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African nightshade and African spinach: A neglected and underutilized resource with significant potential to manage plant-parasitic nematodes

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This work is dedicated to my family for their love, endless support,
and encouragement.

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Abstract

African indigenous vegetables (AIV) are essential for dietary diversification and ensuring nutritional requirements for people in sub-Sahara Africa. AIV have been largely marginalized by agriculture research, yet they are hardy and tolerant to varying environmental conditions. Plant-parasitic nematodes particularly root-knot nematodes (RKN: *Meloidogyne* spp.) and cyst nematodes (CN: *Globodera* and *Heterodera* spp.) cause severe yield reduction on most cultivated crops and are of high economic importance. Despite the significance of nematode surveys and diagnosis, the occurrence and correct identity of RKN and potato cyst nematodes (PCN) on AIV such as African nightshade (*Solanum* spp.) and African spinach (*Amaranthus* spp.) remains largely unknown. In Chapter 2 and 3, a survey was conducted in Kenya and a DNA barcode based assay was used to identify RKN and PCN species. Our survey revealed that *S. villosum* exhibited high root galling whereas on *S. scabrum*, *A. cruentus*, and *A. dubius* root galling was rare or very low. Moreover, soil collected from the rhizosphere of *S. villosum* and *S. scabrum* contained few cysts of PCN and no developing PCN females were observed on the roots of growing plants. The resulting RKN and PCN mitochondrial DNA haplotypes are globally distributed, indicating that areas of high native nematode species richness (RKN species) are not resistant to colonization by alien nematode species (PCN species). In this context we detected RKN - PCN co-infection in potato and RKN - RKN co-infection in tomato and *Parthenium hysterophorus* (an invasive weed in Africa). In Chapter 3, the dynamics of RKN and PCN on *A. dubius*, *A. cruentus*, *S. scabrum*, and *S. villosum* over 2 years was studied in a field experiment at KALRO, Kenya. The effects of AIV crop species on RKN and PCN soil infestation were evaluated using susceptible *S. lycopersicum* or *S. tuberosum*. After the successive cultivation of *A. dubius* and *S. scabrum* our results show that RKN soil infestation decreased by 85%, whereas *S. scabrum* and *S. villosum* decreased PCN by more than 80%. When cropping susceptible crops, after three seasons of successive cultivation of these AIV, galling index and number of developing PCN females measured on susceptible crops decreased by more than 75%. Wilting incidences and RKN-PCN co-infection incidences also decreased significantly. In Chapter 4, the resistance mechanism of African nightshade and African spinach to RKN and PCN species was studied. We showed that successful parasitism was impaired by localized root tissue necrosis and disintegration during the early stages of nematode infection in resistant African nightshade and African spinach. Notably, *A. dubius* (broad leaf) showed full resistance to *M. enterolobii*, a highly pathogenic nematode known for overcoming plant resistance in most cultivated crops. For PCN, both *S. scabrum* and *S. villosum* stimulated PCN hatching but not their reproduction with a similar mechanism of resistance as proposed before. These findings reveal that nematode resistant AIV evolved cellular self-destruction of root tissue as a mechanism for defense against RKN and PCN. In that way, a cell suicide process orchestrates the containment, starving, and expulsion of parasitic nematodes. Inevitably, the information generated in this study is important in breeding programmes, designing crop rotation schemes, and cropping systems in order to avoid yield losses caused by high RKN and PCN soil infestation. This will help to support the implementation of a productive and effective integrated pest management strategy that is needed to meet the nutritional requirements of people in sub-Saharan Africa.

Zusammenfassung

In Afrika heimische Blattgemüse (AIV) sind essentielle Komponenten einer reichhaltigen und ausreichenden Ernährung der Einwohner sub-Sahara Afrikas. Obwohl AIV ausdauernd und tolerant gegen wechselnde Umweltbedingungen sind, wurden sie von der agrarwissenschaftlichen Forschung bisher weitgehend ignoriert. Pflanzenparasitäre Nematoden, insbesondere Wurzelgallennematoden (RKN: *Meloidogyne* spec.) und Zystennematoden (CN: *Globodera* and *Heterodera* spp.) verursachen schwerwiegende Schäden an einer Vielzahl von Nutzpflanzen und sind von hoher ökonomischer Bedeutung.Trotz der wichtigen Rolle, die Untersuchungen zum Vorkommen von Nematoden und diagnostischen Ansätze spielen, ist bisher wenig über das Vorkommen und die korrekte Identität von RKN und CN an AIV wie z.B. dem Afrikanischen Nachtschatten (*Solanum* spec.) und Afrikanischem Spinat (*Amaranthus* spec.) bekannt. In Kapitel 2 und 3 der vorliegenden Arbeit wird eine Studie beschrieben, in der Zysten- und Wurzelgallennematoden in Kenia in einem Barcode-System identifiziert wurden. Die Studie ergab, dass *S. villosum* stark von Wurzelgallennematoden (RKN) befallen wurde, während *S. scabrum* und *A. dubius* nur wenig infiziert war. Boden aus der Rhizosphäre von *S. villosum* und *S. scabrum* enthielt nur wenige Zysten von Kartoffelzystennematoden (PCN) und es wurde keine Neuentwicklung von Zysten an Wurzeln dieser Pflanzen beobachtet. Die analysierten RKN und PCN Haplotypen mitochondrialer DNA sind weltweit verbreitet, was darauf schließen lässt, dass Gebiete, in denen viele Arten von RKN heimisch sind, dennoch von nicht-heimischen PCN invadiert werden können. In diesem Zusammenhang wurde auch beobachtet, dass RKN-PCN Koinfektionen an Kartoffel und RKN-RKN Koinfektionen an Tomate und *Parthenium hysterophorus*, einem invasiven Neophyten in Afrika auftraten. In Kapitel 3 der Arbeit wurde in einem Feldversuch am KALRO, Kenia, die Populationsdynamik von RKN und PCN an *A. dubius*, *A. cruentus*, *S. scabrum*, and *S. villosum* über 2 Jahre hinweg untersucht. Der Effekt des Anbaus von AIV auf die Bodenverseuchung durch RKN und PCN wurde durch den Anbau und die Befallsanalyse anfälliger Tomate und Kartoffeln analysiert. Nach wiederholtem Anbau von *A. dubius* and *S. scabrum* reduzierte sich die Bodenverseuchung durch RKN um 85%, die Werte von PCN fielen durch Anbau von *S. scabrum* and *S. villosum* um mehr als 80%. Die Entwicklung von PCN Weibchen an anfälligen Pflanzen reduzierte sich durch den dreimal wiederholten Anbau von resistenten AIV um 75%. Auch der Welkeindex und RKN-PCN Koinfektionen gingen deutlich zurück. In Kapitel 4 wurde der Resistenzmechanismus von Afrikanischem Nachtschatten und Afrikanischem Spinat gegenüber RKN und PCN Spezies untersucht. Es konnte gezeigt werden, dass die parasitäre Entwicklung der Nematoden durch lokale Nekrosen und Gewebeauflösung während der frühen Phasen des Infektionsverlaufs in den resistenten Pflanzen blockiert wurde. Interessanterweise zeigte *A. dubius* (breitblättrig) vollkommene Resistenz gegenüber *M. enterolobii*, einem hoch pathogenen Nematoden, gegen den die allermeisten Nutzpflanzenarten keine Resistenz aufweisen. *S. scabrum* and *S. villosum* stimulierten den Schlupf von PCN Larven, boten ihnen jedoch aufgrund des beschriebenen Resistenzmechanismus keine Grundlage für eine Weiterentwicklung. Die Untersuchungen ergaben, dass nematodenresistente AIV über einen zellulären Mechanismus verfügen, der bei Resistenzreaktionen gegen RKN und PCN zur Selbstzerstörung von Wurzelgewebe führt. Dieser Mechanismus führt zur Abwehr und zum Verhungern der Nematodenlarven. Zweifellos sind diese Informationen wichtig für Zuchtprogramme und die Entwicklung von Fruchtfolgen und Anbausystemen, um künftig die Ertragsverluste durch RKN und PCN zu reduzieren. Auf diesem Wege kann einerseits die Implementierung von produktiven und effektiven Systemen für integrierte Bekämpfung, die notwendig sind, um den Nahrungsbedarf für die Menschen in sub-Sahara Afrika decken zu können.

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Acronyms

AIV	African indigenous vegetables
CABI	Centre for Agriculture and Bioscience International
COI	Cytochrome c oxidase subunit 1
DAMPs	Danger-associated molecular patterns
DNA	Deoxyribonucleic acid
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization
IFAD	International Fund for Agricultural Development
IFPRI	International Food Policy Research Institute
IPM	Integrated pest management
KALRO	Kenya Agricultural & Livestock Research Organization
NAD5	NADH dehydrogenase subunit 5
NBS-LRR	Nucleotide-binding site leucine-rich repeat
PAMPs	Pathogen-associated molecular patterns
PCN	Potato cyst nematodes
PCR	Polymerase chain reaction
RKN	Root-knot nematodes
SDG	Sustainable Development Goal
UN	United Nations
USDA	United States Department of Agriculture
WHO	World Health Organization

Chapter 1

General introduction

1. The role of AIV in combating malnutrition and diseases

The reliance on a very few crop species for energy, with 84% of calories coming from just 17 crops (West et al., 2014), is the primary reason for the dual burden of malnutrition. The national food supplies have become more homogenous, with South/Southeast Asian regions diets dominated by white rice. Micronutrient deficiency prevalence is at around 30% (Beal et al., 2017). In Africa, the adoption of calorie-dense but nutrient-poor foods (e.g. maize, wheat, and rice) during 1979−1993 resulted in marked declines in micronutrient in diets (Beal et al., 2017; Forouzanfar et al., 2015). The massive success of the Green Revolution was as a result of package deal of seeds, fertilizers, and energy, but with very few crop species. It is now clear that a new package is urgently needed to deal with future agriculture challenges, one that is tailored across different environmental, social, and health outcomes. This will require diversifying our current cropping systems currently dominated by limited crop species and to demonstrate that diversified farming can be financially competitive with the current monocultures. The cultivation and consumption of traditional crops is one successful way of adapting to numerous challenges facing our food systems. Traditional crop species can act as a safety net against various agro-ecological stresses. Nutrition strategies are required in order to have healthy and capable farming families so that they can contribute to the world's dietary diversity.

Nutrients from fruits and vegetables form an essential component of the diet. The World Health Organization (WHO) report on global strategy on diet, physical activity, and health strongly encouraged the consumption of more fruits and vegetables. For a healthy lifestyle a minimum daily intake of 400 g of fruits and vegetables is recommended (WHO, 2006). In sub-Saharan Africa the production of fruit and vegetables remains very low making Africa a hotspot for food insecurity and malnutrition. In Africa over 50 million children are emaciated and the majority of undernourished is found in sub-Saharan Africa. Every year an additional of 3 million children die from undernutrition associated causes (Black et al., 2013; IFPRI, 2017). Simultaneously, of recent sub-Saharan Africa is experiencing a rise of chronic diseases such as cardiovascular disease, obesity, and various forms of cancer (Forouzanfar et al., 2015). In response to that the international community has set up ambitious goals to eliminate malnutrition in all its forms. Consequently, this saw 2016 the start of a UN Decade of Action on Nutrition designed to implement the global effort to fight malnutrition (Bhutta, 2016).

In some parts of Africa some families relies on the lesser appreciated and little known indigenous vegetables as well as other native food plants as their diet (Guarino, 1997). These indigenous vegetable crops are popularly known as African indigenous vegetables (AIV) and they are mainly associated with smallholder farmers. Neglecting these crop plants has compromised sustainable food production and human health in Africa. AIV are cheap source of a variety of nutrients and phytochemicals that are important for human health. These include vitamins, minerals, proteins, low GI (glycaemic index) carbohydrate, carotenoids, fibre, antioxidants, and other nutraceuticals with health-promoting benefits (Al-Gubory, 2017; Massawe et al., 2016; Uusiku et al., 2010). Dietary improvement through the consumption of AIV have been suggested as an effective strategy for reducing micronutrient deficiency because they are readily available and cheap source of nutrients. In fact AIV such as African nightshade (locally known as mnavu or managu in East Africa) and African spinach (locally known as dodo or terere in East Africa) are more nutritious than some of the recently introduced exotic vegetables (Table 1 and 2).

1.1 Nutritional composition of AIV

AIV such as African nightshade (422 µg/100 g), African spinach (537 µg/100 g), jew mellow (329 µg/100 g), cowpea (537 µg/100 g), spider flower (434 µg/100 g) are rich in vitamin A which is normally of animal origin (Van Jaarsveld et al., 2014; Jimoh et al., 2018). In Northen Ghana the consumption of AIV (*Ceiba* spp. and *Manihot* spp.) increased the preschool children attendance due to significant increase of retinol status (Takyi, 1999). Some AIV are rich in folic acid which is present in smaller amounts in grain and cereal crops commonly cultivated in Africa. They include African eggplant (118 µg/100 g), cowpea (129 µg/100 g), spiderflower (121 µg/100 g), African spinach (75 µg/100 g), cassava (118 µg/100 g), and jute mallow (118 µg/100 g). Their folic acid is comparable or more to the introduced commercial vegetables such as broccoli and spinach (Stadlmayr et al., 2012). AIV such as cowpea (57 mg/100 g), African eggplant (79 mg/100 g), taro (52 mg/100 g), African nightshade (120 mg/100 g), baobab (47 mg/100 g), and African spinach (60 mg/100 g) are the best source of vitamin C (Abukutsa-Onyango, 2003; Jimoh et al., 2018; Stadlmayr et al., 2012). In fact their vitamin C content is comparable to tropical fruits such as oranges which are considered to be excellent source of vitamin C (Phillips et al., 2018).

Apart from vitamins, AIV are also a rich source of essential minerals. In a preliminary

assessment of AIV collected in KwaZulu-Natal, South Africa to establish their mineral content (Ca, Fe, Na, Mn, Mg, Zn and P) and antioxidant levels. Twelve AIV namely *M. balsamina, Amaranthus dubius, Amaranthus hybridus, Amaranthus spinosus, Cucumis metuliferus, Cleome monophylla, Asystasia gangetica, Cucurbita tribola, Solunum scabrum,* and *Physalis viscosa* had mineral concentrations exceeding 1% of plant dry weight and these were much higher than typical mineral concentrations in exotic leafy vegetables (Odhav et al., 2007). In many parts of Africa nursing women, pregnant, and young children are at high risk of Ca malnutrition. Calcium deficiency in the same population of elderly people leads to osteoporosis and osteopenia. Some AIV contributes significantly to the daily intake of Ca (Stadlmayr et al., 2012; Uusiku et al., 2010). These are African eggplant (332 mg/100 g), moringa (434 mg/100 g), baobab (313 mg/100 g), goose foot (226 mg/100 g), jute (291 mg/100 g) and cowpea (265 mg/100 g). Iron deficiency, anaemia, and infections causes high morbidity and mortality in children under the age of 5 (Jonker et al., 2017). This is especially more prevalent in poor families because they cannot afford animal based protein. These include African nightshade (7.2 mg/100 g), amaranth (5.1 mg/100 g), cassava (6 mg/100 g), goose foot (6 mg/100 g) and cowpea (5 mg/100 g). Recently a study revealed that there is inadequate intakes of zinc ranging from 51% to 99% in both younger and older children (Harika et al., 2017). Some AIV such as goose foot with zinc content of 19 mg/100 g may contribute substantially in many parts of Africa (Uusiku et al., 2010).

AIV are a rich source of plant-based bioactive compounds phytonutrient and natural antioxidants with potent antioxidative activities. A wide array of phytochemicals including alkaloids, flavonoids, tannins, saponins, steroids, and phenols are found in AIV such as *Solanum scabrum, Corchorus olitorius, Cleome gynandra, Amaranthus dubius,* and *Crotalaria ochroleuca*. Their leaf extract have high free radical scavenging properties (Mibei et al., 2017; Ndhlala et al., 2017; Neugart et al., 2017), thus these bioactive antioxidant compounds can protect biological organs and tissues against free radicals induced oxidative stress. The protection of biological organs from free radicals is crucial for cell redox homeostasis and organ structural integrity and function. Thus, their consumption promotes health and prevents the onset of the risk of development and progression of noncommunicable human illness such as cancer, cardiovascular, autoimmune, inflammatory, and neurodegenerative diseases (Al-Gubory, 2017; Ndhlala et al., 2017).

Table 1. Nutritional value of raw and cooked (boiled and drained) African spinach (amaranth) leaves, compared to other leafy vegetables commonly grown in sub-Saharan Africa

Nutrients/Leafy vegetable	Green cabbage Cabbage, raw Value per 100 g	Chinese cabbage Cabbage, raw Value per 100 g	English spinach Spinach, raw Value per 100 g	African spinach Amaranth, raw Value per 100 g	Amaranth, cooked Value per 100 g
Protein (g)	1.28	1.20	2.86	2.46	2.11
Minerals					
Calcium (Ca; mg)	40	77	99	215	209
Iron (Fe; mg)	0.47	0.31	2.71	2.32	2.26
Magnesium (Mg; mg)	12	13	79	55	55
Phosphorus (P; mg)	26	29	49	50	72
Potassium (K; mg)	170	238	558	611	641
Sodium (Na; mg)	18	9	79	20	21
Zinc (Zn; mg)	0.18	0.23	0.53	0.90	0.88
Copper (Cu; mg)	0.019	0.036	0.130	0.162	0.158
Manganese (Mn; mg)	0.160	0.190	0.897	0.885	0.861
Vitamins					
Vitamin C (mg)	36.6	27.0	28.1	43.3	41.1
Riboflavin (mg)	0.040	0.050	0.189	0.158	0.134
Niacin (mg)	0.234	0.400	0.724	0.658	0.559
Vitamin B-6 (mg)	0.124	0.232	0.195	0.192	0.177
Folate (total; mcg)	43	79	194	85	57
Vitamin A, RAE1 (mcg)	5	16	469	146	139
Vitamin K (mcg)	76	42.9	482.9	1140	-
Lipids					
Fatty acids (total saturated; g)	0.034	0.043	0.063	0.091	0.050
Cholesterol (mg)	0	0	0	0	0

Source: USDA National Nutrient Database for Standard Reference, Release 23 (2010) http://www.nal.usda.gov/fnic/foodcomp/search/ [1]The recommended dietary allowance (RDA) for vitamin A is measured in retinol activity equivalents (RAE).The body obtains vitamin A from retinol and carotenoids. One RAE is equal to 1 mcg of retinol; 12 mcg of beta-carotene; 24 mcg of other vitamin-A precursor carotenoids

Table 2. Nutritional leafy value of African nightshade in comparison to other leafy vegetables commonly grown in sub-Saharan Africa

Nutrients\Leafy vegetable	Green cabbage	English spinach	African spinach	African nightshade	Southern pea
	Cabbage[a]	Spinach[a]	Amaranth[a]	Mnavu[ab]	Cowpea[a]
Protein (g)	1.28	2.86	2.46	4.3	1.5
Minerals					
Ca (mg)	41	133	380	194	265
Fe (mg)	0.6	3.1	6.2	3.5	5.1
Mg (mg)	12	53	93	25	60
P (mg)	37	45	58	75	61
K (mg)	317	502	602	430	475
Na (mg)	12	87	13	3	6
Zn (mg)	0.2	0.53	0.72	0.8	0.5
Vitamins					
Vit A (µg)	8	409	241	5.8	150
Vit E (mg)	0.15	2.31	0.24	2.3	2.36
Folate (µg)	48	176	79	70	129
Vit C (mg)	54	36	45	75	57
Cholesterol (mg)	0	0	0	0	0

Values per 100 g fresh leaves from: [a]Stadlmayr et al., 2012, [b]Keding and Yang, 2009

2. Biology of African spinach

African spinach (Amaranthaceae: *Amaranthus* spp.) are herbaceous, short-lived annuals. Plants are upright and sparsely branched with thick and fleshy grooved stems. African spinach varieties cultivated as leafy vegetables have relatively large leafy area compared to other varieties. The shape and colour of the leaf shows high variability: from green or red to purplish with the pigment betalain. The genus *Amaranthus* is highly diverse consisting of more than 70 species. Commonly cultivated species in Africa are *A. dubius* and *A. cruentus* (Figure 1A and B), but other species such as *A. tricolor* and *A. viridis* are also cultivated (Mureithi et al., 2017). African spinach belongs to the fast growing plants and its photosynthesis is very efficient. The photosynthetic pathway is C4 and it can achieve higher yield under drought conditions. A recent study demonstrated that the yield and nutritional composition of amaranth is less affected by future rise in summer temperatures compared to other crops (Hwang et al., 2018). African spinach is one of the crop that has been earmarked for utilization to support food security and climate change mitigation (Alemayehu et al., 2015). In fact a recent study showed that drought stress enhances

nutritional and bioactive compounds, phenolic acids and antioxidant capacity of *A. tricolor* leafy vegetable (Sarker & Oba, 2018). African spinach can be grown in marginal areas, with the ability to grow in various soil type and some varieties can tolerate pH as high as 8.5 as well as acidic soils. However the ideal pH is 5.5-7.5. The crop requires less nitrogen compared to maize but it response very well to fertilization. Most of the African spinach species are day neutral. African spinach is rich in morphological diversity and frequent hybridization, a variety of morphotypes are recognized. Thus, morphological, biochemical, molecular, and cytogenetical parameters are required for proper identification and understanding species/variety relationships (Das, 2016). African spinach is monoecious, but interspecific hybrids occur frequently as a result of cross pollination of two different species. However, 90% of the hybrids between grain and leaf amaranth are sterile suggesting a big genetic difference (Das, 2016). In Africa, little effort has been devoted towards the development of hybrids with desirable traits. The desirable traits in African spinach of significance are: time of flowering, determinate vs indeterminate growth pattern, leaf characteristics, taste and nutritive value, pest and disease tolerance, stress tolerant and seed pigment. Propagation of African spinach is mainly by direct seeding of small black or brown seeds. A seeding rate of 2 g per m^2 is recommended. Growth is rapid under humid and warm weather conditions. After 3 – 4 weeks seedlings are big enough for consumption or for transplanting.

3. Biology of African nightshade

African nightshade (Solanaceae: *Solanum* spp.) is a group of AIV with a wide distribution across Africa. Inaccurate identification lead to confusion over the species (Ogg et al., 1981). For example the commonly used term "*S. nigrum* agg." in describing African nightshade is misleading in the absence of specimen vouchers. Like most *Solanum* spp, flowers of members of the African nightshade are insect-pollinated mainly by bees (De Luca & Vallejo-Marín, 2013). In Africa, the leaves and berries of African nightshades are eaten as spinach and fruits respectively (Keding & Yang, 2009) (Figure 1D). The fruits have a thin skin and a juicy berry and are bird-dispersed (Knapp, 2002; Särkinen et al., 2018). Steroidal glycoalkaloid are very high in unripe fruits and very low in the ripe berries (Cipollini & Levey, 1997a, 1997b). This makes the unripe fruits unattractive to frugivores, insects, and other pathogens (Cipollini & Levey, 1997a). Thus, pests and pathogens

invasion on unripe fruits is restricted. This increases the chances of consumption by animals or birds at maturity stage and dispersal (Särkinen et al., 2018). There are extensive cases of ploidy within *Solanum* spp. The ploidy level in potatoes has been extensively investigated due to their economic importance. The ploidy in potatoes varies: they can be diploid, tetraploid or hexaploid, with the cultivated potato itself (*S. tuberosum* L.) consisting of both diploid and tetraploid populations (Spooner et al., 2007). African nightshades have been the focus of the recent studies and some studies have verified the and vouchered chromosome counts (Edmonds & Glidewell, 1977; Ronoh et al., 2018). The species of African nightshade are self-compatible (Edmonds, 1979; Olet et al., 2011). Natural hybridization between diploids, polyploids, and between polyploids has been reported (Table 3). There is still a lot of confusion regarding the chemistry and toxicity of African nightshade as they do contain nortropane alkaloids known as calystegines (Pigatto et al., 2015). However these compounds like in other Solanaceae plants are only toxic under laboratory experiments with rats (Stegelmeier et al., 2008). A chemical survey study survey indicated that toxic glycoalkaloids are only available in unripe fruits (Cipollini et al., 2002), while these compounds are not found in the leaf and stem (Voss et al., 1993). African nightshade does not tolerate drought conditions as much as African spinach, but they can grow in different soil types. Efforts are on-going to develop varieties that can tolerate drought conditions (Dinssa et al., 2016).

Figure 1. African indigenous vegetables. (A – C) *Amaranthus dubius* – edible leaf, (D) *A. cruentus* – fluorescent flower, (E) *Solanum scabrum* – edible leaf, (F) *S. villosum* – edible leaf, (G) *S. scabrum* – nonedible black berries, and (H) S. villosum – edible orange berries.

Table 3. Combinations of *Solanum* spp. crosses from various studies summarizing possible and unsuccessful hybridizations (Ganapathi, 1986; Heiser et al., 1965; Jacoby & Labuschagne, 2006; Olet et al., 2015; Ronoh et al., 2018).

♀ ♂ =	1	2	3	4	5	6	7	8	9	10	11	12
Diploids												
1.*S. americanum*	++	−	−	+	+	+	+	+	+	+	++	
2.*S. chenopodioides*	+	++	−	+	+	+	+	+	+	+	++	
3.*S. sarrachoides*	−	−	++	−	−	−	−	−	−			
4.*S. nodiflorum*	+		+									
Tetraploids												
5.*S. retroflexum*	+	+	−		++	++				+	+	
6.*S. burbankii*	+	+	−		++			++				
7.*S. florulentum*	+	+	−				++	−	−	−	++	
8.*S. villosum*	++	+	−				++	++		++	++	
9.*S. tarderemotum*	+	+	−				++	++	++		++	
10.*S. memphiticum*	++	+	−				++	++	++	++	++	
Hexaploids												
11.*S. scabrum*											++	
12.*S. nigrum*	+											++

++ = fertile progeny; + = sterile progeny; − = unsuccessful crossing (seedless berries produced).

