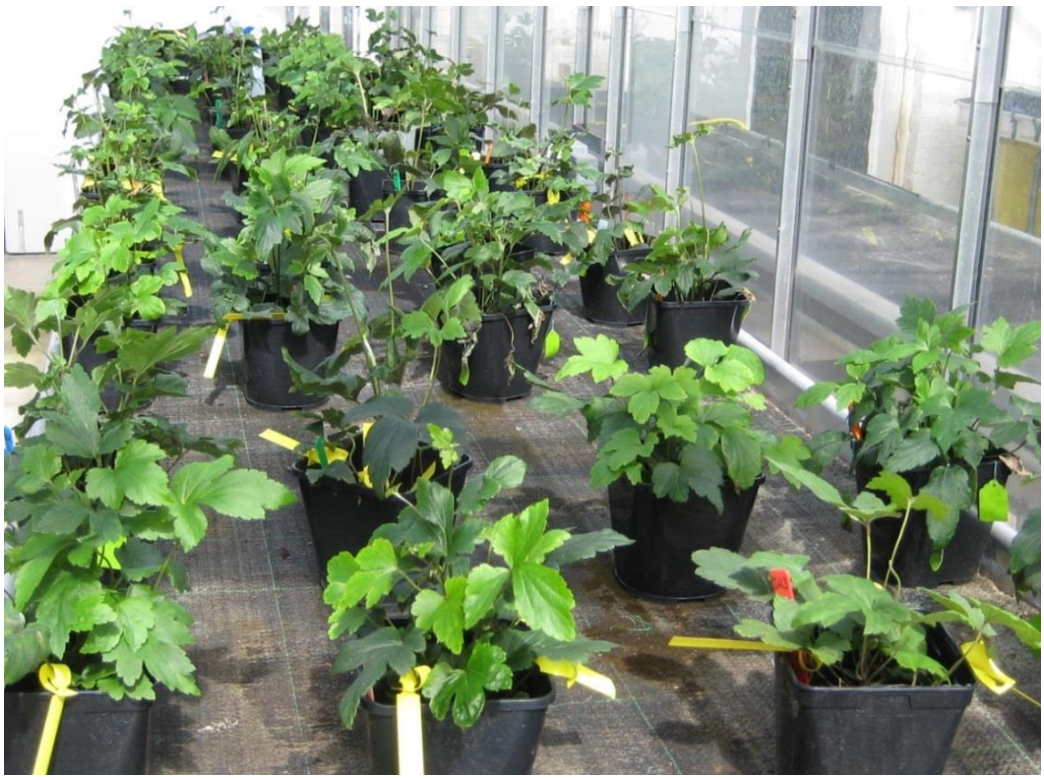

Figure 6.7 Layout of nematode preventative study on *Anemone hupehensis* at SRUC Plant Growth Unit, Edinburgh, UK

6.4.3 Method

Plants were grown in the glasshouse until they had a minimum of 6-8 leaves. Leaf samples were taken at random for extraction from these plants to confirm that they were nematode free. Plants were kept in a glasshouse facility and isolated from other plants with conditions of 20 ± 2°C to 25 ± 2°C. Daily watering was carried out manually on individual plants to prevent water splash in the glasshouse.

Figure 6.8 Layout of experimental block (left) and inoculated (tagged) *Anemone hupehensis* leaf (right) at SRUC Growth Unit, Edinburgh, UK

The study had the same eight treatments and five replicates (Fig. 6.7 & 6.8) as used in the previous field studies - Section 6.2.3.

All other methods used were the same as in Field Studies 1 & 2 (Section 6.2.3).

Visual scoring of nematode symptoms on the 3 inoculated leaves was carried out on each plant at the end of the trial. A value of '1' as 0% was used for clean leaf (no symptoms) and '6' (75-100%) as maximum score per leaf symptoms (Fig. 6.9).

Figure 6.9. Visual symptoms scores on inoculated leaves of *Anemone hupehensis* (+ blue arrows): left leaf – (30%); middle leaf (10%); and right leaf (60%) at SRUC Growth Unit, Edinburgh, UK

Eight weeks after nematode inoculation on the leaves (4 weeks post final product application), sampling of the inoculated leaves for nematode extraction was carried out to obtain the final nematode population per treatment using the method described in (Section 6.2.2).

Data analysis: Statistical analyses are same for all the three field trials. Analysis of data collected was carried out as that used by Jagdale & Grewal, (2002, 2006). Data of nematode populations and leaf symptom score at eight weeks after inoculation (4 weeks post final treatment) were analysed by analysis of variance (ANOVA) using a General Linear Models Procedure (Minitab 16). Variables include treatment, leaf symptom score and final nematode population count. Significant differences between treatments were determined with Fisher's multiple range test at $P < 0.05$.

6.5 Results

6.5.1 **Field Study 1** - Commercial nursery 1 – Oxfordshire, UK

Anemone x hybrida 'Honorine Jobert'**:** There was a significant ($P < 0.05$) difference in the final nematode population between all the treatments and the untreated Control (Table 6. 2).

Table 6.2 Nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Anemone x hybrida* 'Honorine Jobert'. Data are the mean (± SE) of 5 replicates. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

Treatments	**Mean nematode *population/1g of leaf***
Spirotetramat	381.4bc (±15.03)
Abamectin	408.9bc (±15.96)
Azadirachtin	503.5b (±14.95)
ASM	476b (±23.75)
Azad + ASM	323.8cd (±14.41)
Abam + ASM	196.5de (±10.10)
Spiro + ASM	157.8e (±4.46)
Control (Water)	2570.2a (±68.32)

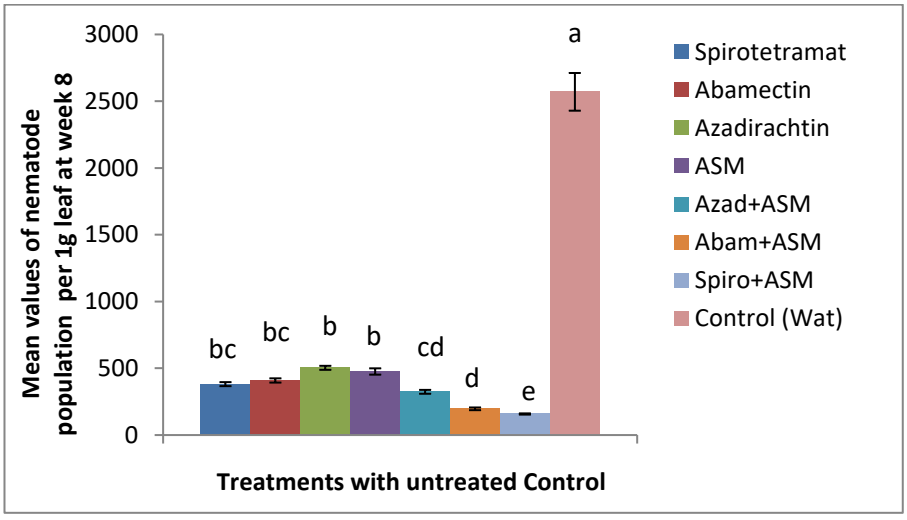

Figure 6.10 Mean (± SE) nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Anemone x hybrida* 'Honorine Jobert'. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

Mean values obtained for nematode population in the leaves range between 157 and 2570 per 1g of leaf (Table 6.2). Spirotetramat + ASM had the lowest mean population (157) while the untreated Control gave the highest mean nematode population of 2570 (Table 6.2). A significantly lower nematode population (P < 0.05) was observed in the leaves in all the treatments compared to the untreated Control (Fig. 6.10; Table 6.2).

The treatment azadirachtin + ASM (mean nematode population of 323.77) was statistically lower (P <0.05) than the spirotetramat, abamectin, azadirachtin and ASM treatments, but significantly higher than the abamectin + ASM and spirotetramat + ASM treatments (Fig. 6.11). In general, there were significant differences (P <0.05) between all the treatments and the untreated Control in nematode mean population (Fig. 6.10).

All the treatments combined with ASM had a significantly lower nematode population than the solo treatments (Fig. 6.11; Table 6.3).

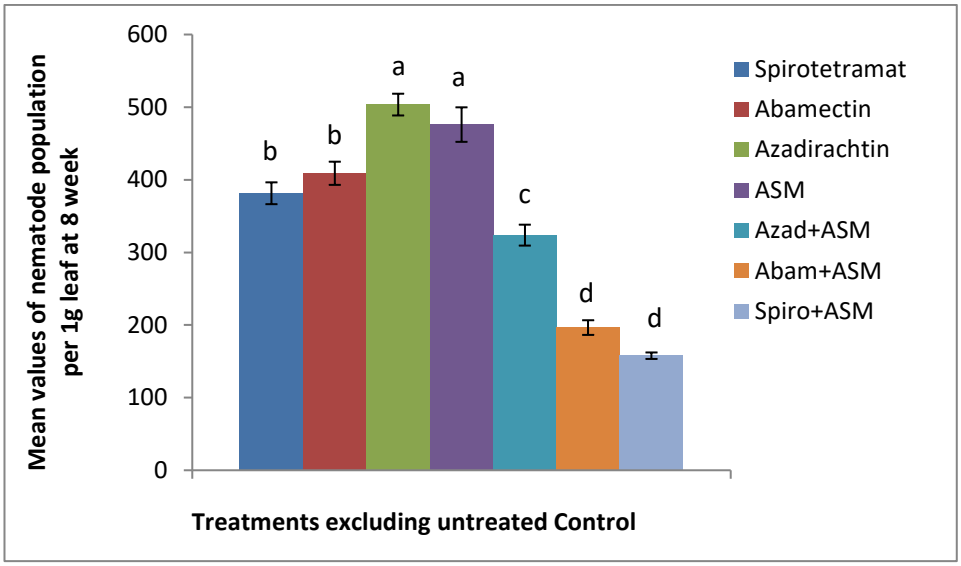

Figure 6.11 Mean (±SE) nematode population from 1g of leaf eight weeks after inoculated with 200 *A. fragariae* on *Anemone x hybrida* 'Honorine Jobert' excluding the untreated Control. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

Table 6.3 Response to ASM ± pesticides on the management of *A. fragariae* inoculated on leaves of *Anemone x hybrida* 'Honorine Jobert' in Field Study 1. Data are mean values of five replicates. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

Treatments	***Mean nematode population 1g of leaf***	
Spirotetramat	381.5	a
vs		
Spiro+ ASM	157.8	b
Abamectin	408.9	a
vs		
Abam+ ASM	196.5	b
Azadirachtin	503.5	a
vs		
Azad+ ASM	323.8	b

6.5.2 **Field Study 2** - Commercial nursery 2 – Herefordshire, UK

Buddleja davidii 'White Profusion'*:* There was a significant (P <0.05) difference in final nematode population between all the treatments and the untreated Control (Table 6.4).

Table 6.4 Value of nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Buddleja davidii 'White* Profusion'. Data are the mean (±SE) of 5 replicates. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05)

Treatments	**Mean nematode *population/1g of leaf***
Spirotetramat	362.2c (±13.01)
Abamectin	475.4b (±37.32)
Azadirachtin	540.7b (±27.44)
ASM	531.7b (±23.11)
Azad+ ASM	141.2d (±8.88)
Abam + ASM	158.6d (±14.61)
Spiro + ASM	72.9d (±10.10)
Control (Water)	2454a (±45.01)

The mean values obtained for nematode population in the leaves range between 72 and 2454 in 1g of leaf (Table 6.4). Spirotetramat + ASM had the lowest nematode population mean value (72.9), while the untreated Control gave the highest nematode population mean of 2454 (Table 6.4).

