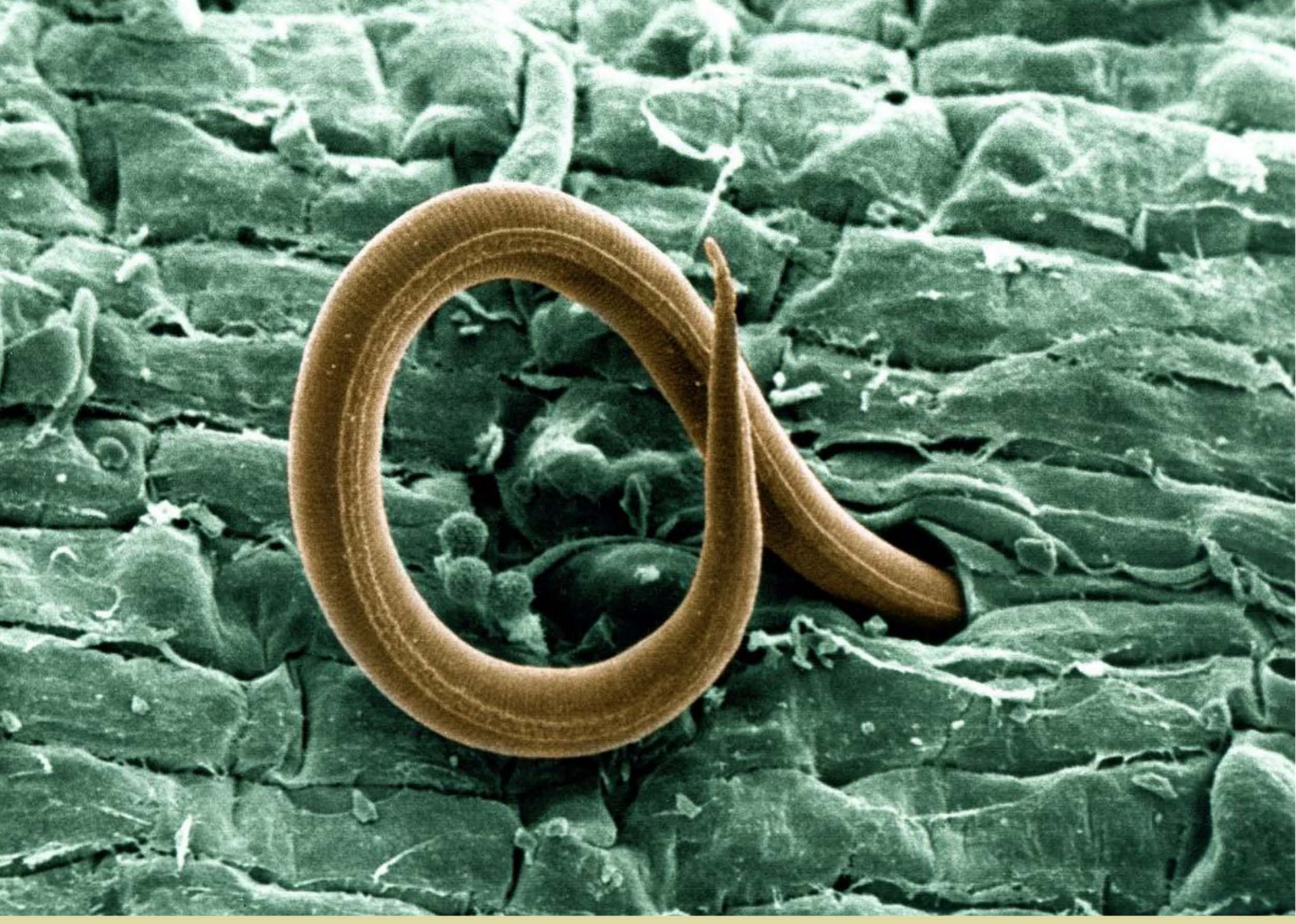


Filipe Maço Santos

Environmentally sustainable solutions for Root-Knot Nematodes control

Dissertação de Mestrado em Biodiversidade e Biotecnologia Vegetal,
orientada por Professora Doutora Cristina Isabel Cabral Galhano e por Professora Doutora Paula Cristina de Oliveira Castro,
apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Agosto 2017

· U C ·

Universidade de Coimbra

Environmentally sustainable solutions for Root-Knot Nematodes control

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob a orientação científica da Professora Doutora Cristina Isabel Cabral Galhano (Escola Superior Agrária do Instituto Politécnico de Coimbra) e da Professora Doutora Paula Cristina de Oliveira Castro (Universidade de Coimbra).

Filipe Maço Santos

Departamento de Ciências da Vida

Universidade de Coimbra

2017

Título: Environmentally sustainable solutions for Root-Knot Nematodes control

Ano: 2017

Autor: Filipe Maço Santos

Orientação Científica: Professora Doutora Cristina Isabel Cabral Galhano e Professora Doutora Paula Cristina de Oliveira Castro

Domínio Científico: Biodiversidade e Biotecnologia Vegetal

Instituição: Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Capa: Nemátode jovem no segundo estádio infecioso (*Meloidogyne incognita*) a iniciar a invasão de uma raíz de um tomateiro no ano de 2013, fotografado por William Wergin e Richard Sayre, colorido por Stephen Ausmus, In: https://upload.wikimedia.org/wikipedia/commons/thumb/e/e6/A_juvenile_root-knot_nematode_%28*Meloidogyne_incognita*%29_penetrates_a_tomato_root_-_USDA-ARS.jpg/1200px-A_juvenile_root-knot_nematode_%28*Meloidogyne_incognita*%29_penetrates_a_tomato_root_-_USDA-ARS.jpg

Agradecimentos

Na realização da presente dissertação de mestrado, contei com o apoio de múltiplas pessoas e instituições às quais estou profundamento grato.

Ao Coordenador do Mestrado em Biodiversidade e Biotecnologia Vegetal, ao Professor António Manuel Santos Carriço Portugal e ao antigo Coordenador do Mestrado em Biodiversidade e Biotecnologia Vegetal, ao Professor Jorge Manuel Pataca Leal Canhoto, agradeço pela oportunidade que me deram para frequentar este Mestrado que muito me ajudou no enriquecimento da minha formação académica e científica.

À Professora Doutora Cristina Isabel Cabral Galhano, pela sua orientação, pelas suas opiniões e críticas, pela colaboração no solucionar de dúvidas e problemas que foram surgindo ao longo da realização deste trabalho e pela paciência, atenção e força que prestou em momentos menos fáceis. Aqui lhe exprimo a minha gratidão.

À Professora Doutora Paula Cristina de Oliveira Castro, o meu sincero agradecimento pela orientação, por todos os estímulos e desafios para a realização deste Projeto, pela sincera amizade e pela total disponibilidade que sempre revelou para comigo. O seu apoio foi determinante na elaboração desta Tese.

Ao Professor Doutor Leonel Carlos dos Reis Tomás Pereira pela partilha do saber na elaboração desta dissertação.

Às Professoras Doutoras Elisabete Maria Duarte Canas Marchante e Hélia Sofia Duarte Canas Marchante pela ajuda na obtenção das sementes necessárias para a realização deste Projeto.

À Escola Superior Agrária do Instituto Politécnico de Coimbra e ao Centro de Ecologia Funcional da Universidade de Coimbra, pela disponibilidade das instalações e material que possibilitou a realização deste trabalho. Agradeço também aos funcionários pela simpatia e por me ajudarem sempre quando precisava de alguma coisa.

Aos meus amigos, Bruno, Cátia, Daniela e também minha conselheira Joana: obrigado por me terem recebido quase todos os dias em vossa casa, por todos os momentos de companheirismo, por me ajudarem com todas as dúvidas que tive, por me apoiarem nos meus momentos mais baixos e por terem conseguido fazer com que este ano tivesse mais de divertido do que de complicado. Ao Gonçalo e ao Duarte, obrigado

pela companhia, pela ajuda na realização de várias tarefas e por toda a motivação que me deram para concluir este projeto.

À Bia, tu mereces uma medalha de ouro, por me aturares todos os dias, por toda a ajuda nas traduções, por me teres apoiado nos momentos menos bons, por me teres incentivado a dar o meu melhor e a nunca desistir. Obrigado por acreditares sempre nas minhas capacidades e por todo o amor e dedicação.

Aos meus pais, por acreditarem sempre em mim e por me incentivarem sempre a fazer mais e melhor perante os desafios. Obrigado pelo amor incondicional e pela forma como ao longo de todos estes anos me ajudaram, sem vocês nada disto teria sido possível. Um obrigado nunca será suficiente, a vocês dedico este trabalho!

Obrigado por tudo!

Table of contents

List of Figures

List of Tables

Abstract

The Plant-Parasitic Nematodes (PPNs) are responsible for considerable damage on worldwide crops, which is evaluated in a value of 157 billion dollars. Among the PPNs, *Meloidogyne* spp. (Göldi, 1892), commonly known as Root-Knot Nematode (RKN), is one of the most important nematode disease, being responsible for devastating crops with high economic importance, causing also a huge impact on international trade, social and economic development. Pesticides, chemical synthetic products, are used to control these plant enemies. However, these toxic compounds are responsible for causing major environmental and human health problems. Therefore, it is essential to find ecofriendly alternative resources for a sustainable agriculture, which was the main aim of this work. This study evaluated, *in vitro*, the nematicidal activity of eight higher plants (*Acacia dealbata*, *Acacia longifolia* and *Vicia faba*) and algae (*Gracilaria gracilis*, *Sargassum muticum* and *Ulva rigida*) extracts on J2 of *Meloidogyne* sp. The activity of each extract was observed at 24, 48, 96 and 168 hours. In each observation time, four replicates of each treatment and of each control, were analysed for immobile and dead nematodes and were subsequently discarded. The extract of *U. rigida* (0.1 g mL^{-1}) was the most effective (98.8% mortality) against *Meloidogyne* sp. J2 after 48 hours of exposure, obtaining 100% mortality at 96 and 168 hours. The second most effective was the extract of oxidized pod of *V. faba* (0.1 g mL^{-1}) with nearly 100% at 96 and 168 hours of exposure. The fresh pod of *V. faba* extract (0.1 g mL^{-1}) was the third most effective, followed by *U. rigida* (0.05 g mL^{-1}) and *A. dealbata* (0.2 g mL^{-1}), respectively. *S. muticum* (0.1 g mL^{-1}), *G. gracilis* (0.1 g mL^{-1}) and *A. longifolia* (0.2 g mL^{-1}) were the least effective. Based on the obtained results, it should be highlighted that *U. rigida*, oxidized and fresh pod of *V. faba* can be explored as potential sources for new ecofriendly biopesticides. Therefore, the nematicidal effects of these species have brought new insight on the development of a promising natural, cheap and environmentally-friendly alternative to chemical synthetic pesticides for RKN management, progressing towards a sustainable development.

Keywords: algae extracts, biopesticides, *Meloidogyne* sp., plant extracts, sustainable development.

Resumo

Os nemátodes parasitas de plantas (NPPs) são responsáveis pela destruição de culturas pelo mundo inteiro, o qual está avaliado num valor de 157 mil milhões de dólares. Neste grupo incluem-se os nemátodes-das-galhas-radiculares (NGR), *Meloidogyne* spp. (Göldi, 1892), os quais são responsáveis pela destruição de culturas com elevado valor económico, causando um enorme impacto no comércio internacional e no desenvolvimento social e económico, sendo considerados uma das principais doenças dos NPPs. Pesticidas, produtos químicos sintetizados, são utilizados para controlar esta doença. No entanto, estes compostos tóxicos são responsáveis por causar grandes problemas a nível ambiental e a nível da saúde humana. Deste modo, é essencial encontrar recursos alternativos e ecossustentáveis para uma agricultura mais amiga do ambiente, sendo este o principal objetivo deste trabalho. Neste estudo foram avaliadas, *in vitro*, a atividade nematicida de oito extratos de plantas superiores (*Acacia dealbata*, *Acacia longifolia* and *Vicia faba*) e algas (*Gracilaria gracilis*, *Sargassum muticum* and *Ulva rigida*), em J2 de *Meloidogyne* sp. A atividade foi observada às 24, 48, 96 e 168 horas. Em cada tempo de observação foram analisadas quatro repetições de cada tratamento e de cada controlo quanto à imobilidade e mortalidade dos nemátodes, tendo sido estas posteriormente descartadas. O extrato de *U. rigida* (0.1 g mL^{-1}) obteve o melhor resultado (98.8% de mortalidade) contra J2 de *Meloidogyne* sp. após 48 horas de incubação, obtendo 100% de mortalidade às 96 e 168 horas. O segundo mais eficaz foi o extrato de vagem oxidada de *V. faba* (0.1 g mL^{-1}) obtendo quase 100% de mortalidade de J2 após 96 e 168 horas de exposição. A vagem fresca de *V. faba* (0.1 g mL^{-1}) foi o terceiro extrato mais eficaz, seguido pela *U. rigida* (0.05 g mL^{-1}) e *A. dealbata* (0.2 g mL^{-1}), respetivamente. *S. muticum* (0.1 g mL^{-1}), *G. gracilis* (0.1 g mL^{-1}) e *A. longifolia* (0.2 g mL^{-1}) foram os extratos menos eficazes. Os efeitos nematicidas da *U. rigida*, da vagem oxidada e fresca de *V. faba* nestes nemátodes mostraram ter capacidade para o desenvolvimento de um novo biopesticida. Portanto, o efeito nematicida destas espécies trouxe uma potencial alternativa natural, económica e ecossustentável, aos pesticidas sintéticos químicos para o controlo dos NGR, progredindo ao encontro de um desenvolvimento sustentável.

Palavras-chave: biopesticidas, desenvolvimento sustentável, extratos de algas, extratos de plantas, *Meloidogyne* sp.

1. Introduction

Agriculture will face multiple challenges in this century. It can be highlighted the need to increase food production using the same crop fields to adopt more efficient and sustainable production methods and to adapt to climate change (Alexandratos & Bruinsma, 2012; FAO, 2017c).

In 2003 the world's population was 6.3 billion. Nowadays, it is estimated that by 2030 the earth will be inhabited by 8.5 billion people (UN, 2015). Such an increase on the demand of food will require an additional agricultural production which should be based on crop productivity improvement, and not in the increase of arable surface taken from rain and temperate forest. This crop productivity improvement can be obtained through a suitable plant enemies' control (diseases, pests and weeds), which are responsible for reducing crop production by an estimated average of 35% (Popp *et al.*, 2013). Pests and diseases affect food production during crop growth, post-harvest and storage (Kulkarni *et al.*, 2009). Thus, elimination of these plant enemies will help reduce world food crisis, thereby improving animal and human health (Dimetry, 2014). Currently, the plant enemies' control is usually achieved by using synthetic chemical pesticides (Cook, 2000; Agrios, 2005). On the other hand, the use of these pesticides (DDT and derivatives, for instance) has led to serious environmental and health problems (Cook, 2000; Dimetry, 2014). This concern was already evident in the Stockholm Convention that took place in 2001, which aimed to eliminate or restrict the production and use of organic pollutants (POPs) such as aldrin, chlordane, DDT, dieldrin, dioxins, endrin, mirex and toxaphene (UNEP, 2010). Over time, reports about the negative effects of synthetic pesticides' use have been growing, leading to an increasing interest and demand for natural-based biopesticides as an environmentally sustainable approach to pest control (Dubey *et al.*, 2010; Lehr, 2014).

1.1. Tomato

Solanum lycopersicum, commonly known as tomato, belongs to the Solanaceae family, which also includes other well-known species such as tobacco, pepper and potato. The *Solanum* genus has its origin in the South America and *S. lycopersicum* is one of the most consumed horticultural crop worldwide due to its high nutritive value and

diversified use (Costa & Heuvelink, 2005; Ayandiji & Omidiji, 2011; Raiola *et al.*, 2014). In fact, tomato is a good source of vitamins A, B and C, mineral salts such as folic acid, potassium and calcium, essential amino acids, iron, dietary fibers, phosphorus, sugars and it is a low caloric fruit (Ayandiji & Omidiji, 2011). There are several beneficial effects on human health associated with tomato. It decreases the risk of chronic diseases, such as cardiovascular disease and cancer, increases skin resistance against harmful ultraviolet radiation and improves bone health, among others (Agarwal & Rao, 2000; Rao & Rao, 2007; Raiola *et al.*, 2014). As tomato is a perennial plant and has a short production cycle, farmers obtain a high yield. Therefore, the production of tomatoes is very economically attractive. Furthermore, as tomato grows easily, it is one of the most studied crops, and it is normally used as a model plant or used to explore its characteristics (Schwarz *et al.*, 2014).