4. Importance of AIV

AIV are underutilized and neglected crops with useful properties, but regarded less important than major world crops. However, they play a significant role in many low-income countries, providing food security and nutrition to consumers, as well as income to resource-poor farmers. Healthy diets provided by AIV give rise to a variety of outcomes. These relate not only to nutrition and health, but also to all the dimensions of sustainability, which in turn link back to the food system drivers. AIV can; (1) prevent malnutrition in all its forms (undernutrition, micronutrient, deficiencies, overweight and obesity), (2) reduce environmental impact - because the demand for certain diets influences water and land use, biodiversity etc., (3) improve the income of smallholders and poor people - because 97 %

of people employed in agriculture lives in low-income countries, (4) improve health for the most vulnerable and, therefore, enhance social equity, which may positively impact vulnerable groups such as those living in poverty, women, children and smallholders. Unhealthy diets and malnutrition slow economic growth and perpetuate poverty via three main routes: direct losses in productivity from poor physical status; indirect losses from poor cognitive function and deficits in schooling; and losses owing to heavy burden health care costs. The economic cost is transgenerational because malnourished mothers are more likely to give birth to malnourished babies, who are in turn more likely to grow up to be malnourished adults (Delisle, 2008; Reinhardt & Fanzo, 2014). Although traditional crops such as AIV contain important macro- and micronutrients, such crops have been largely neglected by both researchers and industry due to their limited economic importance in the global market, but they are still important in traditional farming systems. In Kenya, a low-income country, the production and consumption of AIV have increased greatly in the recent years due to increasing consumer awareness about their health and nutritional benefits (Schippers, 2000). In fact the area under AIV production expanded by 25 % (Cernansky, 2015). Most food retail outlets including supermarkets sell AIV leaves (Ngugi et al., 2007). The increasing awareness about AIV nutritional qualities, changes in lifestyle and availability of cooling-storage facilities have also boosted their consumption levels in urban dwellers.

In Africa, specifically in Kenya and many parts of East Africa, AIV such as African nightshade (*S. scabrum* and *S. villosum*) and African spinach (*A. dubius* and *A. cruentus*) are commonly cultivated by smallholder farmers. Distinctive attributes of African nightshade and African spinach are their adaptation to adverse local climatic conditions, requiring very minimal inputs and superior nutritional properties (Achigan-Dako et al., 2014; Jimoh et al., 2018; Ndhlala et al., 2017; Neugart et al., 2017; Traoré et al., 2017). They are an invaluable plant genetic resource for the agriculture which is currentlly facing an uncertain future. Their cultivation is a way of creating multifunctionality in cropping systems. These functionalities includes microclimate control, yield optimization, erosion control, water and nutrient-use efficient and increased pest and disease control. The rapid growth and all season growth habit makes them compartible with most cropping systems and thus they can be utilized as trap, catch or relay crop.

More recently new challenges have emerged in low-income farming systems further threatening the sustainability of food production in Africa. Reports of introduced pests and

diseases attacking important food crops were reported in Africa. The fall armyworm (*Spodoptera frugiperda*) is rapidly spreading across Africa with an astonishing speed devouring on the main staple crop, maize (CABI, 2017). An outbreak of maize lethal necrosis disease was reported on maize in several African countries (Mahuku et al., 2015). Highly damaging plant-parasitic nematodes such as potato cyst nematodes (PCN: *Globodera* spp.) were recently reported in Kenya parasitizing potato (Mburu et al., 2018; Mwangi et al., 2015) as well as root-knot nematodes (RKN: *Meloidogyne* spp.) which is a chronic problem on several vegetable crops in Africa. Thus, farmers across Africa grapple with many challenges ranging from environmental change, migration, pests, and disease outbreaks. Therefore, it is important for the smallholder farmers to adopt cropping systems that offers adequate nutrition and health, reduce dependence on external inputs, such as chemical pesticides and fertilizers, and environmental stress-resilience and resistance to emerging pests and diseases. African nightshade and African spinach are nutritious crops offering many health benefits and they are adapted to the local conditions.

5. Plant-parasitic nematodes

Nematodes are non-segmented roundworms that are most abundant and speciose existing group of metazoan organisms. Up to now, approximately 23000 nematode species have already been described, but there is still more than one million species yet to be described (Blaxter, 2011). The ubiquitous nature of nematodes put them as key players in crop production, animal, and human health as well as ecosystem equilibrium. Indeed, certain free-living nematodes can be used as environmental indicators to monitor pollution, and play important role in decomposition process in soils (Bongers & Bongers, 1998). Free-living nematodes such as *Caenorhabditis elegans* have become an excellent model organism to study various biological processes in human beings. Next to their usefulness, some nematodes have a strong impact on public health. Intestinal parasites are classified as neglected tropical diseases that infect low-income populations and decrease productivity of young aged generation. More than 1 billion people worldwide are infected by helminth species *Ascaris lumbricoides, Trichuris trichiura,* hookworm species *Necator americanus* and *Ancylostoma duodenale,* and *Strongyloides stercoralis* causing malnutrition and bowel obstruction (Pullan et al., 2014). Other species of parasitic nematodes belonging to the Strongylidae family cause economic losses in ruminant livestock production by inducing

gastroenteritis (Roeber et al., 2013). In this thesis the nematodes that affect vascular plants are dealt with. Globally, plant-parasitic nematodes affect numerous plant families. More than 4100 species plant-parasitic nematodes are known, they cause agricultural losses estimated at $80 billion per year (Nicol et al., 2011). This estimation could be an underestimation, as many growers especially in low-income countries are ignorant about the damage caused by plant-parasitic nematodes. The problem is aggravated by the fact that these nematodes are microscopic soil pathogens and the above-ground symptoms they cause on plants are very unspecific.

Plant parasitic nematodes are obligate parasites of plants that have a worldwide distribution. They are capable of parasitizing roots, leaves, tubers, and corms of thousands of plant species, resulting in poor quality and reduced yield of crops. The most damaging plant parasitic nematodes species are found in the tropical and warm temperate regions. In these regions subsistence agriculture systems predominate. Phylogenetic analyses show that nematodes have evolved independently to be plant-parasites on several occasions in their evolutionary history, within four clades as biotrophic pathogens (Bert et al., 2011; Van Megen et al., 2009) (Figure 3). Plant-parasitic nematodes display diverse interactions with their host plants. All enter the host with the aid of a hollow, protrusible stylet, or mouth spear. The possession of an oral stylet by nematodes is an example of convergent evolution. This oral stylet is used to penetrate cells and injecting some secretions into plant cells. The nematode secreted molecules are the key interface between plant and nematode interactions in order to facilitate feeding. Since nematodes have evolved parasitism on multiple different occasions, different feeding behaviors are observed that range from short term to long term. Migratory ectoparasitic nematodes are short term feeding nematodes and do not enter the host, they only feed briefly on plant roots as they encounter them while roaming in the soil. Nematodes belonging to this group include *Trichodorus* spp., and *Longidorus* spp. are also capable of transmitting plant viruses (Van Hoof, 1968). Migratory endoparasitic causes extensive damage to host tissue as they enter the host and migrate. Such nematode including *Pratylenchus* spp. and *Radopholus* spp. causes massive root tissue necrosis and toppling of banana plants (De Waele & Elsen, 2002). Some nematodes are semi-endoparasites i.e. they have migratory stages, but at some stage of the life cycle they penetrate the host in order to feed. These nematodes induce a feeding site within their host at the sedentary stage e.g. *Rotylenchulus reniformis*. However, the most economically

important nematodes, root-knot nematodes (RKN) and cyst nematodes (CN) are sedentary endoparasites parasites complex feeding structures in the roots. In contrast to migratory ectoparasitic and endoparasitic nematodes, which establish a short term relationship with their host, the sedentary endoparasitic life styles of RKN and CN evolved to have a prolonged dialogue with the host plant. Thus, plant-parasitic nematodes collectively share the capacity to manipulate their host plants during the entire parasitic life stages (Figure 3).

5.1 Root-knot nematodes (RKN: *Meloidogyne* spp.)

Root-knot nematodes are a group of sedentary obligate plant parasites with a global distribution. They have successfully acquired all the necessary tools required to parasitize a wide range of plant families. In former times root-knot nematodes were placed within Heteroderidae, the same as cyst-forming nematodes. Now, with increasing use of molecular markers in systematics it became clear that both groups of sedentary endoparasites were the result of convergent evolution (De Ley & Blaxter, 2004). Consequently the genus *Meloidogyne* is now a standalone subfamily, the Meloidogyninae Skarbilovich, 1959. Currently, there are 101 described species of *Meloidogyne,* although the species status of several species is highly debated (Karssen et al., 2012). The genus is mainly composed of 3 clades (Tigano et al., 2005). The most damaging *Meloidogyne* spp. the so called *M. incognita* group and *M. enterolobii* belongs to clade I (Pagan et al., 2015) . *Meloidogyne incognita* group include the following *M. javanica*, *M. incognita,* and *M. arenaria*. Clade II consists of *M. hapla*, a species restricted to temperate climate, whereas clade III is composed of; *M. fallax*, *M. chitwoodi*, *M. naasi*, *M. graminicola,* and *M. minor* (Holterman et al., 2009). Apart from the main 3 clades the genus includes several early diverging lineages such as; *M. camelliae*, *M. baetica*, *M. coffeicola*, *M. mali*, *M. ichinohei*, and *M. africana* (Holterman et al., 2009; Janssen et al., 2017). It is worth to mention that the *M. incognita* group species abandoned sex long time ago, yet it is composed of the species with wider geographical distribution and greater agriculture impact (Figure 3). Specifically, *M. incognita*, *M. javanica*, *M. arenaria,* and *M. enterolobii* reproduce clonally using mitotic parthenogenesis. Remarkably, species belonging to this group can overcome host resistance quickly than their sexual relatives.

The life cycle of a RKN includes 4 juvenile stages and an adult life stage and takes 3 to 6 weeks to complete depending on the species and environmental conditions (Castagnone-

Sereno et al., 2013). The 4 stages are separated by molts, during which a new developmental stage is attained through cuticle replacement (Figure 4). During parasitism, the nematode maintains a constant dialogue with their host. After embryogenesis the first-stage juvenile develops and second-stage juvenile hatches. The motile second-stage juvenile invades the root in the zone of elongation. The second-stage burrows into the host root tissue causing no obvious damage (Castagnone-Sereno et al., 2013). The second-stage migrate intercellularly into the differentiating vascular cylinder (Wyss et al., 1992). The feeding of the second-stage will transform protoxylem and protophloem permanent feeding site called giant cells. The giant cells will selfishly withdraw nutrients from their neighboring cells and function as the sole food source to the nematode. As a result of feeding the second-stage will moult into third-stage, fourth stage and adult stage. The adult females will produce hundreds of eggs enclosed in a gelatinous matrix. When present, males do not actively feeds on plant tissue.

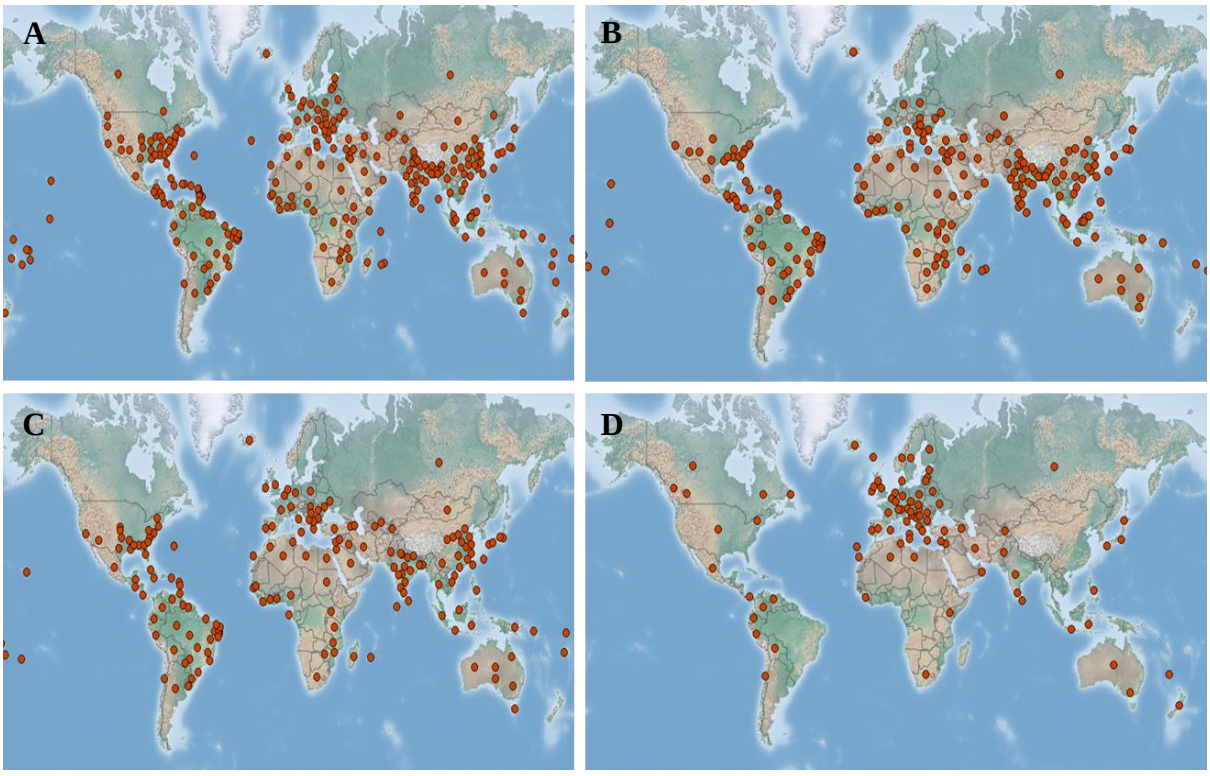

Figure 2. Geographical distribution of root-knot nematode (RKN) and potato cyst nematode (PCN). RKN species: (A) *Meloidogyne incognita*, (B) *M. javanica* (C) *M. arenaria*, PCN species: (D) *Globodera rostochiensis*. Information from the Cookies on Invasive Species Compendium (CABI) data sheet (https://www.cabi.org/isc/datasheet/33245; accessed 31 March 2018).

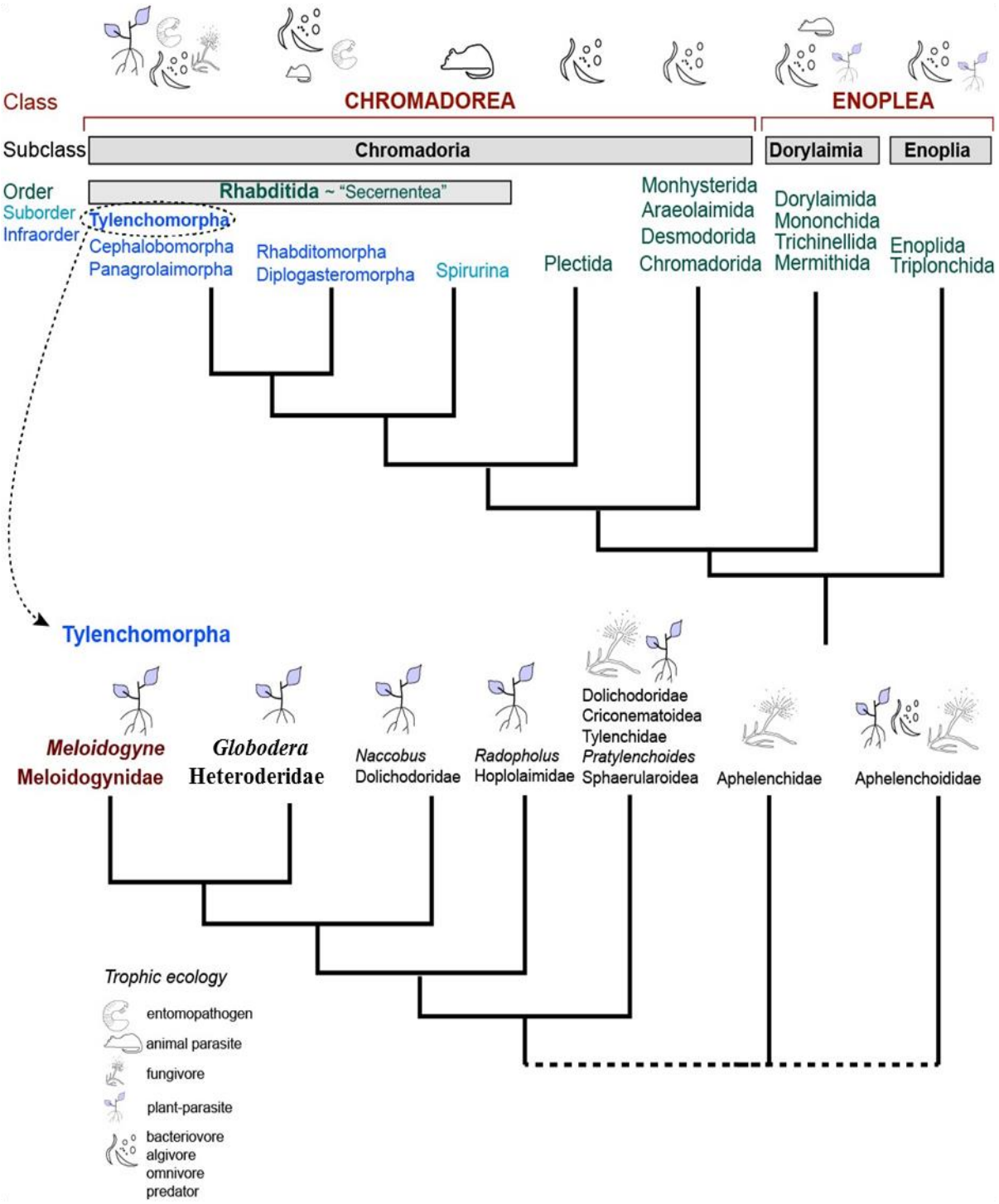

Figure 3. Above: overview of the phylogenetic relationships within the phylum Nematoda. Below: simplified phylogeny of the infraorder Tylenchomorpha, showing the phylogenetic position of the genera *Meloidogyne* and *Globodera*. The different origins of parasitism are shown besides the branches of both trees. Figure modified from Bert *et al.* (2011).

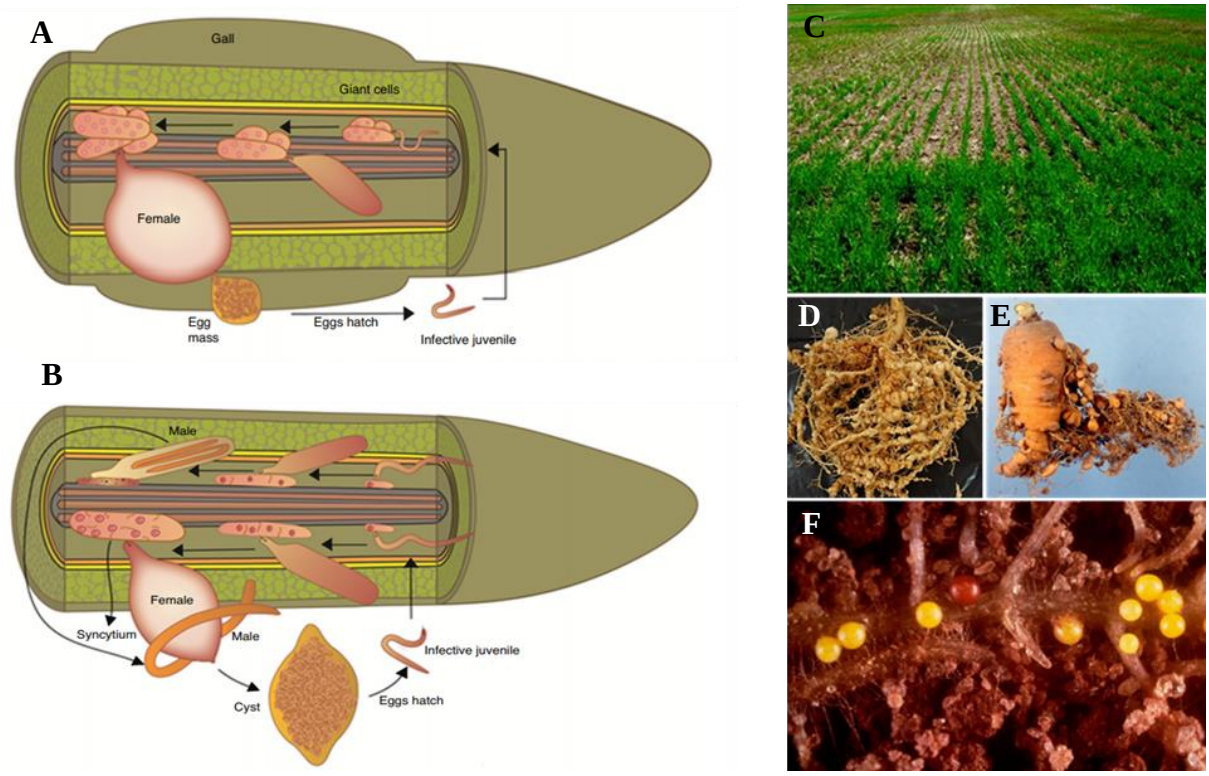

Figure 4. A schematic representation of the life cycle and symptoms of root-knot nematode (RKN) and potato cyst nematode (PCN) on infected plants. (A) RKN life cycle, (B) PCN life cycle, showing various developmental stages, (C) Above-ground symptoms caused by high soil infestations of RKN and PCN on cultivated crops, (D and E) Below-ground symptoms caused by RKN on tomato and carrot respectively, and (F) Below-ground symptoms caused by PCN on potato. Life cycles adapted from Siddique and Grundler (2018).

5.2 Cyst nematodes (CN: *Globodera* spp. and *Heterodera* spp.)

CN are sedentary obligate plant parasites of several crops. They belong to the family Heteroderidae. A total of 6 genera and a total of 99 species are documented, with the largest genera *Heterodera* contributing 82 species and *Globodera* contributing 12 species. These two genera are composed of species of global agriculture importance. Cyst nematodes of great agriculture importance are; *Heterodera glycines*, the soybean cyst nematode and *H. schachtii*, the sugar beet cyst nematode, potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* (Jones et al., 2013; Nicol et al., 2011). PCN such as *G. rostochiensis* and *G. pallida* are quarantine organisms in the EU (EC Directive 2000/29/EC) (EPPO, 2014). Despite the quarantine regulations potato cyst nematode are now distributed globally (Figure 4). Other cysts of agriculture crops are; *H. sacchari*, *H. oryzicola*, *H. elachista*, and *H. oryzae* on rice and *H. zeae* and *H. avenae* on maize. In

wheat-producing countries cereal cyst nematodes are a major problem. Cereal cyst nematode consists of closely related species forming what is referred to as cereal cyst nematode complex, these species includes *H. latipons*, *H. filipjevi,* and *H. avenae* (Nicol et al., 2007).

The life cycle of a cyst nematode starts with hatching of the second-stage juvenile and this can be stimulated by root exudates (also called leachates or diffusates) from a suitable host plant. The dependence on root exudate for hatching varies between cyst nematode species and is tied to the host range. Thus, species such as *H. schachtii* and *H. avenae* with wide host range hatches to a larger extend freely in water. Some species such as *H. glycine* partially depend on root exudates for hatching stimulation. *Globodera rostochiensis* and *G. pallida* are almost entirely dependent on root exudates for hatching (Clarke & Perry, 1977), but a small fraction of second-stage juvenile can hatch spontaneously in the absence of suitable root exudates (Devine et al., 1996). Once the second-stage juvenile hatches it invades the root behind the root tip at the zone of cell elongation. Supported by the secretions the second-stage juvenile pierce cells and enter the cells, thus they migrate intracellularly as opposed to RKN which move intercellularly (Wyss & Zunke, 1986). The second-stage juvenile continue to migrate until it reaches the central cylinder where it selects a cambial or procambial cell to initiate feeding site formation called syncytium (Wyss & Zunke, 1986). After successful formation of a feeding site the nematode continues feeding and molts into third-juvenile and fourth-juvenile, a final moult to become the adult female and male nematodes. The adult females will produce hundreds of eggs inside its body or secreted in a gelatinous matrix (Figure 4E).

6. Economic importance of plant-parasitic nematodes

In 2000, United Nations Millennium Declaration was signed by 189 countries, eight millennium development goals were established for development and poverty eradication. (UN General Assembly, 2000). In particular, the 2nd Sustainable Development Goal (SDG2) targets the end of malnutrition in all forms by 2030. Although, by 2010 undernutrition dropped in other countries, it remained the main contributor to the burden of disease and disability in sub-Saharan Africa. Conceivably, the underlying factors involved are many, nematology can partially contribute to this issue (Ciancio, 2015). Indeed, plant-parasitic nematodes have been reported causing yield losses to many cultivated crops

(Jones et al., 2013; Nicol et al., 2011). Even more worrisome is that plant-parasitic nematodes have adapted to parasitize the majority of cultivated vegetable crops leading to significant yield losses. Yet nutritional recommendations emphasize vegetable consumption for health-benefits associated with their bioactive nutritive molecules such as vitamins, minerals, and anti-oxidants.

While the damage symptoms on host crops by plant-parasitic nematodes is well documented, the data on the economic impact is estimated to be 77-80 billion dollars annually (Jones et al., 2013; Nicol et al., 2011). This figure is underestimation and it does not represent the actual situation on the ground for many reasons (Ciancio, 2015; Jones et al., 2013; Sikora & Fernandez, 2005): (i) plant-parasitic nematodes are microscopic belowground pathogens that causes unspecific aboveground symptoms, that can be confused with abiotic stress symptoms such as drought, nutrient, etc. Other pathogens such as fungi and bacteria cause distinctive aboveground symptoms on their host plants; (ii) plant-parasitic nematodes can enhance replication and transmission of other pathogens such as fungi, bacteria, and viruses (Khan & Siddiqui, 2017; Kyndt et al., 2017), as well as some pests such as aphids (Hoysted et al., 2017), through several mechanisms such as increase infectivity of pathogens by aiding attachment to host cells and dampening plant innate immune response; (iii) In most sub-Saharan Africa the majority of the farmers are unaware of the nematode problem, thus the impact of plant-parasitic nematodes is high in low-income countries (Onkendi et al., 2014), where farmers grapple with many other challenges including other pests and diseases.

In terms of economic loss the two genera dealt with in this thesis, root-knot and cyst nematodes rank first and second respectively (Jones et al., 2013). Root-knot and cyst nematodes infection is a major constraint on efficient crop production. Co-evolution of these nematodes with their hosts led to the emergence of a striking variety of strategies aiming at the evasion of host defenses, colonization of host tissues and, eventually, the formation of a feeding site. RKN and PCN use their host cells as safe houses, providing shelter from a harsh environment, to access nutrients as well as reproduction. The rapid multiplication of nematodes inside root tissues leads to the development of disease symptoms such as root galling and cyst formation on host plants (Bartlem et al., 2013; Perry, 1989; Sijmons et al., 1991). The disease symptoms on host plants may impair efficient water and nutrient uptake by the plant, thus the damage caused by RKN and cyst nematodes is partly dependent on environmental conditions. RKN are difficult to manage:

(i) they are highly polyphagous virtually able to parasitize the majority of flowering plants (ii) the existence of resistance breaking populations, and cyst nematodes have a restricted host range but hatching stimulation host specific. PCN are almost entirely dependent on root exudates for hatching (Clarke & Perry, 1977).