A significantly lower (P <0.05) nematode population was observed in the leaves in all the treatments compared to the untreated Control (Fig. 6.12; Table 6.4).

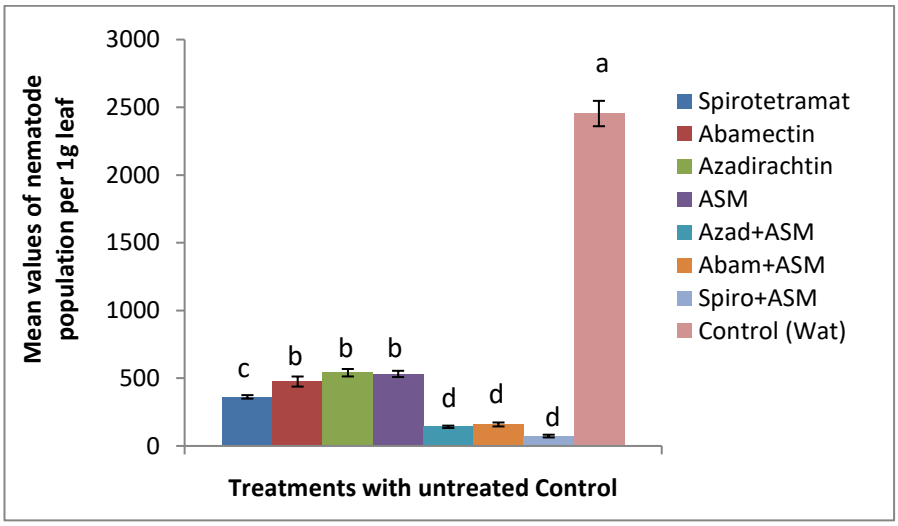

Figure 6.12 Mean (± SE) nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Buddleja davidii* 'White Profusion' with untreated Control. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

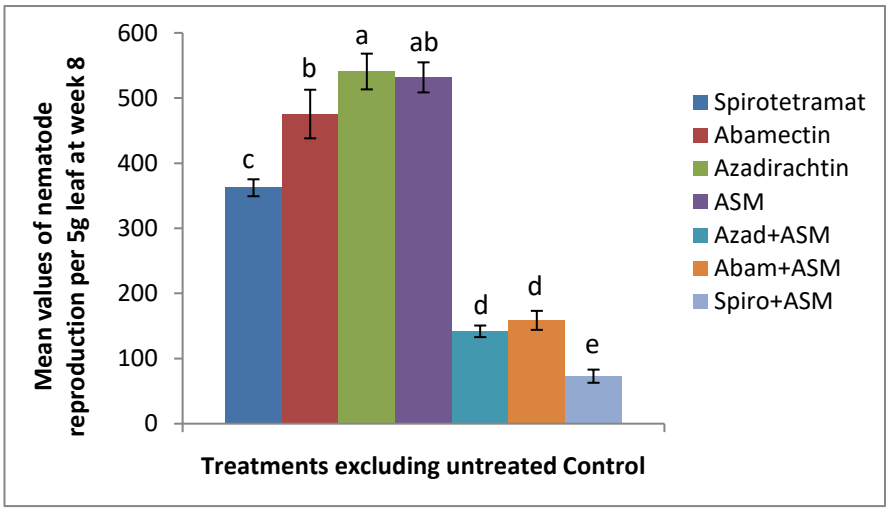

Figure 6.13 Mean (± SE) nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Buddleja davidii* 'White Profusion' excluding untreated Control. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

All the treatments combined with ASM had a significantly lower nematode population than the solo treatments (Fig. 6.13; Table 6.5).
In general, there were significant differences (P <0.05) between all the treatments and the untreated Control in nematode mean population (Fig. 6.12).

Table 6.5 Response to ASM ± pesticides on the management of *A. fragariae* inoculated on leaves of *Buddleja davidii* 'White Profusion' in Field Study 2. Data are mean values of five replicates. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.01).

Treatments	***Mean nematode Population /1g of leaf***
Spirotetramat	362.2 a
vs	
Spiro+ASM	72.9 b
Abamectin	475.4 a
vs	
Abam+ASM	158.6 b
Azadirachtin	540.7 a
vs	
Azad+ASM	141.2 b

6.5.3 Field Study 3 – SRUC Growth Unit Edinburgh, UK

Anemone hupehensis: There was a significant (P <0.05) difference in final nematode population between all the treatments and the untreated Control (Table 6.6).
The mean values for nematode population in 1g of leaves range from 206.5 to 5005 (Table 6.6). The lowest nematode population of 206.5 was obtained from spirotetramat + ASM while the untreated Control gave the highest nematode population of 5005 (Fig. 6.14; Table 6.6). There was a significantly lower (P <0.01) nematode population observed in the leaves in all the treatments compared to the Control (Fig. 6.14; Table 6.6).

Table 6.6 Mean nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Anemone hupehensis*. Data are the mean (±SE) of 5 replicates. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05.

Treatments	**Mean nematode *population/1g of leaf***
Spirotetramat	423.3bc (±24.02)
Abamectin	584.7b (±52.57)
Azadirachtin	609.2b (±27.71)
ASM	576.8b (±52.94)
Azadi + ASM	314.2c (±21.48)
Abame + ASM	292.4c (±15.18)
Spiro + ASM	206.5c (±23.03)
Control (Water)	5005.3a (±119.5)

The nematode population of azadirachtin + ASM (314), abamectin + ASM (292) and spirotetramat + ASM (206) were significantly lower (P <0.05) than the azadirachtin, abamectin and ASM treatments, but with no significant difference with the spirotetramat treatment (Fig. 6.14).

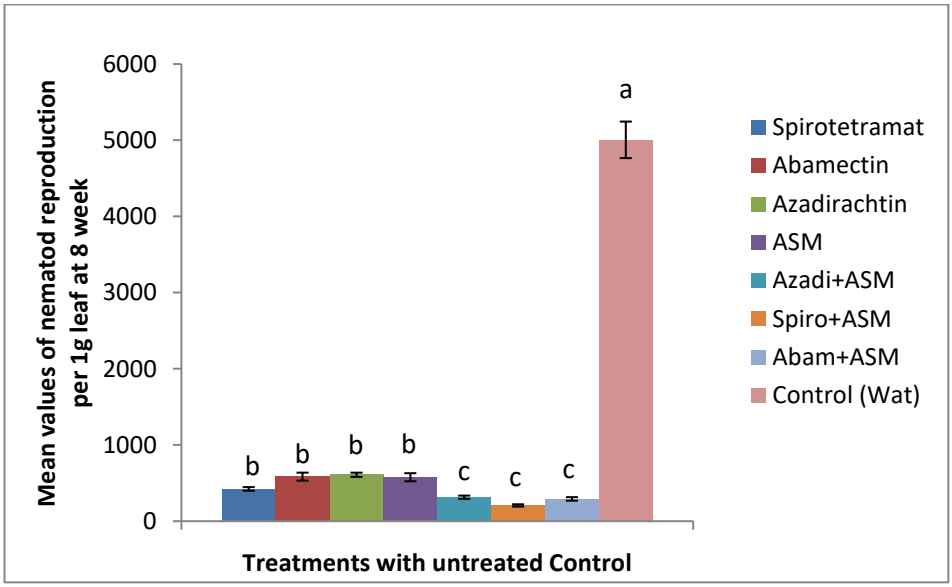

Figure 6.14. Mean (± SE) nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Anemone hupehensis*. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

All the treatments with ASM had a significantly lower nematode population than the solo treatments (Fig. 6.15; Table 6.7).

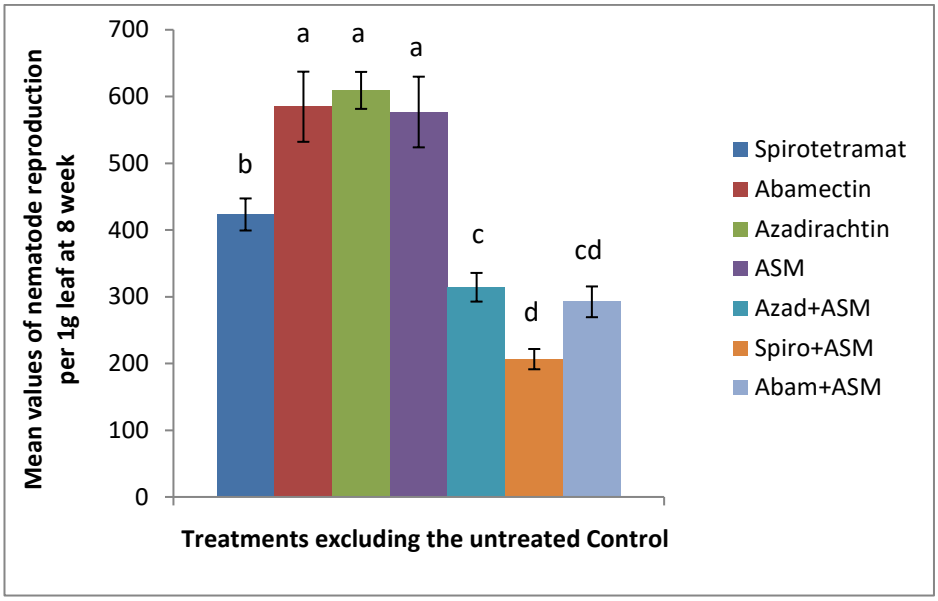

Figure 6.15. Mean (± SE) nematode population from 1g of leaf eight weeks after inoculation with 200 *A. fragariae* on *Anemone hupehensis* excluding untreated Control. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

Table 6.7 Response to ASM ± pesticides on the management of *A. fragariae* inoculated on leaves of *Anemone hupehensis* in a glasshouse study in Location 3. Data are mean values of five replicates. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.01).

Treatments	***Mean nematode population/1g of leaf***
Spirotetramat	423.35 a
vs	
Spiro+ASM	206.48 b
Abamectin	584.7 a
vs	
Abam+ASM	292.4 b
Azadirachtin	609.21 a
vs	
Azad+ASM	314.18 b

In general, there were significant differences (P <0.05) between all the treatments and the untreated Control in nematode mean population (Fig. 6.14; Table 6.6).

6.6 Discussion

The trials at 3 sites have identified products that can play a significant role in the preventative management of LBN infestation on ornamental plants by reducing the appearance of symptoms of nematode infestation through reduction of nematode multiplication in plants. Some of these products also have a curative role (see Chapter 5) by reducing nematode multiplication within already infested plants. Foliar application of these products with or without the elicitor ASM significantly reduced the multiplication capacity of *A. fragariae* on these plants. In addition, this study demonstrated that treatment with all the products evaluated led to a lower population of *A. fragariae* in both glasshouse and outdoor conditions by up to 60% when compared with the untreated Control. The most effective products were treatments in a programme with ASM (spirotetramat + ASM, abamectin + ASM and azadirachtin + ASM.