Tomato production has been increasing over the years and, in 2014, the world production was about 170 million tons (FAO, 2017a). Portugal was the third main producer in EU-28 in 2014, with about 1.4 million tons. Nowadays, one of the most important sectors of agriculture in Portugal is the tomato production (FAO, 2017a).

Tomato yield decrease can be due either to abiotic stress, such as salinity and drought, or biotic stress caused by pests, such as insects (leaf-mining larvae, whiteflies and caterpillars), and diseases caused by bacteria (bacterial canker, bacterial spot, bacterial wilt), fungi (fusarium wilt, early blight, southern blight), viruses (tobacco mosaic virus, cucumber mosaic virus and tomato spotted wilt virus) and nematodes (*Belonolaimus longicaudatus*, root-knot nematode) (Abrantes *et al.*, 2007; Moshe *et al.*, 2012; Smith, 2016).

1.2. Root-Knot Nematodes

The Phylum Nematoda includes a large number of invertebrate animals capable of inhabiting a wide range of environments. They can be free living or obligate plant/animal parasites. The Plant-Parasitic Nematodes (PPNs) are responsible for considerable damage on worldwide crops. Based on their feeding mechanisms, PPNs can be classified into three broad groups: the migratory ectoparasites (e.g., *Xiphinema* spp., *Trichodorus* spp. and *Belonolaimus* spp.), the migratory endoparasites (e.g., *Pratylenchus* spp. and *Radopholus* spp.) and the sedentary endoparasites (*Globodera* spp., *Heterodera* spp. and *Meloidogyne* spp.) (Sijmons *et al.*, 1994; Li *et al.*, 2015).

Damage due to PPNs is evaluated in 157 billion dollars, in the world (Li *et al.*, 2015). Among the PPNs, the *Meloidogyne* spp. (Göldi, 1892) genus, commonly known as Root-Knot Nematode (RKN), is one of the most important nematode disease for crops. It is estimated that every year they cause worldwide crop losses of about 5% and they are capable of parasite over 3,000 plants' species, including fruits, ornamental and vegetable crops, such as tomato, tobacco, rice, sunflower, bean, chickpea, grape, peach, melon, corn, pineapple, carrot, lettuce, papaya and pumpkin (Eisenback & Triantaphyllou, 1991; Hussey & Janssen, 2002; Abad *et al.*, 2003). RKNs are also widely distributed in Portugal. Some of the reported species affecting economically important crops are *M. chitwoodi*, *M. hapla*, *M. hispanica*, *M. javanica*, *M. arenaria*, *M. incognita* and *M. lusitanica* (Abrantes & Santos, 1991; Abrantes *et al.*, 2008; Conceição *et al.*, 2009).

The RKNs are obligate plant parasites and can affect the production and quality of a number of plants with a high economic importance, as mentioned previously. Since RKN need to penetrate roots to feed and reproduce, they affect the nutrient and water uptake and the upward translocation by the root system, decrease the rate of photosynthesis on the leaves and prevent the photosynthetic products passing through phloem to roots. Therefore, plant health is compromised and the plant itself becomes more susceptible to the attack by other pathogens such as pathogenic fungi, virus, and/or bacteria (Hussey, 1985; Carneiro *et al.*, 1999).

The above-ground symptoms exhibited by *Meloidogyne* spp. infected plants may consist in lack of vigor and various degrees of stunting because of the prolonged root stress caused by nematodes. Plants also tend to wilt more readily under water stress and high temperatures than non-infested plants (Hussey, 1985).

RKN life cycle (Fig. 1) includes four juvenile stages and four moults and it takes between 22 and 30 days, depending on host susceptibility, soil temperature, among other factors. The infective stage, the second-juvenile stage (J2), hatches from the eggs and moves through the soil looking for roots of host plants. This stage is extremely important in these nematodes life cycle and in their relation with the host. Any factor that influences this process can have a significant effect in their survival, in the time of duration of the generation and in the capacity of host infection (Moens *et al.*, 2009; Conceição *et al.*, 2012). After root penetration, at the elongation zone, J2 migrate and start to feed on protophloem and protoxylem cells (Karssen & Moens, 2006). These plant cells differentiate into giant cells, providing a permanent supply of nutrients to the nematode,

so the infective juveniles are responsible to induce and develop a permanent feeding site (Vanholme *et al.*, 2004). The infected plant produces a gall around giant cells in response to the presence of the nematode. At this stage, J2 becomes sedentary and, after three successive moults, nematodes reach the third and the fourth-juvenile stages, and finally the adult stage. The ratio females/males depends on several conditions, such as food sources, temperature and water availability. If these conditions are favorable, most of the adults become spherical females, if they are not the number of males increases. The males migrate to the soil but the females keep sedentary in roots and produce a protective gelatinous matrix where eggs are deposited, thus forming up the egg masses. The embryogenic development happens inside the egg resulting in the first-juvenile stage. Then, first-juvenile stage moult into J2, still within the egg (Abad *et al.*, 2003; Karssen & Moens, 2006; Moens *et al.*, 2009).

Meloidogyne spp. reproduction depends on the species; nonetheless, most of them reproduce by obligatory mitotic parthenogenesis. Some reproduce by amphimixis, where the male is necessary to the fertilization, and some reproduce by facultative meiotic parthenogenesis (Chitwood & Perry, 2009).

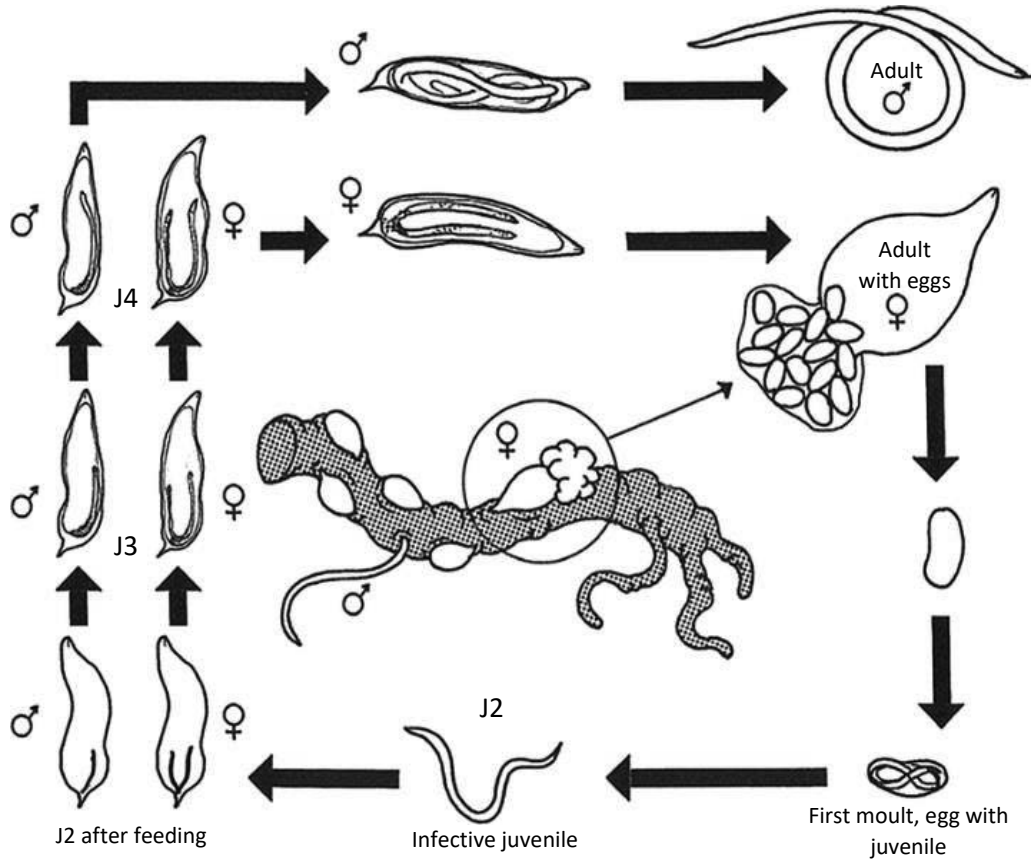


Figure 1 The life cycle of the root-knot nematodes, *Meloidogyne* spp. J2: second-stage juveniles; J3: third-stage juveniles; J4: fourth-stage juveniles. (Adapted from Karssen & Moens, 2006).

1.3. Management and Control of RKN

Firstly, it is essential to understand the difference between the terms management and control. **Management** strategies aim at achieving the effective suppression of the nematode population density by using a combination of control practices. **Control** practices are specific one-time actions available to growers that will reduce nematode populations below the economic damage level (Nyczepir & Thomas, 2009).

In this moment, the most commonly used control practices in agriculture are included in these categories: chemical control, cultural control, host plant resistance and biopesticides (Fig. 2) (Nyczepir & Thomas, 2009).

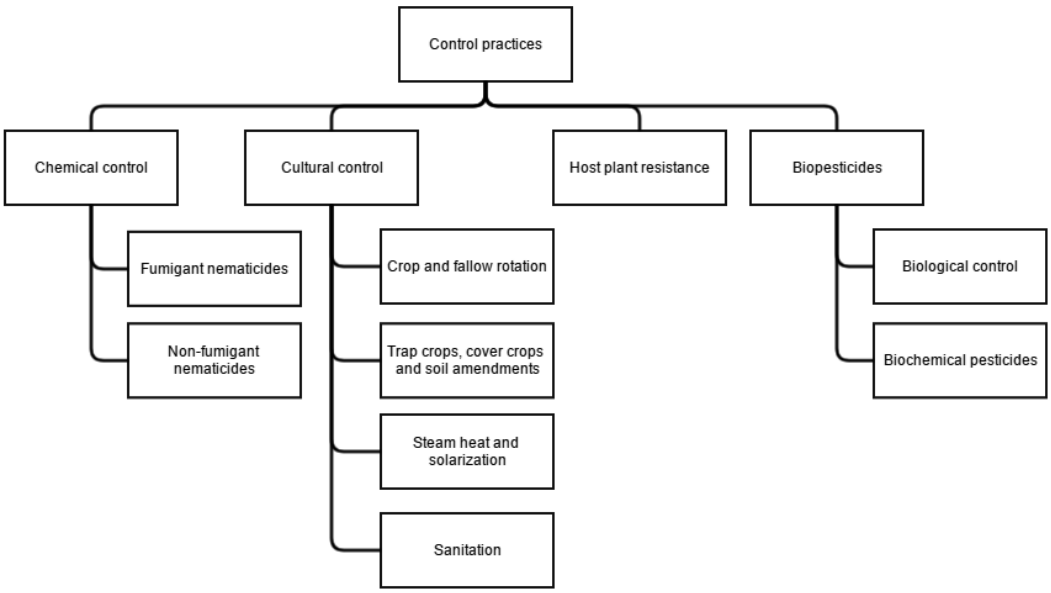


Figure 2 Schematic representation of the different control practices.

Chemical control and host plant resistance are the most used means to control *Meloidogyne* spp. in many annual economically important crops and in several perennial crops in North America (Bridge & Starr, 2007; Nyczepir & Thomas, 2009).

Nematicides used in chemical control are usually categorized according to their application methods in three categories: fumigant, non-fumigant, and those that are derived from naturally occurring biotic sources (Haydock *et al.*, 2006). At the moment, in this chapter, special attention will be given to fumigant and non-fumigant nematicides, which are considered synthetic controls, and not to those derived from naturally occurring biotic sources, which are considered biopesticides (Haydock *et al.*, 2006).

Fumigant nematicides are usually formulated as liquids and they volatilize to a gaseous phase upon entering soil. On the other hand, non-fumigant nematicides, which can be formulated either as granular or liquid materials, are not volatile and they need to be dispersed in the soil through water via irrigation and/or rainfall to be active against nematodes. Non-fumigants nematicides are considered nematostatic, as their effects are reversible and nematodes are not killed. Therefore, it is important that the contact between the nematodes and nematicide lasts for approximately 4-8 weeks, to avoid nematode infection, causing minimal impact on plant growth. (Wright, 1981; Haydock *et al.*, 2006). It is also important to add that non-fumigant nematicides are not so effective in nematode populations suppression as fumigant nematicides because they do not have broad-spectrum activity (Sikora *et al.*, 2005).

For many years, the use of nematicides to control *Meloidogyne* spp. was recommended in pre and/or post-plant phases. However, many of the nematicides have been banned from the market due to environmental and human health awareness issues (Batchelor, 2002). One of them was the 1,2-dibromo-3-choloropropane (DBCP), whose registration as a soil fumigant for all crops was suspended by the US Environmental Protection Agency (EPA) in 1981 (Nyczepir & Thomas, 2009). After this, other nematicides were prohibited, like methyl bromide that was forbidden in USA and Western Europe after January 2005, which causes ozone depletion among other undesirable effects (Nyczepir & Thomas, 2009). Another famous case is the highly toxic aldicarb, which is used to control insects and nematodes. This pesticide was responsible for thousands of poisoning, people and animal's deaths, contamination of soil, foods, rivers and groundwater. For this reasons the European Union banished this product (Zaki *et al.*, 1982; Ruiz-Suárez *et al.*, 2015).

The European Union Authorizations' Directive 91/414/EEC reduced the approved nematicides for use in Europe, as new and existing pesticides active substances must undergo a certification process (Haydock *et al.*, 2006). Hence, the predominance of nematicides has decreased in many regions of the world. Alternatives to chemical control are being increasingly investigated and implemented when possible, and they can include the use of host plant resistance, cultural practices and the application of biopesticides (Nyczepir & Thomas, 2009).

Cultural control includes strategies such as crop and fallow rotation; trap crops, cover crops and soil amendments; exploitation of plant phenology; steam heat and solarization; sanitation (Nyczepir & Thomas, 2009).

Farmers frequently include non-hosts or poor crops to the specific nematode in their crop production schemes to reduce *Meloidogyne* spp. populations (Nyczepir & Thomas, 2009). The key factors needed to assess the economic viability of **crop and fallow rotation** are the identification of nematode species, to know their host range, the number of non-host crops that are possible to cultivate in the geographic region, and the guarantee that rotation crop will not lead to new plant-parasitic nematodes or other plant enemies species establishment (Belair & Parent, 1996; Bridge & Starr, 2007; Nyczepir & Thomas, 2009).

Normally, the crop change reduces the economic return in the short-term because the farmer may have to invest in new equipment and the new crop could be less profitable (Viaene & Abawi, 1998). However, this method has proven to be successful with annual crops. Conversely, it is not adequate to perennials. Sometimes it is necessary to leave the land in fallow (uncropped) for an extended period of time before replanting an agricultural crop. The objective of this method is to suppress nematode populations. The main advantage of fallowing crop land is that the identification/characterization of nematode species becomes relatively unimportant. Many farmers of intensive production systems do not prefer to adopt fallowing because their profit is negative, due to weed control costs and also due to the deleterious soil erosion effects (Nyczepir & Thomas, 2009).

Trap crops are plants used to suppress or directly control nematode populations. These plants must be good hosts allowing the easy infection of the nematode species. After infection, plants are killed before nematodes lay their eggs. This method does not control migratory endoparasites or ectoparasites because they are not “trapped” in roots (Nyczepir & Thomas, 2009). At the same time, trap crops provide others benefits such as enrichment of organic matter, increasing beneficial microbial populations, weed suppression and reducing soil erosion (Melakeberhan *et al.*, 2006). In addition, there are also major advantages of this method that can be pointed out, such as the productivity increase in subsequent cultures and/or nematicides use reduction. On the other hand, most of the considered good trap crops species are plants with unwanted characteristics, like weedy traits or toxicity to the animals (McSorley, 1998).