Most of farmers in low-income countries lack reliable crop protection products, insufficient control strategies, and a lack of awareness of plant-parasitic nematodes leaving most farmers unable to implement effective management strategies. Plant-parasitic nematodes crop damage can destroy crop yields completely, leaving low-income farming families vulnerable to food, nutritional, and livelihood insecurity. Therefore, the management of polyphagous RKN and host specific PCN is a major challenge to most farmers, hence innovative control strategies to diminish the yield loss caused by nematodes is urgently required. The implementation of a successful nematode management strategy requires a proper nematode diagnosis and surveillance mechanisms.

7. Management of plant-parasitic nematodes

7.1 Surveys and identification of plant-parasitic nematodes

Surveying of pests is an indispensable component of crop protection. The nematode surveillance activity is important for (i) implementing an effective nematode management strategy (ii) early detection and prevention of spread of indigenous and exotic nematodes (iii) identifying nematode free areas that can meet the national and international trade requirements. Therefore, a typical nematode survey falls into one of the three categories of detection, monitoring, and delimiting surveys. A detection survey is carried to establish if certain nematode species are present. Detection survey will therefore establish nematode species present and their respect host plants. This information can be used to establish nematode free areas as production sites. Early detection of highly pathogenic nematode pests can minimize their further spread through various management strategies. Monitoring surveys will help to assist in maintaining areas of low nematode prevalence through various nematode management strategies. Delimiting surveys will help to establish the boundaries of an area considered to be infested by or free from a nematode pest. This information is important to establish the nematode invasion or if the nematode pest can be eradicated. It is important to note that failure to detect targeted nematodes does not necessarily mean their absence but it could mean their population density is too low. Nematode detection in such

populations requires an increase in the sample size (Jones, 1955).

Correct nematode diagnosis is a cornerstone for the implementation of a successful nematode management strategy (Onkendi et al., 2014; Sasser et al., 1983). The identification of RKN and CN remains problematic. Although galls of *Meloidogyne* spp., cysts of *Globodera* spp., and *Heterodera* spp. are visible to the naked eye their precise species identity requires morphological and molecular analysis in the laboratory. The analysis is time consuming because it requires nematode extraction from the soil or root tissues before morphological and molecular analysis. Historically, morphology and morphometric characters were used to identify the species from the different genus, giving many problems because both genera are highly conserved in morphology (Hunt & Handoo, 2009). For the genus *Globodera* frequently used morphological characters include mature female and cyst, lateral field morphology and shape of the second stage juvenile tail; a detailed account on the use of morphological characters in *Globodera* can be found in (Subbotin et al., 2010). Within the genus *Meloidogyne* species diagnosis is carried out using the cuticle pattern around the vulva so called perineal pattern. A comprehensive morphological deviation in perineal pattern is described in Whitehead (1968) and Karssen (2002). Taxonomists have also used Transmission Electron Microscopy and Scanning Electron Microscopy (SEM) due to lack of sufficient taxonomic characters in search for more informative features (Ragsdale & Baldwin, 2010). In RKN SEM of the perennial pattern and head morphology allowed a more detailed study (Eisenback, 1985; Karssen, 2002).

Despite the monumental work carried out by several taxonomists in studying nematode morphology, morphological identification remains greatly influenced by the ability of individual nematode genotypes to produce different phenotypes when exposed to different environmental conditions. This includes the possibility of the same nematode species to change phenotypic state in response to environmental change hence morphological identification is hampered by phenotypic plasticity and interspecific similarities (Fusco & Minelli, 2010; Hunt & Handoo, 2009). Morphological characters are dependent on the reproductive strategy of a population. A recent study revealed the presence of different morphotypes within a single species from different locations (Troccoli et al., 2016). Although morphological identification of RKN and CN is sometimes necessary, it is time consuming and it requires great amount of experience as well as reliable and high quality reference material.

In order to complement morphology based identification, numerous nematode identification procedures have been proposed. The differential host preferences tests were used to classify races of RKN and CN species (Cloud et al., 1988; Hartman & Sasser, 1985). Until now there is no cytological or genetic basis to confirm these different host races, suggesting evidence of homological speciation (Castagnone-Sereno et al., 2013). Evidently, it is now widely accepted that epigenetics is at the forefront as the mechanism underlying the success of RKN (Perfus-Barbeoch et al., 2014), thus rendering differential host test unreliable for nematode diagnosis.

Biochemical based diagnostic technique that relies on isozyme profiles are also available. Specifically, Esbenshade and Triantaphyllou (1987) used esterase and malate dehydrogenase isozyme profiles profile variation to differentiate common RKN species. Later, esterase and malate dehydrogenase isozyme profiles variation was used to differentiate other *Meloidogyne* species (Carneiro et al., 2000; Karssen, 2002). Although the isozyme electrophoresis was very useful during the old days, this technique is time consuming and is dependent on stage specific of the nematode. Nevertheless, improvement on biochemical techniques that exploits the use of antibodies either monoclonal or polyclonal may offer a possibility for a cheaper and quicker nematode diagnosis.

Rooted in the recognition that the above mentioned methods are not effective and reliable, PCR based methods targeted DNA sequence molecules are now widely used for the identification of nematodes. Blaxter (1998), pointed out that morphological characters were not enough to give a clear resolution of nematode taxonomy. Therefore several molecular approaches were developed such as restriction length polymorphisms (RFLP) of genomic DNA, satellite DNA, restriction fragment analysis, species specific primers to amplify the sequence-characterized amplified regions (SCAR) in combination with gel electrophoresis, duplex PCR (Adam et al., 2007; Castagnone-Sereno et al., 1999; Fullaondo et al., 1999; Waeyenberge et al., 2009; Zijlstra et al., 2000). With the fast, affordable and accessible sequencing technology now available around the world, DNA barcoding can replace the above mentioned methods. Thus, PCR methods based on informative mitochondrial and ribosomal DNA fragments, coupled with Sanger sequencing are widely used for nematode diagnosis and phylogenetic analyses. In this context a ribosomal gene cluster (18S, ITS, and 28S), mitochondrial genes including COX1 and COXII and the noncoding region between 16S and COXII have been used for phylogenetic analyses and identification (Holterman et al., 2006, 2009; Kiewnick et al., 2014; Pagan et al., 2015). Given that nematode problems

exists mainly in low-income countries with minimal laboratory capacity, novel technologies for rapid diagnosis, continuous surveillance, and real-time tracking of emerging nematode species is required. Thus the significance of accurate nematode species identification as an indispensable tool to inform an effective nematode management strategy is discussed in this thesis.

7.2 Regulatory control to minimize nematode infestations

RKN and CN can be spread easily by human activities such as transfer of infested soil, water and plant debris. At local farm level, it is recommended that all soil attached to agricultural machines and tools must be cleaned to avoid transferring nematodes to the other fields. Quarantine strategy is a very effective preventive approach of most important plant-parasitic nematodes nematode but it is not a curative approach (Nyczepir & Thomas, 2009). Phytosanitary measures are available for the most damaging nematode species in order to reduce their spread. To avoid the introduction of RKN and CN into a field, awareness and regulation are required (Wesemael et al., 2011). Temperate RKN species of *M. chitwoodi* and *M. fallax* and PCN species of *G. rostochiensis* and *G. pallida* are quarantine organisms in the EU (EC Directive 2000/29/EC) whereas a tropical RKN species of *M. enterolobii* is classified under EPPO A2 list (EPPO, 2014). Despite the strict regulations, nematodes with great impact on yield are continuing to expand their territory. Recently, *G. rostochiensis* was reported in Kenya parasitizing potato (Mwangi et al., 2015).

The current advisory programmes on plant-parasitic nematodes do not rely on precise information due to lack of research articles on the impact of preventive approach. Nematode introduction to new farms can occur undetected because nematodes are extremely difficult to detect when in low numbers. In most cases detection can only happen when the soil infestations are already high and with high chances of spread to new fields. All of this establishes a vicious circle between new nematode detection and plant damage. Lack of awareness of nematode presence and damage leaves farmers unprepared and ill-equipped to implement proper management strategies. The approach is more applicable in protected environments since new nematode infestation is only possible through the entrance provided nematode free planting material and water is used. These observations motivated several growers to install airlocks fitted with foot baths at the point of entrance. Nematodes that can survive in planting material (e.g., seeds, bulbs, corms, tubers and

cuttings) thus they can be prevented by several methods such as heating, coating or spraying the planting material with natural nematicides, or using tissue culture to get the planting material when applicable (Bridge, 2000). On the contrary, eliminating nematodes in irrigation water can be a difficult task for the farmer.

7.3 Physical control of plant-parasitic nematodes

This approach is aimed at killing the nematodes by exposing them to irradiation, heat and osmotic pressure etc. Steaming is widely investigated and involves injecting steam into the soil resulting in soil sterilization. During this process most nematodes including other microorganisms found in the steaming layers are killed (Katan, 2000). The effectiveness of this technique depends on soil preparation. The soil must allow deep and uniform penetration of the steam. The application of the steam into the soil requires a boiler and an injecting device. This technique can inject steam under a fleece placed on the soil for up to 20 – 30 cm soil at over 80 ^{0}C (Collange et al., 2011). A solid hood placed on the soil can be used as an alternative to fleece method. This method is only suitable for smaller areas but sealing is not required. Another approach is to use negative pressure technique and this provides better results as steam is forced to enter the soil (Runia, 1983). This technique requires that the pipes are installed permanently in the field. Steaming reduces natural biocontrol processes by indiscriminately killing microorganisms (including non-pathogen ones) (Mcsorley et al., 2006). This technique also requires a lot of heat energy and equipment investment which might not be accessible to the resource poor farmers. Another approach is solarization, this technique makes use of transparent plastics films that will trap solar radiation and converts it into heat energy in the soil. This technique has been widely studied. In warm climatic conditions solarization can increase soil temperature by between 2 – 15 ^{0}C. Therefore its efficacy depends on temperature and duration. Wang and McSorley (2008), established that in a water bath heated above 38 ^{0}C all *M. incognita* second-stage juveniles were completely killed; but it requires less time at 42 ^{0}C than at 39 ^{0}C (48 h at 39 ^{0}C, but only 14 h at 42 ^{0}C). Degree-day is the appropriate measure as temperature alone is not a good measure of efficacy. Thus, more than 75 degree days were required to kill all nematodes at 40 ^{0}C, whereas only 24 ^{0}C were required at 43 ^{0}C (Wang & McSorley, 2008). Because of its dependent on solar energy, solarization must be practiced during the periods with maximum solar radiation in order to achieve maximum soil temperature and duration.

In tropical climates such as in sub Saharan Africa these conditions are fulfilled easily and soil temperatures above 45 ^{0}C can be achieved for longer durations. Although this technique is promising for nematode control (Ozores-Hampton et al., 2004), some failures have been reported (Chellemi, 2002). These failures can be attributed to (i) the majority of nematode eggs are heat resistant (ii) different climatic conditions that can be influenced by soil type and moisture during the solarization period (iii) reintroduction of nematodes after solarization. In the latter case, the upper soil can be re-infested by nematodes from lower soil surface as a result of deep soil tillage practices, nematodes can also be introduced from the planting material, equipment and irrigation water. This renders this technique expensive and not economically viable for the poor small scale farmer.

7.4 Biological control of plant-parasitic nematodes

Natural enemies are promising for plant-parasitic nematodes control. Several nematode antagonists have been reported including fungi and bacteria that parasitize and feed on nematodes, and compounds released by microorganisms, like bacteria, fungi and nematicidal plants. Fungi and bacteria can be classified on their nematophagous and antagonistic characteristics. Some fungi are endoparasites, trappers, toxin producers and egg parasites thus they are called nematophagous fungi. *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) reduces root galling caused by *M. incognita* and *M. javanica* on tomato crops by parasitizing the nematode eggs (Goswami et al., 2006). Kiewnick and Sikora (2006) reported a 66% reduction in root galling and 74% reduction of egg mass formation after a pre-plant application of fungus *P. lilacinum* strain 251 on tomato plants. Later this product was commercialized in several countries for the control of different nematodes. A one application of the egg-parasitic fungi *Pochonia chlamydosporia* (formerly *Verticillium chlamydosporium*) was able to reduce the reproduction of *M. javanica* in lettuce and tomato rotations in glasshouse (Van Damme et al., 2005). A recent study showed the ability of *Purpureocillium lilacinum* and *Verticillium leptobactrum* to reduce the development of *Globodera* spp. in roots by 76% and 83% and in the soil by 61% and 66% respectively (Hajji et al., 2017). The egg parasitic fungi *Purpureocillium lilacinum* and *Pochonia chlamydosporia* are globally distributed with a saprophytic lifestyle and they are commonly found in cultivated soils as well as root surfaces and some invertebrates. Their ability to successfully colonize the rhizosphere and mass production feasibility (Kerry & Hidalgo-Diaz, 2004; Rumbos & Kiewnick, 2006) makes the two fungi species as

potential commercial bionematicides. Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts, capable of colonizing more that 80% of plant species. They are capable of protecting their colonized host plants from biotic stress such as plant-parasitic nematodes (Schouteden et al., 2015). In a split-root experimental set-up, Vos et al., (2012) observed a significant decrease in *M. incognita* or *Pratylenchus penetrans* infection in tomato roots colonized by AMF (*Funneliformis mosseae*). A systemic suppression of *Radopholus similis* and *Pratylenchus coffeae* was also observed in roots colonized by AMF (*Rhizophagus irregularis*) (Elsen et al., 2008). Other fungi such as *Aspergillus* spp. and *Trichoderma* spp. have toxic effect on the nematodes. For example species of *Aspergillus* (*Aspergillus terreus, Aspergillus niger* and *Aspergillus fumigates*) demonstrated high toxicity on second stage juveniles of *M. incognita* (Goswami & Tiwari, 2007; Tripathi et al., 2006). Many studies have also reported the use of *Trichoderma* spp to control cyst nematodes such as *Heterodera* avenae and *Heterodera filipjevi* (Zhang et al., 2016). A recent study suggests that when high endospore concentrations of obligate endoparasitic bacteria *Pasteuria penetrans* are in the root zone they reduce the reproduction of *Meloidogyne* spp. (Bhuiyan et al., 2018).

The above literature suggests that biological control has secured a position among the most sustainable and effective approaches to control nematodes and other pests. However despite its potential, the success of biocontrol of nematodes and other pests remains largely fragmented. A recent study showed that a commercialized product BioAct WG (*Purpureocillium lilacinum* strain 251, Pl251) was not effective at egg parasitism of *M. incognita* eggs at field level in spite of high egg parasitism in vitro (Giné & Sorribas, 2017). Another study showed that the application of entomopathogenic fungus (*Beauveria bassiana*) increases the reproduction of *Ditylenchus destructor* and *D. dipsaci* nematodes on potato (Mwaura et al., 2017). In yet another study the application of biocontrol-strain *Bacillus* sp. JC12GB43 promote the growth of potato pathogens *Phytophthora infestans* and *Fusarium coeruleum* depending on environmental conditions (Cray et al., 2016). These findings suggest complex interactions between biocontrol agent and their environment. In order to improve the efficacy of biocontrol agents there is need for better understanding of abiotic factors (temperature, humidity, soil physical, and chemical properties etc.) and biotic factors (microbial community, biocontrol agent, and host compatibility etc.). The efficacy of most biocontrol agents will remain very low and not economic justified for a farmer to adopt as a control measure until the above factors are taken into consideration.

Another major problem with microbial biocontrol agents is that the pathogen or pest can also develop resistance (Melo et al., 2016; Tabashnik et al., 2013). However these problems can be solved by integrating biocontrol agents and other management strategy elements.

7.5 Chemical control of plant-parasitic nematodes

Historically, chemical control has been the most effective strategy to reduce plant-parasitic nematodes populations in the soil (Jones, 2017; Nyczepir & Thomas, 2009). However, the majority of nematides were banned due to their unprecedented health and environmental outcomes. In some parts of the world specifically on large commercial farms located in low-income countries chemical control is still used to combat nematode and other soil borne pests (Table 3). In South Africa there are more than 50 crop related nematicidal products registered. The following chemical nematicides are still commonly used as soil fumigants in South Africa: dazomet, EDB, furfural, metam potassium and sodium and methyl bromide/chloropicrin (Jones, 2017). In Kenya 1,3-Dichloropropene (Telone® II) is used as a soil fumigant to control nematodes in commercial pineapple fields. Although, the use of soil fumigants by commercial farms have succeeded in controlling nematodes and other soil borne pests to maintain a constant supply of large volumes of foods to local and global markets. However, such practices are generating negative outcomes on multiple fronts: (i) biodiversity losses, (ii) environmental degradation, and groundwater contamination, (iii) human excessive exposure to very toxic chemicals. The majority of commercial farms in these low-income countries are owned by multinational companies where crop uniformity is at the heart of production leading to heavy dependent on chemical inputs. What is required is thus a new approach in agriculture in order to replace the heavy reliance on chemical inputs in order to control nematodes and other pests. Thus several efforts have been made to replace chemical control. These efforts utilize the ability of some alternative approaches to kill nematodes, enhancing the in-soil competitions and interfering with nematode life cycle.

Table 3. Some common chemical nematicides used in Africa (Jones, 2017).

Crop	Active substances registered
Cotton, cruciferae, cucurbit, deciduous fruit, ginger, bean (green), papaya, lawns, and turf	Ethoprophos, Fenamiphos[1]
Banana	Cadusafos, fenamiphos, fosthiazate and oxamyl[1]
Citrus	Cadusafos, ethoprophos, fenamiphos, fosthiazate and terbufos[1]
Grape	Cadusafos and fenamiphos[1]
Groundnut	Fenamiphos, furfural, oxamyl and terbufos[1]
Lettuce, onion and flowers	Ethoprophos and furfural[1]
Paprika and green chilli	Furfural[1]
Peach	Cadusafos, fenamiphos and oxamyl[1]
Pea	Ethoprophos and fenamiphos[1]
Sorghum	Carbofuran[1]
Roses	Fenamiphos, fosthiazate, furfural, foshiazole, cadusafos, fluopyram and azadirachtin[2]
Roses, French beans,	Azadirachtin[2]
Maize	Carbosulfan[2]
Bananas, maize, tobacco, ornamentals, potatoes, pyrethrum, sugarcane, vegetables	Ethoprophos and abamectin[2]
Pineapple, ornamentals	1,3-Dichloropropene and oxamyl[2]

[1]South Africa, [2]Kenya

7.6 Cultural control of plant-parasitic nematodes

These are agronomic practices that are implemented by a farmer in order to reduce nematode problem. A grower can achieve this either by one or combining the following approaches: (1) selection of healthy seed material: by elimination of nematode infested planting material to control problematic nematodes such as PCN, the spiral nematode, the burrowing nematode, root lesion nematode, wheat gall nematode and rice white tip nematode (2) adjusting the time of planting: a nematode requires suitable climatic factors in order to complete its life cycle for example crops planted during the cold season when nematodes are less active are less susceptible to nematode damage (3) fallowing: although

this approach is not economic leaving fields without growing plants exposes nematodes to starvation. (4) manuring: this approach involves the use of farm yard manure, oil seed cake of neem and castor, cultivation of green manure crops promotes nematode suppression through various mechanisms. (5) antagonistic crops: such crops contains some chemicals e.g. marigold (Tagetes spp.) plants contains the α – terthinyl and bithinyl that kills nematodes, mustard contains allyl isothiocyanate which kills the nematodes. (6) resistant crops: probably the most economical way controlling nematodes (7) trap/cover/relay crops: resistant crops can be used as trap, cover, relay or rotational crops in order to suppress nematode soil populations. Resistant crop species/cultivars used as rotational crops appear more promising. A highly resistant crop allows the target nematode to hatch and penetrate the root tissue but later the host plant interferes with the formation of a functional nematode feeding site. In recent years, *Solanum sisymbriifolium* was selected as a potential trap crop for PCN (*Globodera* spp.). This plant species showed hatch stimulation comparable to susceptible potato crop, but no progeny PCN are formed (Scholte, 2000; Timmermans et al., 2006). Recently a resistant pepper was used as a trap cover crop in vegetable production and it reduces RKN infestation in soil by more than 80% (Navarrete et al., 2016). Both plants acts as dead-end traps, attracting *Globodera* spp. (in the case of *S. sisymbriifolium*) and *Meloidogyne* spp. (in the case of pepper) juveniles in the soil, and preventing their progeny from developing. Nevertheless, the two strategies have some limitations (i) apart from reducing PCN soil population densities a trap crop such as *S. sisymbriifolium* does not give any economic value to the farmer (ii) the resistant pepper crop does not imply resistance to all *Meloidogyne* spp. In fact resistance of pepper was only reported to *M. incognita*, *M. javanica* and *M. arenaria* only. In future *Meloidogyne* spp. such as *M. enterolobii* will be a major problem and this must be addressed urgently (iii) the contribution to nutritional security by both trap plants is insignificant. Therefore, holistic strategies are required in order to build long term nematode and other pest management measures whilst securing a healthy agro-ecosystem and securing the livelihoods. Thus this thesis focused on the dynamics of RKN and PCN on African nightshade (*Solanum* spp.) and African spinach (*Amaranthus* spp.), and as potential cover or relay crops to control *Globodera* spp. and *Meloidogyne* spp. Integrating these crop plants in the existing cropping systems is a crop diversification strategy that may promote nematode suppression and at the same time promoting healthy diets. The approach is multidisciplinary that in future it will bring together scientists from different ideology and pedagogy in order to address the current and future food system challenges in low-income countries.

8. Aims

We have chosen RKN and PCN as a subject of this thesis because both nematode groups are known to exist in various farming systems including smallholder farms. The spread of these nematodes has substantially increased due to transport networks and the globalization of agriculture since the beginning of industrial revolution, a pattern likely to continue. Many of these introduced plant-parasitic nematode species causes economic yield losses on important cultivated crops. Despite the economic significance of these plant-parasitic nematodes their identity and dynamics on African nightshade and African spinach remains far from established especially under African conditions. As a result the main aim of this thesis was to identify RKN and PCN species and to study the impact of African nightshade and African spinach on these nematodes in Kenya. In order to achieve this several aspects have to be studied, specifically:

a. As RKN and PCN are known to parasitize several cultivated crops including some African nightshade and African spinach, their identity and damage levels have to be documented particularly under African conditions.

b. As African nightshade and African spinach are important leafy vegetables their effects on RKN and PCN dynamics have to be documented as well as the impact on the subsequent susceptible crops.

c. RKN and PCN infection process on African nightshade and African spinach have to be studied. This will help to understand their nematode resistance mechanism.

The proposed strategy will generate comprehensive data and new insights that will allow evaluating and revising current pest and disease management strategies in Africa and improve the sustainability of agriculture. This should significantly contribute to the management of plant-parasitic nematodes under smallholder settings.

Chapter 2

When global becomes local: Plant parasitic nematodes in AIV cropping systems

Modified from:

Chitambo, O., Haukeland, S, Fiaboe, K.K.M., Kariuki, G.M., Grundler, F.M.W. (2016). First report of the root-knot nematode *Meloidogyne enterolobii* parasitizing African nightshade in Kenya. *Plant Disease* 100:1954 https://doi.org/10.1094/PDIS-11-15-1300-PDN.

Chitambo, O., Haukeland, S., Fiaboe, K.K.M., and Grundler, F.M.W. (2018). First Report of *Meloidogyne hapla* and *Meloidogyne javanica* co-infection on *Parthenium hysterophorus* in Kenya. *Plant Disease* https://doi.org/10.1094/PDIS-06-18-0964-PDN.

1. Abstract

Surveys and correct diagnosis of plant-parasitic nematodes are considered to be the cornerstones for the implementation of an integrated pest management. However, the occurrence and identity of root-knot nematodes (RKN: *Meloidogyne* spp.) is under reported in some countries. We therefore conducted a survey in Kenya to study the occurrence and identity of RKN species. We detected *M. enterolobii* parasitizing African nightshade for the first time in Kenya. *Meloidogyne enterolobii* is considered to be a highly pathogenic plant-parasitic nematode species because it is able to reproduce on varieties of tomato, tobacco, watermelon, and pepper that are resistant to other RKN species. In addition, the pathogenicity and reproductive potential of *M. enterolobii* is higher when compared with other tropical RKN such as *M. javanica*, *M. incognita*, and *M. arenaria*. The resulting mitochondrial haplotypes revealed a human aided dispersal of *M. enterolobii* and other RKN species through agricultural activities. In this context we also detected *M. hapla* and *M. javanica* co-infection on *Parthenium hysterophorus* (an invasive weed in Africa) in Kenya indicating that these nematodes are continuing to spread and they can coexist. We argued that introduced plant-parasitic nematodes might benefit from the naivety of new neighbors or release from natural enemies or new enemies that have not learn to encounter them.

2. Introduction

A hallmark of the Anthropocene is the transportation of species including plant-parasitic nematode beyond their native ranges. During the last century the rate at which humans have spread species from their native ranges have significantly increased (Hulme et al., 2009; Tittensor et al., 2014). The spread of plant-parasitic nematodes has substantially increased due to transport networks and the globalization of agriculture since the beginning of industrial revolution. Many of these introduced plant-parasitic nematode species causes economic yield losses on important cultivated crops (Coyne et al., 2018; Nicol et al., 2011).