These products when applied alone also reduced *A. fragariae* multiplication and symptoms compared to the untreated Control but to a lesser degree.

In all the 3 field studies, lower nematode multiplication of up to 50% was demonstrated in treatment with combinations with the elicitor than all the treatments of stand alone insecticides; results therefore gave significant differences (P <0.05) between insecticide alone (spirotetramat, abamectin, azadirachtin) and when insecticides were combined with elicitor ASM. However when the insecticides were used alone, there was a significant (P <0.05) nematode multiplication reduction and symptoms of *A. fragariae* compared to the untreated Control. Results obtained when the three insecticides were compared with ASM indicate a lower nematode multiplication of 10-15% by abamectin and spirotetramat than ASM, while azadirachtin and ASM were similar in nematode multiplication as a stand alone product.

Results obtained from abamectin with or without the elicitor ASM agreed with the past work of LaMondia (1999) that foliage applications of abamectin reduced *A. fragariae* populations in *Lamium maculatum* plants, although the author used 0.6 ml

litre^{-1} Avid – (0.011g litre^{-1} abamectin), a higher dose than the 0.5 ml/litre^{-1} abamectin (0.009g litre^{-1}) that was used in this study. Chałańska *et al.*, (2017) reported 41.7% reduction of *A. ritzemabosi* in leaves of *Anemone hupehensis* with 2 foliar applications of combined *Allium sativum* and abamectin (same concentration as was used in this study).

Spirotetramat and ASM have not been fully evaluated for their capacities to suppress multiplication of LBN in ornamental plants, although Chałańska *et al.*, (2017) found a combination of spirotetramat and *A. sativum* ineffective in controlling multiplication of *A. ritzemabosi* on infested *Anemone hupehensis* plants. This study is one of the few studies investigating azadirachtin and spirotetramat as potential nematicides on LBN, and the first study evaluating ASM to manage LBN on ornamentals. Foliar applications of spirotetramat, azadirachtin, and ASM at the manufacturer's recommended dose rate to the foliage of *A. fragariae* inoculated plants all caused a significant reduction in the mean nematode multiplication compared to untreated plants, suggesting that reproduction of *A. fragariae* in the leaves was hindered by these products.

Spirotetramat is reported to have both phloem and xylem mobility (ambimobile) in several plant species (Nauen *et al.*, 2008). Foliar application of spirotetramat to nematode inoculated plants had a strong suppressive effect on the multiplication of *A. fragariae* on *Anemone hupehensis*, *Anemone x hybrida* 'Honorine Jobert' and *Buddleja davidii 'White* Profusion' when compared with the untreated Control. This effect is similar to the report of a reduced reproduction of cereal cyst nematode (*Heterodera avenae*) when spirotetramat was applied to the foliage of spring wheat on two nematode infested fields in the United States (Smiley *et al.*, 2011).

Spirotetramat once applied and absorbed inside the leaf is hydrolysed to spirotetramat-enol, the form in which the compound enters the xylem and phloem and is translocated throughout the plant. Spirotetramat-enol is a lipid biosynthesis inhibitor and reduces fecundity and fertility when ingested orally via xylem and phloem by insects (Smiley *et al.*, 2011). This may also be the case with *A. fragariae* and may explain why spirotetramat had low contact mortality in bioassays against *A. fragariae* (see Chapter 2).

Spirotetramat gave more consistent nematode control of *Meloidogyne incognita* and *Heterodera glycines* on tomato and soybean respectively when applied as a curative control rather than preventative approach; this is because the spirotetramat-enol concentration might have dropped below the effective level if applied earlier than nematode invasion (Vang *et al.*, 2016). This report supported the previous findings that spirotetramat does not stop nematode invasion into the plant but the efficacy is noticed when nematodes ingest spirotetramat through the phloem and xylem (Nauen *et al.*, 2008), indicating that nematode invasion must occur, before efficacy is achieved. This current study had repeated applications of spirotetramat with the first application carried out 4 days after nematode inoculation and the second application 14 days later. In Chapter 5, spirotetramat was applied as a curative approach (post nematode invasion) on already LBN infested plants, and both approaches (curative and preventative) demonstrated efficacy compared to the untreated Control.

Spirotetramat is an insecticide registered in the UK for controlling many sucking insect pests including aphids, with an EAMU approval (COP 2011/00954 PP) on ornamental plants.

Foliar application of abamectin at the manufacturers recommended dose rate led to a significant reduction in nematode multiplication when compared with the untreated Control.

Past research work has reported that repeated applications of abamectin reduced both *A. fragariae* and *Ditylenchus dipsaci* populations in *Lamium maculatum* and *Phlox subulata* in the USA (LaMondia, 1999). Young & Maher (2000) demonstrated the activity of abamectin against LBN (*A. ritzemabosi*) *in vitro* and *in vivo*. Abamectin was also reported to have potential to control LBN on hardy nursery stock in the UK (Young & Maher, 2000), while on the contrary, abamectin was found to be ineffective among a range of alternatives evaluated for the control and management of leaf and bud nematodes by Bennison (2007). In recent research, a repeated foliar application of abamectin combined with *A. sativum* extract reduced *A. ritzemabosi in vivo* on *Anemone hupehensis* (Chałańska *et al.*, 2017).

The inconsistent efficacy of abamectin could be influenced by the use of adjuvants and the timing of product application. In this study, early morning application of treatments before sunrise was carried out on pre-irrigated plants about 1 hour after

irrigation. Jagdale & Grewal (2006) noted that at 100% relative humidity, a significant number of nematodes were recorded on the leaf surface, suggesting moisture is required to aid the movement and survival of LBN, while a similar report observed that wet-weather conditions are an important factor for the movement of LBN on host plants (Kohl *et al.*, 2010).

The adjuvant Tween 20 used in this study has a good compatibility with abamectin, (and other products), and there was no phytotoxicity observed due to the use of abamectin.

There have been previous evaluations of azadirachtin for its contact mortality in aqueous suspension on LBN (Chapter 2; An *et al.*, 2017), however, azadirachtin and other neem derived products have not been fully evaluated for their capacity to suppress LBN in ornamental plants. Past research work also observed that Neema, an extracted oil of neem seeds and Neema-plus, a pellet type of seed remnants after oil extraction caused rapid immobilisation of *Meloidogyne incognita* with 1% Neema on root and soil of cucumber plants grown in nematode infected soil (Lynn *et al.*, 2010). Foliar application of azadirachtin A. 1% in this study gave a significantly reduced nematode population compared with the untreated Control. The result in this study is in agreement with suggestions that azadirachtin is a useful tool in integrated nematode management programs (Khalil, 2013). A neem formulated product registered as Azatin (a.i. azadirachtin), for use on protected ornamental plant production, has been approved as insecticide in the UK. However, it should be noted that the approval relates to the control of western flower thrips and onion thrips only.

Results from ASM treatments have confirmed the previous reports in Chapters 4 and 5 that ASM can reduce LBN multiplication and symptoms as individual treatments and when used in combination with other pesticides (Rotifa, 2017). ASM is registered in the UK as Inssimo® for use as a plant growth activator in ornamental plant protection (protected chrysanthemums) (Authorisation Number 0121/2015; MAPP-16870), and as Bion® for use as a plant growth activator on wheat (Authorisation Number: 0590/2003; MAPP-09803). ASM has been reported to induce resistance to pathogens when applied to plants (Kessmann *et al.*, 1994). Previous research work also has showed significant reductions (up to 74%) in root-knot nematode (*Meloidogyne incognita*) reproduction in tomato due to ASM

application (Molinari & Baser, 2010), while a consistently reduced infection of light leaf spot disease (*Pyrenopeziza brassicae*) was obtained on winter oilseed rape by foliar application of ASM during the field trials (McGrann *et al.*, 2017).

This study has confirmed the previous results of Chapters 4 and 5 that foliar application of ASM (3x), on nematode inoculated *Anemone hupenhensis*, or naturally infested ornamental plants significantly reduced the population of *A. fragariae* and exhibited less leaf symptoms when compared with the untreated Control (Rotifa & Evans, 2016; Rotifa *et al.*, 2016).

There was similarity between results obtained from all 3 locations in term of efficacy of nematode management, population reduction and control in response to the application of ASM to all the plants investigated.

Previous researchers have recommended the use of ASM (called Actiguard® in USA) in combinations with fungicides and bactericides in tomato spray programs in North Carolina, for increased plant resistance and reduction of inoculum levels of early blight *Alternaria solani*.(Ivors & Louws, 2007) Other researchers have found that elicitor plus fungicide combinations provided the most consistent disease control of *Rhynchosporium commune* on a barley crop (Walters *et al.*, 2014).

The use of elicitor / pesticide combinations could be important in reducing overall pesticide use, and could prolong the development of resistance to pesticide, since ASM is not directly antimicrobial (Vallad & Goodman, 2004), and subsequently increase pesticide longevity. It could also serve as a valuable tool for IPM control of nematode management. These results will help to build up support for further extension of ASM on protected ornamentals according to industry representative, as the benefit of ASM is not limited to LBN alone.

Chapter 7

Efficacy of novel products as soil (plant media) treatments to prevent migration of leaf and bud nematodes (*Aphelenchoides fragariae*) from infested soil to leaf of *Anemone hupehensis* plants

7.1 Introduction

Aphelenchoides fragariae, are endo and ectoparasites of various plants most especially in the ornamental sector. They often cause serious economic loss to several nursery-grown herbaceous and woody perennials. Common ornamental host plants include anemone, begonia, bergenia, dahlia, ferns, ficus, hibiscus, hosta, salvia, weigela among others (LaMondia, 1999; Kohl *et al.*, 2010; Sánchez-Monge *et al.*, 2015). The nematodes are able to overwinter in dried leaves, soil and dormant crowns (Wallace, 1959; Jagdale & Grewal, 2006). Loss of plants due to LBN in the UK according to Young & Maher (2000) was estimated at £5,000 - £15,000 per annum depending on the size of the nursery; an estimate that must have increased by now. Almost the double estimate of above amount was given recently by growers during personal discussion on the economic loss caused by LBN.

LBN problems have become more common due to the revocation and subsequent loss of effective systemic nematicides (diazinon, aldicarb and parathion), to regulatory issues and environmental concerns (Jagdale & Grewal, 2002; An *et al.*, 2017), increased nursery production and long distance movement of plants (Young & Maher, 2000). Current popular management strategies include sterilisation of equipment and minimal use of overhead irrigation, as water splash can aid movement of nematodes from infested leaves to non infested leaves, and avoiding the use of already infested soil or plant media for planting (Kohl *et al.*, 2010; Chałańska *et al.*, 2017.

Hot water treatment have been used to manage PPN by treating bulbs, bare-rooted plants, dormant crowns, suckers and runners of many economically important crops (Tsang *et al.*, 2001; Fallik, 2004; Coyne *et al.*, 2010). Successful hot water drenching

at 70°C, 90°C and 100°C have been carried out against overwintering *A. fragariae* in pots to prevent migration to the leaves of Hosta plants with no adverse effect on dormant crowns (Jagdale & Grewal, 2004).