Cover crops are those grown together or alternating with cash crop cycles. They prevent soil erosion, improve soil structure (water infiltration and water-holding capacity), improve soil fertility, increase soil organic matter and may also help in insect, weed, and nematode suppression (including *Meloidogyne* spp.) if the crop is properly selected (Timper *et al.*, 2006). Cover crops can suppress nematodes because they only move short distances on their own and if a cover crop is not a host, nematodes cannot migrate to another field. Therefore, some nematodes can starve, helping to reduce their population (Nyczepir & Thomas, 2009).

Soil amendments are elements added to the soil such as organic compost, poultry manure and livestock. Normally, this strategy is used to enhanced the organic content and improve the structure of the soil, increasing nutrients and moisture soil capacity (Nyczepir & Thomas, 2009). This method is rarely used to control root-knot nematodes due to its lower efficiency. In some cases the application of fresh poultry or composted horticultural wastes increased vegetable yields and simultaneously decreased *Meloidogyne incognita* population (Riegel & Noe, 2000).

Steam heat has long been utilized in greenhouse and nursery crop production systems to control plant-parasitic nematodes, other soil-borne pathogens, insects and weeds through soil sterilization. In fact, PPNs die when soil is heated at 45ºC (Sikora & Fernandez, 2005). Wang and McSorley, (2008) found that lower temperatures had lethal effects on *Meloidogyne incognita* eggs and J2 of however, the exposure time increased, which is consistent with the findings of Pullman *et al.*, (1981). Nonetheless, the utilization of steam heat in these days is limited by the high cost of heating fuel, amount of labor required, reduction of beneficial soil biota and change in soil pH (Melander & Jørgensen, 2005).

Soil solarization is normally used to manage root-knot nematodes in field conditions. This technique of increasing soil temperature requires solar energy in high levels without interruptions. Therefore, this method is used in temperate regions of the world (Gaur & Perry, 1991). The temperature necessary to kill the PPNs was already referred in the last technique. The limitations of this method are the size of the area to be treated, the diminishing of pest control efficacy with increasing soil depths, the negative effects on beneficial organisms, and the period of time needed (Freitas *et al.*, 1997; McSorley, 1998).

After using the techniques mentioned above to reduce RKN populations, it is essential to follow good **sanitation practices** to minimize the introduction/dispersion of RKNs into the crops. Sanitation is a cultural control to avoid the movement of RKNs to new production areas or among planting sites (Nyczepir & Thomas, 2009). Some examples of sanitation practices include cleaning equipment utilized in the farm, utilization of inspected and certified plants without nematodes from trustworthy nurseries, avoiding irrigate non-infested crops with runoff water that comes of infested crops and removing infected plants before replantation (Nyczepir & Thomas, 2009).

Host plant resistance consists in the introduction of the cross natural nematode resistance genes into cultivated plant species to improve their resistance to nematodes by plant breeders. Some of the limitations of this method are the lack of durability because of the increased frequency of virulent nematode populations, the time it takes to screen for resistant plant varieties and the time necessary to breed resistance traits into commercial varieties, and the specificity of the resistant host plant, since most of them are only effective against a single *Meloidogyne* species. However, when available, efficient host plant resistant is one of the most effective, economical and environmentally safe strategies (Hussey & Janssen, 2002).

Another interesting way to control RKNs is the use of genetic engineering to develop effective resistance in modern crop cultivars, known as Plant-Incorporated Protectants (PIPs), which are recognized as biopesticides to US EPA. One of the most popular examples of the use of genetic engineering is the introduction, in the plants' genome, of the gene Bt, which encodes a pesticidal protein that occurs naturally in *Bacillus thuringiensis*. In this way, the plant is capable of producing the same pesticidal protein that will help control and/or kill the pest while it feeds on the plant. The US EPA is responsible for controlling the genetic and proteic material introduced in the plant; however, they do not regulate the plant itself (Schnepf *et al.*, 1998; US EPA, 2016). The development of genetically modified plants resistant to *Meloidogyne* spp. is being studied, proving to be benign to the environment and non-target organisms. Furthermore, this approach does not pose a risk to human health, which has been proven by toxicological and allergenicity studies. However, the commercialization of these products will be very difficult because of the currently lack of consumer acceptance of genetically modified plants and European policies (Thomas & Cottage, 2006; Atkinson *et al.*, 2009).

Biological control of nematodes refers to the use of living organisms that are natural nematodes' enemies, such as insects, fungi, bacteria, predatory nematodes and protozoa, or their metabolites, to reduce the damage of nematodes in the crops (Eilenberg *et al.*, 2001; Li *et al.*, 2015). Normally these natural enemies can be found in soils suppressive to nematodes (Oka *et al.*, 2000a). Actually, some microorganisms have effective strategies to kill, digest and trap the PPNs. They usually affect specifically the development stages of the nematode's life cycle. This seems to be an ecological and economical way to reduce the damage of RKNs. However, this method takes more intensive planning and management, which is a significant disadvantage. Another drawbacks of the biological control agents are the long time it takes to be accepted in the market, the existence of natural enemies to each biological control agent and their limited survival in some environmental conditions (Li *et al.*, 2015).

Biopesticides are ecofriendly pesticides which are derived from natural substances such as plants, bacteria, animals and certain minerals (Copping & Menn, 2000).

The biopesticides have different definitions in Europe and in the USA. In Europe, biopesticides are those which are based on natural products (biochemical pesticides) and microbial pesticides; in USA, biopesticides include natural products, microbial pesticides, but also PIPs, which were previously mentioned (Copping & Menn, 2000; Chandler *et al.*, 2008; OECD, 2015; US EPA, 2017).

Biopesticides have several benefits compared to the conventional pesticides. They are usually inherently less toxic and, therefore, safer and more target-specific than conventional broad-spectrum pesticides. They are also more effective in lower quantities and generally decompose faster, which offers lower exposure. Biopesticides can be used in integrated pest management (IPM) programs, reducing the use of conventional pesticides and simultaneously offering potentially higher crop yields (Thakore, 2006; S. Kumar & Singh, 2015; Kamble *et al.*, 2016; US EPA, 2016).

1.4. Biochemical Pesticides

The biochemical pesticides are natural substances, including plant extracts, pheromones and natural insect growth regulators, able to control pests by less toxic mechanisms than synthetic pesticides (Sarwar, 2015; US EPA, 2016). Sometimes it is difficult to determine if the substance corresponds to the established criteria to be classified as a biochemical pesticide. Thus, EPA has established a distinctive committee

to make these kind of decisions (US EPA, 2016). When the biochemical pesticides are incorporated in programs of IPM they reduce the use of conventional pesticides and at the same time crop yields remain high. Therefore, the use of biopesticides and bio-fertilizers is becoming more and more important as an alternative to the conventional pesticides. The main reason to this fact is the increasing consumer awareness and concern about food and environment safety. The production of biopesticides is increasing rapidly and, in the future, it will be superior than the synthetic pesticides' production (Gupta & Dikshit, 2010).

The biochemical pesticides, which occur naturally in nature, have been successfully used to reduce RKNs in various crops. They can have suppressive, repellent or antagonistic effects on RKNs, while others are toxic. This may potentially lead to the production of low-cost bionematicides. The factors that will determine the applicability of the nematicide are their cost-effectiveness and availability (Chitwood, 2002; Coyne *et al.*, 2009).

There are several benefits of biochemical pesticides over synthetic pesticides, some of which have already been already mentioned. The former are more efficient in lower concentrations, which makes them less toxic than synthetic pesticides; they decompose relatively faster and contain new compounds that RKNs are not yet able to inactivate. Furthermore, they are also derived from renewable sources (Chitwood, 2002; Coyne *et al.*, 2009).

Various higher plants (Chitwood, 1992, 1993, 2002; Oka *et al.*, 2000b; Dawar *et al.*, 2007; Abbas *et al.*, 2009), and algae (Chitwood, 2002; Rizvi & Shameel, 2006; Khan *et al.*, 2015), have been found/reported with nematicidal activity. These bionematicides are being screened and evaluated before being commercialized (Haydock *et al.*, 2006).

Plants

Higher plants synthesize and produce secondary metabolites, which are not essential to plant survival but facilitate the primary metabolism in plants, helping in their development and growth (Kutchan & Dixon, 2005). Secondary metabolites normally have an important role in plant defense against pathogens (Stamp, 2003). These chemicals are extremely diverse and thousands of them are included in the major classes, such as flavonoids, monoterpenoids, diterpenoids, alkaloids, and polyphenols (Feeny, 1976).

Naturally occurring compounds in higher plants, such as, polythienyls, isothiocyanates, glucosinolates, cyanogenic glycosides, polyacetylenes, alkaloids, fatty acids and derivatives, terpenoids, sesquiterpenoids, diterpenoids, quassinoids, steroids, triterpenoids, phenolics and miscellaneous have been found to have nematicidal activity (Chitwood, 2002; Echeverrigaray *et al.*, 2010; Abdel-Rahman *et al.*, 2013; Khalil, 2014; Khan *et al.*, 2015).

Nowadays, more than one thousand seventy-five species of higher plants demonstrated to possess pesticidal activity against birds, insects, mites, molluscs and nematodes with agricultural importance (Prakash *et al.*, 2014). Some of these plants were *Acacia auriculiformis, Adhatoda vasica, Tagetes patula, Tagetes lucida, Mentha spicata, Eucalyptus melliodora and Carum carvi* (Prakash *et al.*, 2014). Overall, in the present political and economic environment, there seems to be an excellent opportunity for the development of phytochemical-based biopesticides.

Seaweeds

Macroalgae have long been studied as potential pharmaceutical and biocidal agents (Ara *et al.*, 1999, 2002, 2005; Craigie, 2011). Marine algae are known to stimulate and help fruit, vegetable and other crops' growth, because they contain all the major and minor plant nutrients, presenting biocontrol properties as well (Crouch & Van Staden, 1993; Wu *et al.*, 1997; Khan *et al.*, 2005). Furthermore, they possess many organic compounds, growth hormones or related to them, like gibberellins, precursor of ethylene, betaine and auxins, which affect also other plant growth (Crouch & Van Staden, 1993; Craigie, 2011).

These organisms contain also a wide range of compounds such as phenolic compounds, alkaloids, alginic acids and bromine-containing acetogenins, some of these compounds revealed to have nematicidal activity (Zhao, 1999; Ara *et al.*, 2005). Macroalgae compared to common pesticides exhibited more or similar suppressive effects in the infection by nematodes, decreasing the number of galls and penetration of nematodes in the tomato and sunflower roots. The extract of *Ascophyllum nodosum* (Phaeophyceae) curbed the infection of *Meloidogyne javanica* and *Meloidogyne incognita*, that can be due to γ-aminobutyric acid betaine, glycine betaine and δ-aminovaleric acid betaine (Jenkins *et al.*, 1997; Wu *et al.*, 1997). Hence, marine algae can be a great help to prevent the infection caused by RKNs (Chitwood, 2002; Thirumaran *et al.*, 2009).

1.5. Biopesticides and Sustainable Development

On January 2016, the resolution of the United Nations to transform our world, progressing towards a Sustainable Development came into force. For this purpose, they established 17 main goals, composed of 167 subgoals, which were approved unanimously by 193 member states of United Nations, including several world leaders (UN, 2016a).

Goal number 2 aims to end hunger, improve nutrition, achieve food security and also promote a sustainable agriculture (UN, 2016b). As mentioned before, the biopesticides make an important contribution to sustainable agriculture and to achieve global food security (Kumar & Singh, 2015; Kamble *et al.*, 2016; US EPA, 2016).

The Goal number 15, which aims to stop biodiversity loss, stop and reverse land degradation, combat desertification, and protect, restore and promote the conservation and the sustainable use of terrestrial and other ecosystems (UN, 2016b), will be taken into consideration in this project.

Invasive species are one of the major threats to natural ecosystems and the cause of loss of biodiversity in many parts of the world (Marchante *et al.*, 2015, 2017) . These species are responsible for decreasing the abundance of native species, sometimes even for eradicating them (Bais *et al.*, 2003; Callaway *et al.*, 2005). They are also responsible for many negative impacts on public health, economy and water resources (Pyšek *et al.*, 2012; Simberloff *et al.*, 2013). Plant invasions are normally very expensive and difficult to resolve, sometimes even impossible (Marchante *et al.*, 2017).

This problem has increased the concern of scientists, researchers, land managers, politicians and leaders of countries all over the world (Marchante *et al.*, 2017). In Portugal, national legislation acknowledged the severity of this problem and took measures to minimize the introduction of new plants that can become invasive (Decreto-Lei nº. 565/99, of 21 December). Working with invasive species, by finding them a purpose, will contribute to control their growth and avoid their proliferation in natural ecosystems.

Acacia dealbata Link, a native and woody plant of Australia, was introduced in the Southern Europe in the nineteenth century as an ornamental species. After its introduction it became an invasive species between Portugal and Italy, specially due to the Mediterranean and Atlantic climates (Lorenzo *et al.*, 2010). In fact, nowadays, *A. dealbata* is considered a serious environmental problem, because it has invaded

watercourse, abandoned arable land and native woodland, reducing the autochthonous flora in Europe (Carballeira & Reigosa, 1999; Lorenzo *et al.*, 2008). The main reason for this invasive success is the release of allelopathic compounds during flowering period, corresponding to the germination period of native species (Carballeira & Reigosa, 1999; Lorenzo *et al.*, 2008). It was demonstrated that aqueous extracts of *A. dealbata* during this period inhibit the germination and growth of meadow species and *Lactuca sativa* (Carballeira & Reigosa, 1999). The phenolic compounds present in various *Acacia* species can be responsible for these effects since these compounds probably interfere with several biological processes of understory species (Casal *et al.*, 1985; González *et al.*, 1995). It was also demonstrated that allelopathic compounds released by *A. dealbata* had an effect on soil microorganisms (Lorenzo *et al.*, 2013).

Acacia longifolia, another woody *Acacia* species, also native from Australia, is one of the worst invasive species of plants in Portugal. It was introduced in the Southern Europe in the end of the nineteenth and early twentieth century to reduce sand erosion. However, they are spreading widely mainly due to fire events. Today, it is responsible for invading extensive areas of coastal ecosystems dominated by small shrubs and herbs and transforming this area in a monoculture of *A. longifolia*. This species invasion is responsible for decreasing the plant richness and diversity, altering several plant traits and also changing the soil chemistry and functionality. Therefore they affect the plant communities and their medium-term dynamics (Marchante *et al.*, 2008a, 2008b; Marchante *et al.*, 2015). These impacts can persist over time, making it more difficult and complex to restore the invaded areas (Marchante *et al.*, 2009; Marchante *et al.*, 2011; Le Maitre *et al.*, 2011). Another environmental impact caused by *A. longifolia* invasion is the reduction of forest productivity, mainly in pine plantation in the littoral, which increases the risk of occurring fires. Consequently this provokes a negative impact in Portugal's economy. Unfortunately, chemical and mechanical control methods used in Portugal are very expensive and usually not successful, due to the persistent and extensive, long-lived seed-bank accumulated in the soil, which promotes a quick re-invasion of the areas after the interventions (Marchante *et al.*, 2010). It was also demonstrated that *A. longifolia* has significant effects on the catabolic diversity of soil microbiota, which may have wider consequences on nutrient cycling (Marchante *et al.*, 2008a).

Sargassum muticum (Yendo) Fensholt is a brown macroalgae, native of Japan, which has spread widely along the European coast, being considered an invasive species in Europe, including Portugal (Guiry & Guiry, 2017c). Its large quantity of biomass can represent a viable biotechnological asset in the European resources development programs. Furthermore, *S. muticum* can produce interesting phenolic compounds with low molecular weight (Lann *et al.*, 2012), which have demonstrated anti-microfouling and antioxidant activities (Lann *et al.*, 2008; Plouguerne *et al.*, 2008). This seaweed also contains sterols, which reduce the plasma-cholesterine level in animal experiments (Ruqqia *et al.*, 2015).

The *Sargassum muticum* is used as a potassium and nitrate source for soil fertilization (Milledge *et al.*, 2016). It is also used in heavy metal ion bioremediation to treat wastewaters, due to its biosorption of heavy metals such as nickel, chlophenolic compounds and cadmium (Davis *et al.*, 2003; Lodeiro *et al.*, 2006a, 2006b; Bermúdez *et al.*, 2011).