Plant-parasitic nematodes such as root-knot nematodes (RKN: *Meloidogyne* spp.) are known to have a global distribution (Sasser, 1977). There are nearly 100 valid RKN species that are recognized. For example, *M. incognita*, *M. javanica*, and *M. arenaria* are known to occur in tropical regions whereas *M. hapla* is restricted to temperate regions (Moens et al., 2009). In Africa, *M. incognita*, *M. javanica*, and *M. arenaria* are regarded as the dominant species causing economic damage on crop plants such as potato, tomato, and pineapple (Coyne et al., 2018; Onkendi et al., 2014). In contrast typical tropical RKN species are increasingly being detected in European countries (Maleita et al., 2018; Wesemael et al., 2011) and temperate climate loving species such as *M. hapla* have also been isolated from tropical regions (Meressa et al., 2014; Onkendi et al., 2014). This suggests that agriculture trade and travel is breaking down nematode biogeographic barriers, causing the global distribution of plant-parasitic nematode such as RKN. Emerging species such as *M. enterolobii* have also been reported from Africa (Onkendi & Moleleki, 2013), some European countries (Kiewnick et al., 2008), and South America (Luquine et al., 2018) . In Africa, the resistance breaking *M. enterolobii* has been isolated from Togo, Malawi, Senegal, Nigeria, Democratic Republic of Congo, South Africa, and Burkina Faso (Onkendi et al., 2014). The global distribution of certain RKN species is likely to increase as globalization of the world's economy continues. The establishment of these native species is going to be supported by global climate change by making hostile regions favorable for certain RKN species reproduction.

Despite the economic importance of RKN, most of the information to date is from high-income countries (Wesemael et al., 2011), with relatively little data from low-income farming systems. Until now there are no reports of *M. enterolobii* from East-Africa yet it is very likely to be present. Moreover, the impact of RKN species multiple infections is not

reported. Lack of such information makes low-farming income farming systems more vulnerable to RKN. This is because farmers in low-income countries are highly depended upon the harvest for their livelihoods, nutrition, and food security (Perrings, 2005; Wiggins et al., 2010), therefore the unprecedented spread of RKN is profoundly worrying. This poses a significant challenge to the ongoing management strategies. In this study we carried a survey to document the presence and impact of emerging RKN species. Documenting the impact of emerging RKN species is pivotal to improving management, prevention, and risk assessment tools. The implementation of prevention and risk assessment tools will allow scientific based policies to be put in place, thus minimizing the spread of plant-parasitic nematodes and support efficient management strategies.

3. Results and discussion

3.1 First report of the root-knot nematode *Meloidogyne enterolobii* parasitizing African nightshade in Kenya

African nightshades (*Solanum* spp.) are important leafy vegetables in many parts of eastern, western, central, and southern Africa (Keding et al. 2007). In Kenya, sustainable production of African nightshades faces a twin challenge from both above- and belowground pests. Root-knot nematodes (RKN; *Meloidogyne* spp.) are belowground pests capable of parasitizing many hosts including African nightshade, leading to severe yield loss and sometimes total crop failure. A survey was carried out in Kenya between May and July 2015 to determine the presence and incidences of RKN infecting the African nightshade *Solanum scabrum*. In the field, this nightshade exhibited the following symptoms: leaf yellowing, leaf drop, and stunted growth. Symptomatic African nightshade plants isolated from Yatta, Machakos County showed very large galls in comparison with those commonly associated with *Meloidogyne incognita* and *M. javanica* infected African nightshade plants. Population densities of infective second stage juveniles in the soil ranged from 100 to 750 individuals per 100 cm^3 soil. To characterize the *Meloidogyne* species, single adult females (n = 20) were picked from galled nightshade roots for morphological analysis. Female perineal patterns were similar to those in the first description of *M. enterolobii* (Yang and Eisenback 1983); however, some samples deviated from the original description by showing a moderately high to high dorsal arch. Therefore, DNA was

extracted separately from 10 single females and PCR was used to amplify a 420-bp fragment of cytochrome oxidase I (COI) of the mitochondria (Derycke et al. 2010). The PCR products (represented by accession no. KT936633) were sequenced and aligned with sequences in GenBank. BLAST analysis resulted in 100% identity to the sequence of an *M. enterolobii* isolate from China (GenBank Accession No. JX683714). Using DNA of the same females, species identification was also confirmed using PCR species-specific SCAR primer set MK7-F/MK7-R (Tigano et al. 2010). No amplification was produced with the specific primers for other tropical species (*M. javanica*, *M. incognita*, and *M. arenaria*). The same results were obtained from females cultured on *S. scabrum* in the greenhouse. *M. enterolobii* is considered to be a highly pathogenic plant-parasitic nematode species because it is able to reproduce on varieties of tomato, tobacco, watermelon, and pepper that are resistant to other RKN species. In addition, the pathogenicity and reproductive potential of *M. enterolobii* is higher when compared with other tropical RKN such as *M. javanica*, *M. incognita*, and *M. arenaria* (Kiewnick et al. 2009). To our knowledge, this is the first report of *M. enterolobii* in Kenya.

3.2 First Report of *Meloidogyne hapla* and *Meloidogyne javanica* co-infection on *Parthenium hysterophorus* in Kenya

Parthenium hysterophorus L. (Asteraceae), is an annual or short-lived perennial herbaceous noxious weed native to North and South America. Since its introduction in Kenya, Parthenium has spread across many parts of the country and has become a menace to agriculture. Stunted and wilted Parthenium plants with globular galled roots marked with profuse roots were observed in a field in Tigoni, Kiambu County (average temperature 15.3°C, Koeppen-Geiger Climate Classification Cfb). Nematodes were extracted from root zone soil using the Baermann tray and population densities of infective second stage juveniles ranged from 300 to 980 individuals per 100 ccm soil. Mature females and their corresponding egg masses were handpicked from a single infected plant root. The posterior part of 20 adult *Meloidogyne* females was used for morphological analysis, while the respective anterior part was carefully stored in ethanol for molecular analysis. Analysis of perineal patterns (Eisenback et al. 1980) revealed 13 (65 %) females of *Meloidogyne hapla* and 7 (35 %) females of *M. javanica*. To confirm pathogenicity, infection assays with one or two species were performed. Egg masses from pure cultures each containing between 410 - 530 eggs were inoculated onto individual Parthenium plants growing in sterile soil in

a greenhouse maintained at an average temperature of about 25 °C. For mono-infection two egg masses from one species, for co-infection one egg mass of *M. hapla* and one of *M. javanica* were used. Fifteen plants per treatment were inoculated. At 45 days post-infection, the number of egg masses developed on Parthenium inoculated with *M. hapla* only was 129 ± 19.5 per root system, while this number was 188 ± 23.6 when *M. hapla* + *M. javanica* were co-inoculated. On Parthenium plants inoculated with *M. javanica* only, no egg masses or galls were observed, indicating that *M. hapla* facilitates infection of *M. javanica* on this plant. There was no clear difference in root galling on the mono-infected and co-infected plants. To confirm species identification of *M. hapla* and *M. javanica*, molecular analysis was performed on females extracted from the field and the green house. DNA was extracted from ethanol-preserved females (n = 10), and PCR carried out using species-specific SCAR (sequence-characterized amplified region) primer set JMV1(5′-GGATGGCGTGCTTTCAAC-3′/JMV (5′- AAAAATCCCCTCGAAAAATCCACC-3′) for *M. hapla* and Fjav (GGTGCGCGATTGAACTGAGC) /Rjav (CAGGCCCTTCAGTGGAACTATAC) for *M. javanica* (Zijlstra et al. 2000), which produced the expected fragments length of 440 bp and 670 bp, respectively. To further confirm species identification of *M. hapla*, the same DNA (n = 10) was amplified targeting the mitochondrial DNA region between COII and 16S rRNA gene and sequenced using primers C2F3 (GGTCAATGTTCAGAAATTTGTGG) / 1108 (TACCTTTGACCAATCACGCT) (Powers and Harris 1993). Species identification *M. javanica* was re-confirmed by amplifying the mitochondrial NAD5 gene and sequenced using primers NAD5F2 (TATTTTTTGTTTGAGATATATTAG)/NAD5RI (CGTGAATCTTGATTTTCCATTTTT) (Janssen, et al. 2016). The PCR products (represented by Accession No. KX137039 and KY436071) were sequenced and aligned with sequences in GenBank. BLAST analysis resulted in 99 - 100% identity to the sequence of *M. hapla* and *M. javanica* respectively. The same perineal pattern and PCR results were obtained from females isolated from the greenhouse experiment. There were no differences in the appearance of the galls between the treatments. To our knowledge, this is the first report of co-infection by *M. hapla* and *M. javanica* on Parthenium. We showed that *M. hapla* is able to infect Parthenium and facilitates infection by *M. javanica*. Our findings hint on the complexity of the interaction of *Meloidogyne* spp. and their hosts. Since Parthenium is drought tolerant and can aggressively colonize disturbed sites, it will facilitate survival and further spread of both nematode species. Co-infections amongst *Meloidogyne* spp. and other plant parasitic nematodes will be a subject of further research in order to precisely

understand the disease severity and the evolutionary trajectories of virulent nematode populations.

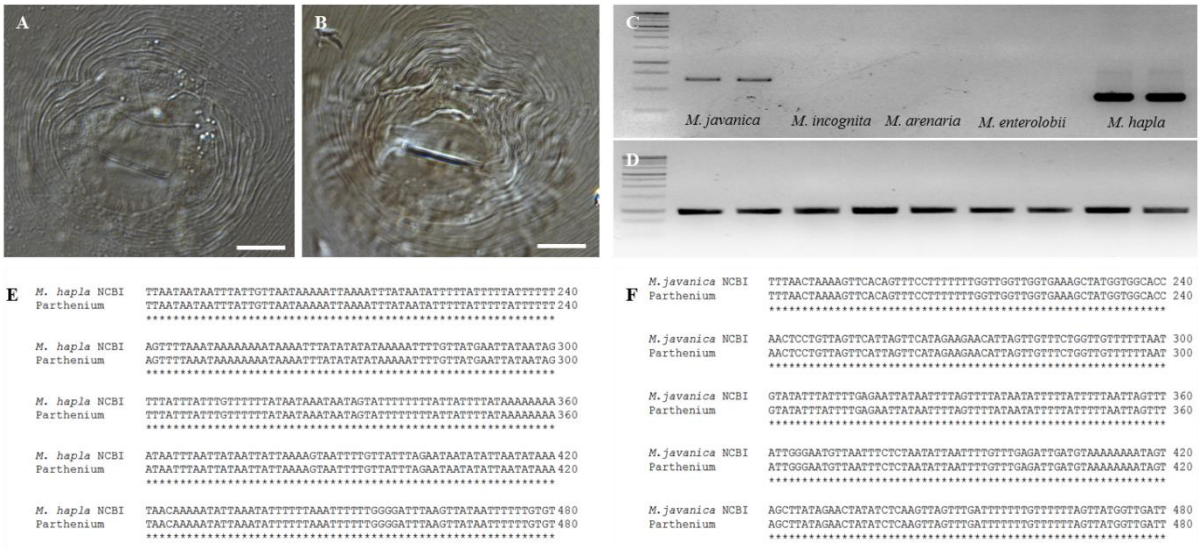

Figure 1. Morphological and molecular identification of *M. hapla* and *M. javanica on* Parthenium. Photomicrographs of female perineal pattern of *M. hapla* (**A**) and *M. javanica* (**B**) isolated from single Parthenium. The same perineal pattern results were obtained from females isolated from the greenhouse experiment. **C**, PCR DNA bands of sequence-characterized amplified regions (SCAR) of *M. javanica* and *M. hapla* isolated from single parthenium weed plant. The same SCAR-PCR results were obtained from females isolated from the greenhouse experiment. **D**, PCR DNA bands of NAD5 and the noncoding region between 16S and CoxII of females isolated from parthenium weed plant. **E**, 16S and CoxII DNA sequence alignment confirming *M. hapla* isolated from coinfected plants and monoinfected plants. **F**, NAD5 DNA sequence alignment confirming *M. javanica* isolated from coinfected parthenium plants. The same DNA sequences were obtained from females isolated from the greenhouse experiment. Scale bar = 25 µm.

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Chapter 3

African nightshade and African spinach decrease root-knot nematode and potato cyst nematode soil infestation in Kenya

Modified from:

Chitambo, O., Haukeland, S., Fiaboe, K.K.M., Kariuki, G.M., Grundler, F.M.W. 2019. African nightshade and African spinach decrease root-knot nematode and potato cyst nematode soil infestation in Kenya. Plant Disease https://doi.org/10.1094/PDIS-07-18-1193-RE.

1. Abstract

Plant-parasitic nematodes, particularly root-knot nematodes (RKN: *Meloidogyne* spp.) and cyst nematodes (CN: *Globodera* and *Heterodera* spp.) cause severe yield reduction in most cultivated crops and are of high economic importance. African nightshade (*Solanum* spp.) and African spinach (*Amaranthus* spp.) are important African indigenous vegetables (AIV) as a rich source of nutrition and income. However, their host status to plant-parasitic nematodes remains largely speculative. Therefore, a survey was conducted which revealed that *S. villosum* exhibited high root galling whereas on *S. scabrum, A. cruentus,* and *A. dubius* root galling was rare or very low. Additionally, soil collected from *S. villosum* and *S. scabrum* root rhizosphere contained few cysts of potato cyst nematodes (PCN) and no developing PCN females were observed on the roots of growing plants. Therefore, we studied the dynamics of RKN and PCN on *A. dubius, A. cruentus, S. scabrum,* and *S. villosum* over 2 years in field experiment. The effects of AIV crop species on RKN and PCN soil infestation were evaluated using susceptible *S. lycopersicum* or *S. tuberosum*. After first, second and third cultivation of *A. dubius, A. cruentus,* and *S. scabrum.* RKN infestation of the soil decreased by more than 85%, whereas *S. scabrum* and *S. villosum* decreased PCN densities by more than 80%. When cropping susceptible crops, after three seasons of successive cultivation of these AIV, galling index and number of developing PCN females measured on susceptible crops decreased by more than 75%. Wilting incidences and RKN-PCN co-infection incidences also decreased significantly. Here, we present data that support the development of a novel cropping system including African spinach and African nightshade, which reveals a high potential to manage RKN and PCN in an environmentally friendly, effective, and productive way.

2. Introduction

Plant parasitic nematodes, particularly tropical root-knot nematodes (RKN: *Meloidogyne* spp.) and cyst nematodes (CN: *Globodera* and *Heterodera* spp.) are plant pathogens of high economic importance causing severe yield losses in most cultivated crops. The life cycle of RKN and CN includes phases of survival in the soil, invasion of plant roots, and development inside root tissues. On susceptible host plants, rapid multiplication of nematodes inside root tissues leads to the development of disease symptoms such as root galling and cyst formation, respectively (Bartlem et al. 2013; Huang 1985; Perry 1989; Sijmons et al. 1991). This is associated with the formation of specific feeding cells from which they withdraw nutrients for the entire parasitic phase. As nematode-induced disease symptoms may impair water and nutrient uptake by the plant (Jones 1981), yield losses of up to 30% have been reported on several crops such as potato, tomato, eggplant, and melon (Nicol et al. 2011). Yield loss caused by RKN and CN compromise the sustainability of crop production and is an obstacle for attaining food security.

RKN and CN are obligate root parasites that have evolved highly sophisticated parasitic relationships with their host plants which are based on the formation of specific feeding sites (Hussey and Grundler 1998). The biology of RKN and CN is similar consisting of developmental stages, egg, four juvenile stages, and the adult stage. However, potato cyst nematodes (PCN) tend to be much more host specific and require host stimulus for egg hatching (Clarke and Perry 1977). The parasitic stage of RKN and PCN is entirely dependent on a suitable host plant and is highly vulnerable to the risk of starvation in the absence of a suitable host plant.

Numerous factors have contributed to the widespread occurrence of RKN and PCN in smallholder cropping systems. In Africa, smallholder cropping systems are complicated and often characterized by a simultaneous cultivation of crop species that supports development of RKN and PCN. This is aggravated by lack of awareness coupled with lack of proper nematode diagnostics. Thus, most farmers are unprepared and ill-equipped to respond effectively to RKN and PCN problem. Consequently, RKN and PCN population densities have increased and their spread facilitated through the distribution of contaminated planting material, irrigation water, rainfall runoff, soil attached to farming implements, animal hooves, and footwear. In addition, intercontinental exchange of propagating material and trade has facilitated the global spread of highly damaging nematode species. This is well

illustrated by the introduction of *G. rostochiensis* and *G. pallida* into Kenya (Mburu et al. 2018; Mwangi et al. 2015). Human aided distribution of nematodes is further supported by the wide spread of RKN such as *M. arenaria, M. incognita,* and *M. javanica* in Africa and across the world (Onkendi et al. 2014; Wesemael et al. 2011). In addition, reports of some RKN species such as *M. enterolobii* are also on the rise (Chitambo et al. 2016; Coyne et al. 2018; Onkendi et al. 2014). The occurrence of PCN in smallholder farms is worrisome because RKN is already a heavy burden (Coyne et al. 2018). Accordingly, the presence of RKN and PCN threatens low-income farming systems which are essential for food production and livelihood.

Considering the above-mentioned situation, diminishing the yield loss caused by RKN and PCN is urgently required. The use of nematicides to control plant-parasitic nematodes has been gradually restricted due to undesirable effects on health and environment (Zasada et al. 2010). Nevertheless, several techniques such as soil tillage, plant-derived nematicidal compounds, sanitation, heat-based methods, biological control, green manure, trap crops cover crops and host resistance, are available to support the management of RKN and PCN (Collange et al. 2011; Bélair et al. 2016; Pickup 2016; Trudgill et al. 2014; Zasada et al. 2010). However, implementing these control methods alone is often not sufficient. RKN that are capable of multiplying on resistant tomato and pepper varieties (Djian-Caporalino et al. 2011; Kiewnick et al. 2009) and certain populations of PCN are capable of multiplying on resistant potato varieties (Fournet et al. 2018). Recently, biological control products have been released to combat nematode problems, but their effects are not always reliable and consistent (Cray et al. 2016; Mwaura et al. 2017; Ward et al. 2012). Innovative strategies to control RKN and PCN are therefore urgently required.

In some African countries, there is renewed interest in African indigenous vegetables (AIV) because of their role in food and nutrition security. AIV such as African spinach (Amaranthaceae: *Amaranthus* spp.) and African nightshade (Solanaceae: *Solanum* spp.) are produced by farmers for food, nutrition, and livelihood security (Cernansky 2015; Dinssa et al. 2016; Gruber 2017; Ukam et al. 2016; Moyo et al. 2017; Neugart et al. 2017). The host status of African spinach and African nightshade to RKN and PCN varies in response to infestation by a range of species and environmental conditions. Several studies demonstrated that *Amaranthus* species such as *A. cruentus* are poor hosts for RKN (Ferris et al. 1993; Nchore et al. 2013; Rodríguez Kábana et al. 1988). A screening of non-tuber bearing Solanaceous plants showed that *S. nigrum* species were resistant to PCN (Scholte

2000). Meanwhile, some studies indicate that species of African nightshade and African spinach might act as alternative hosts for RKN and PCN (Boydston et al. 2010; Kokalis-Burelle et al. 2012; Rott et al. 2011). This created a conundrum regarding the precise host status of *Amaranthus* spp. and *Solanum* spp. to RKN and PCN particularly under African conditions.

Here, we performed a field survey and detailed field trials to study the impact of nematodes on cultivation of AIV. The objectives of the current work were (1) to determine if *Solanum* spp. and *Amaranthus* spp. are hosts for RKN and PCN, (2) to determine the identity of RKN and PCN parasitizing *Solanum* spp. and *Amaranthus* spp., (3) to determine the population dynamics of RKN and PCN on *Solanum* spp. and *Amaranthus* spp., and (4) to determine the potential of *Solanum* spp. and *Amaranthus* spp. to manage RKN and PCN.

3. Materials and methods

3.1 Plant-parasitic nematode survey of AIV in Kenya

African nightshade and African spinach are amongst the key AIV that have been targeted for promotion in Africa for smallholder farmer agroecosystems. We therefore conducted a survey during the period in June and August of 2015 to study RKN and PCN root symptoms and soil infestation. Soil and root samples were collected from a total of 25 farms. At each farm, approximately 0.2 ha of land used for vegetable production was sampled. The following numbers of farms were visited in different counties: 4 farms in Kiambu county, 3 farms in Nyandarua county, 4 farms in Machakos county, 6 farms in Kakamega county, 5 farms in Murang'a county, and 3 farms in Busia county. The following crops were sampled; African nightshade (*S. villosum* and *S. scabrum*), African spinach (*A. dubius* and *A. cruentus*) potato (*S. tuberosum*), and tomato (*S. lycopersicum*). Root and soil samples collected from different counties were analyzed for occurrence of RKN and PCN. Crop damage levels were also determined.

RKN and PCN associated with each crop species were determined by uprooting the entire plants. Twelve plants for each crop species at each farm. From this material, 12 samples of roots and adhering soil were taken from a root zone at about 15 cm depth. RKN infestation was assessed as number of galls per plant and it was described on a scale of 0 to 5, (Taylor and Sasser 1978), where 0 = no galls; 1 = 1 to 2; 2 = 3 to 10; 3 = 11 to 30; 4 = 31 to 100;

and 5 = more than 100 galls. PCN infestation was assessed by counting the number of females developed on the roots. Soil samples were mixed thoroughly and sieved before collecting five 100 cm^3 subsamples for nematode extraction. RKN J2 were extracted immediately, for PCN, the soil was air dried before cyst extraction. For RKN J2 extraction a modified Baermann technique was used. RKN J2 were distinguished from other plant-parasitic nematodes by their typical morphology (Jepson 1987). RKN J2 were counted in 5 cm^3 counting chambers under a 50× magnification stereo microscope (Leica MZ12, Nussloch, Germany). PCN were extracted using a Fenwick can. Briefly individual subsamples of 100 cm^3 of soil were rinsed and cysts collected on the second sieve (250 µm) were transferred to a filter paper. After drying, cysts were counted using a magnification len. Cysts, 10, from different crop species were crushed separately in water, and three aliquots of each egg suspension were enumerated under a dissecting microscope at 25-50× magnification.Viability of eggs per cyst was assessed visually according to a standard protocol (Anonymous 2017).

Samples for RKN morphological analysis were analyzed within 72 hours after field collection. Identity of RKN females was assessed using the perennial pattern (Eisenback et al. 1980). Perennial pattern were prepared from 20 females per county. For PCN cysts, cyst shape and colour were used to discriminate PCN from other cyst nematodes. Mature females of RKN and PCN stored in absolute ethanol (99%) were used for molecular analysis. In order to confirm the morphological results NADH dehydrogenase subunit 5 (NAD5), and Cytochrome c oxidase I (COX1) were amplified and sequenced to determine species identity of RKN and PCN Amplification and sequencing of RKN and PCN was carried out on 15 samples per crop. Briefly, genomic DNA extracted from females. A single adult female nematode was immersed in 60µl of sterile water and it was thoroughly crushed using a sterile tooth pick. Thereafter, DNA was extracted using worm lysis buffer (WLB, 10 mM Tris HCL pH 8.0, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DDT, 0.45% Tween 20) and proteinase K. PCR amplification was carried out using Taq DNA polymerase (Qiagen, Germany), with 3µl of extracted nematode genomic DNA and 0.5mM of each primer. Primers NAD5F2 (TATTTTTTGTTTGAGATATATTAG) and NAD5R1 (CGTGAATCTTGATTTTCCATTTTT) were used to amplify the NAD5 gene (Janssen et al. 2016). COI gene was amplified using primers JB3 (TTTTTTGGGCATCCTGAGGTTTAT) and JB4.5 (TAAAGAAAGAACATAATGAAAATG) (Derycke et al. 2010). The PCR amplification

conditions were as follows: initial denaturation 94°C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min, with a final extension at 72°C for 7 min. The PCR product was visualized on 1% gel stained with GelGreen (Biotium, USA). Each PCR amplicon was purified and subsequently submitted for direct Sanger sequencing (GATC Biotech, Germany).

3.2 Impact of AIV cultivation on population dynamics of RKN and PCN and subsequent nematode management in tomato and potato

The field trials were carried out at an experimental station at Kenya Agricultural & Livestock Research Organization (KALRO 1.1518°S; 36.6852°E) from 2015 to 2017. This site has a climate classified as warm and temperate. The climate at KALRO is considered to be Cfb according to the last revision of Köppen-Geiger climate classification (Kottek et al. 2006). The average temperature is 15.3 °C and the average annual rainfall is 1263 mm. The sites had natural infestation of PCN and RKN. At the RKN site the following species were present: *M. incognita*, *M. javanica*, *M. arenaria*, *M. enterolobii* and *M. hapla* as well as undescribed *Meloidogyne*. At the PCN site, *G. rostochiensis* and *G. pallida* were present, as well as undescribed *Globodera*. Both field trials had similar experimental parameters and were conducted across the following seasons: 1st growing season August to November 2015, 2nd growing season February to May 2016, 3rd growing season August to November 2016, 4th growing season March to June 2017.

The experiment was a randomized complete block design with main plots measuring 10 × 10 m. The main plots were subdivided into subplots of 3 × 3 m. The plots were maintained and used in each growing season. AIV and tomato seeds were sourced from Simlaw Seeds Company Ltd, (Nairobi, Kenya). Seed potatoes were sourced from the seed production unit of KARLO (Tigoni, Kenya). AIV and tomato seeds were sown and raised in a nursery bed for one month before being transplanted in the field at a planting density of 14 plants/m². Chitted potato tubers were planted at 10 plants/m². Well decomposed cow manure was incorporated at a rate of 4 kg/m² before planting. The seedlings were irrigated after transplanting to enhance their establishment. Thereafter, the crop was managed in accordance to the normal farmer's practices. During the dry spell, supplemental irrigation was applied.

The experiment consisted of two phases. In the first phase, the impact of AIV on the

population dynamics of RKN and PCN was considered. African nightshade (*S. scabrum*, and *S. villosum)* and African spinach (*A. dubius* and *A. cruentus*) were selected for inclusion in the experiment because of their widespread cultivation in the region. The crops were grown for three successive seasons in main plots (i.e first, second, and third growing seasons). In the second phase of the experiment the effect of cultivating AIV for three successive seasons on RKN and PCN management was assessed by the cultivation of susceptible crops (*S. lycopersicum* cv. Moneymaker and *S. tuberosum* cv. Shangi) in the fourth growing season. The following cropping sequences were adopted to assess effects of AIV cropping system on RKN: (1) 3 seaseons *A. dubius* -followed by- *S. lycopersicum* (2) 3 seasons *S. villosum* -followed by *S. lycopersicum* and (3) 3 seasons *S. scabrum* –followed by *S. lycopersicum*. The following cropping sequences were adopted to assess the effects of AIV cropping system on PCN: (1) 3 seasons fallow -followed by *S. tuberosum*, (2) 3seasons *A. dubius* -followed by *S. tuberosum* (3) 3 seasons *S. villosum* -followed by *S. tuberosum* and (4) 3 seasons *S. scabrum* –followed by *S. tuberosum*. Plots that were previously under *A. cruentus* were not included in second phase. Collection of data on J2 soil population density, galling index, and number of viable cysts was determined as described above. Visual assessment on plant health was also collected. Plants were considered wilted if they are slightly wilted, wilted, severely wilted or nearly dead. To confirm the presence of bacterial wilt (*Ralstonia solanacearum*) on the wilted plants we placed the cut crown in water. Bacteria ooze from the exposed vascular elements of wilted plants in 8 - 12 min, forming milky strands flowing into water confirmed the presence of *Ralstonia solanacearum* (Riley et al. 2002). The presence of galls and developing PCN females on the same plant was used to assess RKN-PCN co-infection. Plants were considered co-infected if RKN and PCN females were observed on the roots of the same plant. The number of flowers per plant was counted from the same treatments after assessing wilting and co-infection incidences.