Caliente liquid mustard comprises isothiocyanates derived from mustard, and capsicums derived from chilli pepper, has been reported previously for the suppression of PPN (Ramirez *et al.,* 2009), weeds (Brown & Morra, 1995), and pathogenic fungi (Kirkegaard *et al.,* 1996). These compounds act as natural bio-fumigants (Brown & Morra, 1995), and soil-borne pest and diseases are suppressed due to the biocidal effect of isothiocyanates derived from glucosinolates (Kirkegaard *et al.,* 1996). However, due to the impact of these bio-fumigant compounds on non-target species, some beneficial organisms could be affected (Ibekwe, 2004; Ramirez *et al.,* 2009). On a positive note, the incorporation of *Brassica juncea* (Indian mustard), *Eruca sativa* (Nemat) and *Raphanus sativus* during both winter and summer seasons reduced significantly populations of *Globodera pallida* on potato field trials (Ngala *et al.,* 2015).

Nemanator, made up of liquid molasses, fertilizer blend (concentrate) and bacteria strains (*Bacillus thuringiensis*) is currently undergoing evaluation for nematicidal activity in the USA by OceanGrown Incorporation. Among the success previously recorded by *B. thuringiensis* on insects include toxicity against caterpillars (Lepidoptera), beetles (Coleoptera) and mosquitoes (Diptera) among others, but the bacteria recorded low or no toxicity against other animals (Schnepf *et al.,* 1998). Wei *et al.* (2003) reported that *B. thuringiensis* demonstrated toxicity efficacy against free-living nematodes that infect animal and plants in laboratory bioassays.

Fluopyram controls plant pathogenic fungi through disease development inhibition in several fruit, vegetable and field crops, and has shown positive control over several isolates of *Botrytis cinerea* which had earlier resisted control to Boscalid fungicide in strawberry (Amiri *et al.,* 2014). Fluopyram (pyridinyl-ethyl benzamide*)* was developed by Bayer CropScience from the succinate dehydrogenase inhibitor (SDHI) group, and labelled as a fungicide with broad activity against various fungi and shown to inhibit disease development on plants (Wang *et al.,* 2017). The potential role of fluopyram (coded as SC400 in this project; Table 7.1) for the management of

PPN was reported by Faske & Hurd (2015) against *Meloidogyne incognita* and *Rotylenchus reniformis* on tomato roots in the USA. *Caenorhabditis elegans* when exposed to fluopyram above 10ppm in laboratory bioassays for 24hrs did not recover (Heiken, 2017). Fluopyram significantly reduced nematode populations in soil and soybean plants inoculated with *Heterodera glycines* and *Meloidogyne incognita* (Heiken, 2017).

Flocter (*Bacillus firmus-strain* I-1582), a wettable powder (WP) is a biological nematicide, used for the treatment of free living soil nematodes with no recorded adverse effect on field plants (Hitzberger *et al.*, 2014). Flocter was debuted by Bayer CropScience in Italy for the control of major nematode pests on carrots, tomatoes, aubergines, melons and tobacco (Hitzberger *et al.*, 2014). This wettable powder formulation is recommended for use in greenhouse and field as part of IPM programme.

Registration of garlic as a pesticidal product in the European Union market has been challenging due to the biochemical complexity of garlic, and inconsistent results of garlic based materials (Anwar *et al.*, 2016). Sulphur compounds present in garlic have potential as nematicides or insecticides against nematodes of carrot and parsnip in the UK, and root knot nematodes of carrot & tomato in Italy (Anwar *et al.*, 2016). ECOspray, a UK based business has obtained a source of a chemically consistent garlic extract, and subsequently obtained approval as crop protection product, with increasing evidence that food grade garlic extract can cause mortality to nematodes and other important agricultural pests (Anwar *et al.*, 2016). Garlic based insecticides have now been approved in Denmark and Norway as ECOguard for cabbage root fly control, and ECOspray in the UK obtained regulatory approval for a product called 'Eagle Green Care' as a liquid nematicide for pest control on elite sports turf (MAPP No.14989). Other approved garlic extract products include NEMguard granules (MAPP No. 15254) for carrot and parsnip, NEMguard PCN Granules (MAPP No. 17377) approved for potato, and NEMguard DE (MAPP No. 16749) to control root knot nematodes (*Meloidogyne* spp.) and stem & bulb nematodes (*Ditylenchus dipsaci*) on outdoor bulb onion; and against free living nematodes on outdoor garlic, leek, fodder beet and red beet. There are however, some concerns regarding the use

of garlic extracts as a nematicide due to their effects on non-target organisms such as the nematode *Phasmarhabditis hermaphrodita*, which is a biopesticide used for slug management, which exhibited high mortality due to the presence of polysulfides (Anwar *et al.*, 2009).

An *et al*, (2017) evaluated a range of products through soil drenching against *A. fragariae*, and the results showed 50–80% mortality. Two products: Pylon (chlorfenapyr) and Nemakill (containing cinnamon, clove and thyme oil) caused 100% mortality in soil within 7–42 days post treatment (An *et al.*, 2017). Some chemicals such as oxamyl 10%, diazinon (Diazinon 4E), trichlorfon (Dylox 6.2G), ethoprophos (Mocap 10G) and peroxyacetic acid (Zerotol) have been investigated for efficacy on *A. fragariae* through soil drenching of infested Hosta plants, and led to over 70% population reduction in the soil and leaves from 15-45 days after treatment (Jagdale & Grewal, 2002). Oxamyl has been reported to cause reduction in *A. fragariae* populations of 72.2% in leaves and 76.9% reduction over control in soil around infested Hosta plants at 45 days after treatment (Jagdale & Grewal, 2002). The efficacy of oxamyl as a nematicide against free living nematodes has long been established, and is known to work systemically against target pests (Wright *et al.*, 1980; Whitehead *et al.*, 1984; Osborn *et al.*, 2010). Oxamyl has been reported to cause reduction in LBN (*A. fragariae*) populations of 72.2% in leaves and 76.9% reduction over control in soil around infested Hosta plants at 45 days after treatment (Jagdale & Grewal, 2002) compared with this study of 91% reduction over control in soil. While oxamyl is unavailable in the USA (Walker *et al.*, 1997; Jagdale & Grewal, 2002), its availability to some growers in the UK during this study was also restricted. The last EAMU of oxamyl, for outdoor ornamental plant production in the UK, which ended December 31st 2017, only allowed use on outdoor ornamentals when applied by a mechanical granule applicator, hence cannot be safely carried out by hand-held equipment. Presently, oxamyl (which has been reported to have incidental control of *A. fragariae*) can no longer be used by growers of protected ornamentals and containers as the EAMU for oxamyl ended at the end of 2017. Although oxamyl had a long term restriction during this study, its selection along with the candidate treatments was to serve as standard nematicide.

There is therefore a need to identify products that could be applied to the soil or plant media to prevent migration of LBN from infested soil to the plant foliage, for effective management of this important pest. This subject is targeted as treatment that could be adopted for use in soil of stock beds, but most especially, in the container grown plant media. Following the inoculation methods tested in Chapter 3 of this thesis, products selected for this study were evaluated using the successful tool identified in Chapter 3. Candidate products were considered based on the following criteria: products with potential information from the literature, products that have showed potentials to control nematode though with some controversial reports, products with accidental control on LBN when used for their approved pests, and more importantly, based on results from the previous laboratory bioassays of this project (Chapter 2).

The objective of the study in this Chapter was to evaluate the efficacy of available chemical, and other environmental friendly products such as biological and plant extract products to prevent migration of *A. fragariae* from plant media to the leaves of *Anemone hupehensis*.

7.2 Materials and methods

7.2.1 Materials

A bioassay was developed to evaluate the efficacy of several products (Table 7.1) for preventing the spread of *A. fragariae* inoculated in soil (plant media) to *Anemone hupehensis* plants in glasshouse conditions of 25° ± 2°C.

Nematodes: The nematode species (*A. fragariae*) were from the same source as reported in Chapter 2, Section 2.2.2. Nematodes were isolated from infested evergreen fern (*Woodwardia fimbrata*) and multiplied on the leaves of clean *Anemone hupehensis*. Nematodes were extracted as described in Chapter 2 - Section 2.2.2.

Plants: Certified nematode-free (plug plants) *Anemone hupehensis* var. japonica ‘Prinz Heinrich’ (henceforth referred to as *Anemone hupehensis)*, were obtained as rootstock from a commercial nursery, Jackdaws' Field Nursery, Horsham, West Sussex, UK.

Products: The seven products evaluated were (i) Allyl isothiocyanate & capsicum chilli pepper, (ii) Oxamyl – used as the standard nematicide (iii) Garlic extract, (iv) *Bacillus firmus*-strain I-1582, (v) Molasses liquid- fertilizer blend and bacteria mix, (vi) Fluopyram and (vii) Water as control (Table 7.1).

Table 7.1 Pesticide products evaluated in plant media inoculated with *Aphelenchoides fragariae*.

			Concentration level	
Product name	*Active ingredients*	*Manufacturers*	*Rate/ha*	*Dose/1L*
Caliente LM	Isothiocyanates and capsicum	Plant Health Care UK	50L/500L/ha	100ml/L
SC 400	Fluopyram	Bayer CropScience	0.625L/300L/ha	2.1ml/L
Nemanator	Molasses&bacteria	OceanGrown USA	37.9L/500L/ha	0.76ml/L
Vydate10G	Oxamyl	DuPont	55kg / ha	11g/L
EcoGuard	Garlic	ECOspray	20L/ha	10:240/L
Flocter	*Bacillus firmus*	Bayer CropScience	80kg/500L/ha	16g/L

7.2.2 Methods

Plants were grown in 2 litre pots of moist (100%) peat based compost 'M3 Fisons Levington, UK' (henceforth referred to as soil) containing NPK and pH 6.0, sterilised (autoclaved) by 'Prior-Clave UK' machine at 121°C for 30 mins. Plants were allowed to grow until they had a minimum of 6-8 leaves (Fig. 7.1), and were maintained in a glasshouse at 25°C ± 2°C. All test plants had pot saucers through which manual watering were carried out to avoid splash and cross contamination. Pots were adequately spaced (50cm) in-between plants to prevent canopy touch. Leaf samples were taken from plants at random for extraction to confirm that they were nematode free. Plants were kept in a glasshouse and isolated from other plants in the glasshouse. Plants were laid out in a randomised block design with seven treatments and six replicates (Fig. 7.1).

Figure 7.1 Layout of soil treatment experiment of *A. fragariae* with novel products on *Anemone hupehensis* in the glasshouse

The required dose rates of the products or water (as Control) were applied as a drench around the plant in the 2 litre pots of autoclaved moist soil. Twenty four hours after the application of the soil treatments, 1000 mixed stages of live nematodes (*A. fragariae*) in 3ml of water were dispensed using a glass pipette around the surface of the soil between edge of the pot and the plant in each pot, with 3ml distilled water as the Control treatment. The plants were watered daily (~80ml/pot) and left in the glasshouse for 10 weeks.