El-Deen & Issa (2016) demonstrated that *Sargassum muticum* also has activity against *Meloidogyne incognita*.

The appreciation of **endogenous resources** will develop a local economy and promote sustainable development by conserving and improving the biodiversity. Therefore, this process involves expanding the region´s capacity to add value to production. This will allow to absorb and retain the economic excesses locally generated and to attract excesses generated in other regions (Natário *et al.*, 2016).

Gracilaria gracilis is a red algae, Rhodophyta, with over 150 currently recognised species (Guiry & Guiry, 2017a). The seaweeds of this genus are very important for biotechnological and industrial uses and are considered economically valuable resources because of their capacity to reach high yields of commercially valuable biomass and the presence of phycocolloids, the main source of agar, which is a gelatinous non-toxic colloidal carbohydrate present in the cell wall and intercellular spaces of the seaweed. The agar can be used in the preparation of ice creams, soups, cosmetics, bacteriological samples, jellies and food (Capo *et al.*, 1999; Ghosh *et al.*, 2011). Compounds with antibiotic, antioxidant, antifungal, and antiviral activities have been detected in genus *Gracilaria* (Mazumder *et al.*, 2002; Souza *et al.*, 2012; Kumar *et al.*, 2013).

Gracilaria gracilis can be found in significant quantities in the coast of Portugal. Nowadays, in this country, it is used as a source of agar and used directly as food in animal aquaculture (Guiry & Guiry, 2017a). Extracts of *G. gracilis* showed antimicrobial activity (Tuney *et al.*, 2006). Rizvi & Shameel (2006) demonstrated that methanol extract of *G. gracilis* has some activity against *Meloidogyne javanica*. Thus, *G. gracilis* will also be studied in this work, as it is a potential nematicide and exists in great quantity in Portugal, as mentioned before, which could be a way of improving the country's economy.

Ulva rigida has a worldwide distribution in temperate and warm seas, and it is present in large quantities in the coast of Portugal (Guiry & Guiry, 2017b). This macroalgae is rich in carbohydrates, fibre, protein, minerals and vitamins and has low lipid content. It is often utilized as a fresh sea vegetable by many island cultures and it is used in animal feeding because it is an excellent source of nutrients, promoting a balanced diet (Taboada *et al.*, 2010).

There are several important reported activities for this species: antigenotoxicity (Celikler *et al.*, 2008, 2009), antihyperglycemic (Celikler *et al.*, 2008), antileishmanial (Sabina *et al.*, 2005), immunomodulating (Leiro *et al.*, 2007), antibacterial (Febles *et al.*, 1995; Ali *et al.*, 2009; Trigui *et al.*, 2013), and antioxidant (Yildiz *et al.*, 2012; Trigui *et al.*, 2013). Moreover, Khan *et al.* (2015) verified a moderate effect on *Meloidogyne javanica*. For the same reason referred before, *U. rigida* will also be studied because it exists in great quantity in Portugal and has potential as a nematicide.

Circular Economy is also an issue that is intimately related with sustainability. Over the last years the concept and development of circular economy model has been changing, improving the dominant economic development model "take, make and dispose" (Ness, 2008; European Commission, 2014). The latter one is responsible for the negative effects on the natural ecosystems integrity and on the economy stability, which are essential for humanity´s survival. The European Commission defines circular economy as a way to preserve the value added in products for as long as possible, with the aim of eliminating wastes. It allows to maintain the resources in the economy when the product has reached the end of its life, in order to keep it in productive use and create further value (European Commission, 2014).

Circular economy can become a new business model that leads to a more sustainable development (Mathews & Tan, 2011; Naustdalslid, 2014). Sustainable development involves simultaneous consideration of technology, economy, environment and social aspects, as well as the interaction between these aspects (FAO, 2017b). All this contributes to the circular economy (Birat, 2015).

The circular economy can help society reach wellbeing and increased sustainability at low or no energy, material and environmental costs. Therefore, the circular economy has the potential to understand and implement a new method/pattern to improve the society (European Commission, 2014).

Faba bean (*Vicia faba* L.) is one of the most important cultivated legume crops in the world. Their seeds contain vitamins, fibers, nutrients and many other compounds capable of fighting cholesterol and probably mitigate Parkinson´s disease or even cancer. It has the ability to fix nitrogen and control diseases and weeds in agriculture (Hou *et al.*, 2015). The amino acid called L-dopa is also present in faba bean and is responsible for the stimulation and excitation of the brain. Recent studies indicate L-dopa as a beneficial drug to combat brain diseases (Topal & Bozoğlu, 2016).

Portugal is one of the most important producers of bean in Europe, mainly in Beira Alta, Beira Litoral, Beira Baixa and Trás-os-Montes and Alto Douro, regions with a production of 3037 tons in 2016 (INE, 2017). The faba bean is cultivated mainly for seed consumption. Therefore, the pod is a common waste resulting from the faba bean processing to obtain its seeds, representing 60% of the complete fruit (Cordeiro, 2016).

The antioxidant agents present in plant are responsible for the stabilization of fatty acids through a reaction with free radicals, preventing radicals to react with oxygen atoms. Over time, these antioxidant agents begin to lose their capacity to react with free radicals and start to react with oxygen, turning the pod darker in color, a process designated by oxidation (Degáspari & Waszczynskyj, 2004).

Besides the properties referred above, *Vicia faba*'s seed also showed very interesting antifungal activity due to the protein chitinase (Wang *et al.*, 2012); and antihypertensive, antimicrobial, antioxidant and anticancer activities due to vicilin and legumin, two storage proteins (Ferreira, 2013). Given all those properties, it may also have nematicidal activity against RKNs.

1.6. Objectives

Taking into account all the above mentioned, the main general goal of this study was to find natural chemicals (biochemical pesticides) that could be effective alternatives to synthetical chemicals in *Meloidogyne* sp. control. Moreover, this work aims to contribute: a) to a better knowledge and increase value of some terrestrial plants and Portuguese native species of marine macroalgae; b) to Circular Economy, converting agroindustrial wastes as raw material; and c) to control an invasive Portuguese species.

2. Material and methods

2.1. Preparation of plant extracts

Table 1 summarizes all the plant extracts used in this work to study their potential as nematicides/nematostatic against *Meloidogyne* sp.

Table 1 Plant extracts and their respective concentrations tested for nematicidal/nematostatic activity on *Meloidogyne* sp.

Plant species	**Used plant part**	**Extract concentration (g/mL)**
Acacia dealbata	Seeds	0.2
Acacia longifolia	Seeds	0.2
Sargassum muticum	Thallus	0.1
Gracilaria gracilis	Thallus	0.1
Ulva rigida	Thallus	0.1 and 0.05
Vicia faba	Fresh and Oxidized pod	0.1

2.1.1. Extracts of *Acacia dealbata* and *Acacia longifolia*

Acacia longifolia and *Acacia dealbata* seeds were gently provided by Hélia Marchante (Professor at ESAC-IPC and researcher at CFE) and Elisabete Marchante (Researcher at CFE).

Both *Acacia longifolia* and *Acacia dealbata* extracts were obtained through a solid-liquid extraction method using distilled water as solvent. Firstly, using a mortar with pestle, 30 g of seeds were macerated in 150 mL of distilled water, to obtain a concentration of 0.2 g mL^{-1} (Figs. 3A and 3B). The macerate was transferred to an Erlenmeyer and it was stirred for 48 hours. To remove bigger particles, the extract was sieved to a laboratory bottle. Then, it was centrifuged (ROTANTA 460 R- Hettich Zentrifugen) at 11500 rpm, for 25 minutes. This step was repeated until the extract was

duly clear and then filtered through a filter paper Whatman Nº1, using a vacuum pump system, in order to obtain a clear solution to facilitate nematode observation during the *in vitro* assays (Fig. 3C). The extracts were stored in a refrigerator at 4ºC, until they were used.

A)  B) 

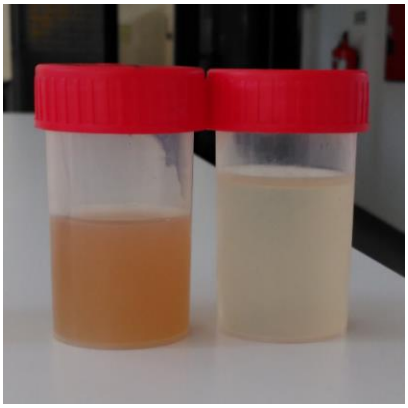
C) 

Figure 3 (A) Seeds of *Acacia longifolia*; (B) Seeds of *Acacia dealbata*; (C) Extract of *A. dealbata* (left) and *A. longifolia* (right) filtered, at a concentration of 0.2 g/mL.

2.1.2. Extracts of algae

Sargassum muticum (Fig. 4A), *Gracilaria gracilis* (Fig. 4B) and *Ulva rigida* (Fig. 4B) were collected in "Baía de Buarcos" in Figueira da Foz (Fig. 4C). This area is essentially dominated by a rocky substrate with marine pools, totally exposed to the sea undulation and has a reduced slope. The seaweeds were handpicked and washed with seawater to remove sand particles, debris and epiphytes. They were kept in plastic bags in an ice box to maintain temperature, avoiding algae degradation, until transportation to the laboratory.

A) 

B) 

C) 

Figure 4 (A) *Sargassum muticum* in Baía de Buarcos of Figueira da Foz; (B) *Ulva rigida* (green) and *Gracilaria gracilis* (red) in Baía de Buarcos of Figueira; (C) Baía de Buarcos of Figueira da Foz.

In the laboratory, algae were washed with tap water, to get rid of the salts from the surface. The water was drained off to remove the excess from the algal material with a blotting paper. Then, they were dried in an oven at 55ºC for 24 hours. After this procedure, using a mortar with pestle, 30 g of each seaweed were macerated in 300 mL of distilled water to obtain a concentration of 0.1 g mL^{-1}. Then, the procedure followed was similar to that described above (2.1) (Fig. 5).

Ulva rigida extract was tested firstly at 0.1 g mL^{-1}. After this procedure, 30 mL of the extract of *U. rigida* (0.1 g mL^{-1}) were diluted with 30 mL of distilled water, to obtain the concentration of 0.05 g mL^{-1}, and centrifuged at 11500 rpm during 25 minutes to homogenize the solution (Fig. 5D). This second concentration extract was prepared based on the results obtained with the higher concentration of this extract. The extracts were stored in the fridge at 4ºC, until they were used.

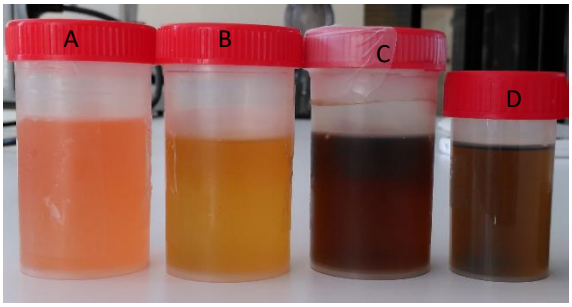


Figure 5 Extract of *Gracilaria gracilis* (A), *Sargassum muticum* (B) filtered at a concentration of 0.1 g/mL and *Ulva rigida* filtered at a concentration of 0.1 g/mL (C) and 0.05 g/mL (D).

2.1.3. Extracts of *Vicia faba*

Fresh and oxidized pod of *Vicia faba* extracts were prepared using lyophilized material gently provided by Inês Seabra (Professor at ESAC-IPC) (Figs. 6A and 6B). Unfortunately, it was not possible to obtain a concentration of 0.2 g mL^{-1}, due to the lack of sufficient of lyophilized material. A solid-liquid extraction method using distilled water as solvent was also used, in this case. Firstly, 3 g of lyophilized material of fresh pod and 1.5 g of lyophilized material of oxidized pod, were added to 30 and 15 mL of distilled water, respectively, to obtain a concentration of 0.1 g mL^{-1}. The plant material was stirred until dissolved, for 30 minutes. Then, the subsequent procedure was similar to that above described (2.1).

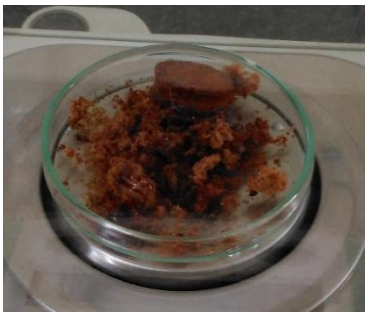
A)  B) 

Figure 6 (A) Extract of fresh pod of *Vicia faba* lyophilized; (B) Extract of oxidized pod of *V. faba* lyophilized.

2.2. RKN population maintenance

Meloidogyne sp. isolate was obtained from infected greenhouse tomato plants (Fig. 7A-C). Egg masses were picked with fine-tipped forceps and the isolate was maintained in "Tiny Tim" tomato plants (Fig. 7D).

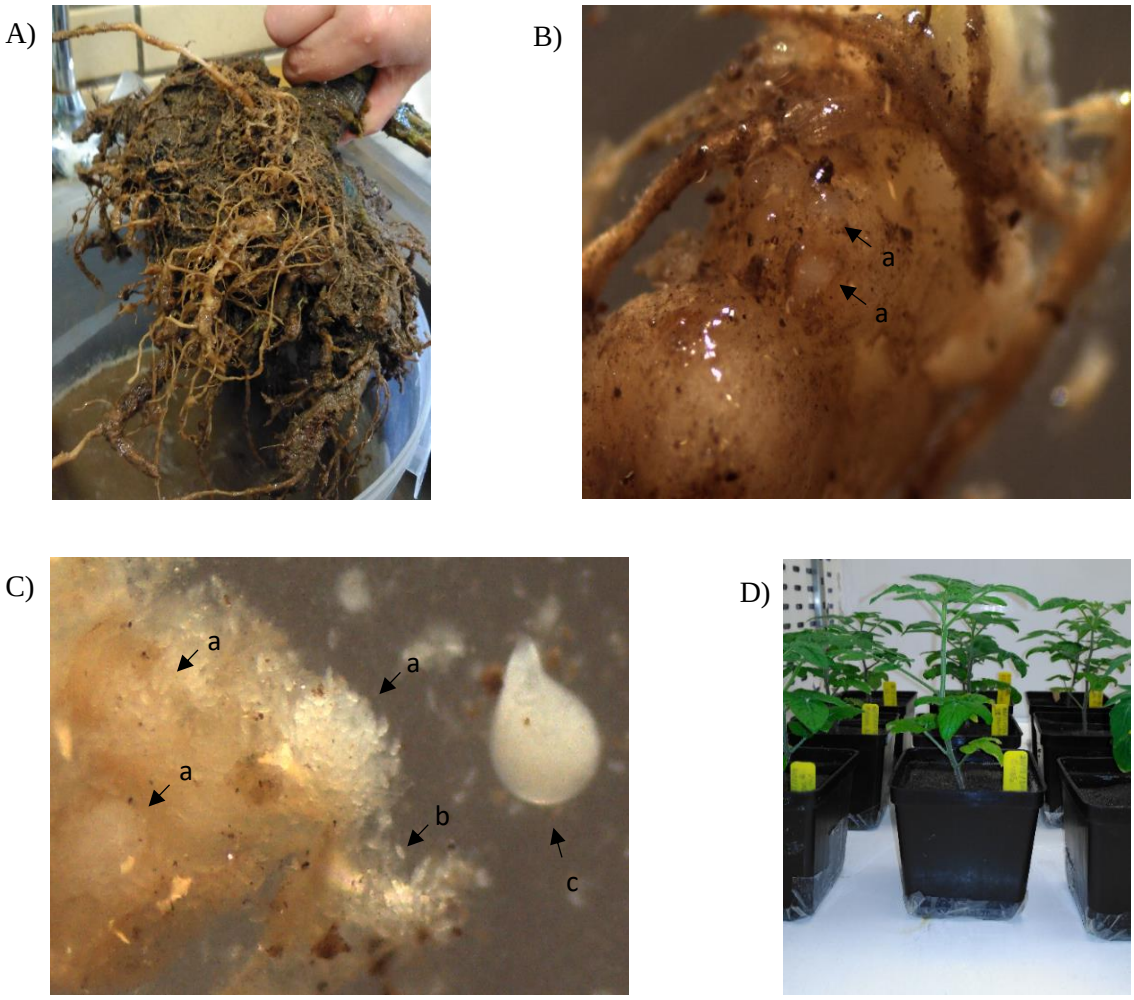


Figure 7 Root-knot nematode population maintenance: (A) Tomato roots infected with *Meloidogyne* sp.; (B) Tomato root with *Meloidogyne* sp. egg masses on the surface of a gall. a: egg masses; (C) Some life cycle stages of *Meloidogyne* sp. a: egg masses; b: egg; c: female; (D) Population of "Tiny Tim" tomato used to maintain *Meloidogyne* sp. population.