3.3 Data and statistical analysis

During the survey RKN crop damage was categorized as a proportion of plants with galling index ≤ 1 and > 1. For PCN, crop damage was expressed as proportion of plants with developing PCN female nematodes and those without. RKN and PCN crop damage was then expressed as a percentage of total number of plants sampled per individual crop species. Visited farms were analyzed at county level. Wilting incidences were calculated for

each treatment as the proportion of wilted plants expressed as a percentage of total number of plants sampled. RKN-PCN co-infection incidence was calculated as the proportion of plant roots simultaneously infected by both RKN and PCN expressed as a percentage of a total number of plants sampled. Nematode density data was $\log_{10}$ (x+1) transformed before analysis in order to meet normality and constant variance assumptions. Repeated measures analysis of variance was used to test the effect of AIV on abundance of J2 of RKN and PCN viable eggs and galling index. Analyses of variance (ANOVA) were conducted to assess the impact of AIV on developing PCN female nematodes, wilting incidences, galling index and number of flowers on subsequent susceptible crop. A P value ≤ 0.05 was considered statistically significant. All statistical analyses were performed using SigmaPlot v. 12.5 (Systat Software, San Jose, CA, USA).

Nematode DNA sequences were first queried via Standard Nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to examine whether the sequence would match any species in the database (Altschul et al. 1990). ClustalW (www.ebi.ac.uk/Tools/msa/clustalo/) was used for a detailed comparison of obtained DNA sequences with related reference sequences of related species. Phylogenetic analyses were conducted using *MEGA* version 6 and maximum likelihood analyses were conducted with 5000 bootstrap replicates under the GTR + I + G model according to Tamura et al. (2013).

4. Results

4.1 Plant-parasitic nematode survey of AIV in Kenya

Galling index and number of adult PCN females were used to assess nematode severity on different crop species. Consistently, *S. lycopersicum, S. villosum,* and *S. tuberosum* plants were associated with galling index of > 1, whereas for *A. dubius, A. cruentus,* and *S. scabrum* plants were associated with galling index of ≤ 1 across the Counties studied (Fig. 1A - C). A further examination of the soil collected from the crop root rhizosphere across the Counties showed no statistical differences in RKN J2 population densities (Fig. 1D - F). There was low number of RKN J2 in soil extracted from the root rhizosphere of *A. dubius, A. cruentus,* and *S. scabrum* despite a consistent galling index of ≤ 1 across the Counties. Similarly across the Counties, *S. tuberosum* and *S. lycopersicum* plants were associated with developing PCN females on their roots and in contrast no developing PCN females were recorded on *A. dubius, A. cruentus, S. scabrum,* and *S. villosum* (Fig. 2A - C). There

was no statistical difference in the number of viable PCN eggs/cyst extracted from the root rhizosphere of *S. tuberosum*, *S. lycopersicum*, *S. scabrum,* and *S. villosum* (2D - F). Although, no developing PCN females were observed on *A. dubius* and *A. cruentus* the number of viable PCN eggs/cyst extracted from their respective root rhizosphere was high.

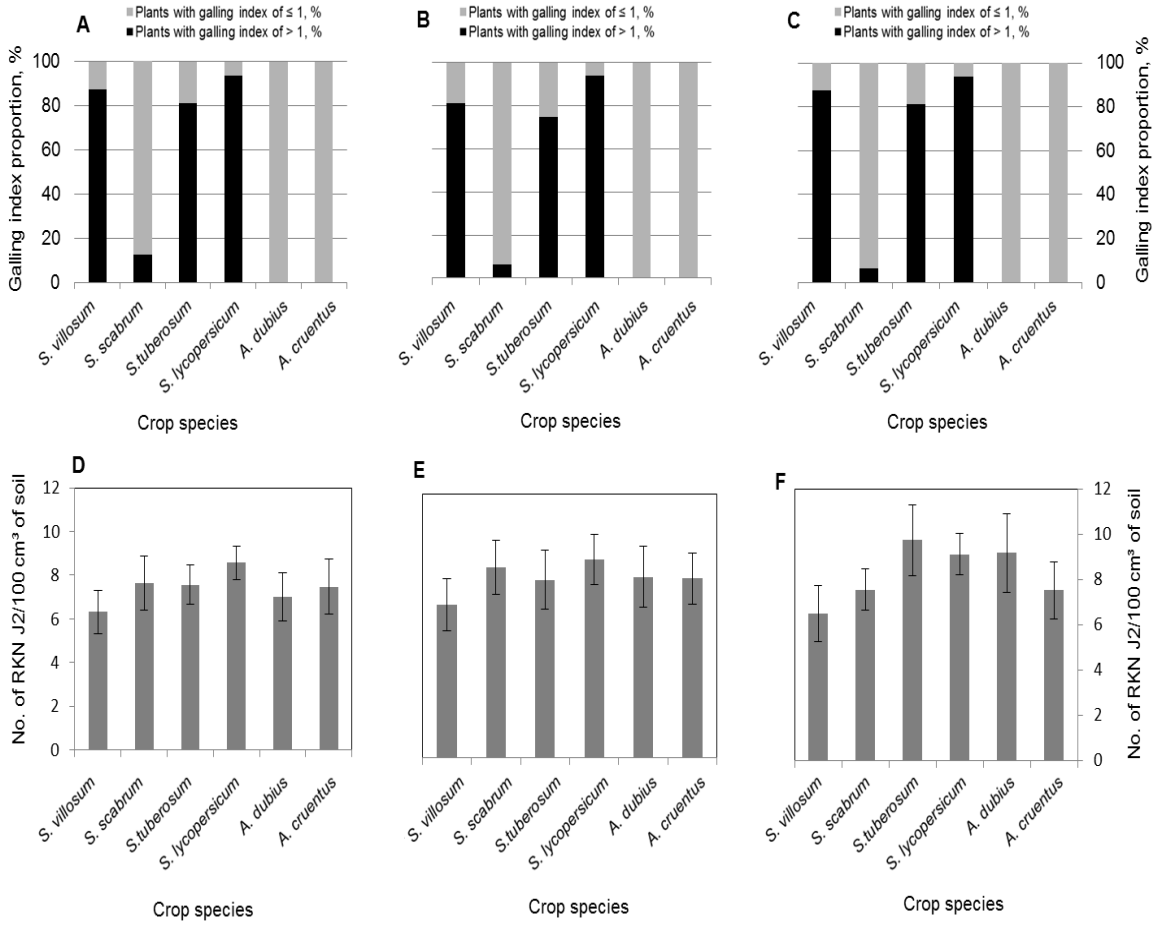

Figure 1: Root galling proportion (A) Murang'a county (B) Machakos county, and (C) Kakamega county. The corresponding root-knot nematode (RKN) soil infestation levels of second-stage infective juveniles (J2) isolated from rhizosphere of different crops (D) Muranga County (E) Machakos County, and (F) Kakamega County. Values of the bars with different letters are significantly different at $P \leq 0.05$. A - *Amaranthus*, S - *Solanum*, RKN - root-knot nematodes, J2 - second-stage infective juveniles.

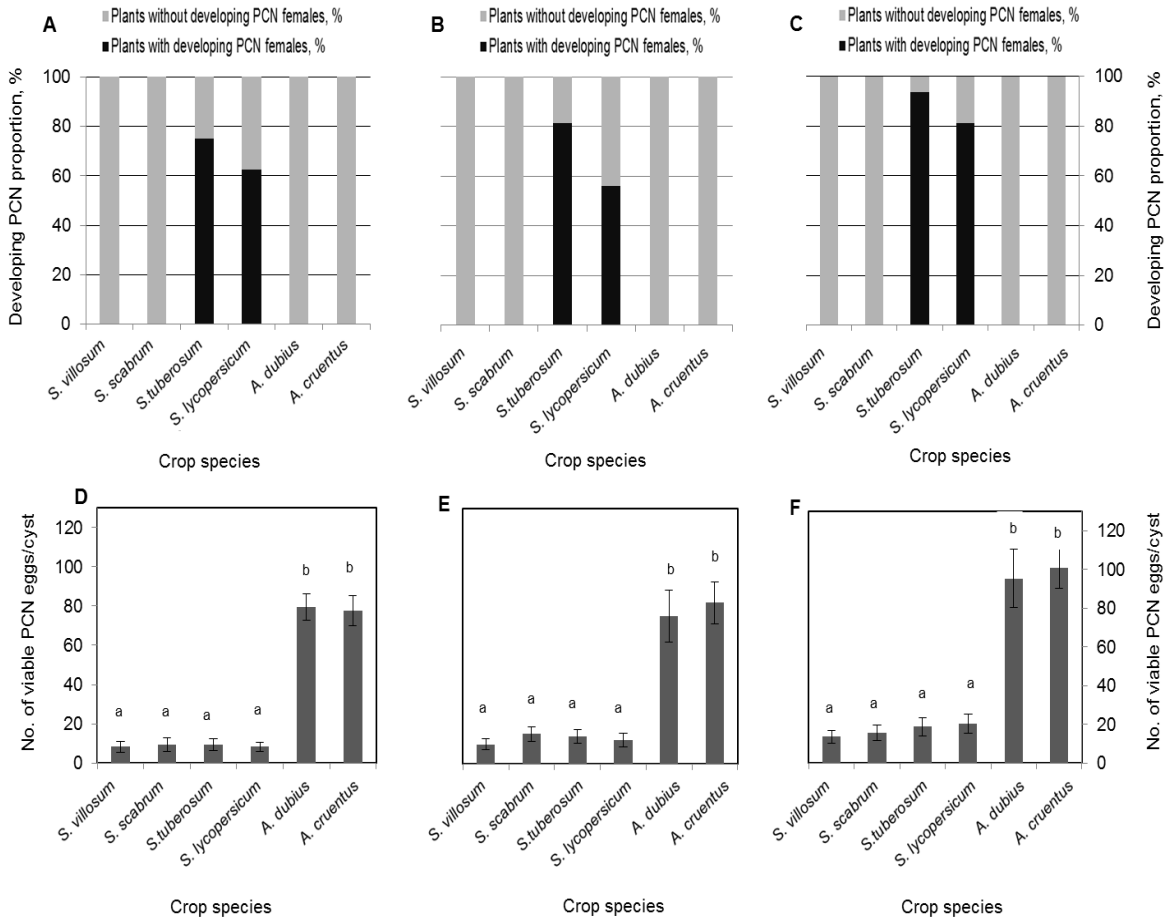

Figure 2: The proportion of plants with developing potato cyst nematodes (PCN) females (A) Kiambu county, (B) Murang'a county, and (C) Nyandarua county. The corresponding PCN soil infestation levels isolated from rhizosphere of different crops (D) Murang'a County (E) Kiambu county, and (F) Nyandarua County. Values of the bars with different letters are significantly different at $P \leq 0.05$. A - *Amaranthus,* S - *Solanum,* PCN - potato cyst nematodes.

A morphological comparison of female perineal pattern was used to differentiate RKN isolated from different crop species. There was no clear morphological difference between some RKN female perineal patterns. Specifically, there was morphological overlap between *M. javanica, M. arenaria,* and *M. incognita* perineal patterns. RKN female perineal patterns from pure cultured samples ranged from the general lateral ridges that divide the dorsal and ventral striae observed on *M. javanica* to high, squarish dorsal arch that is normally observed on *M. incognita* (Fig. 3A - C). *Meloidogyne hapla* female patterns were characterized by flattened ovoidal shape and subcuticular punctations in the smooth tail terminal area and the lateral ridges were absent (Fig. 3D). Female perineal patterns of *M.*

enterolobii were round to dorso-ventrally ovoid. Lateral lines were not distinguishable (Fig. 3E). Some of the perineal patterns of a sample of RKN females from *S. villosum* and *S. lycopersicum* did not conform to the normal description of other RKN. These perineal patterns were characterized by very fine striae and very low dorsal arch (Fig. 3F); it could not be assigned to a described species. The spherical brown cysts isolated from the soil and pale yellow females observed on the roots were identified as *G. rostochiensis* or *G. pallida.* In some samples the cysts were light brown to brown in color and subspherical raising the possibility of an undescribed *Globodera* sp. which did not conform to the normal description of *G. rostochiensis* or *G. pallida.*

DNA sequence blasting and sequence alignment of COI gene identified the following RKN species (Table 1; Fig. 3H); *M. hapla* parasitizing *S. lycopersicum, S. tuberosum, S. villosum,* and *S. scabrum* (accession No. KX137039, MH399800 - MH399802), *M. enterolobii* parasitizing *S. lycopersicum, S. tuberosum, S. villosum,* and *S. scabrum* (accession No. KT936633, MH399803 - MH399805) and an associated *Meloidogyne* sp. parasitizing *S. lycopersicum,* and *S. villosum* (accession No. MF351699). This region failed to differentiate *M. javanica*, *M. incognita,* and *M. arenaria*. Therefore DNA sequence blasting and sequence alignment of NAD5 gene was used to differentiate these species. The sequence alignment of NAD5 gene identified *M. javanica* parasitizing *S. lycopersicum, S. tuberosum, S. villosum,* and *S. scabrum* (accession No. KY436071, MH399831 - MH399837), *M. arenaria* parasitizing *S. lycopersicum, S. tuberosum, S. villosum,* and *S. scabrum* (accession No. MH399824 - MH399830), and *M. incognita* parasitizing *S. lycopersicum, S. tuberosum, S. villosum, S. scabrum, A. dubius,* and *A. cruentus* (accession No. MH005027, MH399838 - MH399845). Phylogenetic analysis based on the COI gene sequence revealed an associated *Meloidogyne* sp. closely related to *M. africana* (Fig. 3H).

Three PCN species were identified based on COI DNA analysis (Table 1; Fig. 3G), *G. rostochiensis* (accession No. MF773722, MH399815 - MH399817), *G. pallida* (accession No. MH399818 - MH399820), and an associated *Globodera* sp. (accession No. MG438286, MH399821 - MH399823), which is closely related to *G. ellingtonae*.

Table 1. Root-knot nematodes (RKN) and potato cyst nematodes (PCN) species identified from different crops using mtDNA-based technique

	African spinach		**Tomato**	**Potato**	**African nightshade**		**Sequences (NAD5/COI)**
Crop species	*A. dubius*	*A. cruentus*	*S. lycopersicum*[a]	*S. tuberosum*[b]	*S. scabrum*	*S. villosum*[a]	
County			Nematode species[c]				Accession numbers
Kakamega	×	×	Ma, Mi, and Mj	-	×	Ma, Mi, and Mj	MH399836, MH399835, MH399834, MH399833, MH399843, MH399842, MH399841, MH399825
Kiambu	Mi	Mi	**Ma**, **Me**, Mh, **Mi**, Mj, Msp., and Gr	**Ma**, Me, Mh, Mi, Mj, **Gr**, Gp, and Gsp.	Me and Mj	Ma, Me, **Mh**, Mi, **Mj**, and Msp.	MH005023, MH005027, MH005026, MH005025, MH399805, MH399832, MH399802, MH399817, MH399820, MH399823, MH399822, MF322782
Machakos	×	×	**Ma**, Me, **Mi**, and Mj	-	Me and Mi	Ma, **Me**, **Mi**, and **Mj**	MH399837, MH399845, MH399844, MH399824
Murang'a	×	Mi	Ma, Me, Mh, Mi, Mj, and Gr	Ma, Me, **Mh**, **Mi**, Mj, and Gr	Mj	Ma, **Me**, Mh, **Mi**, and Mj	MH399832, MH399831, MH399829, MH399828, MH399839, MH399838, MH399803, MH399801, MH399816, MF773722
Nyandarua	×	×	**Ma**, **Mh**, Mi, **Mj**, and Gr	**Ma**, Mh, **Mi**, **Mj**, Gr, and Gp	×	Ma, Mh, Mi, and Mj	MH399827, MH005024, MH399830, MH399800, MH399815, MH399818

[a]RKN multiple species infection were detected.

[b]RKN-PCN co-infection were detected.

[c]Ma = *Meloidogyne arenaria*, Me = *M. enterolobii*, Mh = *M. hapla*, Mi = *M. incognita*, Mj = *M. javanica*, Msp. = *Meloidogyne* sp, Gr = *Globodera rostochiensis*, Gp = *G. pallida*, Gsp. = *Globodera* sp. Species in bold were detected in combination from a single plant.

× = no RKN or PCN were detected from the roots.

- = no crop was observed.

S = *Solanum*

A = *Amaranthus*

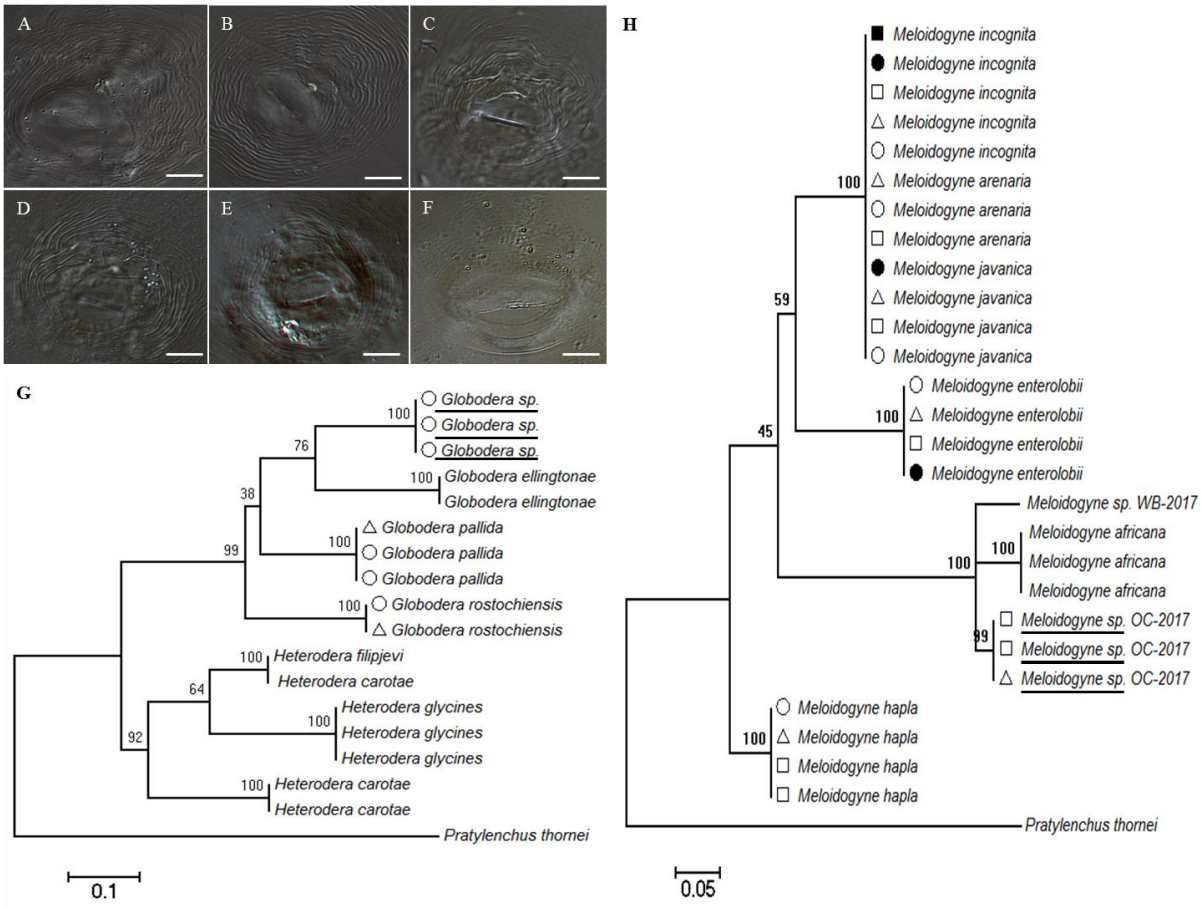

Figure 3: Determination of root-knot nematodes (RKN) and potato cyst nematode (PCN). (A-F) Perineal pattern of RKN isolated from different crop species: (A-C) *Meloidogyne incognita*, *M. javanica*, and *M. arenaria* (D) *M. hapla*, (E) *M. enterolobii* and (F) *Meloidogyne* sp. (G) Phylogenetic tree based on mitochondrial cytochrome oxidase I (COI) sequences of *Globodera* and *Heterodera* spp., an associated *Globodera* sp. is marked by underline. (H) Phylogenetic tree based on mitochondrial COI sequences of RKN; an undescribed *Meloidogyne* sp. is underline. Values above branches are Maximum Likelihood bootstrap values. For details on phylogenetic reconstruction see Materials and Methods. The following symbols represent the host plant from which the adult nematodes were extracted: ○ *S. tuberosum*, □ *S. villosum*, ■ *A. dubius*, ● *S. scabrum* and Δ *S. lycopersicum*. A - *Amaranthus*, S - *Solanum*. Scale bar = 25 µm.

4.2 Impact of AIV cultivation on population dynamics of RKN and PCN and subsequent nematode management in tomato and potato

Our survey results showed that RKN parasitism was very low on *A. dubius*, *A. cruentus*, and *S. scabrum*. A field trial was conducted at a site that had natural soil infestation of *M. incognita*, *M. javanica*, *M. hapla*, *M. enterolobii*, and an associated *Meloidogyne* sp. At the beginning of the experiment no significant differences in RKN population densities existed

among the plots assigned to different crop treatments (Fig. 4A). By the end of the first season, population densities of RKN were significantly increased under *S. villosum* and were significantly reduced under *A. dubius, A. cruentus,* and *S. scabrum* (Fig. 4A). In season 2 and 3, these dynamics continued. However the successive cultivation of susceptible *S. villosum* promoted RKN soil infestation and root galling (Fig. 4A and B) and severe wilting and root galling (s 1D and E). The two species of African spinach achieved a similar RKN suppressive effect therefore plots under *A. cruentus* were not considered in the next experiment. After three successive seasons of cultivating AIV, a RKN susceptible *S. lycopersicum* cv. money maker planted under AIV plots showed different galling index and number of flowers at 6 weeks after planting. The galling index was lower in *S. scabrum* and *A. dubius* and the number of flowers was higher compared to *S. villosum* (Fig. 4C).

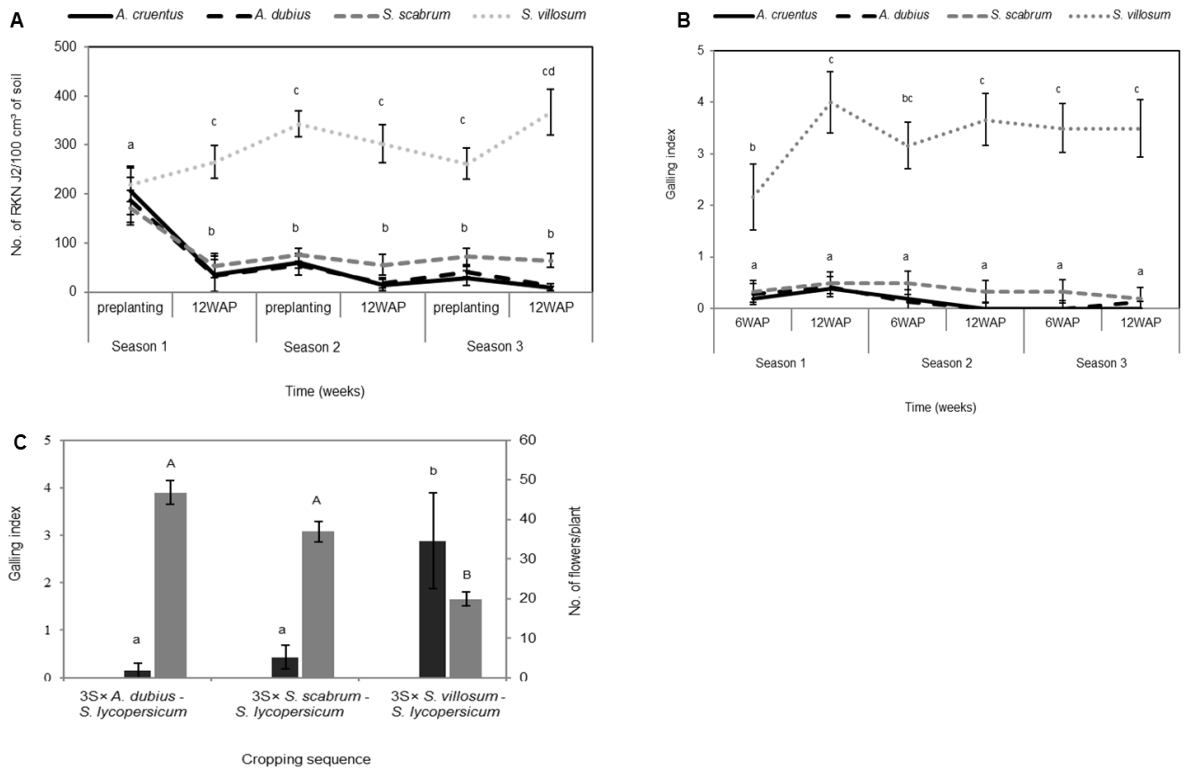

Figure 4: Impact of African indigenous vegetables (AIV) cultivation on population dynamics of root-knot nematodes (RKN) and subsequent nematode management in tomato. (A) RKN population densities under AIV cultivation for three successive seasons; (B) Root galling on AIV crops; (C) Galling index and the number of flowers on *S. lycopersicum* at 6 weeks after planting following three successive (3S) cultivation of AIV. Values of the bars with different letters are significantly different at $P \leq 0.05$ using Tukey post-hoc multiple comparisons test. Error bars represent standard deviation of mean. In section C dark bars represent primary axis and light bars represent secondary axis. A - *Amaranthus*, S - *Solanum*.