Observations were made at 5 and 8 weeks post-nematode inoculation to assess nematode survival in soil and populations in the leaves. Three leaf samples per plant were randomly collected at the base, middle and growing region of the plant for nematode extraction. Nematode extraction was carried out as described in Chapter 2, – Section 2.2.2. Soil assessment was undertaken at week 8 to determine the survival of *A. fragariae* in soil. Soil (30g) was collected from the base of the plant up to the root zone from each pot, mixed thoroughly and 10g sub-samples collected from the total 30g soil sample per pot. The 10g sub-samples of compost were used for nematode extraction using the modified Baermann funnel technique (Southey, 1993). Soil in the extraction plate (350-µm openings) lined with Kimwipes tissue (11cm x 21cm - Box size code number KC34155 by Kimberly-Clark Professional; Kimtech Science, West Malling, Kent, ME19 4HA, United Kingdom) was placed on top of glass funnel with a piece of rubber tubing attached to the stem and closed by clamp. The funnel is filled with water to collect nematode that swim down through water

tube (Fig. 7.2). This was left for 72h in the laboratory condition of 21°C ± 2°C, and water suspension containing nematodes was collected in a beaker for counts. Nematode numbers were expressed per 10g soil and per 1g of leaf (Jagdale & Grewal, 2002; An *et al.*, 2017).

Data analysis: Analysis of data collected was carried out as described by An *et al.*, (2017). Data from the mean nematode population from this study were subjected to analysis of variance using One-way ANOVA at each observation. Data from overtime observations (5 and 8 weeks) were analysed using repeated measures ANOVA with treatments and time as subject factors. Significant differences between treatments were determined with Tukey's mean comparison at $P < 0.05$. All the data analyses were performed in Minitab (Vs.17).

Figure 7.2 Picture showing nematode extraction in progress using the modified Baermann funnels technique for soil samples in the laboratory

7.3 Results

Experiment 7.3.1. Efficacy of novel products as soil treatments to prevent migration of leaf and bud nematodes (*Aphelenchoides fragariae)* from infested soil to leaves of *Anemone hupehensis* plants

Results from the leaf samples indicate that there were significant differences ($P < 0.05$) in nematode infestation between all the applied soil treatments and the untreated Control after 5 weeks (Fig. 7.3) and 8 weeks (Fig. 7.4).

The lowest level of leaf infestation by nematodes at week 5 was recorded from the Garlic extract treatment where a mean of 2 nematodes/g leaf was recorded, while a mean of 463 nematodes/g leaf were recorded from the untreated Control (Fig. 7.3). All the treatments had significantly lower nematode numbers in the leaf compared to the untreated Control (P <0.05).

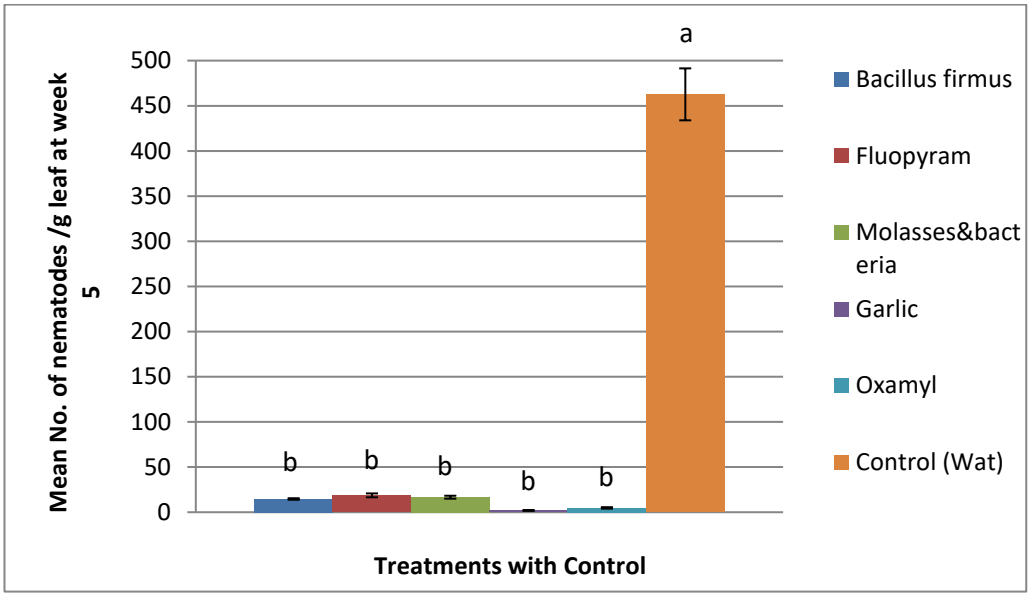

Figure 7.3. Nematode population (per 1g leaf) of *Anemone hupehensis* in nematode inoculated soil 5 weeks after soil drench. Each bar represents the mean (+SE) of six replicates. Bars with same letter are not significantly different (Tukey's multiple range test, P <0.05).

The lowest level of leaf infestation by nematodes at week 8 was recorded from the oxamyl treatment where a mean of 7.8 nematodes/g leaf was recorded, while a mean of 1382 nematodes/g leaf was recorded from the untreated Control (Fig. 7.4).

All the treatments had significantly lower nematode numbers in the leaf compared to the untreated Control (P < 0.05).

There was no result from the treatment using Isothiocyanates & capsicum as the product was phytotoxic, with phytotoxic symptoms appearing a few days after treatment (Fig. 7.5).

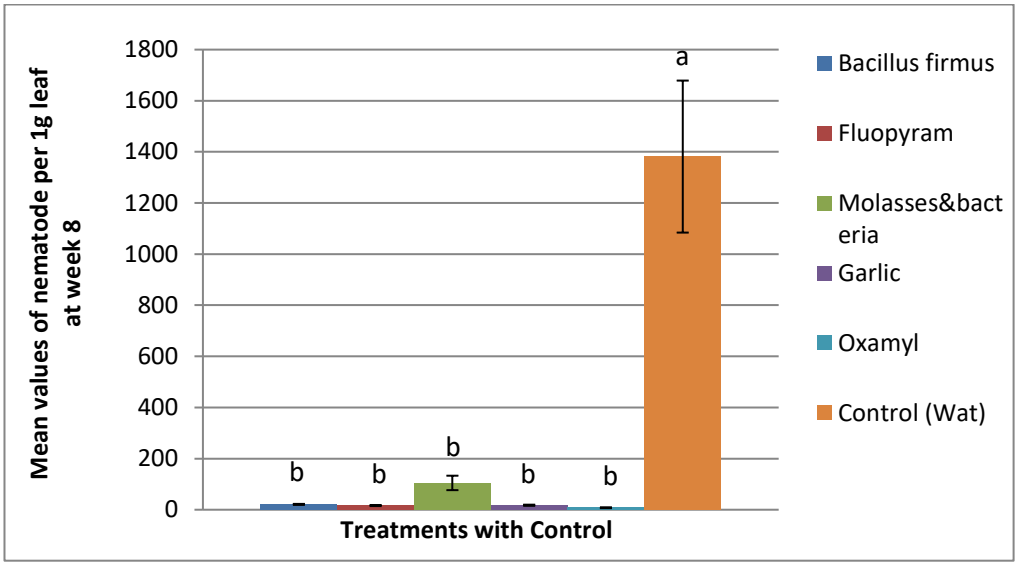

Figure 7.4 Nematode populations (per 1g leaf) of *Anemone hupehensis* 8 weeks after nematode inoculation in soil. Each bar represents the mean (+SE) of six replicates. Bars with same letter are not significantly different (Tukey's multiple range test, P <0.05).

Figure 7.5 Phytotoxic effect of Isothiocyanates & capsicum treatment (front pot arrowed) in (*A. fragariae*) inoculated soil of *Anemone hupehensis* plants a few days after treatment

Results from the assessment of the nematode numbers in soil 8 weeks after nematode inoculation, show that Isothiocyanates & capsicum (which was phytotoxic to the plant), had the lowest nematode number with a mean value of 2.8 nematodes per 10g

soil. The highest mean nematode number was obtained from the untreated Control with 57.5 nematodes per 10g soil (Table 7.2).

Table 7.2. Effect of products applied as a soil drench on *Aphelenchoides fragariae* in the soil. Data are the mean (± SE) nematode value of 6 replicates from 10g of soil 8 weeks post-soil infestation with nematodes. Columns followed by the same letter are not significantly different (Fisher's multiple range test, P <0.05).

	Mean nematode population / 10g soil
Treatment	Mean (± SE)
Bacillus firmus	14.33 (± 1.54) cd
Fluopyram	22.83 (± 3.24) c
Molasses&bacteria	38 (± 6.57) b
Garlic	21 (± 3.65) c
Oxamyl	4.67 (± 0.83) de
Isothiocyanates & capsicum	2.83 (± 0.99) e
Control (water)	57.5 (± 7.59) a

Treatments with *B. firmus*, fluopyram and garlic indicate no significant difference between them, likewise oxamyl and Isothiocyanates & capsicum (Table 7.2). All the treatments excluding Control were significantly different from Molasses & bacteria treatment in nematode number. There was a significantly lower nematode number in all the treatments compared with the Control (P <0.05; Table 7.2).

7.4 Discussion

The results from seven products evaluated as soil treatments demonstrated that all the treatments significantly (P <0.05) reduced the LBN population (in soil and leaves) when compared with the untreated control.

Isothiocyanates & capsicum had a phytotoxic effect on plants in this study (Fig. 7.5), hence no results of leaf infestation by nematodes was possible, although the number of nematodes in soil was significantly reduced. Results from field and greenhouse bioassays with mustard extracts demonstrated a significant reduction of the infection rate of entomopathogenic nematodes (EPN) on *Galleria mellonella* larvae (Ramirez *et al.*, 2009), although *Heterorhabditis* species was more susceptible to the negative

impact of mustard green manures than *Steinernema* species. Similarly, susceptibility of different species of PPN (*Meloidogyne javanica*, *Tylenchulus semipenetrans*) to mustard bio-fumigants (*Brassica juncea*) varies (Zasada & Ferris, 2003). However, isothiocyanates & capsicum demonstrated efficacy as a potential soil applied nematicide as it led to the lowest number of nematodes in treated soil after 8 weeks. Application of isothiocyanates & capsicum could be utilised so that product is applied a few weeks prior to the introduction of plants to the soil to minimise the risk of any toxicity. In laboratory bioassays (Chapter 2; Rotifa & Evans, 2015), Isothiocyanates & capsicum recorded 95% nematode mortality of *A. fragariae* in a water bioassay after 72h of exposure. The contact mortality results and the results from the soil tests suggest that the nematicidal properties of isothiocyanates & capsicum may have a role to play in the management of LBN in the soil stage of the nematode life cycle. The results in this study indicate that the populations of inoculated *A. fragariae* in soil after treatment by isothiocyanates & capsicum and oxamyl were not significantly different (P <0.05), which confirms that isothiocyanates & capsicum if managed carefully has potential as a nematicide treatment.

Bacillus thuringiensis containing in the Molasses & bacteria treatment is a widely used insecticide with toxicity targeted at the intestine of insects through the insecticidal action of the crystal proteins of the various strains (Wei *et al.*, 2003). Although, Molasses & bacteria in this current study demonstrated less efficacy compared with the other products, the mean nematode number was significantly (P <0.05) lower in both soil and leaves compared with the untreated Control.