Tomato seeds were uniformly distributed on filter paper, moistened with distillated water covering the surface base of a Petri dish that was placed in a chamber at 25ºC. After germination, seeds were transferred, individually, to plastic cups with 5.5 cm of diameter and a capacity of 75 cm^3, containing a mixture of sterilized soil and sand in a proportion of 1:1. The plastic cups were placed in a growth chamber with a temperature around 23ºC, relative humidity around 60% and a photoperiod of 12 hours. Plants were daily watered and fertilized weekly with a nutrient solution (Nutrea®) in a concentration of 200 mL/hL.

When tomato seedlings had two pairs of “true leafs”, they were transferred, individually, to plastic pots with 9.5 cm of diameter, a capacity of 450 cm^3 containing a mixture of sterilized soil and sand in a proportion of 1:1. Then, each pot was inoculated with ten egg masses which were obtained from the infected tomato roots mentioned above and maintained in the growth chamber at the same conditions. Sixty days after inoculation, the egg masses were removed and the procedure was repeated.

2.3. Nematode inoculum

Meloidogyne sp. J2, the infective juveniles, were obtained from egg masses removed from infected tomato roots. Egg masses were put in a small sieve, prepared with a square of nylon, about 30 μm of mesh, tied with an elastic to a rigid plastic ring, with 2.5 cm diameter and 0.9 cm of height. The sieve was placed in a sterilized Petri dish, with 5 cm diameter, containing tap water. Between the sieve and the bottom of the Petri dish was placed a small square of plastic net. Thereby, it was formed a space between the Petri dish and the sieve, to allow the J2 get out of the egg to the water in the Petri dish. After this, the Petri dish with egg masses were put in a chamber at 25ºC. Second-stage juveniles hatched in the first 24 hours were discarded, to be sure that only 24 hours-old J2 were used in the study (Fig. 8).

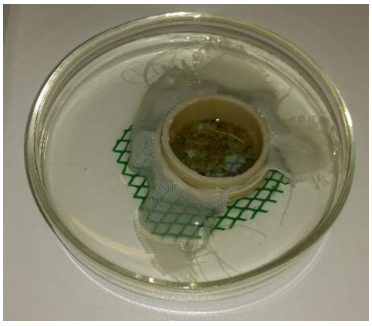


Figure 8 Petri dish to obtain *Meloidogyne* sp. J2 from egg masses.

2.4. Nematicidal and Nematostatic activity bioassays

Bioassays were performed in excavated glass blocks previously disinfected with 96% ethyl alcohol. In each block, it was added 0.5 mL of each of the different treatments. An eyelash stuck to a Pasteur pipette was used to transfer 20 J2 *Meloidogyne* sp. to each block. The eyelash was disinfected prior to use in each treatment.

To avoid potential extract evaporation, all blocks were placed in a humid chamber prepared by placing a net on the bottom of a plastic box and adding water nearly to the emersion of the net. Glass blocks were put on the net and the box, after being covered, was placed in the dark (Fig. 9). Number of immobile and dead nematodes were monitored at 24, 48, 96 and 168 hours, after J2 exposure to the treatment, using a stereoscopic microscope (Leica Zoom 2000 Stereozoom Microscope, 7x to 30x Magnification). In each observation time, four replicates of each treatment and of each control were analysed for immobile and dead nematodes and were subsequently discarded. Nematodes were considered dead when they were motionless and remained immobile after being touched several times in different parts of the body with an eyelash and still kept immobile during one hour after being transferred to distilled water. Nematodes were considered immobile when they moved after being touched with an eyelash or regained mobility after one hour of being in distilled water.

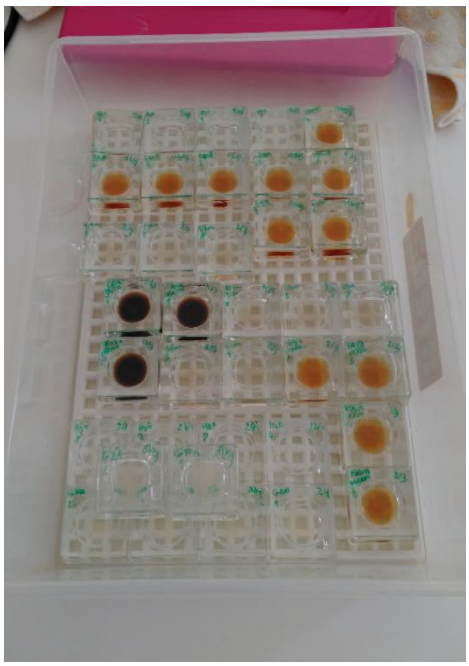


Figure 9 Humid chamber with the bioassay glass blocks.

Distilled water was used as negative control and a commercial nematicide, Nemacur (0.6% v/v), whose active substance is fenamiphos was used as positive control. It was made four replicates for each treatment and observation time, to have independency among observation times. Thus, sixteen replicates were made for each treatment.

The percentage of J2 mortality observed in the glass blocks after 24, 48, 96 and 168 hours was corrected by eliminating the natural death in the negative control (water control) according to the Schneider-Orelli formula (Püntener, 1981):

$$\% \text{ of Corrected Mortality} = \frac{\% \text{ Mortality in Treament} - \% \text{ Mortality in Control}}{100 - \% \text{ Mortality in Control}} \times 100$$

The lethal and immobilization activity were classified as follows: strong, mortality/immobility >80%; moderate, mortality/immobility 80-61%; low, mortality/immobility 60-41%; very low, mortality/immobility 40-10%; no activity, mortality/immobility <10% based on the scale adopted by Kong *et al.* (2006).

Data were also subjected to a one-way and two-way analysis of variance (ANOVA), followed by Tukey test, using a significant level of $p = 0.05$ (Zar, 1996).

3. Results

As dead can be considered the highest nematicidal activity of a treatment, firstly, only the dead nematodes, and hence the nematicidal activity, will be taken into consideration. Nevertheless, the nematostatic activity is also important in nematode control, since immobile nematodes cannot infect the roots. Nematostatic activity will be analysed later in this section

After 24 hours exposure, none of the extracts demonstrated lethal activity against *Meloidogyne* sp. J2 (Fig. 10), only the commercial nematicide was effective, with 100% mortality, as expected. However, after 48 hours of exposure (Fig. 11), it was observed a strong mortality effect, above 98%, for the *Ulva rigida* extract (0.1 g mL^{-1}), followed by the oxidized pod of *Vicia faba* (23.8%) and the fresh pod of *V. faba* (7.5%). All the other extracts did not cause significant J2 mortality. Therefore, the extract of *U. rigida* (0.1 g mL^{-1}) was the most effective against *Meloidogyne* sp. J2, having comparable mortality to that of the commercial nematicide, after 48 hours of exposure.

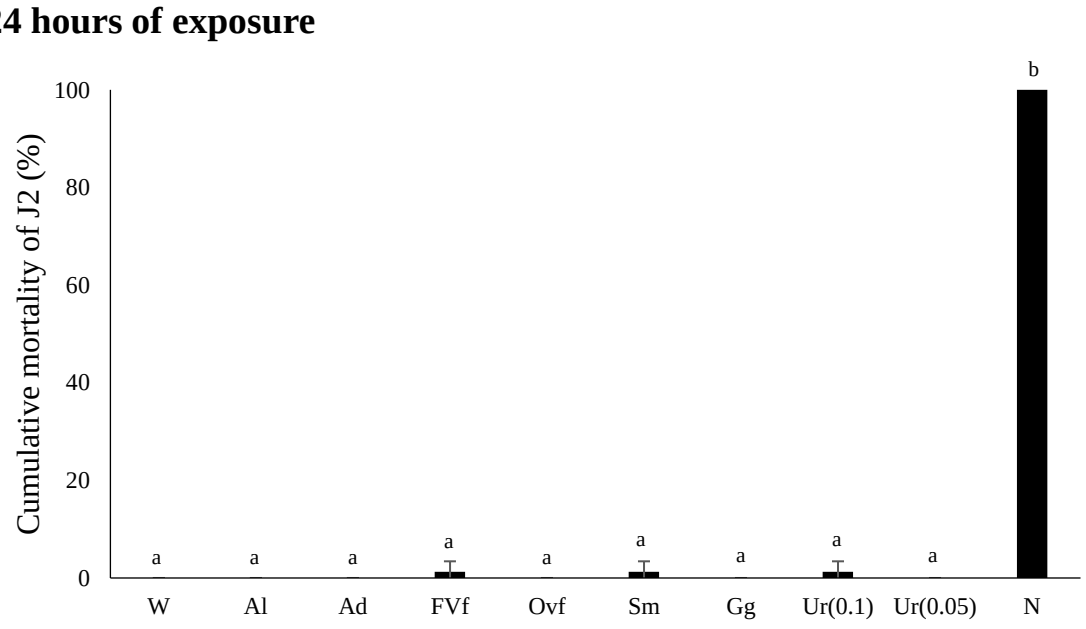


Figure 10 Percentage of cumulative mortality of *Meloidogyne* sp. second-stage juveniles (J2) after 24 hours exposure to different extracts. Results are means of four replicates. Columns with the same letter do not differ significantly according to Tukey test (P≤ 0.05). W: Water; Al: *Acacia longifolia* (0.2 g/mL); Ad: *Acacia dealbata* (0.2 g/mL); FVf: Fresh pod of *Vicia faba* (0.1 g/mL); OVf: Oxidized pod of *Vicia faba* (0.1 g/mL); Sm: *Sargassum muticum* (0.1 g/mL); Gg: *Gracilaria gracilis* (0.1 g/mL); Ur(0.1): *Ulva rigida* (0.1 g/mL); Ur(0.05): *Ulva rigida* (0.05 g/mL); N: Nemacur (0.6% v/v).

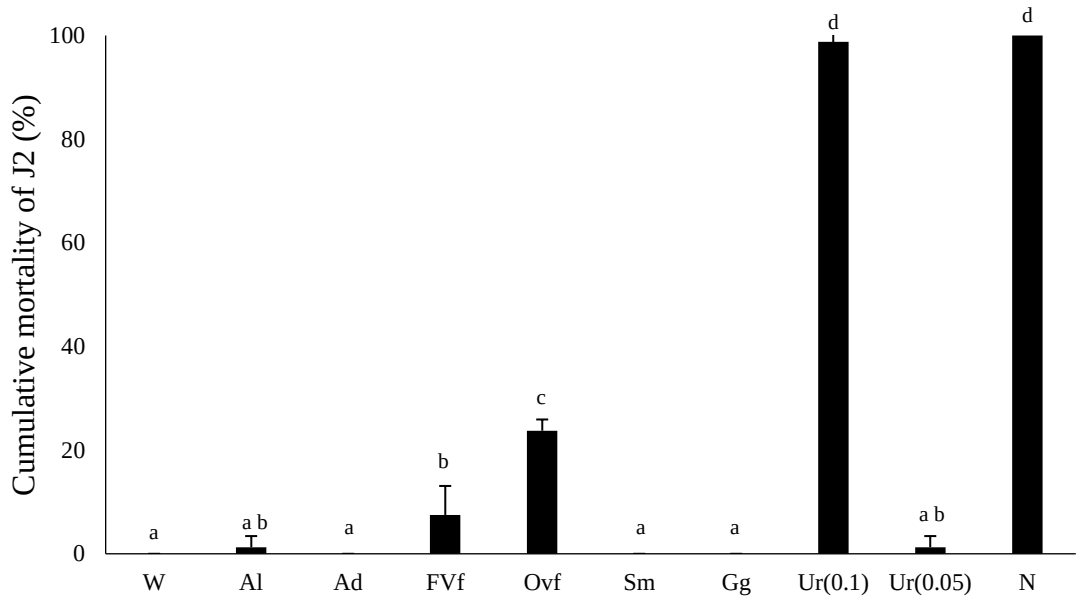


Figure 11 Percentage of cumulative mortality of *Meloidogyne* sp. second-stage juveniles (J2) after 48 hours exposure to different extracts. Results are means of four replicates. Columns with the same letter do not differ significantly according to Tukey test (P≤ 0.05). W: Water; Al: *Acacia longifolia* (0.2 g/mL); Ad: *Acacia dealbata* (0.2 g/mL); FVf: Fresh pod of *Vicia faba* (0.1 g/mL); OVf: Oxidized pod of *Vicia faba* (0.1 g/mL); Sm: *Sargassum muticum* (0.1 g/mL); Gg: *Gracilaria gracilis* (0.1 g/mL); Ur(0.1): *Ulva rigida* (0.1 g/mL); Ur(0.05): *Ulva rigida* (0.05 g/mL); N: Nemacur (0.6% v/v).

At 96 hours exposure (Fig. 12), *Ulva rigida* extract (0.1 g mL^{-1}) caused 100% mortality. Another extract that also had a strong activity was the oxidized pod of *Vicia faba* (93.8%), which had an increase of 70% of mortality, from 48 to 96 hours. Thus, both *U. rigida* (0.1 g mL^{-1}) and oxidized pod of *V. faba* extracts had a comparable behavior to the commercial nematicide. Therefore, based on these results, both extracts should be better studied to confirm their potential as natural nematicides for RKN. The fresh pod of *V. faba* extract demonstrated a moderate nematicidal activity (67.5%), which might also be considered as a potential nematicide. On a lower level of nematicidal activity, there was *U. rigida* (0.05 g mL^{-1}) and *Acacia dealbata* extracts, the later one having less nematicidal activity than the former. The extracts of *Acacia longifolia*, *Sargassum muticum* and *Gracilaria gracilis* did not have relevant activity against *Meloidogyne* sp. J2.

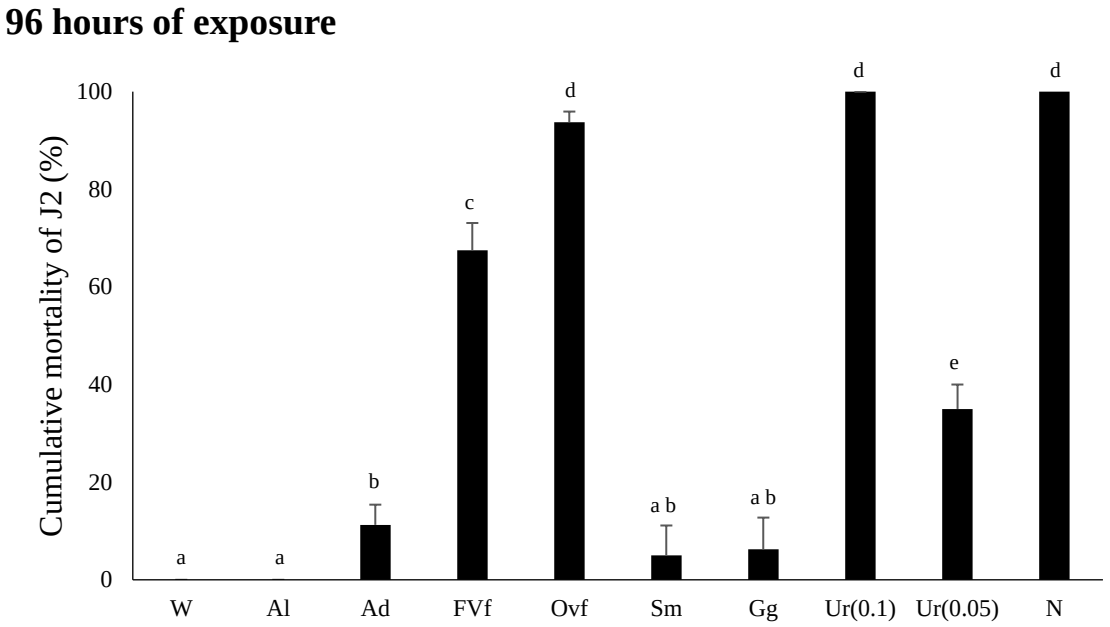


Figure 12 Percentage of cumulative mortality of *Meloidogyne* sp. second-stage juveniles (J2) after 96 hours exposure to different extracts. Results are means of four replicates. Columns with the same letter do not differ significantly according to Tukey test (P≤ 0.05). W: Water; Al: *Acacia longifolia* (0.2 g/mL); Ad: *Acacia dealbata* (0.2 g/mL); FVf: Fresh pod of *Vicia faba* (0.1 g/mL); OVf: Oxidized pod of *Vicia faba* (0.1 g/mL); Sm: *Sargassum muticum* (0.1 g/mL); Gg: *Gracilaria gracilis* (0.1 g/mL); Ur(0.1): *Ulva rigida* (0.1 g/mL); Ur(0.05): *Ulva rigida* (0.05 g/mL); N: Nemacur (0.6% v/v).