In our survey, no adult PCN females were observed on *S. scabrum* and *S. villosum*, and very few viable PCN eggs were found in cysts extracted from the soil surrounding the roots of these plants. A field trial was conducted at a site that had natural soil infestation of *G. rostochiensis, G. pallida*, and an undescribed *Globodera* sp. The same site also had a natural infestation of RKN. At the beginning of the experiment (season 1), no significant differences in PCN population densities existed among the test plots assigned to different crops. By the end of season 1, population densities of PCN (measured as viable eggs/cyst) were significantly lower on *S. scabrum* and *S. villosum* compared under *A. dubius* and *A. cruentus* or fallow (Fig. 5A). In season 2 and 3, these dynamics continued. After three successive seasons of cultivation of AIV, a PCN susceptible *S. tuberosum* cv. Shangi showed different responses in wilting incidences, number of PCN females and number of flowers at 6 weeks after planting. The incidences of wilting on *S. tuberosum* was significantly reduced in *S. scabrum* and *S. villosum* test plots (Fig. 5B and s1A), but wilted and stunted *S. tuberosum* plants were observed under fallow and *A. dubius* (S 1B). The number of PCN females was lower in *S. scabrum* and *S. villosum* compared to *A. dubius* and fallow (Fig. 5C). The effect of AIV on RKN-PCN co-infection also varied. Potato plants with both root galls and PCN females were observed (S 1C). RKN-PCN co-infection on *S. tuberosum* was significantly reduced in *S. scabrum, S. villosum,* and *A. dubius* and the number of flowers was higher in *S. scabrum* (Fig. 5D).

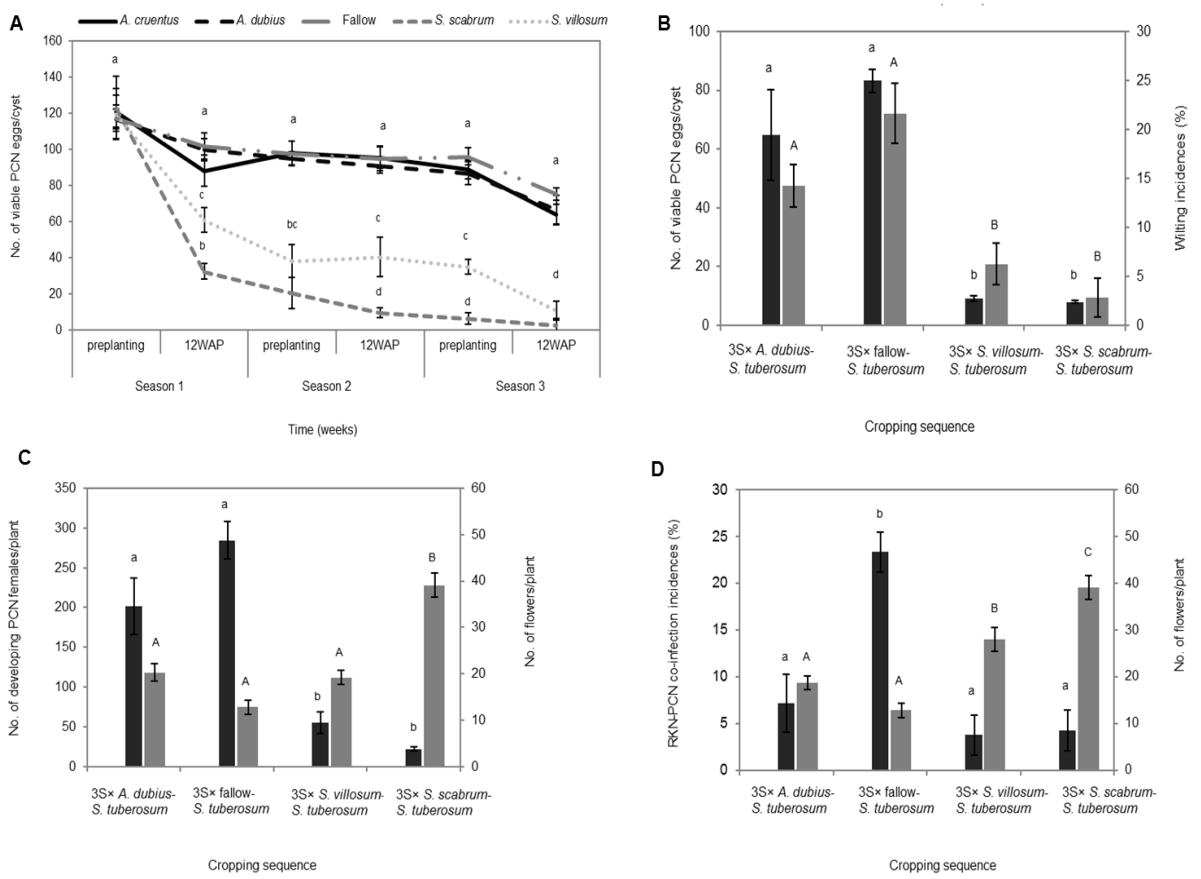

Figure 5: Impact of African indigenous vegetables (AIV) cultivation on population dynamics of potato cyst nematodes (PCN) and subsequent nematode management in potato. (A) PCN population densities under AIV cultivation for three successive seasons; (B) PCN population densities and wilting incidences on *S. tuberosum* at 6 weeks after planting following three successive (3S) cultivation of AIV; (C) Number of developed PCN females and the number of flowers on *S. tuberosum* at 6 weeks after planting following three successive (3S) cultivation of AIV; (D). Co-infection incidences and the number of flowers on *S. tuberosum* at 6 weeks after planting following three successive (3S) cultivation of AIV. Values of the bars with different letters are significantly different at $P \leq 0.05$ using Tukey post-hoc multiple comparisons test. Error bars represent standard deviation of mean. In section B, C and D dark bars represent primary axis and light bars represent secondary axis. A - *Amaranthus*, S - *Solanum*.

5. Discussion

Parasitism of crops by RKN and PCN is a major constraint for food production. In Africa, smallholder cropping systems are complicated and often characterized by simultaneous cultivation of crop species that support development of RKN and PCN. Hence, the current farming system increases the economic impact of these nematodes.

AIV including African nightshade (*S. scabrum* and *S. villosum*) and African spinach (*A.*

dubius and *A. cruentus*) are neglected and underutilized crops, but have been a part of farming practices and nutrition in traditional societies in Africa. However, there is a lack of information on their host status to RKN and PCN, and so far no study has focused on the impact of these crops on RKN and PCN dynamics. Here, we demonstrate that reintroduction of African nightshade and African spinach into cropping systems can be used to reduce RKN and PCN populations and yield effects on following susceptible crop species.

Implementation of an effective management strategy to control plant-parasitic nematodes requires accurate nematode species identification and their respective host plants (Taylor and Sasser 1978). Thus, in this study we first characterized the different RKN and PCN infecting *S. scabrum*, *S. villosum*, *S. lycopersicum*, *S. tuberosum*, *A. dubius*, and *A. cruentus*. We employed both morphological and molecular approaches to identify the RKN and PCN species. Current morphological identification procedures were able to differentiate some, but not all of the RKN. Despite morphological identification failing to give a clear resolution to separate tropical RKN species such as *M. javanica, M. arenaria* and *M. incognita,* the other RKN, *M. hapla, M. enterolobii,* and an associated *Meloidogyne* sp. were clearly separated from each other by using the perineal patterns (Eisenback et al. 1980). The widely used barcode gene COI reliably differentiated *M. hapla, M. enterolobii,* and *Meloidogyne* sp. from the other tropical RKN. The recently identified NAD5 gene fragment DNA marker (Janssen et al. 2016), allowed a reliable identification of the most common tropical RKN, *M. javanica, M. arenaria,* and *M. incognita*. The phylogenetic position of an undescribed *Meloidogyne* sp. indicates a closer relationship with *M. africana* which was previously reported on coffee (Janssen et al. 2017). Furthermore, COI gene sequence identified *G. rostochiensis* and *G. pallida* and reliably differentiates the undescribed *Globodera* sp. from the other PCN. The presence of *G. rostochiensis* and *G. pallida* parasitizing *S. tuberosum* was recently reported in Kenya (Mburu et al. 2018; Mwangi et al. 2015). The phylogenetic position of the undescribed *Globodera* sp. indicates a closer relationship with *G. ellingtonae* which was previously reported on potato (Handoo et al. 2012). Remarkably, most RKN and PCN lineages identified in the current study have a global distribution favoring the hypothesis that spread was aided by humans through agriculture (Castagnone-Sereno et al. 2013).

We detected nematode species such as *M. hapla, G. rostochiensis,* and *G. pallida*, which are usually found in temperate climates, in a moderate tropical climate. It shows that these

nematode species have ability to successfully compete with tropical species. It underlines that temperate nematode species have to be considered as pathogens in tropical and sub-tropical management systems. Temperate RKN such as *M. hapla* have already been reported in sub-tropical conditions (Chitambo et al. 2018; Meressa et al. 2014) indicating the ability of these nematodes to change their temperature or climate preferences. This underpins the need for a proper nematode diagnosis and that plant resistance to several species of RKN and PCN is necessary for an effective nematode control under the current situation in many parts of Africa.

RKN were mainly associated with *S. villosum, S. lycopersicum,* and *S. tuberosum* indicating that RKN are capable of causing damage on these crop species. In fact, they had been previously reported as good hosts for RKN (Nchore et al. 2012; Onkendi et al. 2014; Sikora and Fernandez 2005; Sikora et al. 2018). By contrast, *A. dubius* and *A. cruentus* showed resistance to the studied RKN species and only *M. incognita* were able to induce very few galls on these species. In the literature, the host status of *Amaranthus* spp. to RKN is not clear. Previously, it was shown that several *Amaranthus* species were resistant to RKN (Babatola and Awoderu 1986; Reddy et al. 1980). Later, Ferris et al. (1993) found that *A. caudatus*, *A. hypochondriacus*, and *A. cruentus* were non-hosts to *M. chitwoodi*, and *A. retroflexus* was rated as a poor host for *M. chitwoodi*. In contrast, a recent study indicated that *A. tricolor* supports *M. incognita* reproduction (Vaingankar et al. 2018). This suggests that the genus *Amaranthus* is highly diverse and is composed of many species and possibly varieties that vary in response to *Meloidogyne* infection. In principle, there are three types of plant responses to *Meloidogyne* infection (i) susceptibility – indicated by nematode development and plant damage; (ii) resistance – causing low root galling in *A. dubius* and *A. cruentus* resulting in low nematode reproduction; (iii) tolerance – showing low reduction of root and shoot traits but strongly supporting nematode development. The latter was described in a recent study which demonstrated that *A. tricolor* genotype IC-0598184 performed well after infection by *M incognita* but remained with a high burden of *M. incognita* infection (Vaingankar et al. 2018). The fact that *A. dubius and A cruentus* were resistant to RKN identified in this study make them ideal candidates for RKN management.

PCN identified in this study were only associated with *S. lycopersicum* and *S. tuberosum*, but not *S. scabrum* and *S. villosum* indicating resistance in these crops. *Solanum scabrum* and *S. villosum* belongs to the Solanaceae family and Scholte (2000) reported the ability of non-tuber bearing Solanaceae plants to stimulate PCN hatching. In contrast, non-

Solanaceae plants such as *A. dubius* and *A. cruentus* do not have an effect on PCN hatching. Thus, after successive cultivation of African nightshade the number of developing PCN observed on *S. tuberosum* was reduced. Dandurand et al. (2013) used a resistant trap crop, *Solanum sisymbriifolium* to control PCN, and this approach decreased PCN cyst infestation in the soil by more than 90%. Related nightshade belonging to the non-tuber bearing species in the *Solanum* genus have demostrated the ability to stimulate PCN hatching and to prevent further development of PCN. We found a similar effect of the analyzed nightshades in our study.

Simultaneous occurrence of two or more different nematode species renders host resistance deployed against one species ineffective, because another species can overcome the resistance. It is known that tomato cultivars carrying *Mi-1.2* gene introgressed from *Solanum peruvianum* are resistant to *M. incognita, M. javanica* and *M. aranaria*, but not *M. enterolobii* (Kiewnick et al. 2009). In Africa, multiple species of RKN infections have been reported (Chitambo et al., 2018; Kolombia et al., 2017), indicating that nematode multiple infections are ubiquitous in Africa, but too often ignored. Our results indicate that *A. dubius* and *A. cruentus* are resistant to the studied RKN species including *M. enterolobii.* This species has been reported to overcome resistance of most cultivated crops carrying resistance genes against other RKN, including resistant cotton, sweet potato, tomatoes (*Mi-1* gene), soybean (*Mir1* gene), potato (*Mh* gene), sweetpepper (*Tabasco* gene), bell pepper (*N* gene), and cowpea (*Rk* gene) (Berthou et al. 2003; Brito et al. 2007; Castagnone-Sereno 2012; Cetintas et al. 2008; Yang and Eisenback 1983).

Our studies indicate that AIV resistant to RKN and/or PCN are ideal cover crops for management of both of the groups of nematodes or can be used as rotational crops, relay crops etc. as well. Integrating these crops in smallholder cropping system as cover crops, rotational crops or relay crops has several advantages including nematode control and dietary diversification. Elsewhere, cover crops are used in various production systems to provide many benefits such as pest and disease management, addition of organic matter to soil, and increased productivity of cash crops. For example the use of cover crops in the Brassicaceae family such as oilseed radish, white mustard, and winter rapeseed decreased sugar beet cyst nematode population densities (Lelivelt and Hoogendoorn 1993; Wen et al. 2017).

In summary, we have shown that accurate diagnosis of RKN and PCN will help provide

proper implementation of an effective management decision. We identified *A. dubius* and *S. scabrum* with the ability to suppress RKN species identified in this study, whereas *S. scabrum* and *S. villosum* suppress the identified PCN species. *Solanum scabrum* suppresses both RKN and PCN identified in this study. According to our results these crop species can be used to manage RKN and PCN. We recommend that growers intending to simultaneously control PCN and RKN should use *S. scabrum*. Although *S. villosum* was able to suppress PCN, it is highly susceptible to RKN and should not be used where RKN are detected. This finding is a major relief to the resource constrained smallholder farmers who are overburdened by plant-parasitic nematodes, pests and diseases as well as nutritional challenges. The reintroduction of AIV species into the existing cropping systems may be a way of promoting agro-biodiversity to improve resilience to plant-parasitic nematodes, pests and diseases as well as dietary diversification in Africa. Therefore, this approach can be used as a simple management strategy for RKN and PCN in an environmentally friendly, effective, and productive way.

6. Acknowledgement

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7. Supplementary information Chapter 3

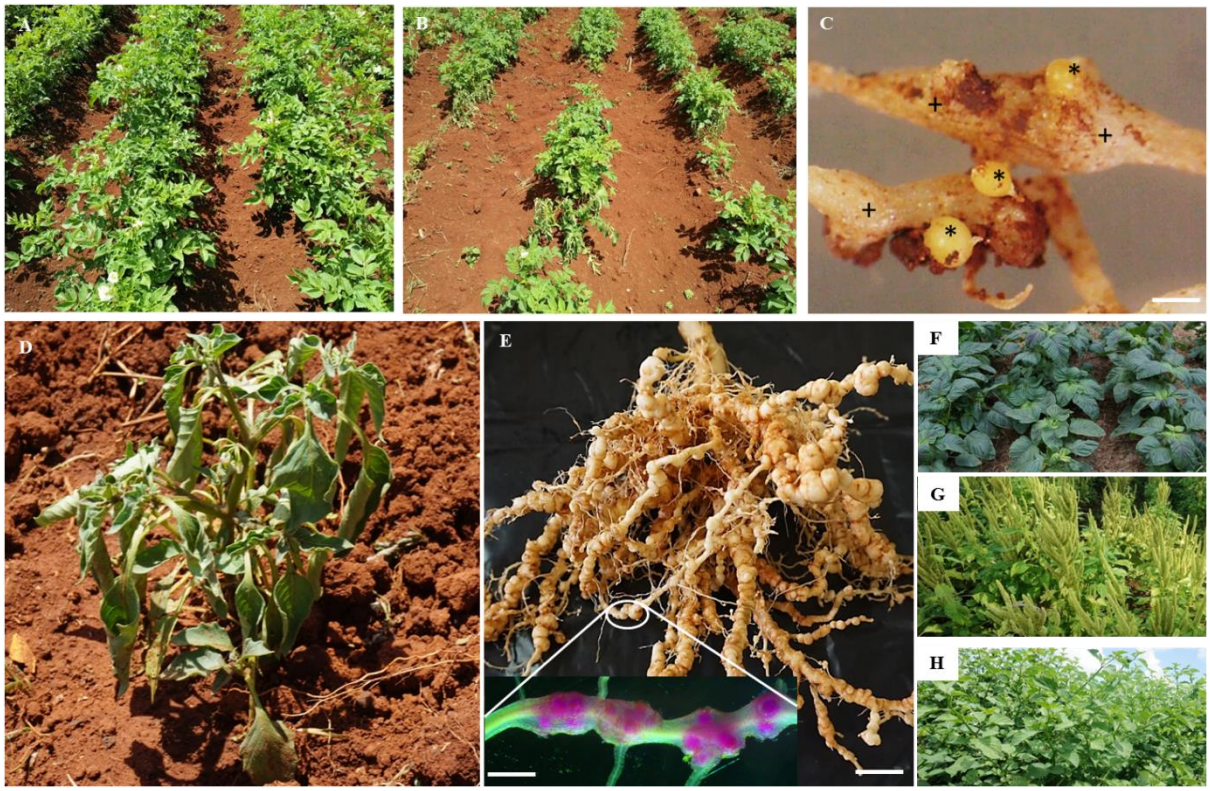

Figure S1: The effect of cultivating African nightshade on the subsequent potato crop; (A) healthy potato plants on the plot previously planted with *Solanum scabrum*; (B) wilting and stunted plants due to potato cyst nematodes (PCN) infection on plots that were previously fallow; (C) Root-knot nematodes - potato cyst nematodes (RKN-PCN) co-infection on potato root. Scale bar = 1 cm. Effects of continuous cultivation of susceptible crops; (D) aboveground symptoms on *S. villosum* showing severe wilting; (E) belowground symptoms showing severe root galling on *S. villosum* root system Scale bar = 5 cm. Insert: RKN females stained with Sodium hypochlorite solution - acid fuchsin-glycerin technique in root galls at higher magnification Scale bar = 1 cm. The appearance of selected crop species; (F and G) African spinach (*A. dubius* and *A. cruentus*) and (H) African nightshade (*S. scabrum*). A - *Amaranthus*, S - *Solanum*. + = root galls induced by RKN, * = adult female of PCN.

Chapter 4

Host status and resistance mechanism of selected African nightshade and African spinach against *Meloidogyne* spp. and *Globodera* spp.

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Modified from:

Chitambo, O., Haukeland, S., Fiaboe, K.K.M., Kariuki, G.M., Grundler, F.M.W. Host status and resistance mechanism of selected African nightshade and African spinach against *Meloidogyne* spp. and *Globodera* spp. *Manuscript in preparation*.

1. Abstract

Plant parasitic nematodes particularly root-knot nematodes (RKN: *Meloidogyne* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.) require suitable host plants for nutrition and completion of their life cycle. They cause economic damage on the parasitized plants. African nightshade (*Solanum* spp.) and African spinach (*Amaranthus* spp.) are important leafy vegetables in many parts of Africa for food, nutrition, and livelihood security. Although RKN and potato cyst nematode (PCN: *Globodera* spp.) resistance has been observed in some species of African nightshade and African spinach, how these plants interfere with nematode infection process is still unknown. Here, we show that successful parasitism was impaired by localized root tissue disintegration during the early stages of nematode infection in resistant African nightshade and African spinach. Nematode infected roots of *S. scabrum* (broad leaf) and *A. dubius* (broad leaf) exhibited high localized root tissue disintegration and were resistant to all species of RKN used in this study. Notably, *A. dubius* (broad leaf) showed full resistance against highly pathogenic *M. enterolobii*. For PCN, both *S. scabrum* and *S. villosum* stimulated PCN hatching but not their reproduction with a similar resistance mechanism as before. We propose that during the course of evolution plants including AIV evolved to direct root tissue necrosis and disintegration to orchestrate the containment, starving, and expulsion of nematodes. The ability of resistant AIV to autonomously induce localized root necrosis and disintegration reveals that maintaining root tissue integrity is very important for successful nematode parasitism. Thus AIV-protective responses to RKN and PCN include two equally significant tasks: expulsion of nematode and root tissue repair. Inevitably, the information generated in this study is important in breeding programmes, designing crop rotation schemes, and cropping systems to avoid yield losses caused by high RKN and PCN soil infestation.

2. Introduction

Root-knot nematodes (RKN: *Meloidogyne* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.) are the most economically damaging group of plant-parasitic nematodes (Jones et al., 2013), because of the negative impact their parasitism has on farming systems. Vegetables crops are the most susceptible host plants (Collange et al., 2011). RKN and potato cyst nematodes (PCN) are known to occur in farming systems in Africa, posing a significant threat to crop production by small-holder farmers (Coyne et al., 2018; Onkendi et al., 2014). The restriction of certain chemical nematicides due to their environmental impact and on human and animal health (for example in Europe, Directive 2009/128/EC), has intensified research on alternative nematode management. Integrated pest management practices which combine cultural methods and host plant resistance are the most promising options for nematode management under small-holder farms.

RKN and PCN have a similar biology consisting of a distinct egg, juvenile stages (J1), (J2) the infective stage, (J3), (J4) and adult stages. However, PCN tend to be much more host specific and require host stimulus for egg hatching. The J2 of RKN and PCN are attracted to stimuli from suitable host roots and they penetrate the root through the cortex where they enter the vascular cylinder. In the vascular cylinder, RKN become sedentary and select five to eight vascular cells to differentiate into their feeding cells, termed giant cells, whereas PCN induce the formation of multicellular feeding sites called syncytia (Jones, 1981; Steinbach, 1974; Wyss et al., 1992). The ability of RKN and PCN to survive in the host plant is the result of adaptation or co-evolution between host plant and the nematode (Hussey & Grundler, 1998), thus despite their large size (compared to other pathogens) they are able to survive inside root tissue. It is therefore necessary for these nematodes to locate a suitable host plant and install an immunoregulatory environment (Goverse & Smant, 2014). Otherwise, the host plant would be able to generate an effective anti-nematode response that will result in containment, starving, and expulsion of nematodes.

The African nightshade (Solanaceae: *Solanum* spp.) and African spinach (Amaranthaceae: *Amaranthus* spp.) are nutritious crops cultivated in tropical sub-Saharan Africa that represent the bulk of African indigenous vegetables (AIV) (Cernansky, 2015; Gido et al., 2017; Maundu et al., 2009). These crops are traditionally grown by small holder farmers and continue to be maintained by sociocultural preferences, however their response to RKN remains not well documented and to some extend neglected by formal research. The degree

of susceptibility among African nightshade and African spinach varies in response to infestation by a range of species and races of RKN. Susceptibility to RKN among African nightshade (*S. villosum and S. nigrum*) has been reported in previous studies (Nchore et al., 2013). In a field trial contacted in Kenya, *S. villosum* increased RKN soil infestation and root galling on the subsequent susceptible crop, but *S. scabrum* was found to have an opposite effect. Susceptibility to RKN was reported in *A. caudatus*, *A. hypochondriacus* and *A. tricolor* (Reddy et al., 1980). *A. retroflexus* was more susceptible to *M. javanica* than to *M. incognita* and *M. arenaria* (Kokalis-Burelle & Rosskopf, 2012). In contrast *A. cruentus* was found to be a poor host for several RKN (Nchore et al., 2013; Ntidi et al., 2016; Rodriguez-Kabana et al., 1988). In Kenya, our recent study showed that *A. dubius* significantly reduced RKN soil infestation and root galling on the subsequent susceptible crop.

PCN are more host specific thus trap crops which are non-host crops have been successfully used to reduce their population densities. PCN trap crops stimulate egg hatching but do not support nematode reproduction because the J2 cannot successfully parasitize plant roots. Scholte (2000), showed that *Solanum sisymbriifolium* Lam. was effective as potato at inducing PCN egg hatching but not the subsequent nematode development and reproduction. The screening of 90 accessions of Solanaceae (non-tuber bearing) demonstrated PCN egg hatching stimulatory effect and resistance (Scholte, 2000). Thus, PCN egg hatching stimulatory effect by Solanaceae (non-tuber bearing) is well documented. In an experiment contacted in Kenya African nightshade decreased PCN soil infestation and infection on the subsequent susceptible potato crop.

Studying the host status and resistance mechanism against nematodes on AIV is of great importance because it enables the selection of suitable varieties in crop rotations and helps breeders to select for a desired feature for the breeding programme. So far the mechanism of AIV resistance against RKN and PCN remains unknown, although much work has been done to elucidate them in other plants (Cai et al., 1997; Vos et al., 1998; Williamson & Hussey, 1996; Williamson & Kumar, 2006). Here we performed laboratory and pot experiments to study nematode development and reproduction. This was achieved by (1) evaluating the AIV early response to RKN and PCN infection (2) studying the reproduction of RKN and PCN on AIV.

3. Materials and methods

3.1 Nematode inoculum and planting material

Pure RKN cultures of *M. incognita*, *M. javanica*, *M. arenaria*, *M. enterolobii* and *M. hapla* were maintained on susceptible tomato cv. Money maker. These cultures were used in all experiments. Forty eight hours old second stage juveniles (J2s) of the nematodes were extracted by a method according to (Whitehead & Hemming, 1965), standardized, concentrated and used for inoculating the test plants. Briefly, nematode eggs were extracted by cutting the tomato roots into10 - 20 mm sections and ground them in 0.5% NaOC1 for four minutes. The homogenate was then washed with distilled water through a series of mesh sieves and the nematode eggs were collected on a 25 µm sieve. For *G. rostochiensis*, cysts were obtained from stock cultures maintained on potato cv. Shangi in a greenhouse maintained at25 ± 4 ^{o}C. Cysts were extracted from the soil according to (Seinhorst, 1964). For hatching the cysts were soaked for one week in tap water and egg suspensions were made. The eggs were then exposed to hatching agent extracted from young African nightshade plants. Five widely cultivated AIV in Kenya; *S. scabrum* branched (S.cb), *S. scabrum* non-branched (S.cn), *S. villosum* (S.v), *A. dubius* broad leaf (A.db), and *A. dubius* narrow leaf (A.dn) were used in this experiment in order to study their response to RKN and PCN. Susceptible tomato cv. Money maker and potato cv. Shangi were used as positive controls.