In this study, fluopyram significantly reduced nematode numbers in the soil and hindered multiplication of *A. fragariae* in leaves of *Anemone hupehensis*. Fluopyram is sold in the USA as a nematicide called ‘Velum Prime’ and ‘Velum Total’ (Bayer CropScience, Research Triangle Park, NC). These products are registered in the USA for fungi control on crops such as potato, tomato, cabbage and broccoli, while Velum Prime in particular is labelled as a nematicide for root-knot nematodes, cyst nematodes and other free living nematodes (Bayer CropScience, Research Triangle Park, NC). The use of fluopyram as a soil treatment greatly suppressed root-knot

nematode (*Meloidogyne incognita*) populations on lima bean (*Phaseolus lunatus*) with no phytotoxicity effect (Jones *et al.*, 2017).

Considering the performance of fluopyram in this current study and the ability to prevent nematode migration from soil to the leaves with the significant reduction of nematode populations in soil and leaves, fluopyram has shown promise for the management of LBN. A fluopyram product is currently under trial in the UK for use as a nematicide in potatoes.

Garlic extract has demonstrated significant activity by reducing nematode numbers in the soil and multiplication of *A. fragariae* in leaves of *Anemone hupehensis* in this study. The major constituents of garlic oils are allium, diallyl disulphide and trisulphide, and these have demonstrated toxic effects on pine wood nematode *Bursaphelenchus xylophilus* in laboratory bioassays (Park *et al.*, 2005). Concentrations of raw garlic straw '*Allium sativum'* - 1%, 2% and 4% - increased the mortality of *Meloidogyne incognita* from 9.8 to 36.6%, and equally reduced hatching activity in laboratory bioassays and pot studies (Gong *et al.*, 2013).

In Chapter 2 of this thesis, garlic extract at a reduced dose of the manufacturer's recommended rate demonstrated the highest nematicidal activity with over >75% mortality in laboratory bioassays against LBN (*A. fragariae*). Results obtained from bioassays in Chapter 2 led to the investigation of its efficacy when applied to soil (Rotifa, 2015a). Garlic extract has demonstrated an effective control of *A. fragariae* in soil along with a reduction in leaf infestation, consequently meriting further study as an option to manage LBN.

Results obtained during this soil treatment study confirmed the previous studies that oxamyl was effective against LBN (Young & Maher, 2000; Bennison, 2007). Oxamyl treated soil demonstrated the lowest nematode population of *A. ritzemabosi* on leaves of *Anemone hupehensis* in a recent field experiment (Chałańska *et al.*, 2017). This current study is in support of previous results on oxamyl with a significantly (P <0.05) reduced mean population of *A. fragariae* in both leaf and soil samples compared to the Control (Rotifa & Evans, 2016). Oxamyl had the lowest nematode mean numbers in all the treatments evaluated in both leaf and soil samples at week 5 & 8 respectively.

Oxamyl has been reported to cause reduction in LBN (*A. fragariae*) populations of 72.2% in leaves and 76.9% reduction over control in soil around infested Hosta plants at 45 days after treatment (Jagdale & Grewal, 2002) compared with this study of 91% reduction over control in soil.

Oxamyl 10% (as Vydate 10G-55kg/ha) used as standard nematicide, as at the time of this study had an EAMU for outdoor ornamental plant production which ended 31st December 2017, targeting insect pests and stem and bulb nematode, but not for LBN.

This current subject has not received much attention in the area of control of *Aphelenchoides* spp. through infested soil/plant media up the plant foliage; hence it is a novel study which highlights potential products to replace oxamyl in which its EAMU had already expired. Considering the results obtained from this study, most of the products investigated reduced nematode infestation on leaves and reduced the nematode population in infested soil (Rotifa & Evans, 2016). The products demonstrated potential as soil treatments to infested soil, and is in support of the opinion of Jagdale & Grewal, (2002) that treatment of LBN (*A. fragariae*) in infested soil is more effective than treatments applied to nematode infested leaves. The treatment on infested leaves as carried out in previous Chapters such as the curative treatment of Chapter 5, had results of percentage nematode reduction averaging between 70-80% over the Control, as compared to this study with a reduction over the Control of 91%. In the General Discussion, a concept of a twin treatment involving both soil and foliar treatments as an approach for LBN will be outlined for further investigation.

Results as demonstrated in this study highlighted the potential of each candidate treatment in preventing nematode migration from infested plant media to the plant leaves. However, treatments should be used in combination with other preventative methods such as hygiene, as part of integrated control programme.

These products could be used in commercial conditions especially in container grown plants, and in stock beds, for both preventative and curative treatments; while maintaining a high level of hygiene in an IPM approach.

Chapter 8

General discussion

8.1 Introduction

The major objectives of this thesis were to develop novel management strategies for LBN management in ornamental plants, by preventing migration from infested soil and management of infested plants in the growers' nursery; restricting continuous multiplication of nematodes in the leaves of already infested plants, and to investigate the potential of using elicitors of plant resistance to confer systemic resistance by plants to LBN infestation.

Existing techniques for LBN management were adapted, and new methods developed, particularly in the area of nematode inoculation to assess the efficacy of soil inoculation or direct inoculation of nematodes on the leaves for the purpose of screening novel products against *A. fragariae*. Experimental approaches included the use of water bioassays, glasshouse and commercial field trials. Insecticides, plant extracts and other options were evaluated along with products (elicitors) that induce plant defences to evaluate whether elicitors can confer resistance against LBN. Elicitor products were evaluated alone and in combination with other insecticides. This thesis has demonstrated an approach for the practical application of induced resistance products (elicitors) for use in ornamental plants for protection against LBN as a curative and preventative method of LBN control.

Products with potential for use as soil / plant media treatments were evaluated for preventing the migration of *A. fragariae* from infested soil to plant stems and subsequently to the leaves. These products included chemical and plant extracts.

The concluding part of this thesis deals briefly with the key outcomes arising from the research activities in this PhD project and suggests management strategies that could be adopted for the integrated management of *A. fragariae* and other LBN in general. It concludes with key messages, suggestions for future research work and overall conclusions.

8.2 Importance of LBN

LBN are a significant foliar pest of ornamental plants (over 1104 host species from 126 botanical families) whose feeding results in angular-shaped blotches on the leaves which are usually delimited by veins in most plants and often accompanied by leaf distortion (Kohl *et al.*, 2010; Kohl, 2011; Sánchez-Monge *et al.*, 2015; Bennison & Maulden, 2016). Following nematode penetration into leaves through stomata, large sections of the affected leaf become chlorotic, which later form necrotic lesions usually surrounded by large veins (Lehman, 1996; Jagdale & Grewal, 2006). As well as having an endoparasitic lifestyle, *A. fragariae* is also an ectoparasite, especially in closed buds (De-Waele, 2002). The affected parts of plants will lose their aesthetic value and consequently lead to economic loss to growers (Kohl *et al.*, 2010). It is difficult for ornamental plants to command a good market value when the infestation is visible, while avoidance is the best suggestion.

The two major approaches for *A. fragariae* management are to avoid infestation arising in the first place, and to manage the infection when symptoms are observed. However, it is very difficult to examine any commercial nursery and not find signs of nematode infestation. This is not surprising as the transfer of plants from nursery to nursery and across country borders increases the risk of nematode spread. With the attendant economic loss in term of infested plants, the cost of (increasingly ineffective) control, and the possibility of further spread to healthy plants, it is necessary to tackle the issue of LBN infestation, because once it is established in the nursery, considering its life cycle, multiplication rate (where populations of up to 15,000 nematode per leaf can be found (Lambert & Bekal, 2002), and their strategy of survival in dried leaf debris for many years, the elimination of LBN can be challenging (De-Waele, 2002: Singh *et al.*, 2013).

8.3 Various approaches adopted in this thesis

Nematicides and insecticides previously used for LBN management in the UK have recently been withdrawn, or restricted due to environmental and safety issues; (Jagdale & Grewal, 2002; Bennison, 2007; An *et al.*, 2017). This thesis highlighted results of laboratory bioassays of current (at the time of investigating this work)

available chemicals and bio-pesticide products, and the results demonstrated the potential of several products as alternatives to oxamyl, which up to the end of 2017 had an EAMU for use in ornamental crop production, but with issues regarding operator safety and application. Isothiocyanate & capsicum, garlic extract and abamectin caused the highest nematode mortality after 72hrs of exposure to *A. fragariae* in a contact bioassay. The results indicate that the hydrolysation of sulphur compounds found in *Allium sativum* (raw garlic) to a variety of isothiocyanate compounds have nematicidal activity against *A. fragariae,* as well as other pathogens (Choi *et al.*, 2007). Allyl isothiocyanate and capsicum contained in the product Isothiocyanates & capsicum has activity against *A. fragariae,* as well as root-knot nematode (*Meloidogyne incognita*) on tomato (Bawa *et al.*, 2014) and several other nematode species including *Caenorhabditis elegans*, *Heterodera schachtii*, *Xiphinema americanum* and *Globodera rostochiensis* (Lazzeri *et al.*, 1993; Donkin *et al.*, 1995; Lord *et al.*, 2011). Toxicity of abamectin to LBN has previously been reported by Young & Maher (2000), An *et al.* (2017) and Chałańska *et al.* (2017) and was confirmed in this study. Products that work systemically (spirotetramat, azadirachtin and oxamyl) had moderate levels of contact mortality, but were still carried forward for further investigation as their modes of action may not be due to direct mortality but through impacts on fecundity (Wright *et al.*, 1980; Lynn *et al.*, 2010; Smiley *et al.*, 2011).

This was followed by the highlight of methods adapted and modified for nematode inoculation to the soil and direct inoculation to leaves, a useful tool for screening resistance to LBN. The successful multiplication of *A. fragariae* in leaves of *Anemone hupehensis* and *Weigela florida* plants by the direct inoculation techniques, and the migration of inoculated nematodes from infested soil with subsequent multiplication in the leaves of *A. hupehensis* proved to be an effective method to identify potential products to apply to leaves or the soil (Rotifa & Evans, 2016). Results of inoculation methods are in agreement with the earlier reports of Jagdale & Grewal (2006) and Zhen *et al.* (2012). These methods were successfully used in the subsequent experiments of this thesis. In addition, investigations have identified a correlation between the visual assessment of lesion symptoms of *A. fragariae* damage on the leaves of *A. hupehensis* and *W. florida,* and the corresponding

nematode population within the leaf. However, the author encourages nil tolerance for plants with symptoms in the nursery.

The products identified in this thesis were investigated for both preventative and curative purposes. If symptom is observed on plant, and still within reasonable level of treatment as indicated in this thesis, then curative approach could be used after such plants might have been isolated. Plant isolation is important to prevent chances of further infestation and spread while treatment is being carried out. Curative treatment is suggested provided the level of infestation on leaves is not more than 15% total leaf area damage. Upon treatment, further nematode multiplication will be prevented while newly emerging leaves will be free of infestations. This curative approach is important to some growers who are willing to salvage some plants termed 'important and exotic' should they be found with symptoms, and provided their degree of infestation is within the treatable category. This simple symptoms guide can therefore be of benefit to growers as a useful management aid to provide a decision making approach on whether to treat infested plants or discard them based on severity of visual lesions (symptoms) on leaves. In addition, evaluation of plant resistance is a vital reason for the symptom key, while this visual guide key is equally useful to assess the effectiveness of control treatments.