At 168 hours of exposure (Fig. 13), the effect of the *Ulva rigida* extract (0.1 g mL^{-1}) was equivalent to that observed at 96 hours and the effect of oxidized pod of *Vicia faba* had a very small increase (from 93.8% to 96.3%). The fresh pod of *V. faba* extract (80%), which had an increase of 12.5% when compared to 96 hours of exposure, demonstrated again a moderate to strong activity. The effect of *U. rigida* (0.05 g mL^{-1}) extract was almost equivalent to that observed at 96 hours, with an increase of only 1%. The *Acacia dealbata* extract showed lower level of nematicidal activity once more, despite increasing from 11.3% to 22.5%. Another extract with lower nematicidal activity was *Sargassum muticum* extract (11.3%). In the others two extracts, namely *Acacia longifolia* and *Gracilaria gracilis*, the mortality was residual, comparable to that occurred in the negative control, water.

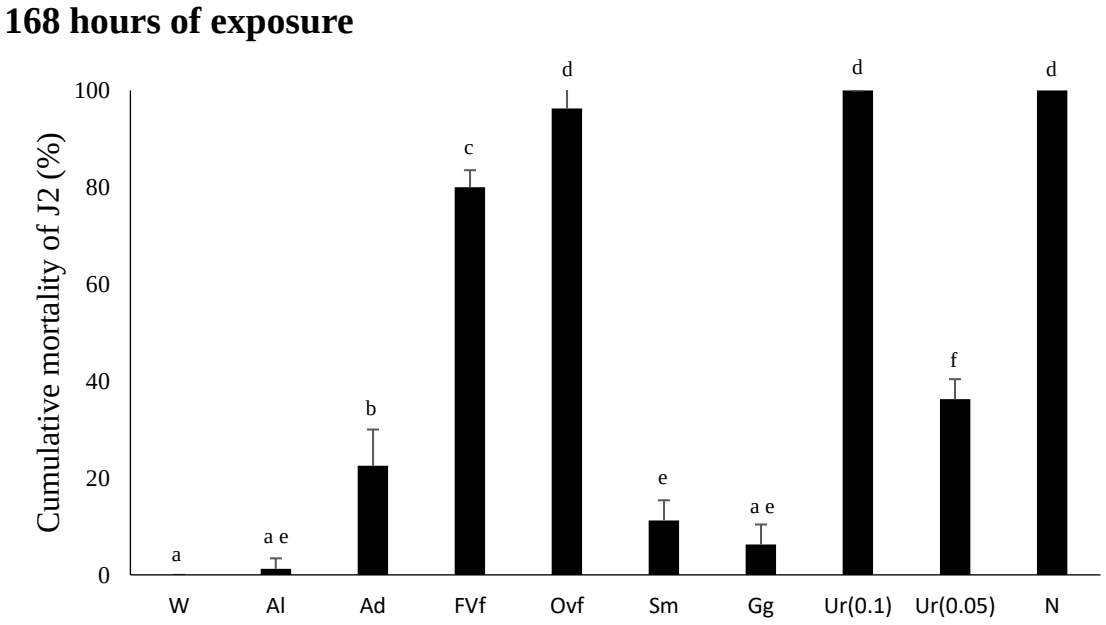


Figure 13 Percentage of cumulative mortality of *Meloidogyne* sp. second-stage juveniles (J2) after 168 hours exposure to different extracts. Results are means of four replicates. Columns with the same letter do not differ significantly according to Tukey test (P≤ 0.05). W: Water; Al: *Acacia longifolia* (0.2 g/mL); Ad: *Acacia dealbata* (0.2 g/mL); FVf: Fresh pod of *Vicia faba* (0.1 g/mL); OVf: Oxidized pod of *Vicia faba* (0.1 g/mL); Sm: *Sargassum muticum* (0.1 g/mL); Gg: *Gracilaria gracilis* (0.1 g/mL); Ur(0.1): *Ulva rigida* (0.1 g/mL); Ur(0.05): *Ulva rigida* (0.05 g/mL); N: Nemacur (0.6% v/v).

To sum up, it can be said that, in terms of mortality, the *Ulva rigida* extract ($0.1\ g\ mL^{-1}$) had an effect on this *Meloidogyne* species similar to that caused by the commercial nematicide, except for the first observation time, 24 hours. Regarding the mortality caused by oxidized pod of *Vicia faba* extract it was similar to those two treatments at 96 and 168 hours exposure, which may potentially lead to a good nematicide against RKN species.

To better understand the simultaneous effect of both factors, extracts and time, on *Meloidogyne* sp. J2, the results of the two-way ANOVA analysis are presented in table 2. In this analysis, it is possible to take into account that observation in each time is independent.

Table 2 Two-way ANOVA analysis results of plant extracts over time on *Meloidogyne* sp. J2 cumulative mortality.

Treatment	Extract concentration (g/mL)	Time (hours)	Mean ± SD *
Water	---	24	0 ± 0 [a]
		48	0 ± 0 [a]
		96	0 ± 0 [a]
		168	0 ± 0 [a]
Acacia longifolia	0.2	24	0 ± 0 [a]
		48	0.3 ± 0.43 [a b]
		96	0 ± 0 [a]
		168	0.3 ± 0.43 [a b]
Acacia dealbata	0.2	24	0 ± 0 [a]
		48	0 ± 0 [a]
		96	2.3 ± 0.83 [b]
		168	4.5 ± 1.5 [c]
Fresh pod of *Vicia faba*	0.1	24	0.3 ± 0.43 [a b]
		48	1.5 ± 1.12 [b]
		96	13.5 ± 1.12 [d]
		168	16 ± 0.71 [e]
Oxidized pod of *Vicia faba*	0.1	24	0 ± 0 [a]
		48	4.75 ± 0.43 [c]
		96	18.8 ± 0.43 [f]
		168	19.3 ± 0.83 [f]
Sargassum muticum	0.1	24	0.3 ± 0.43 [a b]
		48	0 ± 0 [a]
		96	1 ± 1.22 [a b]
		168	2.3 ± 0.83 [b]
Gracilaria gracilis	0.1	24	0 ± 0 [a]
		48	0 ± 0 [a]
		96	1.3 ± 1.23 [a b]
		168	1.3 ± 0.83 [a b]
Ulva rigida	0.1	24	0.3 ± 0.43 [a b]
		48	19.8 ± 0.43 [f]
		96	20 ± 0 [f]
		168	20 ± 0 [f]
Ulva rigida	0.05	24	0 ± 0 [a]
		48	0.3 ± 0.43 [a b]
		96	7 ± 1 [g]
		168	7.25± 0.83 [g]
Nemacur (0.6% v/v).	---	24	20 ± 0 [f]
		48	20 ± 0 [f]
		96	20 ± 0 [f]
		168	20 ± 0 [f]

***** - Results are the means and standard deviation of four replicates with 20 nematodes each. Results with the same letter do not differ significantly according to Tukey test ($P \leq 0.05$).

It can be noticed that this analysis corroborated the results presented above.

In order to better understand the influence of each extract on this *Meloidogyne* species, the nematostatic effect, that is J2 immobility, is now also analyzed linked with mortality data.

Effects of *Acacia longifolia* extract

The *Acacia longifolia* (0.2 g mL^{-1}) extract had a moderate J2 immobilization effect after 24 hours of exposure (61.3% - 76.3%) and almost no lethal activity was observed during the experiment. Between the 96 and 168 hours of exposure the percentage of immobilized J2 decreased without the increase in mortality, which may be due to an adjustment of the J2 to the extract of *A. longifolia* (Fig. 14).

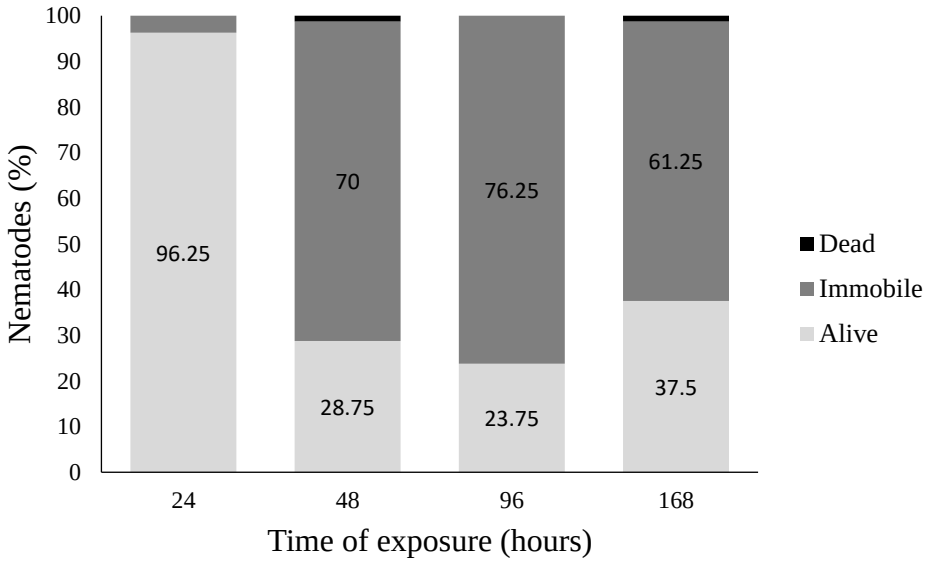


Figure 14 Effect of *Acacia longifolia* extract (0.2 g/mL) on *Meloidogyne* sp. J2 after 24, 48, 96 and 168 hours' exposure.

Effects of *Acacia dealbata* extract

The effects of the *Acacia dealbata* extract on J2 at a concentration of 0.2 g mL^{-1} is represented in Figure 15. Forty eight hours after exposure, a very low percentage of immobility effect (16.3 %) was observed. At 96 hours of exposure, the immobility decreased probably because immobile nematodes died. In fact, mortality started to be noticeable at this period of time (11.3%) and increased up to 22.5% at the end of the experiment (168 hours), when the immobility was 63.8%. Considering the nematostatic and the nematicidal activities, as well as, the total of immobile and dead nematodes at 168 hours, the *A. longifolia* extract had a strong activity against this J2 species (86.3%) (Fig. 15).

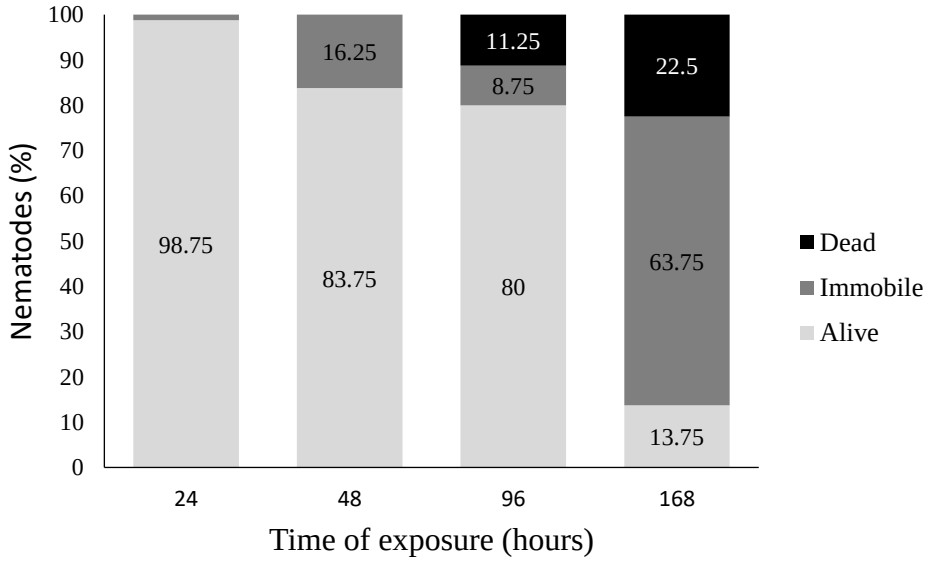


Figure 15 Effect of *Acacia dealbata* extract (0.2 g/mL) on *Meloidogyne* sp. J2 after 24, 48, 96 and 168 hours' exposure.

Effects of fresh and oxidized pod of *Vicia faba* extracts

Regarding the fresh pod of *Vicia faba* extract (Fig. 16), after 24 hours of exposure, a moderate effect was observed: 72.5% of J2 were immobile. This percentage increased to 88.8%, at 48 hours, which can be considered a strong nematostatic effect. Furthermore, mortality started to increase to 7.5% and after 96 hours it increased to 67.5%, considered a moderate mortality effect. It is also important to highlight that, after 48 hours of exposure, less than 4% of nematodes were alive, meaning with normal mobility. In fact, at 48 hours exposure, most of the J2 were immobile or dead. Until the end of the experiment, it was observed a gradual decrease of immobility, corresponding to an increase of mortality. The nematicidal effect increased with increasing time of J2 exposure to the extract (Fig. 16).

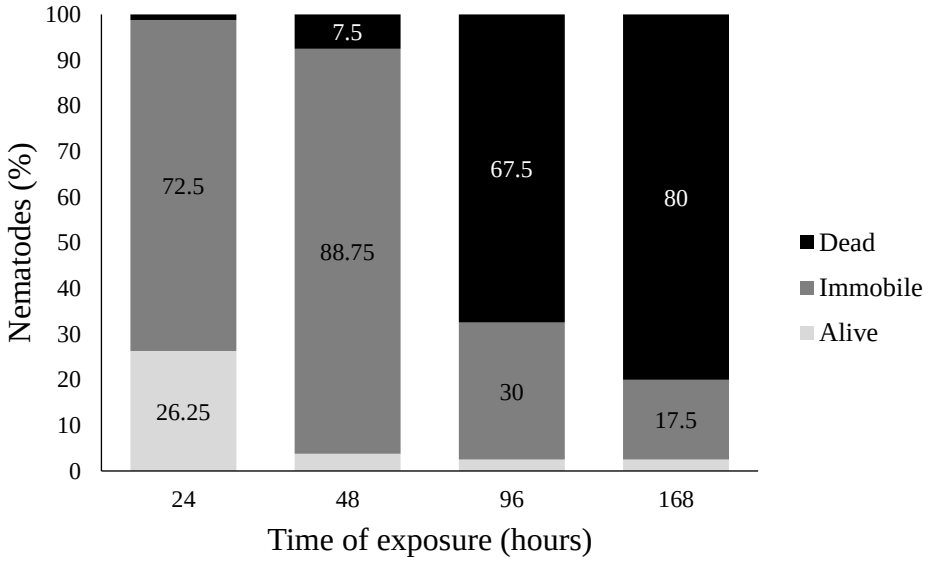


Figure 16 Effect of fresh pod of *Vicia faba* extract (0.1 g/mL) on *Meloidogyne* sp. J2 after 24, 48, 96 and 168 hours' exposure.

A strong nematostatic effect was observed after 24 hours of exposure to oxidized pod of *Vicia faba* extract (0.1 g mL^{-1}) (Fig. 17). This percentage decreased over time while the number of dead J2 increased. After 96 hours of exposure almost 94% of the J2 were already dead. Therefore, the strong nematostatic effect was converted in a strong nematicidal activity (Figs. 17 and 18).