3.2 Laboratory experiment

AIV seeds were disinfected with 0.05% (w/v) sodium hypochlorite (NaOCl) for 10 minutes and 70% (v/v) ethanol for 1 minute followed thorough rinsing (4 times) with autoclaved distilled water. Before planting the sterile seeds were allowed to dry on filter paper. Sterile seeds of AIV were cultivated on Murashige and Skoog (MS) medium with a 16hr light and 8hr dark cycle at 25 °C. Two seeds were planted per petri dish (9cm). At 20 days after planting, the seedlings were inoculated with sterilized 300 - 320 freshly hatched J2 nematodes per plant. The nematodes were inoculated onto the surface of MS medium in petri dishes (9 cm).

3.3 Pot experiment

Eighteen days old seedling of each AIV was transplanted into a 15 cm diameter pot containing autoclaved sand: soil (2: 1). Five days later 2000 J2 of RKN were introduced at the rhizosphere by making three holes with glass rod and immediately covered to prevent desiccation. The treatments were replicated six times and arranged in completely randomized design. The treatments were watered lightly with about 200 ml of water as required and maintained at 25 ± 4 ^{o}C for 60 days.

3.4 Evaluation for nematode parasitism and AIV response

The plant early response to nematode infection was recorded at different time points (12, 48, and 72 hours post inoculation) by counting the number of disintegrating infected root tip segments expressed as percentage of the non-disintegrated infected root tips. The development of nematodes inside root samples and external egg masses were visualized by the acid fuschin according to (Bybd et al., 1983). The number of adult female nematodes, egg masses and galls per plant was recorded at 20 days and 60 days post infection. Egg masses were visualized by the acid fuschin staining as before. Female nematodes, egg masses and galls were counted with a DM2000 dissection microscope (Leica Microsystems) and imaging was carried out using Leica DM4000 inverted microscope (Leica Microsystems) fitted with an Olympus C-5050 digital camera.

3.5 Statistical analysis

Data from each experiment were analysed separately by analysis of variance using SigmaPlot 12.5. Means for percentage root disintegration, counts of egg masses, eggs and number of galls were transformed by $\log_{10}(x+1)$ before statistical analysis, and means were separated using the Tukey–Kramer honest significant difference test ($P \leq 0.05$).

4. Results

4.1 Root tip disintegration and reduced root penetration interferes with RKN and PCN parasitism on AIV

To characterize the early response of AIV to RKN infection, an in vitro nematode infection system was used which allowed us to follow nematode development under the light microscope (Figure 2A and B). Plant response to J2 invasion of AIV root was assessed at 12, 48 and 96 hours post infection. Percentage of disintegrated root tips was significantly high on *A. dubius* broad leaf, *S. scabrum* broad leaf and *S. scabrum* narrow leaf compared to *A. dubius* narrow leaf and *S. villosum*. Although not statistically different, at 96 hours post infection the percentage of disintegrated root tips was higher on *A. dubius* broad leaf, *S. scabrum* broad leaf and *S. scabrum* narrow leaf whereas on *A. dubius* narrow leaf and *S. villosum* the percentage of disintegrated root tips was reduced (Figure 2C - F). As a result the AIV that showed high percentage of disintegrated root tips had reduced number of nematodes inside their roots (Figure 2).

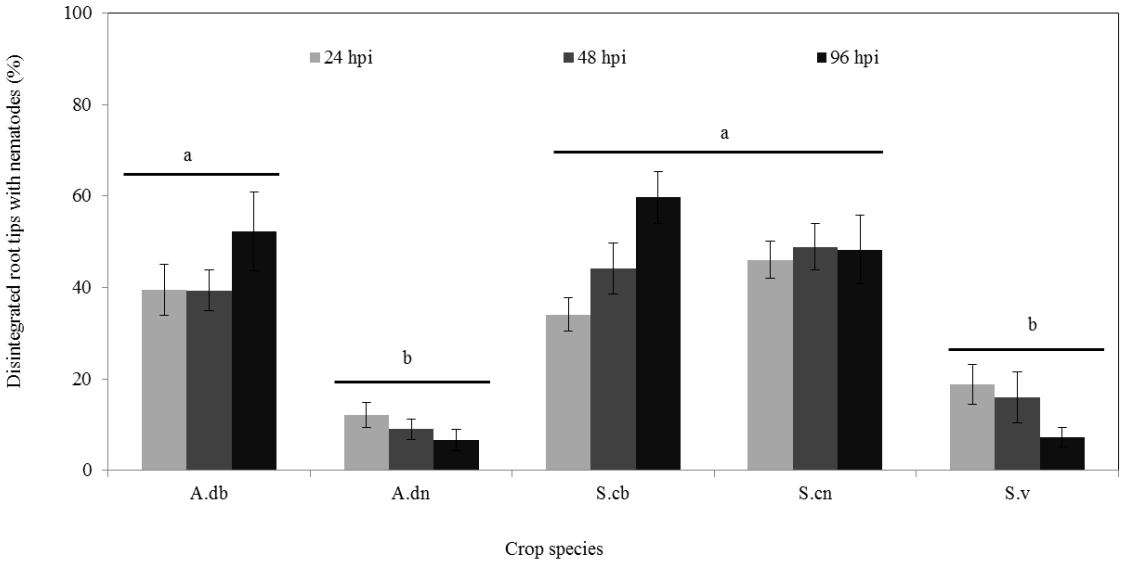

Figure 1. Percent of disintegrated root tips of African indigenous vegetables (AIV) at 48 hours post infection by *Meloidogyne incognita*. Values of the bars with different letters are significantly different at $P \leq 0.05$ using Tukey post-hoc multiple comparisons test. Error bars represent standard deviation of mean. A.db = *A. dubius* broad leaf, A.dn = *A. dubius* narrow leaf, S.cb = *Solanum scabrum* broad leaf, S.cn = *Solanum scabrum* narrow leaf and S.v = *Solanum villosum.*

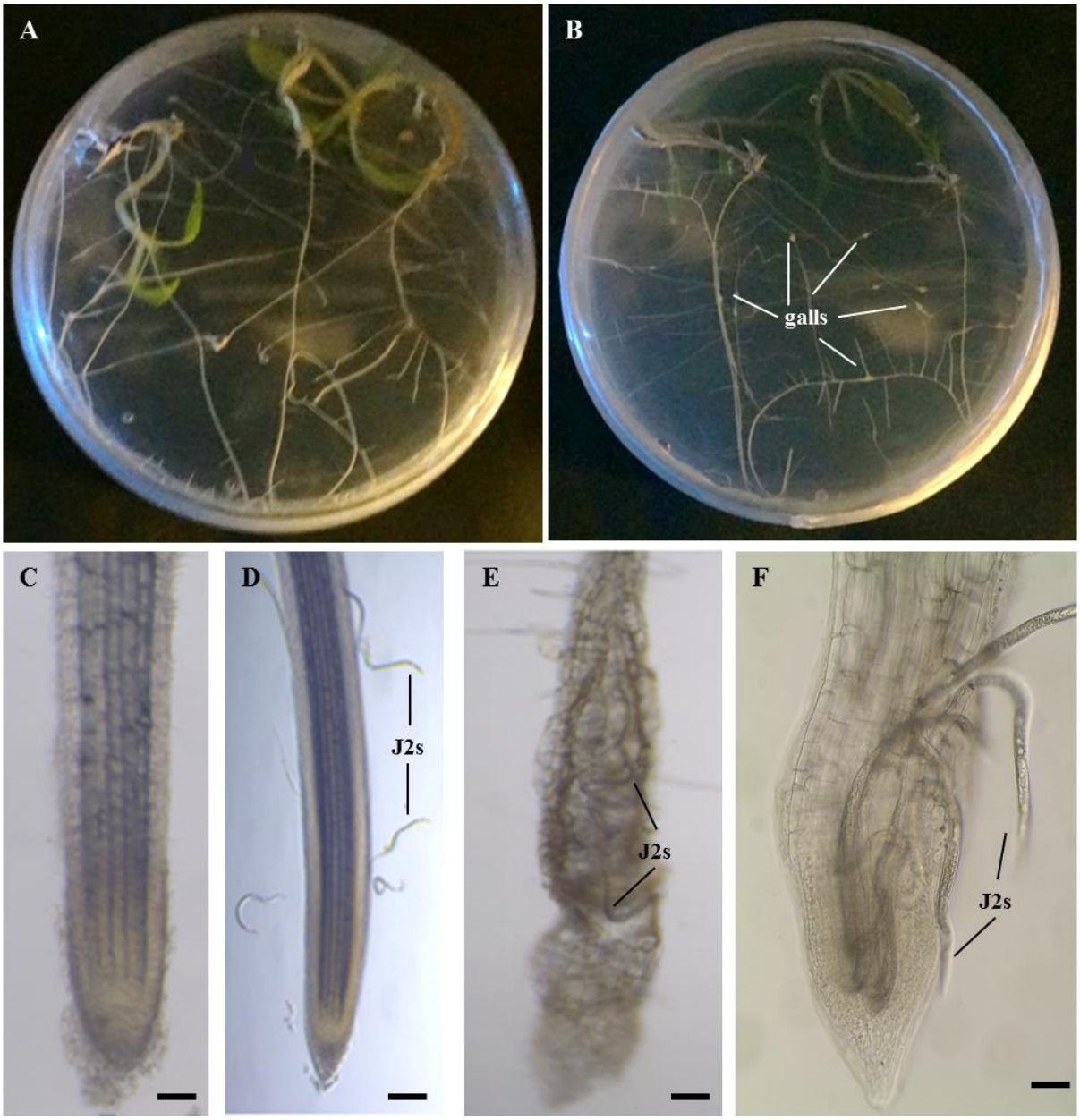

Figure 2. African indigenous vegetables response to root-knot nematode (RKN) infection: (A) Uninfected roots of S.cn cultured on MS medium, (B) Development of root galls induced by *M. incognita* at 20 days post inoculation on S.v, (C) Uninfected root of S.cb, (D) *M. incognita* J2 attracted to S.cb roots 8 hours post inoculation, (E) S.cb root tip disintegration 48 hours post infection with *M. incognita*, (F) Intact root tip of S.v 48 hours post infection by *M. incognita*. S.cb = *Solanum scabrum* broad leaf, S.cn = *Solanum scabrum* narrow leaf and S.v = *Solanum villosum.*

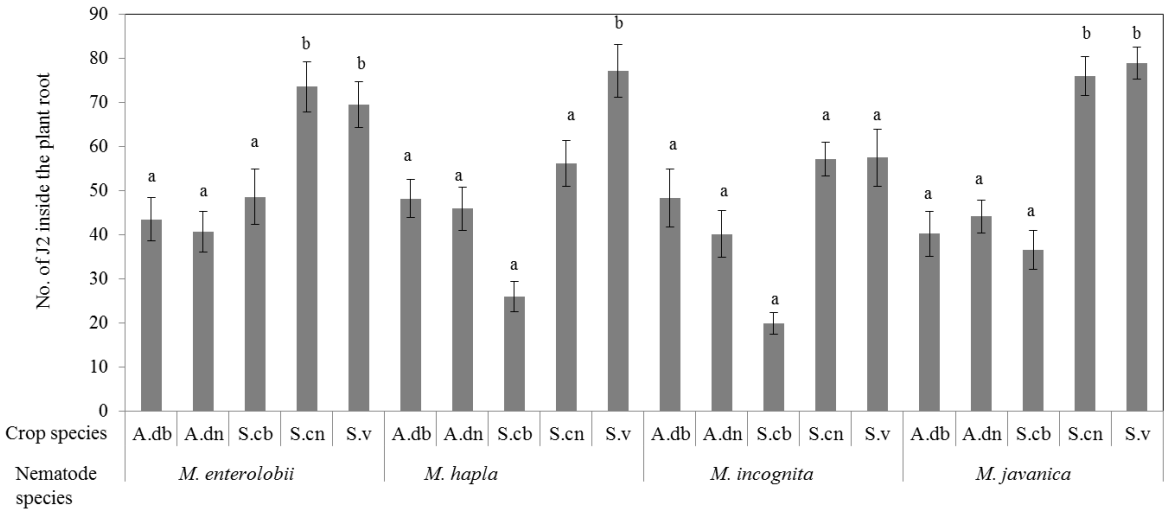

Figure 3. Number of juveniles penetrating the five African indigenous vegetables (AIV) at 48 hours post inoculated with 300 infective juveniles (J2) of different root-knot nematode (RKN). Values of the bars with different letters are significantly different at $P \leq 0.05$ using Tukey post-hoc multiple comparisons test. Error bars represent standard deviation of mean. A.db = *Amaranthus dubius* broad leaf, A.dn = *Amaranthus dubius* narrow leaf, S.cb = *Solanum scabrum* broad leaf, S.cn = *Solanum scabrum* narrow leaf and S.v = *Solanum villosum.*

4.2 Specific genotypes of AIV species hinders RKN reproduction

In order to assess the impact of AIV on RKN reproduction a pot experiment was carried out. The level of reproduction was measured as the number of egg masses per plant. *S. villosum* supported the highest reproduction of all RKN species used in this study (Figure 4). However, the reproduction of RKN was genotype dependent on the other AIV. The number of *M. enterolobii* egg masses was significantly high on *S. scabrum* (narrow leaf) compared to *S. scabrum* (broad leaf) and the response was the same with *M. javanica* (Figure 4). The two genotypes of *A. dubius* (narrow leaf and broad leaf) showed a similar trend. Consistently with nematode reproduction (number of egg masses), the galling index (RKN root symptoms) was high on *S. villosum*. Galling index was genotype dependent on the other AIV, with the other genotypes showed no signs of root galling (Figure 5). Typical root galling on susceptible AIV is shown on below (Figure 7B and C)

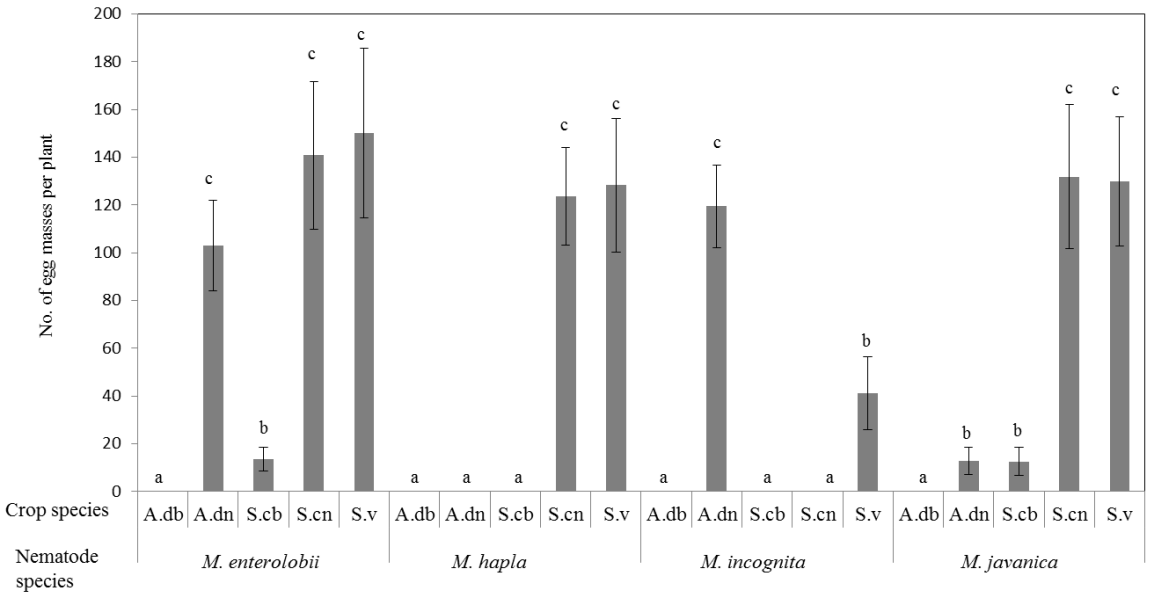

Figure 4. Number of root-knot nematode (RKN) egg masses per plant at 60 days post inoculation on African indigenous vegetables (AIV). Values of the bars with different letters are significantly different at $P \leq 0.05$ using Tukey post-hoc multiple comparisons test. Error bars represent standard deviation of mean. A.db = *Amaranthus dubius* broad leaf, A.dn = *Amaranthus dubius* narrow leaf, S.cb = *Solanum scabrum* broad leaf, S.cn = *Solanum scabrum* narrow leaf and S.v = *Solanum villosum.*

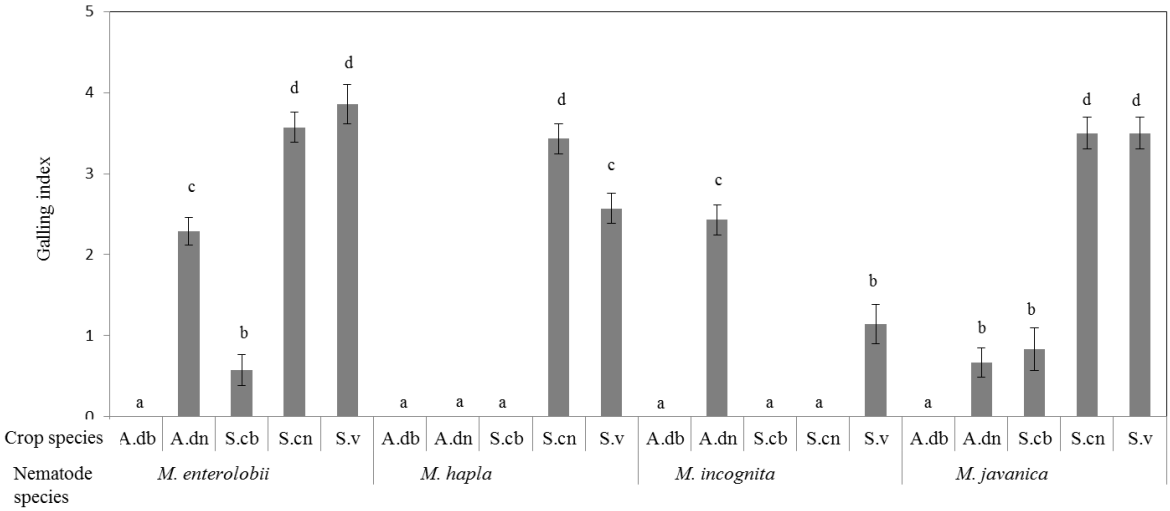

Figure 5. Galling index at 60 days post inoculation on African indigenous vegetables (AIV). Values of the bars with different letters are significantly different at $P \leq 0.05$ using Tukey post-hoc multiple comparisons test. Error bars represent standard deviation of mean. A.db = *Amaranthus dubius* broad leaf, A.dn = *Amaranthus dubius* narrow leaf, S.cb = *Solanum scabrum* broad leaf, S.cn = *Solanum scabrum* narrow leaf and S.v = *Solanum*

villosum.

4.3 African nightshade blocks PCN reproduction

In order to assess the impact of AIV on RKN and PCN reproduction a pot experiment was carried out. African nightshade (*S. scabrum* and *S. villosum*) used in this study stimulated PCN hatching comparable to potato (data not shown). At 48 hours post infection the number of nematodes inside the African nightshade roots was significantly low compared to potato. At 60 days post infection there was no adult female nematodes or cysts attached to the African nightshade roots (Figure 6). No cysts were found in African nightshade posts. Adult PCN female nematodes and cysts attached to potato root are shown below (Figure 7D).

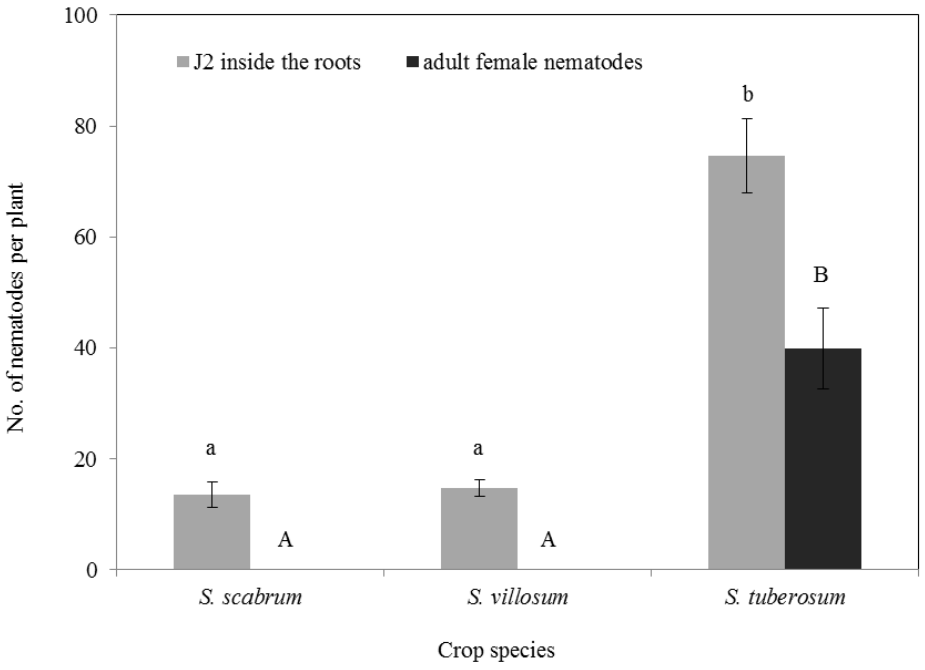

Figure 6. Number of *Globodera rostochiensis* infective juveniles penetrating at 48 hours post inoculation and adult *G. rostochiensis* at 50 days post inoculation on African nightshade (*Solanum scabrum* and *S. villosum*) and potato (*S. tuberosum*). Values of the bars with different letters are significantly different at $P \leq 0.05$ using Tukey post-hoc multiple comparisons test. Error bars represent standard deviation of mean.

Figure 7. Nematode induced symptoms on susceptible host plants at 90 days post infection; (A) resistant plant root system, (B) galls on S.cn induced by *M. javanica*, (C) galls induced by *M. incognita* on A.dn, (D) *G. rostochiensis* females and cysts on potato roots. A.dn = *Amaranthus dubius* narrow leaf, S.cb = *Solanum scabrum* broad leaf, S.cn = *Solanum scabrum* narrow leaf.

5. Discussion

Use of resistant cultivars as nematode management tool in low-income farming systems is very promising because of its affordability and no hazardous effects on human health and the environment. The present research reports on the reaction and host status of AIV to RKN and PCN based on the ability of nematode to reproduce on different species and genotypes. The AIV species and genotypes showed significant variations in their response to nematode infection.

Several of the tested AIV had varying degrees of resistance against the selected species of RKN. *Solanum scabrum* (broad leaf) and *A. dubius* (broad leaf) has shown a spectrum of resistance against the four RKN species tested. However, the other AIV showed a variation in resistance emphasizing the importance of nematode species and population identification when implementing a management strategy. Analysis of AIV early response to RKN and PCN infection showed that root tip disintegration perturbs the nematode infection process. It is well documented that RKN root invasion is achieved, in most cases, by destroying epidermal and subepidermal cells, while intercellular invasion between epidermal cells is less frequent. However, inside the root the J2 orients themselves always in the direction of the root-tip and migrates towards it between cortical and meristematic cells without causing any damage (Wyss et al., 1992). In this study we observed severe root tip disintegration when nematodes were inside the root tissue of resistant AIV. This suggests that some factors within AIV roots interfere with the orientation of J2 during the migration phase or a strong defence response is mounted after the detection of nematode secretions by plant surveillance mechanism.

Fargette et al. (1994) proposed that variations in gall formation and reproduction of RKN on different plant species and genotypes are due to differences in their genetic makeup or due to presence of genes which confer resistance or susceptibility. In resistant AIV nematodes were unable to cause infection and reproduce properly. In order to allow the formation of a feeding site in the vascular parenchyma, the host plant must attract, allow penetration of the epidermis and migration through the cortex. The feeding site would ensure uninterrupted supply of essential nutrients for the developing nematodes to allow reproduction (Abad et al., 2009; Wyss et al., 1992). Resistant AIV could harbour various resistant genes that would interfere with one or more critical steps required for successful parasitism of nematodes. Thus, we observed high nematode reproduction on susceptible AIV compared to resistant AIV. Resistant AIV could also have an elegant way to detect deviations from the root tissue integrity in order to deal with present onslaughts and avoid it in the future, if possible. The management of root tissue disintegration due to nematode infection in resistant AIV is central to their evolutional success, and arguably root tissue disintegration exists to forestall successive assaults by the same or related nematode. We thus propose that during the course of evolution plants including AIV evolved to direct root tissue repair machinery not only to repair disintegrated root tissues but also to orchestrate the containment, killing and expulsion of nematodes.

African nightshade (*S. villosum*) cultivated by some small-scale farmers in Kenya was found to highly susceptible to all RKN species used in this study. Previous studies identified *S. villosum* and other related African nightshade that support high RKN reproduction (Nchore et al., 2013; Sikora et al., 2018). Care should be given not to grow such AIV species in RKN infested although African nightshade are resistant to PCN tested in this study. *Solanum scabrum* (narrow leaf) was found to be resistant against *M. incognita* but surprisingly it was found highly susceptible to *M. javanica*. This may indicate that the *M. javanica* population used in this study might have been dominated by virulent individuals which were able to overcome the contained resistance genes. This might be also responsible for the high reproduction of *M. javanica* on *S. scabrum* (narrow leaf). African spinach species used in this study were found to be resistant against several RKN species. The absence of egg masses on some AIV indicates that some of them are immune for the tested species of RKN. Interestingly, *A. dubius* (broad leaf) was found to be immune to the tested population of *M. enterolobii*. *Meloidogyne enterolobii* is notoriously known for its ability to reproduce on host plants carrying resistance genes against other RKN, including

resistant cotton, sweet potato, tomatoes (*Mi-1* gene), soybean (*Mir1* gene), potato (*Mh* gene), sweetpepper (*Tabasco* gene), bell pepper (*N* gene), and cowpea (*Rk* gene) (Berthou et al., 2003; Brito et al., 2007; Castagnone-Sereno, 2012; Cetintas et al., 2008; Fargette & Braaksma, 1990; Yang & Eisenback, 1983). To date very few non-hosts for *M. enterolobii* are have been documented, including sour orange, peanut, grapefruit and garlic (Brito et al., 2004). It is now widely known that non-tuber bearing Solanaceae plants have the ability to stimulate PCN hatching. In fact, Scholte (2000) reported the ability of non-tuber bearing species in the *Solanum* genus to stimulate PCN hatching and to prevent further development of PCN.