In accordance with previous reports on the use of ASM products against pathogens on several crops, author also considered the potential of elicitor treatment to induce resistance by plants against LBN invasion. This thesis has been able to investigate the potential of acibenzolar-S-methyl (ASM) to reduce multiplication of *A. fragariae* as a preventative measure or a curative treatment (Rotifa, 2017). Although deeper understanding of the mode of mechanism of ASM against LBN in plants is still required, this thesis has successfully identified a novel approach using elicitors to reduce LBN multiplication in ornamental plants in both glasshouse and commercial conditions. Two seasons of field trial assessments using the identified (potential) products along with ASM against naturally infested plants and on inoculated plants have identified viable alternatives/replacement to oxamyl. There was no observed effect in the growth of elicitor treated plants during this project.

Products were applied as a foliar application after 1 hour pre-irrigation of plants was carried out to in order to stimulate nematode migration to the leaf surface (Richardson & Grewal, 1993; Jagdale & Grewal, 2006). The method of leaf wetness was adopted to optimise efficacy of the products by targeting any nematodes that may be on the leaf surface due to the elevated leaf moisture levels; while early morning application of products before sunrise was to prevent breakdown of active ingredients of some contact treatments such as abamectin (earlier reported with controversial efficacy). Both strategies did work, as the results indicate that all the products hindered multiplication of *A. fragariae*. These methods were adopted to manage LBN on both naturally infested and inoculated plants in the glasshouse and commercial conditions, using curative and preventative approaches. These results also confirmed the efficacy of abamectin and highlighted the potential for spirotetramat as viable treatments against LBN (LaMondia, 1999; Smiley *et al.*, 2011; Chałańska *et al.*, 2017). Treatment with a combined ASM and insecticide programme improved performance in reducing nematode multiplication than when the insecticides or ASM were used alone (Rotifa, 2015a; Rotifa & Evans, 2016). This substantiated the observations by Ivors & Louws, (2007); Walters & Heil, (2007) and McGrann *et al.* (2017) that combinations of elicitor treatment and pesticides improve the suppression of pathogens on plants. The novel use of ASM against *A. fragariae* supports its potential to manage LBN, and corroborates observations on various plant pathogens and PPN such as *Meloidogyne incognita* (Cole, 1999; Molinari & Baser, 2010; Walters *et al.*, 2014).

As well as evaluating products on already infested plants in this thesis (a curative approach), a preventative approach was evaluated on plants inoculated with nematodes after the first product treatment. Similar results of lower levels of nematode multiplication on the inoculated ornamental plants were achieved by these products. All the products successfully reduced nematode multiplication compared with the untreated Control. Combined treatment programmes with ASM (especially spirotetramat + ASM) were very effective at reducing nematode multiplication. Again this demonstrates that ASM can be combined with other insecticides as a novel treatment for LBN management. Results of both field trials corroborated earlier reports of ASM as a novel plant protection product that mimics the pathogen-

host interaction and thereby results in systemic acquired resistance in plants (Kessmann *et al.*, 1994; Cole, 1999; Molinari, 2011; McGrann *et al.*, 2017).

Soil investigation demonstrated the potential for novel products as soil treatments to limit nematode migration from infested soil into plants. These products not only reduced the nematode population in the soil, but also limited migration from the soil to the leaves of *Anemone hupehensis*. The available standard product, oxamyl, demonstrated its efficacy as a nematicide; however, garlic extract also demonstrated significant effects on nematodes in the plant media and consequently reducing leaf infestation. Nematicidal properties of garlic extracts have been reported against *Meloidogyne incognita* and *Bursaphelenchus xylophilus* on carrot, tomato and pine (Park *et al.*, 2005; Anwar, 2009; Gong *et al.*, 2013).

Isothiocyanate & capsicum demonstrated a high level of contact mortality in the bioassays as outlined in this thesis. However, it was toxic to the plants when applied to the soil, but did significantly reduce the nematode population in the soil. Isothiocyanates may have a role to play as a soil treatment prior to the introduction of plants (Rotifa & Evans, 2016).

Fluopyram and *Bacillus thuringiensis* both reduced nematode populations in the soil and limited plant invasion. *Bacillus thuringiensis* (Bt), the active ingredient of Molasses & bacteria, has been reported to have toxicity against insects and free living nematodes (Wei *et al.*, 2003). Fluopyram has been reported to have activity against pathogenic fungi, *Meloidogyne incognita* and *Rotylenchus reniformis* (Amiri *et al.*, 2014; Heiken, 2017; Jones *et al.*, 2017). Both of these products have potential to be used for the management of LBN as soil treatments to limit plant invasion.

8.4 Novelty of research and contribution to scientific knowledge

Although various studies have been carried out using ASM as a plant protection product against diseases on crops such as tomato, barley, oil-seed rape and chrysanthemums (Cole, 1999; Ivors & Louws, 2007; Walters *et al.*, 2014; McGrann *et al.*, 2017), this thesis is the first study to report on the use of elicitor treatments for the management of LBN on ornamental plants, both as a curative and preventative

management, and in combination with other pesticides for an improved efficacy against *A. fragariae* (Rotifa, 2017). One of the major advantages of elicitors according to Vallad & Goodman (2004) is the absence of any direct antimicrobial activity when compared with normal traditional pesticides; which means that the systemic resistance is a direct activation of plant defences, a factor which could assist the avoidance to developing pesticide resistance by *A. fragariae*. The results demonstrated by the use of ASM (and to a lesser extent Regalia) reduced nematode multiplication when compared with the untreated Control. This is in support of consistently reduced infection of light leaf spot disease (*Pyrenopeziza brassicae*) on winter oilseed rape by foliar application of ASM (McGrann *et al.*, 2017). Furthermore, results obtained when elicitors were combined with insecticides led to an improved reduction of nematode multiplication compared to results from insecticides alone. The effectiveness of combined elicitor + insecticides might have resulted from elicitors increasing plant resistance against further nematode multiplication, while insecticides, known to reduce inoculum levels, have acted on nematode population, either by systemic or contact action, thereby leading to a significant reduction of nematode levels compared with sole candidate treatments (Bennison *et al.*, 2018). This is also in agreement with past reports that combinations of ASM with fungicides and bactericides increased plant resistance and reduction of fungus inoculum levels of *Alternaria solani* in tomato spray programs (Ivors & Meadows, 2016).

The use of spirotetramat to manage LBN has not been fully investigated previously; while the results in this thesis consolidated previous reports of using abamectin to manage LBN (LaMondia, 1999; Young & Maher, 2000; An *et al.*, 2017). Likewise, information on azadirachtin to manage LBN is rare, especially under commercial conditions. This study has revealed the potential of azadirachtin to manage *A. fragariae* as a preventative approach under field conditions (Rotifa & Evans, 2016; Rotifa *et al.*, 2016). Azadirachtin has recently received approval in the UK in the form of the product Azatin (Certis) for the control of western flower thrips and onion thrips in protected ornamental, tree nursery and perennial crops.

This thesis incorporated an additional technique of 'leaf wetness' prior to application of treatments to facilitate nematode attraction to the leaf surface, subsequently increasing the efficacy of contact-acting products. Various presentations of the author on the modification of treatment's timings were widely accepted by the growers during their technical discussion group meetings organised by the AHDB (Rotifa, 2015a).

An evaluation of leaf symptom visual rating assessment as a management tool has shed light on nematode infested plants and will be helpful for better identification of LBN symptoms on leaves against other symptoms likely caused by bacterial or fungal diseases (Chapter 1; Bennison *et al.*, 2018). Using a simple visual correlation between nematode population and degree of lesions, the symptom rating assessment could assist growers to take immediate action on infested plants. This relationship can be adopted as a useful management guide in taking management decisions based on the extent of visual lesion symptoms showing on the leaves, growers may decide either to treat to make them viable for sale (if symptoms are >15% LAD), or discard infested plants with ≥15% LAD. If the infestation level on leaves is getting to ≥15% LAD, such plants should be discarded immediately as no control treatments beyond that level of infestation would be worthwhile (Bennison *et al.*, 2018). However, to maintain a relatively LBN free plants, cultural control practices as an integral part of IPM programme with a high level of hygiene is very important, along with a preventative approach such as application of ASM to prime the plants before the onset of LBN symptoms. Treatments will be most effective at the first signs of symptoms when the plant is actively growing in the field. Available curative control measure as highlighted in this thesis should be applied as soon as symptoms are seen. This information formed part of the developed factsheet of leaf and bud nematodes management in hardy nursery stock published by AHDB Horticulture UK (Bennison *et al.*, 2018).

At the commencement of this project, many growers had little knowledge on the management of LBN in term of symptoms, dispersal and spread; leading to a continuous economic loss in the ornamental industry. It is worth mentioning that this inadequate identification knowledge was also observed during the author's visit to the USA. Despite enormous publications in the USA, symptom identification is still

a challenge as witnessed during my interaction with growers during my visit to the United States of America courtesy of David Miller's Travel Award (Rotifa, 2015b). Visit was made at a later stage of this thesis to the University of Tennessee, laboratory of Nematology Knoxville, and the Botanical Garden where LBN infested plants donated by the American Hosta Society (AHS) are being monitored. Ongoing research works to manage *A. fragariae* on ornamental plants were highlighted during this visit as well as the importance of cultural control with high level of hygiene in the nursery (Rotifa, 2015b). Peroxyacetic acid has been successfully tested in both bioassays and field trials in the USA. However, as a contact pesticide, peroxyacetic acid has a limited ability to penetrate in to the leaf tissue. New products under experimental investigation during this visit include Nemakill, oil based organic pesticide with active ingredients as Cinnamon oil (32%), Clove oil (8%) and Thyme oil (15%). This product was said to have potential management of *A. fragariae* in both bioassays and glasshouse while field investigation is ongoing.

My field visit to two commercial nurseries at the outskirts of Knoxville revealed that some growers had little knowledge about identification of symptoms caused by LBN. Wrong treatments had been used to manage infestation of symptom on plants. When treatments proved ineffective on many occasions, plants were destroyed according to one of the growers during the visit. Some associations such as AHS have funded nematode research hence farmers involved are familiar with the symptoms. Farmers who are not part of such associations may unlikely be familiar with LBN symptoms and identification, thereby resulting in misdiagnosis. The experience and findings already gathered during the course of this project (in field and glasshouse) were utilised to enlighten growers during the on-farm visits to the nurseries in the USA (Rotifa, 2015b). I hereby recommend a continous enlightement programmes to highlight the need for growers to have good knowledge of LBN symptoms and identification to assist management practices. There was no research undertaken during the visit to the USA and it did not influence the experimental approaches reported in this thesis.

Substantial information have been made to scientific audience and horticultural growers from this project (see page viii).

8.5 Research limitations

Unfortunately, this project could not undertake a detailed study of how ASM induces the self defence mechanism of the plants and subsequently hinders the multiplication of nematodes on both naturally infested plants and inoculated leaves of ornamental plants. Molecular tools could assist to identify what specific set of genes are being activated in the plants through systemic acquired resistance pathways following the application of ASM. This could be useful for screening a susceptible (wide) range of host plants, and potentially be used as a tool for the selection and breeding of nematode-resistant varieties.