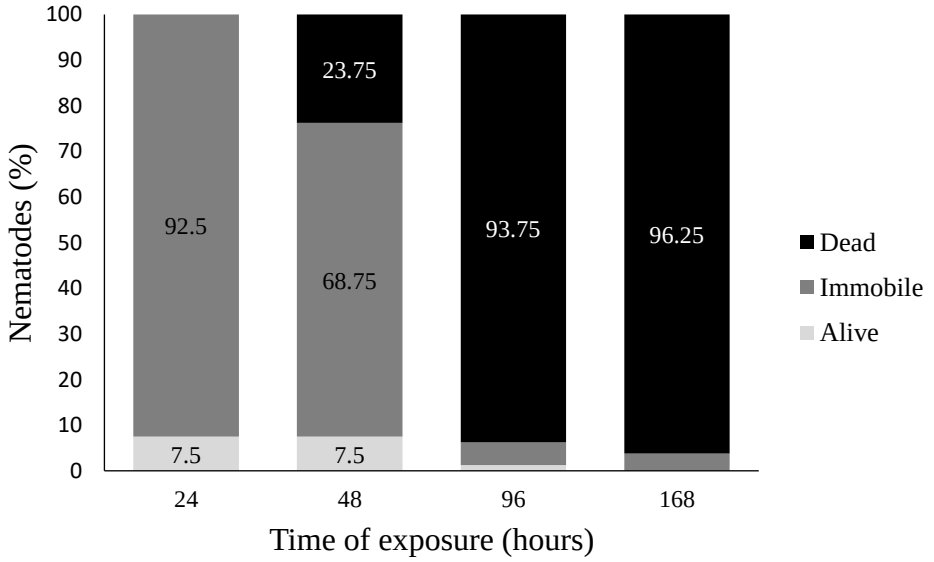


Figure 17 Effect of oxidized pod of *Vicia faba* extract (0.1 g/mL) on *Meloidogyne* sp. J2 after 24, 48, 96 and 168 hours' exposure.

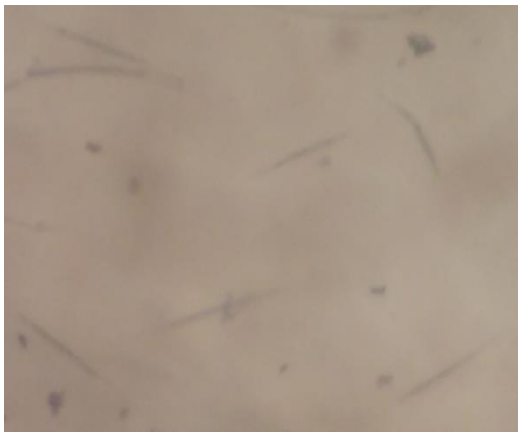


Figure 18 Dead *Meloidogyne* sp. J2 one hour after being transferred to distilled water. These J2 were exposed for 96 hours to oxidized pod of *Vicia faba* extract.

Effects of *Sargassum muticum* extract

The *Sargassum muticum* extract, at a concentration of 0.1 g mL^{-1}, had a considerable quick effect on this *Meloidogyne* species J2, after 24 hours of exposure, with 95% of immobility (Figs. 19 and 20A). However, this effect was considerably reduced to 16.3% at 48 hours (Fig. 20B), slightly increasing to 18.8% at 168 hours. Very low mortality (11.3%) and immobilization of J2 (18.8%) was obtained at 168 hours (Fig. 19).

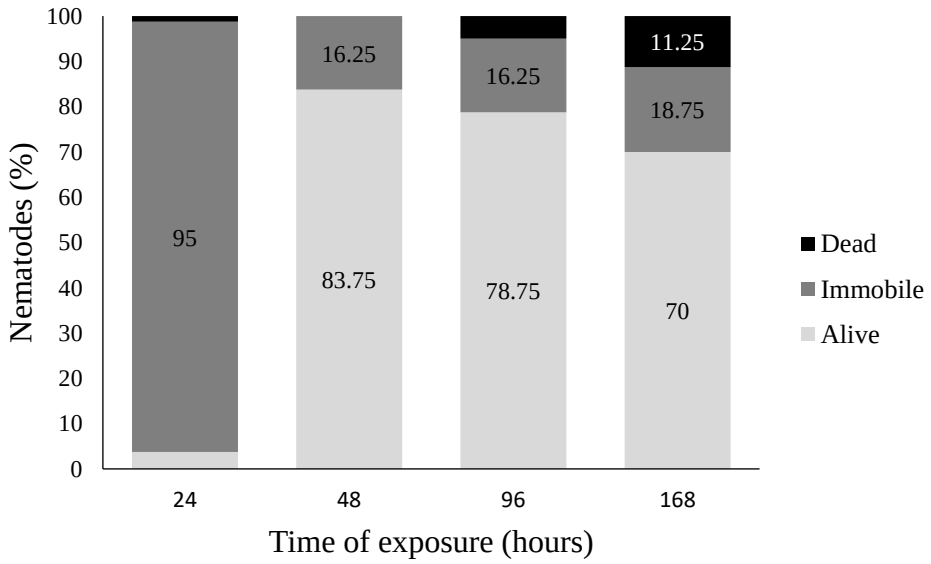


Figure 19 Effect of *Sargassum muticum* extract (0.1 g/mL) on *Meloidogyne* sp. J2 after 24, 48, 96 and 168 hours' exposure.

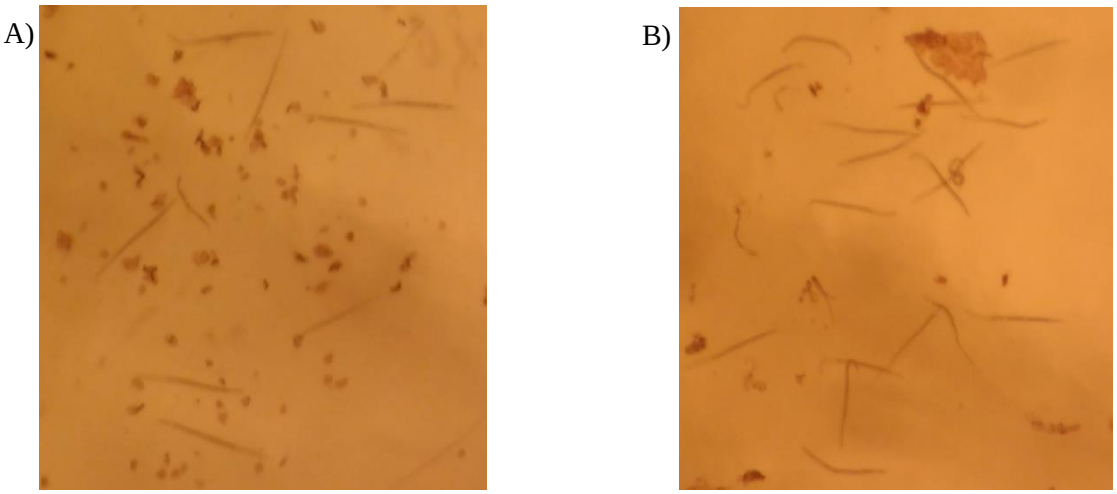


Figure 20 Effect of *Sargassum muticum*: (A) J2 of *Meloidogyne* sp. after 24 hours of exposure to *S. muticum*; (B) J2 of *Meloidogyne* sp. after 48 hours of exposure to *S. muticum*.

Effects of *Gracilaria gracilis* extract

The *Gracilaria gracilis* extract, at a concentration of 0.1 g mL^{-1}, on J2 represented in Figure 21, showed a low nematostatic effect (15%) at 24 and 48 hours, although it increased at 96 hours to 36.3%. At this time, mortality was also observed (6.3%). At 168 hours, the percentage of immobile J2 increased again to 50%. However, it was considered a weak effect, in accordance to the established scale. Mortality values did not increase.

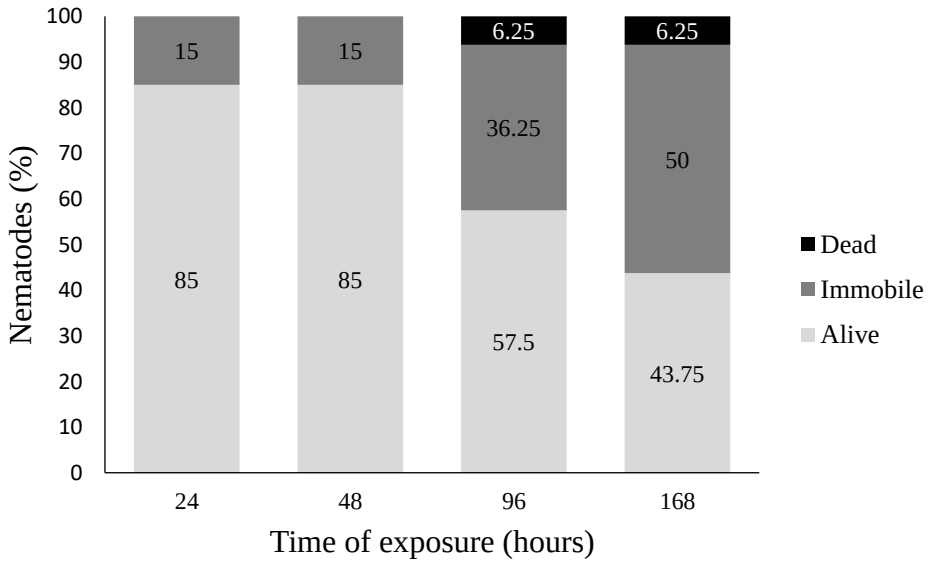


Figure 21 Effect of *Gracilaria gracilis* extract (0.1 g/mL) on *Meloidogyne* sp. J2 after 24, 48, 96 and 168 hours' exposure.

Effects of *Ulva rigida* extract

The effect of *Ulva rigida* extract on J2 at 0.1 (A) and 0.05 g mL^{-1} (B) concentrations are represented in Figure 22. A strong nematostatic effect was observed after 24 hours of exposure from the higher concentration extract of *U. rigida* (0.1 g mL^{-1}) and a strong lethal activity, 98.8%, was obtained at 48 hours, reaching 100% at 96 hours. Therefore, *U. rigida* (0.1 g mL^{-1}) extract had the same effect as the commercial nematicide, after 48 hours of exposure as mentioned before (Figs 11, 12 and 13). Figure 23A shows all the dead J2, with a straight form. Besides that, they did not recover mobility even after one hour in distilled water (Fig. 23B) and probied with a needle. Figure 23C shows all the nematodes alive, moving and with a typical sinusoidal form ("S"-shaped).

Regarding *Ulva rigida* extract, at 0.05 g mL^{-1}, in a general way, it was observed a significant lower nematostatic and nematicidal effect. At 24 hours, 10% of J2 were immobile and this nematostatic effect gradually increased until the end of the experiment (41.3%). However, a significant increase on mortality occurred from 48 hours (1.3%) to 96 hours (35%). At 168 hours, if immobility and mortality were considered together, this concentration extract was very near to be considered as having a strong effect (Fig. 22).

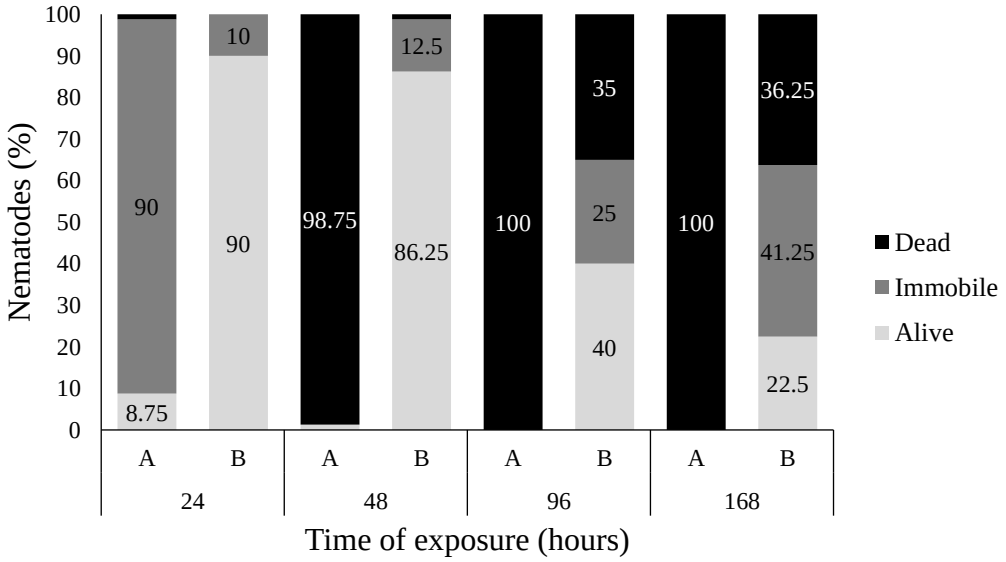
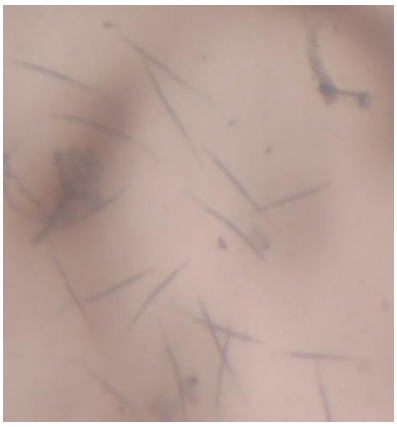
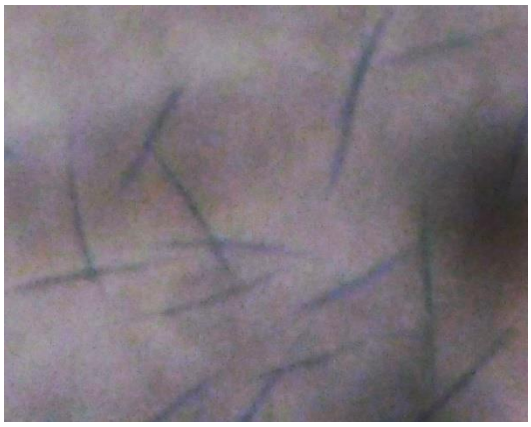


Figure 22 Effect of *Ulva rigida* extract at different concentrations (0.1 and 0.05 g/mL) on *Meloidogyne* sp. J2 after 24, 48, 96 and 168 hours' exposure, A = *U. rigida* with a concentration of 0.1 g/mL; B = *U. rigida* with a concentration of 0.05 g/mL.

A)



B)



C)

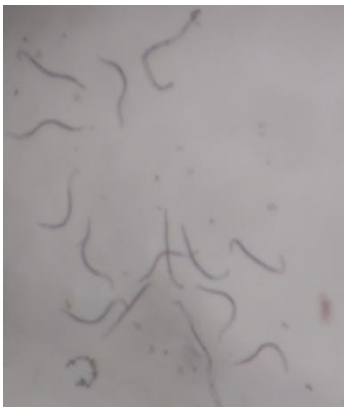


Figure 23 (A) *Meloidogyne* sp. J2 after 48 hours of exposure to *Ulva rigida* (0.1 g/mL); (B) *Meloidogyne* sp. J2, exposed 48 hours to *U. rigida* (0.1 g/mL) one hour after being transferred to distilled water; (C) *Meloidogyne* sp. J2 after 168 hours of exposure to water (negative control).

4. Discussion

After the results presentation and their direct discussion, a deeper analysis will be done subsequently.

As indicated by many other researchers (Oka *et al.*, 2000b; Chitwood, 2002; Kong *et al.*, 2006; Abbas *et al.*, 2009; Ntalli *et al.*, 2010; Abdel-Rahman *et al.*, 2013; Khan *et al.*, 2015), the results obtained in this work demonstrated that, in fact, higher plants and algae extracts could be a source of naturally occurring nematicides for *Meloidogyne* spp. control. This fact was more evident for the extracts that had the strongest *in vitro* nematicidal activity: *Ulva rigida* (0.1 g mL^{-1}), oxidized and fresh pod of *Vicia faba*. The *Acacia dealbata* (seeds) and *U. rigida* (0.05 g mL^{-1}) extracts also demonstrated a great result regarding nematicidal and nematostatic effect.

It can also be observed that, in a general way, J2 mortality increases with the increase of exposure time, that is, nematicidal effect is directly affected by duration of exposure. This finding is in accordance with other authors observations (Rizvi & Shameel, 2006; Ntalli *et al.*, 2010; Martins *et al.*, 2015).