RKN are highly polyphagous in nature and PCN tend to be persistent in the soil, hence it is often very difficult to come up with an effective crop rotations in infested fields and such decision must be taken carefully (Wesemael et al., 2011). Our data showed that the tested AIV were poor to very good hosts to RKN species used. The studied AIV have been simultaneously grown by small-holder farmers including the RKN susceptible ones. Over time this encouraged built up of RKN in the soil. Therefore, the information generated by this study is important in designing crop rotation schemes and cropping systems to avoid yield losses caused by high RKN and PCN soil infestation.

6. Acknowledgement

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Chapter 5

General discussion

1. Diagnosis of plant-parasitic nematodes

Farmers in every region of the world struggle to protect their crops from plant-parasitic nematodes as well as other pests and diseases. In low-income countries the situation is direr due to inconsistent diagnostic procedures, insufficient knowledge of consistent management strategies, and unreliable access to crop protection products. Thus, most farmers are unprepared and ill-equipped to respond effectively to nematode problem. Hence, the economic impact of plant-parasitic nematodes is huge in low-income farming families who rely mostly upon harvest for their livelihoods and food. Accurate diagnosis of plant-parasitic nematodes is a pre-requisite for a successful implementation of management options. The limited ability to accurately identify plant-parasitic nematodes is likely to result in the inappropriate use and misuse of control measures, such as crop rotation, genetic resistance, or synthetic chemicals. However, the morphology of RKN and PCN is extremely conserved. The majority of nematode morphological traits are not taxonomically informative and they are associated with feeding and reproduction mechanism. Nematode morphology is rather dynamic, convergent evolution is widespread in nematodes rendering the determination of homological nematode morphological traits extremely difficult and impossible (Ragsdale & Baldwin, 2010). Consequently, morphological identification of nematodes specifically from the genera *Meloidogyne* is a time consuming task, requiring a great amount of expertise and high quality reference material. Morphological identification of nematode from the genera *Globodera* can also be challenging. To illustrate this the two species of genera *Globodera* were classified as one species until the 1970s, when they were divided into two species (Stone, 1972). With a trend towards cheap and rapid molecular approaches were introduced to complement morphological diagnosis (Powers, 2004). Thus in this thesis DNA barcoding was used to identify nematodes from the genera *Meloidogyne* and *Globodera* (Chapter 2 and 3). This information is important as it forms the basal tier of the integrated nematode management. This involves nematode surveillance actions before and after planting. Effective surveillance and diagnosis allows the farmers to prepare and equip effectively in order to respond effectively to potential nematode problem as our study have shown that *M. enterolobii* was not known in Kenya before as well as the new record of PCN in Kenya.

2. Mitochondrial genes and nematode identification

In this study mitochondrial DNA barcoding proved to be very reliable for RKN and PCN (Chapter 2 and 3). The following barcode genes (i) cytochrome oxidase I (COI) and (ii) NADH dehydrogenase subunit 5 (NAD5) were used throughout this study. Mitochondrial DNA is often referred to as workhorse for evolutionary studies. Using mitochondrial DNA as barcode genes have the following advantages (i) ease of isolation and manipulation because the ratio of its copies to nuclear genome is high (ii) increased range of evolutionary rates due to protein genes + ribosomal genes + AT rich region (iii) clonal and hence genetics simple because they are maternally inherited (iv) rapid change accommodates examining closely related organisms due to high mutation rate (v) clonal and hence genetics simple because genetic recombination is rare. Specifically, the ability of mitochondrial genes such as COI to give a clear nematode species resolution was also demonstrated in other eukaryotes (Hebert et al., 2003). Recently, it was demonstrated that COI can be used as a universal barcode gene for several nematodes (Derycke et al., 2010; Janssen et al., 2017; Troccoli et al., 2016). More importantly mitochondrial NAD5 was successful used to identify root-knot nematodes belonging to the *M. incognita* complex (Janssen et al., 2016).

3. Global distribution of plant-parasitic nematodes

This research generated several mitochondrial haplotypes of RKN and PCN that revealed the geographical widespread of these nematodes. The mitochondrial haplotypes of RKN such as *M. incognita*, *M. javanica*, *M. arenaria*, *M. enterolobii*, and *M. hapla* were found to have a worldwide distribution (Chapter 2 and 3). Similarly, Janssen et al. (2016) found that RKN COI/NAD5 haplotypes were globally distributed. Within PCN a similar trend was observed. The global distribution of identical mitochondrial haplotypes favors the hypothesis that the global distribution was aided by human through agriculture practices and global trade networks, and most likely does not predate agriculture development (Castagnone-Sereno et al., 2013). We argued that if global distribution predates agriculture development then we would expect a much bigger variation in mitochondrial haplotypes from distant locations. For RKN reproducing by mitotic parthenogenesis this will be very true because different populations that have single nucleotide polymorphism would have remained. In sexual reproducing species, such as PCN, similar mitochondrial haplotypes indicates that the populations are not reproductively isolated. The distribution patterns of

PCN was previously reported to be mainly facilitated by anthropogenic activities including agriculture development (Banks et al., 2012; Blacket et al., 2018; Boucher et al., 2013; Plantard et al., 2008). Thus, globalization can impose major plant health challenges and plant-parasitic nematodes are emerging as the winners. Because of agriculture development activities such as international crop exchange, human aided dispersal has contributed to observed global distribution of many species of RKN and PCN. For example, PCN has become a concern in some parts of sub-Saharan Africa such as Kenya (Coyne et al., 2018; Mburu et al., 2018; Mwangi et al., 2015), threatening low-income farming families who depend upon the harvest for their livelihoods, nutrition, and food security. At continental level, mitochondrial haplotypes of highly damaging nematode species such as *M. enterolobii* are widespread (Chitambo et al., 2016; Coyne et al., 2018; Kolombia et al., 2016; Onkendi et al., 2014), including RKN species such as *M. paranaensis* (Terra et al., 2018), emphasizing the importance of human aided dispersal of plant-parasitic nematodes. On the other hand, typical tropical RKN are increasingly found in Europe and other cooler places (Bellé et al., 2016; Gerič Stare et al., 2017, 2018; Maleita et al., 2018; Wesemael et al., 2011). With the rapid globalization and the continued burden of imported cases of RKN and PCN to non-endemic countries, prioritizing nematode diagnosis, surveillance and control efforts is required. Many countries have adopted strict pest surveillance techniques for detecting quarantine nematodes. The aim of surveillance is to facilitate early detection of nematode incursions, so that their further spread is restricted and timely measures can be taken for their eradication. This approach has helped to reduce further spread of some nematode species in some parts of the world through careful trade control, but not in low-income countries (Nicol et al., 2011). Farmers in low-income countries lack awareness of new nematode pest and effective management strategies, leaving them ill-equipped to respond effectively to existing and new nematode pests.

4. Human aided nematode breaking down of biogeographical barriers as the main driver of multiple infections

Biological invasions by alien nematode species are one of the primary ways in which human activities are contributing towards human-induced environmental change. Our research indicates that alien nematode species richness is a consequence of a combination of anthropogenic factors and biotic acceptance of introduced nematodes into areas already

rich in native nematode species. We detected alien nematode species such as *M. hapla, G. rostochiensis*, and *G. pallida*, which are usually found in temperate climates, in a moderate tropical climate. This indicates that areas of high native nematode species richness are not resistant to colonization by alien nematode species. In this context alien nematode species are capable of establishing viable populations and subsequently spread in their new location. This is well-illustrated by the recent reports of alien PCN nematode species in many parts of Kenya (Coyne et al., 2018; Mwangi et al., 2015). In literature it is reported that alien species can adversely affect the native species to extinction (Clavero & García-Berthou, 2005), however our research indicates that alien and native nematode species can coexist. This is well supported by the global distribution of mitochondrial haplotypes of RKN and PCN, implying that multiple nematode infections are plausible (Chapter 2 and 3). Given the often inconspicuous nature of the plant-parasitic nematode symptoms on plants and lack of clear morphological differentiation among the species, nematode multiple infections are underestimated. However, with molecular tools available to study plant-parasitic nematodes (Seesao et al., 2017), we now know that multiple infection exists (Chitambo et al., 2018; Kolombia et al., 2017), and may alter the within-host parasitism. Changes in within-host infection dynamics under multiple infection (Mideo, 2009; de Roode et al., 2005), may have significant impact for between-host dynamics and spread of nematodes. To support this we reported that *M. hapla* facilitates the establishment and reproduction of *M. javanica* on a host plant *Parthenium hysterophorus* (Chitambo et al., 2018). Since our data demonstrates that introduced nematode species can establish in new environment, these established nematode populations can therefore act as the source of additional secondary introductions making the nematode invasion process a self-reinforcing process. Thus access to consistent and effective plant-parasitic nematode protection products is required. . Our research indicates that the mitochondrial haplotypes generated in this study are widespread underpinning the need for an effective nematode surveillance and control efforts in low-income countries.

5. Borderless plant-parasitic nematodes meet AIV

AIV including African nightshade (*S. scabrum* and *S. villosum*) and African spinach (*A. dubius* and *A. cruentus*) are neglected and underutilized crops, but have been a part of farming practices and nutrition in traditional societies in Africa. In low-income countries

nutritional imbalances are increasing, characterized by growing diet-related, non-communicable diseases and persistent undernutrition (NCD Risk Factor Collaboration, 2016; Stevens et al., 2012). In these countries small-scale farmers faces structural inequalities that results in marginalization and oppression that have contributed to the destruction of indigenous food systems and favors global agribusiness interests and commodity speculation. AIV have been downgraded to neglected and underutilized by modern agricultural systems that promote cultivation of a very limited number of crop species; in fact four crops - maize, soybean, rice, and wheat - account for ~56% of the protein and ~60% of the calories that humans consume directly from plants (Jacobsen et al., 2015; Lenne & Wood, 2011). AIV are indispensable in reducing food and nutrition insecurity in low-income farming families (Gahukar, 2014; Gido et al., 2017; Mayes et al., 2011), but have been relegated to the sidelines. Thus, there has been public awareness of these species through organizations such as the Global Plan of Action and Convention on Biological Diversity in 1992 (Virchow, 2003). A number of organizations, including Federal Ministry for Economic Cooperation and Development (BMZ), funded the projects "Horticultural Innovation and Learning for Improved Nutrition and Livelihood in East Africa" and "AIV-IPM Project" which focused on investigating, and promoting these crops as a strategy to improve livelihoods and nutrition of low-income countries. It's a timely quest. More recently new challenges have emerged in low-income farming systems further threatening the sustainability of food production in Africa. Reports of introduced pests and diseases attacking important food crops were reported in Africa. The fall armyworm (*Spodoptera frugiperda*) is rapidly spreading across Africa with an astonishing speed devouring on staple crops such as maize (Day et al., 2017). An outbreak of Maize Lethal Necrosis disease was reported on maize in several African countries (Mahuku et al., 2015). For example the economic impacts of alien species: Maize Lethal Necrosis, *Chilo partellus*, *Parthenium hysterophorus*, *Tuta absoluta* and *Liriomyza* spp. in six low-income countries under mixed maize is estimated at annual losses of US$0.9-1.1 billion; and future annual losses are expected to increase (Pratt et al., 2017). Highly damaging plant-parasitic nematodes such as PCN (*G. rostochiensis* and *G. pallida*) were recently reported in Kenya parasitizing potato (Mburu et al., 2018; Mwangi et al., 2015). PCN are considered quarantine pests of potatoes in many parts of the world and are subject of stringent regulations in most countries (EPPO/CABI, 1997). More worringly, polyphagous RKN including *M. enterolobii* continues to expand into new regions rapidly (Chitambo et al., 2016; Coyne et al., 2018; Onkendi et al., 2014), threatening to destroy crop yields

completely. Thus, farmers in low-income countries grapple with many challenges ranging from environmental change, migration, pest and disease outbreaks. Therefore, it is important for the smallholder farmers to adopt cropping systems that offers adequate nutrition and health, reduce dependence on external inputs, such as chemical pesticides and fertilizers, and environmental stress-resilience and resistance to emerging pests and diseases. African nightshade and African spinach are nutritious crops offering many health benefits and they are adapted to the local conditions. However, their host status to plant-parasitic nematodes was unknown. Thus, this research was carried out within "AIV-IPM Project" after the realization that plant-parasitic nematode problem in low-income farming systems is intractable and is a major constraint to attaining food and nutrition security. There was a lack of information on identity of RKN and PCN species on these AIV, their host status to RKN and PCN, and the impact of these crops on RKN and PCN dynamics. Traditionally these interlinked components are studied in a siloed context. This undermines the importance of nematode diagnosis and its link to the implementation of an effective management strategy. Our research has shown that accurate diagnosis of RKN and PCN will help provide proper implementation of an effective management decision. We identified *A. dubius* and *S. scabrum* with the ability to suppress RKN species identified in this study, whereas *S. scabrum* and *S. villosum* suppress the identified PCN species (Chapter 3). *Solanum scabrum* suppress both RKN and PCN identified in this study (Figure 6). Therefore, AIV that showed RKN and PCN suppression can be used as ideal cover crops for management of both of these groups of nematodes or rotational crops, or relay crops etc. Integrating these crops in low-income cropping system as cover crops, rotational crops or relay crops has several advantages including nematode control and dietary diversification. Elsewhere, cover crops are used in various production systems to provide many benefits such as pest and disease management, addition of organic matter to soil, and increased productivity of cash crops. For example the use of cover crops in the Brassicaceae family such as oilseed radish, white mustard, and winter rapeseed decreased sugar beet cyst nematode population densities (Lelivelt and Hoogendoorn 1993; Wen et al. 2017). Moreover, it is now well established that intercropping increases parasitism of pests (Khan et al., 1997; Turlings & Erb, 2018). AIV with the ability to suppress nematodes allows the smallholder farmers to manage plant-parasitic nematodes in a productive way while meeting the nutrition requirements in low-income countries where large inequalities in food availability exist. The cultivation AIV is a way of promoting locally adapted crop species that will lead to sustainable intensification (figure 6).

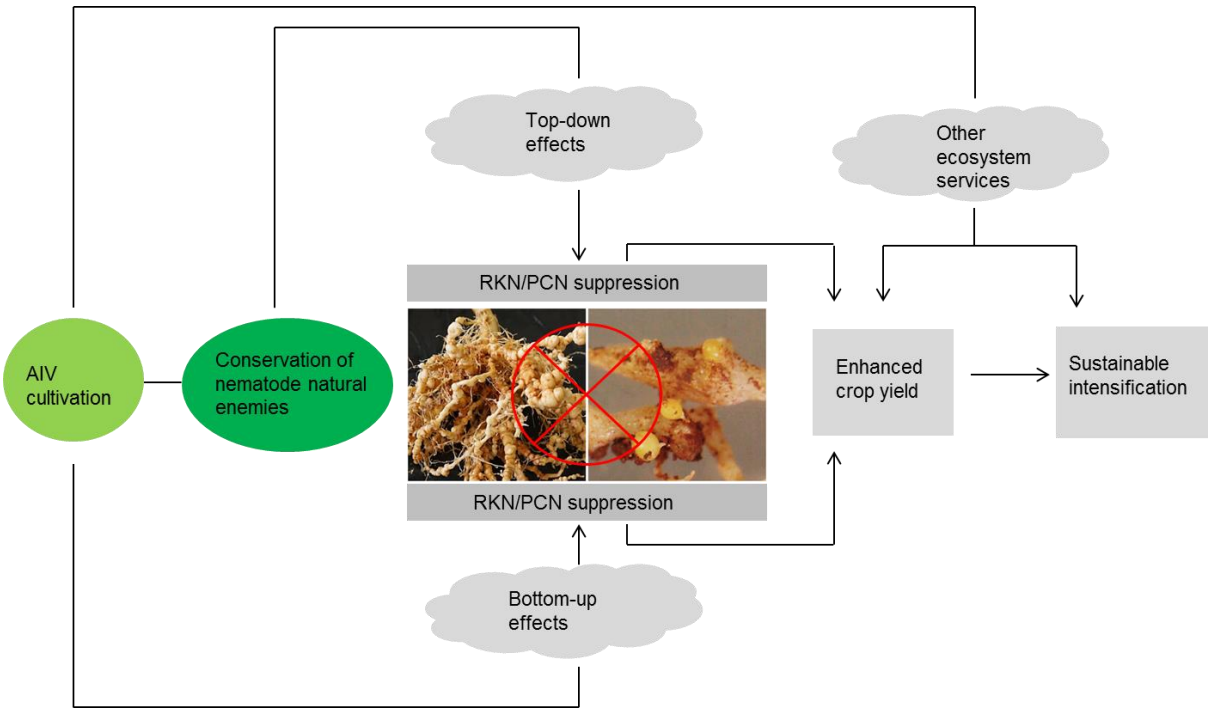

Figure 6. The impact of African indigenous vegetables (AIV: African nightshade and African spinach) cultivation under low-income farming system. AIV such as *Amaranthus dubius*, *A. cruentus*, and *Solanum scabrum* can suppresses root-knot nematodes (RKN) whereas *S. scabrum* and *S. villosum* can suppress potato cyst nematodes (PCN). The mechanistic of nematode suppression can be bottom-up through the direct interference with nematode lifecycle (this study) or top-down through conservation of nematode natural enemies (not investigated). The outcomes are RKN and/or PCN suppression and enhanced yield that can lead to sustainable intensification. This sustainable intensification is strongly linked to nutrition performance by the cultivation of nutrient dense AIV. The cultivation of AIV is a way of strengthening agro-biodiversity in order to enhance the resilience of cropping systems thereby promoting balanced and healthy diet to local consumers.

6. AIV root tissue necrosis and disintegration orchestrate the containment, starving, and expulsion of parasitic nematodes

The ability of RKN and PCN to survive in the host plant is the result of adaptation or co-evolution between host plant and the nematode (Hussey & Grundler, 1998), thus despite their large size (compared to other pathogens) they are able to survive inside root tissue. It is therefore necessary for these nematodes to locate a suitable host plant and establish an immunoregulatory environment which ensures their survival and reproduction (Goverse & Smant, 2014). Otherwise, the host plant would be able to generate an effective anti-nematode response that will result in containment, starving, and expulsion of nematodes.

Root tissue necrosis and disintegration observed on resistant AIV species is an indication of plant cell death in response to nematode infection (Chapter 4). The potential beneficial outcomes of root necrosis and disintegration could be: (1) the removal of an intracellular environment for nematodes, (2) direct nematicidal activity of released components and (3) the amplification and propagation of anti-nematode response. The removal of intracellular niche for the nematode mean that a functional feeding site cannot be established hence the nematode cannot obtain the necessary nutrients it needs for development and survival. The removal of intracellular environment could be initiated by the presence of intracellular receptors which contain nucleotide-binding site (NBS) and a C-terminal leucine rich repeat (LRR) region. There are a number of NB-LRR disease resistance (R) genes that are known to protect plants against a myriad of pathogens including nematodes (Vos et al., 1998; Williamson & Hussey, 1996; Williamson & Kumar, 2006). Tomato genes *Hero A* and *Mi-1* confer broad spectrum against several RKN species (Milligan et al., 1998; Vos et al., 1998) including several pathotypes of PCN species (Ernst et al., 2002). In contrast, *Gpa* and *Gro- 4* isolated from potato (Paal et al., 2004), confers resistance to limited range of pathotypes of a single PCN species. In our study one species of African nightshade, *S. scabrum* showed full resistance against RKN and PCN species. Recent mapping studies have indicated that genes conferring resistance to various pathogens, including RKN and PCN are often organized in clusters. For example, genes conferring resistance to RKN species (*N* and the *Me* genes), bacterium *Xanthomonas campestris* pv. *vesicatoria* and potyviruses PVY (0) and PVY and *Phytophthora capsici* (two QTLs) have been mapped to the same region of the pepper P9 chromosome (Djian-Caporalino et al., 2007; Tai et al., 1999; Thabuis et al., 2003). Thus in our study resistant AIV may contain several R genes which are involved plant innate immune response that serve as surveillance proteins to protect them from several nematode species and other pathogens (chapter 4). Several studies have demonstrated that R genes initiate an early hypersensitive response like reaction that can prevent the successful formation of a functional nematode feeding site (Dropkin et al., 1969; Goverse & Smant, 2014; Kyndt et al., 2014; Postma et al., 2012; Williamson & Kumar, 2006). The possibility of anti-nematode activity of intracellular components released during root necrosis is possible. The AIV used in this study are known to have high concentrations of secondary plant metabolites (Neugart et al., 2017; Ronoh et al., 2018), and it known that such compounds e.g. pyrrolizidine alkaloids and steroid glycoalkaloides possess antimicrobial, insecticidal, and nematicidal properties (Chowański et al., 2016; Jared et al., 2016; Thoden et al., 2007, 2009; Tingey, 1984). Root tissue

necrosis inevitably release intracellular damage-associated molecular patterns (DAMPs) which can amplify and propagate anti-nematode response. DAMPs activate innate immune cell receptors acting similarly to pathogen-associated molecular patterns (PAMPs) when exposed to the extracellular environment. Damage-associated responses of the host is known to contribute to defence against nematodes (Holbein et al., 2016; Shah et al., 2017). Hence, a vicious loop might be created whereby root necrosis generates DAMPs and DAMPs create pro-necrosis state. Our study has shown that resistant AIV have the ability to induce localized root tissue necrosis and disintegration thereby thwarting nematode infection.

7. General conclusion

The transport of plant-parasitic nematode species beyond their native ranges by human activities is violating biogeographical boundaries and resulting in the global reorganization of plant-parasitic nematodes. The resulting plant-parasitic nematode invasion have plagued farmers and is a major threat to crop yields of low-income families who depend upon the harvest for their livelihoods and food. The problem is aggravated by the reliance on a very limited number of crop species that support the reproduction of theses nematodes. Thus, RKN and PCN are increasingly becoming a global plant health concern due to their rapid geographical spread and increasing yield loss on most cultivated crops. Our research demonstrated that alien PCN and RKN are capable of establishing viable populations and subsequently spread in their new location. We established that areas of high native nematode species richness are not resistant to colonization by alien nematode species and highlight the possible outcomes to global environments from introduced nematode species. The global distribution of mitochondrial haplotypes show that patterns of nematode invasion are governed to a large extent by agricultural activities connecting source areas for non-native species and the dispersal of those species through human activities e.g. the recent introduction of PCN and first report of RKN; *M. enterolobii* in Kenya. In the globalized economy, it is very difficult to avoid the transportation of quarantine nematodes. Thus the current strategy to minimize the transportation of quarantine nematodes has not been effective enough to keep up with the mobile society. For instance, it is highly probable that PCN species detected in Kenya managed to invade one place, survive, and then were transported to other places within the country. Similarly RKN species such as *M. hapla* and

M. enterolobii could have been introduced via the same pathway. This indicates that a bridgehead effect is a major driver for parasitic nematode transportation to new geographic locations e.g. through trading and exchanging of planting materials such as potato seeds. This underpins the need for developing a prognostic framework to improve risk assessment, diagnosis, surveillance, and biosecurity for quarantine nematodes invasions in low-income countries. The current study demonstrated that nematode diagnosis of RKN and PCN will help provide proper implementation of an effective management decision. Accurate nematode identification, maintaining surveillance, and establishing rigorous monitoring will allow appropriate quarantine actions to be taken. For example, the detection of damaging nematode species such as *M. enterolobii* and *G. rostochiensis* in Kenya will require the implementation of timely scientific, technical, and policy responses. Thereafter, actions leading to effective management to prevent further spread and limit the impacts of these nematodes are required. We identified *A. dubius* and *S. scabrum* with the ability to suppress RKN species identified in this study, whereas *S. scabrum* and *S. villosum* suppress the identified PCN species. *Solanum scabrum* suppress both RKN and PCN identified in this study. According to our results these crop species can be used to manage RKN and PCN. We recommend that growers intending to simultaneously control PCN and RKN should use *S. scabrum*. Although *S. villosum* was able to suppress PCN it is highly susceptible to RKN and should not be used where RKN are detected. This finding is a major relief to the resource constrained smallholder farmers who are overburdened by plant-parasitic nematodes, pests and diseases as well as nutritional challenges. The reintroduction of AIV species into the existing cropping systems is a way of promoting agro-biodiversity to improve resilience to plant-parasitic nematodes, pests and diseases as well as dietary diversification in low-income farming systems. AIV such as African spinach and African nightshade is a representation of the many untapped local varieties that can be exploited as resource-efficient, resilient food value chains that can provide safe, affordable, and nutritious food. Therefore, this approach can be used as a simple management strategy for RKN and PCN in an environmentally friendly, effective, and productive way.

8. Outlook

- Improved diagnosis, monitoring, and surveillance is urgently needed as it will reveal greater levels of parasitic nematode invasion especially that the risks of invasion are shifting rapidly owing to growing transportation networks, landscape

transformation, new agriculture technology, climate change, and geopolitical events. This will provide a strong incentive to study and predict future nematode distributions as well as their impact on crop production. This requires development of interdisciplinary coordination of expertise and response at local, regional, and global level.

- Although plant-parasitic nematodes co-infections have been established, the implication of this co-existence on nematode establishment, spread, and host plant damage in the new environment remains largely unknown. It is important to understand the life history traits of RKN and PCN and how they interact with each other and the impact on crop yields.

- Genomics-assisted breeding can be used to introgress loci of nematode resistance into other related crop species that are susceptible to parasitic nematodes. Genomics-assisted breeding techniques can also be used to improve the traits of AIV such as leaf yield and grain yield.

- Multi-environmental participatory testing and end-user evaluation of the resistant AIV in order to address the persistent problem of plant-parasitic nematodes while simultaneously tackling the problem of food and nutritional security and soil and water conservation under low-income farming systems.

- To preserve AIV biodiversity and enhance their utilization, participatory approach in the form of dialogue among farmers, researchers and final consumers is needed. This will help to address the current challenges in low-income farming systems that range from biotic stress (e.g. plant-parasitic nematodes), abiotic stress, and malnutrition as stated above. However this scenario requires an accurate identification and knowledge of the involved AIV species. AIV identified by their common name (e.g., African nightshade or African spinach or amaranth) could comprise of a number of species, hybrids, and varieties. Efficient and effective analytical tools such as DNA-based methods can be used to accomplish this mission. This will lead to commercialization and support AIV traceability along the entire supply chain resulting in new market opportunities for novel food products at local and global scale. DNA based identification of AIV will also support the fair and equitable sharing of benefits of genetic resources arising from their exploitation.

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