8.6 Future recommended research

There are other areas that could be considered for future research which arose upon completion of this thesis. Further work is necessary to screen more emerging products for nematicidal activity as carried out in the bioassays. There are new products such as Nemakill (an oil mixture containing Cinnamon oil, Clove oil and Thyme oil) and Pylon (chlorfenapyr) which have demonstrated significant mortality against *A. fragariae* in aqueous solution and as soil drench treatments (An *et al.*, 2017). Information on these products came too late for their inclusion in this study, but they warrant further study. One of the drawbacks of the laboratory bioassay is that it only evaluates contact mortality. As demonstrated in this study, products such as spirotetramat had low contact mortality, but when used on plants had a significant effect in reducing nematode multiplication. Relying on laboratory contact mortality bioassays alone may let promising compounds such as spirotetramat and azadirachtin 'slip through the net', so studies on infested plants are a key component of the screening process for new products for activity against *A. fragariae* and LBN in general.

There is need to investigate other available elicitor products for their potential in limiting nematode multiplication in ornamental plants. Such elicitors that warrant evaluation include laminarin (Vacciplant), Sitko-SA, Softguard (Chitiosan) and Reysa (*Reynoutria sachalinensis)*. Limited results in this study demonstrated positive results from a single application of Reysa.

The combinations of elicitors with pesticides warrant further study for the management of LBN. Further work is also required to consider varying dose rates of the pesticide and elicitor products to fully evaluate their cost effectiveness. For example, a reduced dose rate of either the pesticide or elicitor (if effective) would reduce the overall cost accrued from the use of product combinations. The use of elicitor/pesticide combinations could be valuable in reducing overall pesticide use, and could delay resistance to pesticides arising and increase the longevity of their use for LBN management.

More work is needed to investigate the mechanism of action of elicitors in ornamental plants. It is reported that when plants are treated with ASM, a broad spectrum systemic resistance is developed by the plant against any infection by pathogens, a process called systemic acquired resistance (Lawton *et al.*, 1996; Pieterse & Van Loon, 2007). Molecular tools could assist to identify what specific sets of genes are activated following the increase of salicylic acid in plant as a result of SA treatment by ASM and other potential elicitors. Identification of specific genes activated in plants by elicitors and screened for in nematode susceptible plants, and if such specific genes are already present, they could be 'switched on' or primed to induce resistance against nematode infection.

As the author did not have the opportunity to investigate further into EPN along with other management strategies that were used in this project, there is need to investigate the use of EPN as a management option for LBN control both in the glasshouse and under commercial conditions. EPN could be applied as a biocontrol treatment to nematode infested soil to suppress nematode populations from migrating into the leaves of clean / healthy plants through their toxic metabolites produced by the nematodes or from their symbiotic bacteria. Although Bennison (2007) found *Steinernema carpocapsae* ineffective in controlling leaf and bud nematodes with a 5-spray programme of 500 million/1000m^2, Jagdale & Grewal (2008) used 100 wax moth (*Galleria mellonella)* cadavers infested with 100 infective juveniles of *S. carpocapsae* per cadaver, mixed into the planting medium contained in 346 cm^2 size plastic pots, to successfully suppress *A. fragariae* in the soil of infested Hosta plants as a curative and preventative approach. Using the same plant species as evaluated in

this thesis, EPN such as *S. carpocapsae* and *S. feltiae* could potentially be applied as a treatment to manage LBN. This can be used as a preventative and curative approaches as described in this thesis.

Field trials of soil treatments such as *Bacillus firmus*, fluopyram, garlic extract and molasses & bacteria (*B. thuringiensis*) are required to demonstrate the prevention of infection via nematode infested soil in a range of ornamental plants under commercial conditions. This would complement the glasshouse study carried out with above products in this project. In addition, the combined approach of soil treatments with foliar treatments as an overall management approach for *A. fragariae* requires evaluation and validation.

The leaf symptom visual rating assessment as carried out in this thesis could be extended to more ornamental plant species considering the wide host range of *A. fragariae* and other economically important species such as - *A. ritzemabosi* and *A. besseyi*. Extending the leaf rating by symptom severity assessment will be necessary to consolidate the findings in this thesis which are based only on *Anemone hupehensis* and *Weigela florida*.

8.7 Key messages and treatment guidelines

- **Key messages**

Cultural control methods are an important component in the management of LBN within integrated pest management (IPM) programmes. The most effective of these is a programme of a high level of crop hygiene, especially as LBN can survive for several years in infested leaf debris (Jagdale & Grewal, 2006; Kohl *et al.*, 2010) and can overwinter in soil (*A. fragariae*), infested dried leaves and dormant buds until conditions are favourable, then migrate to the leaves to initiate infestation (Jagdale & Grewal, 2006). Cultural control programmes which involve a high level of hygiene should be observed always. This should include the removal and destruction of infested plants and debris, avoiding replanting in nematode-contaminated soil, sterilisation of pots and equipment, and adequate spacing between plants (>30cm) to avoid nematode spread through canopy touch (Rotifa & Evans, 2016).

Also, if possible, growers should limit the use of overhead irrigation and misting systems which could aid in the transfer of nematodes from infested plants to healthy plants, thereby creating ideal conditions for nematode infestation and spread. This is important to avoid economic loss which could range from £10,000 - £30,000 per annum (Grower's personal communication), depending on the size of the nursery; an amount similar to an estimated loss value suggested by one of the growers during this project.

While making sure that certified nematode free planting material are used to prevent spread (Ward & Hockland, 1996; Coyne *et al.*, 2010), newly purchased plants should be quarantined in a separate area of the nursery for a few weeks (4-6 weeks ideally). This is to allow the observation of any traces of LBN symptom development before plants are mixed with the current nursery stock. In addition, since a high number of *A. fragariae* can develop in plants before visual symptoms are seen, it is recommended that preventative treatments are carried out regularly in the nursery as part of integrated management practices. This will involve regular treatment of all plants with the products evaluated in this thesis, and the same measure could be applied to the newly delivered stock of plants. These actions will serve as a protective treatment for non-infested (clean) plants, and will also discourage further nematode multiplication on infested but asymptomatic plants. These practices will enable a curative and a preventative approach to nematode management in the nursery (Rotifa *et al.,* 2016). Growers should always be vigilant about traces of nematode symptoms on leaves for immediate action as necessary in accordance with the recommendations outlined in this thesis. Foliar spray of the products identified in this thesis should be used to control nematodes on plants with less than 15% leaf damage area. Immediate treatment at the appearance of symptoms when plants are actively growing would bring effective management (Rotifa & Evans, 2016).

The author would recommend a continuous enlightenment campaign among the growers on the management of *A. fragariae*. This is to improve on the initial observation of the poor level of awareness on symptoms of LBN among the growers in the UK, and other areas such as the USA. However, with the opportunity to enlighten growers during some technical discussion meetings organised by AHDB

(Project sponsor), and Horticulture Group of the Society of Chemistry and Industry (SCI), level of awareness on management strategies of LBN has improved among the growers in the UK (Rotifa, 2017). Such awareness should be maintained through technical meetings with growers by the AHDB, and other horticultural groups. The symptoms guide would also help to improve on the lack of recognition and evaluate the treatments adopted. In general, all these recommendations will likely increase early detection of symptoms, forestall potential dispersal and spread of *A. fragariae*, which could ultimately lead to overall improved management practices in the nurseries.

Products identified in this thesis are recommended to be used as both preventative and curative approaches, in accordance with the recommendations outlined in this thesis (preventative / curative treatment of plants, or discard highly infested plants) and in accordance of the product label or as an EAMU. These products and approaches can be utilised as a key component of IPM of LBN in ornamentals.

- **Guidelines for LBN management**

- Watch out for susceptible plant species with LBN symptoms, separate them from the stock for immediate treatment or disposal
- High levels of hygiene should be maintained in the nursery. This should include getting rid of all leaf debris on soil, planting media, beds and ground cover matting between crops to discourage nematode spread within nursery.
- Sterilisation of pots and equipment (trowel, pruning shears/pruning saw, scissors) by chemicals or heat treatment should be done regularly before use.
- Avoid the use of overhead irrigation to reduce nematode dispersal and spread.
- Practice adequate plant spacing, from between 30cm – 100cm space between plants to avoid canopy touch.
- If possible avoid planting susceptible plant species in any areas of the nursery previously exposed to infested plants.
- Use a prophylactic foliar spray programme of plant protection products outlined in this thesis to maintain less than 15% leaf area damage.
- For the best result using a curative approach, apply treatments at the first sign of symptoms on plants, especially during an actively growing period.

Nematode infected plants with more than 15% leaf area damage should be destroyed as they are unlikely to provide an economically viable outcome with plant protection products.

- In addition to the curative treatment on any suspected infested plants, preventative treatments should be carried out regularly in the nursery using the products outlined in this thesis as a component of integrated management practices.
- Quarantine new stock of plants to assess potential symptoms for about 4-6 weeks before they are mixed with other nursery plants, with subsequent preventative treatments as previously suggested.

8.8 Conclusions

- The insecticides azadirachtin, spirotetramat and abamectin demonstrated effective management of LBN on a range of ornamental plants within a foliar application programme. Spirotetramat and abamectin currently have UK approval for the management of other pests on ornamentals. An azadirachtin product (Azatin) also has approval for use on protected ornamentals against insect pests.
- The elicitor acibenzolar-S-methyl - ASM demonstrated effective management of LBN on a range of ornamental plants as a foliar application, and successfully reduced nematode multiplication when used alone and in a programme in combination with other products.
- ASM in combination with azadirachtin, spirotetramat or abamectin enhanced the management of LBN on a range of ornamental plants. The above products have an effect on plants by limiting nematode reproduction. Consequently they can limit nematode multiplication in already infested plants, and limit reproduction in asymptomatic plants before symptoms develop. Results from this study are being used by the industry representatives to build up support for ASM to obtain an Extension of Authorisation for minor use on ornamental plants for LBN.

- As a technical resolution to the critical area of confusion concerning the efficacy of abamectin for the management of LBN, this thesis obtained positive results from the early morning application of abamectin to avoid potential breakdown of product by sunlight, and the adoption of leaf wetness to increase efficacy of contact products on the management of *A. fragariae*, which are known to operate both endo- and ectoparasitically on host plants.
- Development of a symptom severity rating test demonstrated that leaf symptoms are an accurate indication of the population of leaf nematodes in the leaves at any given time, hence an ideal aid to growers' decision making on the management of nematode infested plants.
- Soil treatments with various products significantly reduced the infestation of *Anemone hupehensis* via the soil route of nematode infection. *Bacillus firmus*, fluopyram, garlic extract, molasses & bacteria and oxamyl were all effective and can play a role in nematode management should they be available for use in ornamentals.
- My shadow training during this project on the molecular identification of my model species (*A. fragariae*) shed more light on the usefulness of molecular techniques when dealing with *Aphelenchoides* species or any kind of PPN. Molecular tools can help to detect nematode infestation early enough especially on asymptomatic leaves, leading to nematode diagnosis and correct confirmation of nematodes to the species level.
- This project has facilitated an increase in the level of awareness on the damage done to ornamental plants by LBN through conferences, technical discussion meetings with the growers, and nursery visits in the UK and abroad (USA). In addition, some of the results obtained on the management of *A. fragariae* have been disseminated through AHDB organised technical discussion grower's meetings, technical journals and conferences (page vii), while part of the results has been used for AHDB Horticulture 'Factsheet on leaf and bud nematodes' for the UK ornamental growers.

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