Another issue that should be considered relevant in this study is the use of water as a solvent instead of using organic solvents. Water is an ecofriendly, sustainable, and low-cost solvent. Furthermore, it facilitates the recovery and recycling of the inorganic matters contained in biomass for eventual use as fertilizers (Caboni *et al.*, 2013).

Elbadri *et al.* (2008) reported that the extracts obtained from seeds probably cause higher mortality on RKN J2 than bark and leaf extracts from various higher plant species. Leocádio (2016) also found that *Acacia dealbata* seed extracts had stronger activity than leaves extract on *Bursaphelenchus xilophylus*. In the present study, a weak action was observed for *A. longifolia*'s seeds. After 168 hours of exposure, *A. dealbata* showed a stronger result than *A. longifolia*, as almost 64% were immobile and 22.5% were dead after 168 hours of exposure. Therefore, the majority of nematodes were affected by the extract. The observed differences on the effects of both species of *Acacia* may be a consequence of the secondary compounds present in the seeds of *A. dealbata* (Lorenzo *et al.*, 2010, 2013). Further studies are needed on the chemical composition of *A. dealbata* and *A. longifolia* seeds, in order to better understand the difference between the secondary metabolites of these seeds and to learn which of them are interfering with the physiological processes of J2 of RKN. This would be a way of helping controlling

invasive species, while simultaneously taking into account the global project to change the world.

Positive results were also obtained with oxidized and fresh pod of *Vicia faba* extracts, revealing this type of plant material to be very promising for the control of this harmful biotic agent. In both extracts almost 100% of J2 were dead or immobile, and thus unable to eat or reproduce, after 48 hours of exposure. The oxidized pod of *V. faba* after 96 and 168 hours had the same effect as the positive control (Figs. 12 and 13). Nonetheless, the oxidized plant material had a higher potential in controlling the nematodes than the fresh pod of *V. faba*. As the fresh pod has more phenolic compounds than the oxidized pod (Lee, 1992), the effect on J2 could be due to other kind of compounds or the fact that compounds present in the fresh pod extract may interact in an antagonistic way, cancelling out the potential effect of each of the isolated compounds. It should be evaluated the effect of this extract in the field since it can probably have a positive result due to its *in vitro* nematicidal effect shown before. This could improve Portugal´s economy, one of the most important producers of bean in Europe. On the other hand, converting agroindustrial wastes (pods) to raw materials, as a nematicidal source, adding value to those wastes, will promote sustainable development and circular economy (Naustdalslid, 2014; European Commission, 2014; FAO, 2017b).

Sargassum muticum did not show a strong activity to control *Meloidogyne* sp. J2, which is in agreement with the research of El-Deen & Issa (2016). Besides, the extract used in this work was even less effective. This could be due to the utilization of different species of *Meloidogyne* in each research, as highlighted by Galhano (2005) and Al-Banna *et al.*, (2003), or to the variations in chemical composition triggered by seasonal changes (Gorham & Lewey, 1984).

Although *Sargassum muticum* extract did not have an evident negative effect on this J2 RKN species, its potential effects should be taken into consideration. At 24 hours, near 100% of the nematodes were immobilized (Figs. 19 and 20A), which reveals a strong nematostatic effect in the control of *Meloidogyne* sp. Still, at 48 hours, 83.8% of the nematodes were alive (Figs. 19 and 20B). This recovery in mobility by J2 may be due to a short period of action of *S. muticum* extract, or to the ability of *Meloidogyne* sp. to adjust to the extract. It might be interesting to combine this with other extracts, such as those from *Acacia longifolia* or from *Vicia faba* pod, in order to obtain better results in nematode control. This way, by first applying the *S. muticum* extract, the majority of

nematodes would become immobile and possibly more susceptible to the effects of the mentioned above extracts, which might lead to a higher mortality as a result of the synergetic effect of the different extracts. Therefore, it may be used as a potential biopesticide in the future and, consequently, it may be a way of controlling its invasion.

Gracilaria gracilis did not show a strong activity on this RKN J2 species. Rizvi & Shameel (2006) also obtained a weak J2 mortality on *Meloidogyne javanica* with methanol extract of *G. gracilis* at a concentration of 0.1 g mL^{-1}. However, their results showed stronger activity than the observed in the present work. This could be explained by the use of different solvents, with different polarities, for the extract preparation. Khan *et al.*, (2015) studied the nematicidal activity of water and methanol extracts of 32 seaweeds against *M. javanica* J2. For each species, the methanol extract was, most of the times, more effective than the water extract, which supports the difference mentioned above. Another reason for this difference could be chemical composition differences due to different geographic locations (Spoehr & Milner, 1949).

The success of *Ulva rigida* as a potential nematicide might be due to the presence of terpenoids in its composition (Mezghani *et al.*, 2016), which are known to have nematicidal activity (Chitwood, 2002; Abdel-Rahman *et al.*, 2013; Khalil, 2014). There were significant differences in mortality between *U. rigida* at 0.1 g mL^{-1} and *U. rigida* at 0.05 g mL^{-1}, the later being less effective than the former, which infers a dose-response relationship that is also demonstrated by other authors (Pandey *et al.*, 2000; Martins *et al.*, 2015). Martins *et al.* (2015) observed that the higher concentration of carvone caused higher mortality on *Meloidogyne javanica*. Pandey *et al.*, (2000) also observed higher mortality of *Meloidogyne incognita* J2 by increasing concentration of several essential oils, such as those present in *Eucalyptus citriodora*, *Eucalyptus hybrida*, *Mentha arvensis*, *Mentha piperita*, *Mentha spicata*, *Ocimum basilicum*, *Pelargonium graveolens* and *Cymbopogon martini*.

Khan *et al.* (2015) tested *Ulva rigida* on *Meloidogyne javanica*, after 72 hours of exposure at a concentration of 0.1 g mL^{-1} achieving a 73% of J2 mortality. The present study supports this finding the macroalgal species to be used as a potential biocide, since it was found 100% mortality of for the same period. The reason for the difference in the mortality values between the two studies may be explained by the different species of *Meloidogyne* causing the disease. It is known that different species of *Meloidogyne* react differently to the same extract (Al-Banna *et al.*, 2003; Galhano, 2005). It was not possible

to identify the species used in the present work, but, to effectively compare results and assess the nematicidal effect of these extracts, this is a mandatory step. However, the effect in the genus was clearly observed.

Therefore, the *Ulva rigida* at 0.1 g mL^{-1} extract could be a safer and more target-specific alternative than conventional, broad-spectrum pesticides to control this disease (Kumar & Singh, 2015; Kamble *et al.*, 2016; US EPA, 2016). It showed a positive effect very quickly because it inhibits the ability of almost all J2 to infect plants after 24 hours of exposure and at 48 hours had the same effect of the commercial nematicide, as mentioned before. Since *U. rigida* at 0.1 g mL^{-1} extract had a very positive result in controlling RKN J2, the possibility of producing a biopesticide is higher. Therefore, this project can contribute to increasing Portugal´s economy since *U. rigida* exists in great quantity in the country.

There is some important future research that should be carried on: to identify the *Meloidogyne* species; to confirm in field the nematicidal effect of those with more promising results; to determine the mode of action of each extract; to isolate and identify active compounds and study their toxicity on several organisms commonly used in ecotoxicological pesticide effects, as well as evaluate the safety of these extracts on human health; to test the nematicidal activity extracts on other nematode species; and to ensure the development of costly competitive formulations.

5. Conclusion

Considering the emerging problems resulting from the use of synthetic chemical pesticides, it is essential to find ecofriendly alternatives for a more sustainable agriculture. The effects of *Ulva rigida*, oxidized and fresh pod of *Vicia faba* on *Meloidogyne* sp. observed in this work bring new insights for the development of a promising natural, cheap and environmentally-friendly alternative nematicides, to control the problematic RKN, which are causing significant economic losses in Portugal and all over the world. As raw material used in those extracts came from Portuguese native species of marine macroalgae and from an agroindustrial waste, this solution will certainly value Portuguese endogenous resources and boost Circular Economy, progressing towards a Sustainable Development by taking into consideration the goals number 2 and 15 established by the United Nations.

6. References

Abad, P., Favery, B., Rosso, M. N., & Castagnone-Sereno, P. (2003). Root-knot nematode parasitism and host response: Molecular basis of a sophisticated interaction. Molecular Plant Pathology, 4(4), 217–224.

Abbas, S., Dawar, S., Tariq, M., & Zaki, M. J. (2009). Nematicidal activity of spices against *Meloidogyne javanica* (Treub) Chitwood. Pakistan Journal of Botany, 41(5), 2625–2632.

Abdel-Rahman, F. H., Alaniz, N. M., & Saleh, M. A. (2013). Nematicidal activity of terpenoids. Journal of Environmental Science and Health, Part B, 48(1), 16–22.

Abrantes, I. M. D. O., Santos, M. C. V., Clara, M. I., Fernandes, J. E., & Marques, M. L. (2007). Principais doenças e pragas do tomateiro e meios de luta. Instituto do Ambiente e Vida, Coimbra, 176.

Abrantes, I. M. D. O., Santos, M. C. V. D., Conceição, I. L. P. M. D., Santos, M. S. N. D. A., & Vovlas, N. (2008). Root-knot and other plant-parasitic nematodes associated with fig trees in Portugal. Nematologia mediterranea, 36, 131–136.

Abrantes, I. M. D. O., & Santos, M. S. N. D. A. (1991). *Meloidogyne lusitanica* n. sp. (Nematoda: Meloidogynidae), a Root-knot Nematode Parasitizing Olive Tree (*Olea europaea* L.). Journal of Nematology, 23(2), 210–224.

Agarwal, S., & Rao, A. V. (2000). Tomato lycopene and its role in human health and chronic diseases. CMAJ : Canadian Medical Association Journal, 163(6), 739–44.

Agrios, G. N. (2005). Control of plant diseases. In: Plant Pathology (G. N. Agrios, ed.). New York: Academic Press, 5th ed., 293–353.

Al-Banna, L., Darwish, R. M., & Aburjai, T. (2003). Effect of plant extracts and essential oils on root-knot nematode. Phytopathologia Mediterranea, 42(2), 123–128.

Alexandratos, N., & Bruinsma, J. (2012). World agriculture towards 2030/2050: the 2012 revision. ESA Working paper No. 12-03, Agricultural Development Economics Division (ESA), Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, 94-122.

Ali, A. I.-B., Ktari, L., Boudabbous, A., & Bour, M. El. (2009). Antibacterial activity of the green alga *Ulva rigida* collected from Tunisian coast: seasonal and geographical variation. Planta Medica, 75(9), 1033-1040.

Ara, J., Sultana, V., Ehteshamul-Haque, S., Qasim, R., & Ahmad, V. U. (1999). Cytotoxic activity of marine macro-algae on *Artemia salina* (Brine shrimp). Phytotherapy Research, 13(4), 304–307.

Ara, J., Sultana, V., Qasim, R., & Ahmad, V. U. (2002). Hypolipidaemic activity of seaweed from Karachi coast. Phytotherapy Research, 16(5), 479–483.

Ara, J., Sultana, V., Qasim, R., Ehteshamul-Haque, S., & Ahmad, V. U. (2005). Biological activity of *Spatoglossum asperum*: a brown alga. Phytotherapy Research, 19(7), 618–623.

Atkinson, H. J., Urwin, P. E., & Hussey, R. S. (2009). Plant Biotechnology and Control. In: Root-knot Nematodes (R.N. Perry, M. Moens, J.L. Starr, eds.). CAB International, Wallingford, UK, 338-356.

Ayandiji, A. O. R., & Omidiji, D. A. (2011). Determinant Post Harvest Losses among Tomato Farmers in Imeko-Afon Local Government Area of Ogun State, Nigeria. Global Journal of Science Frontier Research, 11(5), 23–28.

Bais, H. P., Vepachedu, R., Gilroy, S., Callaway, R. M., & Vivanco, J. M. (2003). Allelopathy and exotic plant invasion: from molecules and genes to species interactions. Science, 301(5638), 1377–1380.

Batchelor, T. A. (2002). International and European community controls on methyl bromide & the status of methyl bromide use and alternatives in the European Community. In: Proceedings of International Conference on Alternatives to Methyl Bromide - The Remaining Challenges (T. Batchelor, J. Bolivar, eds.). European Commission, Brussels, Belgium, 37–48.

Belair, G., & Parent, L. E. (1996). Using crop rotation to control *Meloidogyne hapla* chitwood and improve marketable carrot yield. HortScience, 31(1), 106–108.

Bermúdez, Y. G., Rico, I. L. R., Bermúdez, O. G., & Guibal, E. (2011). Nickel biosorption using *Gracilaria caudata* and *Sargassum muticum*. Chemical Engineering Journal, 166(1), 122–131.

Birat, J.-P. (2015). Life-cycle assessment, resource efficiency and recycling. Metallurgical Research and Technology, 112(2), 206–220.

Bridge, J., & Starr, J. L. (2007). Plant Nematode Biology and Parasitism. In: Plant nematodes of agricultural importance: a colour handbook (J. Bridge, J. L. Starr, eds.). CRC Press, 5–15.

Caboni, P., Saba, M., Tocco, G., Casu, L., Murgia, A., Maxia, A., Menkissoglu-Spiroudi, U., & Ntalli, N. (2013). Nematicidal activity of mint aqueous extracts against the root-knot nematode *Meloidogyne incognita*. Journal of Agricultural and Food Chemistry, 61(41), 9784–9788.

Callaway, R. M., Ridenour, W. M., Laboski, T., Weir, T., & Vivanco, J. M. (2005). Natural selection for resistance to the allelopathic effects of invasive plants. Journal of Ecology, 93(3), 576–583.

Capo, T. R., Jaramillo, J. C., Boyd, A. E., Lapointe, B. E., & Serafy, J. E. (1999). Sustained high yields of *Gracilaria* (Rhodophyta) grown in intensive large-scale culture. Journal of Applied Phycology, 11(2), 143–147.

Carballeira, A., & Reigosa, M. J. (1999). Effects of natural leachates of *Acacia dealbata* Link in Galicia (NW Spain). Botanical Bulletin of Academia Sinica, 40, 87–92.

Carneiro, R. G., Mazzafera, P., & Ferraz, L. C. C. B. (1999). Carbon partitioning in soybean infected with *Meloidogyne incognita* and *M. javanica*. Journal of Nematology, 31(3), 348–355.

Casal, J. F., Reigosa, M. J., & Carballeira, A. (1985). Potential allélopathique de *Acacia dealbata* Link. Revue D'écologie et de Biologie Du Sol, 22(1), 1–12.

Celikler, S., Tas, S., Vatan, O., Ziyanok-Ayvalik, S., Yildiz, G., & Bilaloglu, R. (2009). Anti-hyperglycemic and antigenotoxic potential of *Ulva rigida* ethanolic extract in the experimental diabetes mellitus. Food and Chemical Toxicology, 47(8), 1837–1840.

Celikler, S., Yildiz, G., Vatan, O., & Bilaloglu, R. (2008). *In vitro* antigenotoxicity of *Ulva rigida* C. Agardh (Chlorophyceae) extract against induction of chromosome aberration, sister chromatid exchange and micronuclei by mutagenic agent MMC. Biomedical and Environmental Sciences, 21(6), 492–498.

Chandler, D., Davidson, G., Grant, W. P., Greaves, J., & Tatchell, G. M. (2008). Microbial biopesticides for integrated crop management: an assessment of environmental and regulatory sustainability. Trends in Food Science & Technology, 19, 275–283.

Chitwood, D. J. (1992). Nematicidal compounds from plants. In: Phytochemical Resources for Medicine and Agriculture (H. N. Nigg, D. Seigler, eds.). Plenum Press, New York, 185-204.

Chitwood, D. J. (1993). Naturally Occurring Nematicides. In: Pest Control with Enhanced Environmental Safety (S. O. Duke, J. J. Menn, J. R. Plimmer, eds.). American Chemical Society, Washington, DC, 300–315.

Chitwood, D. J. (2002). Phytochemical based strategies for nematode control. Annual Review of Phytopathology, 40(1), 221–249.

Chitwood, D. J., & Perry, R. N. (2009). Reproduction, physiology and biochemistry. In: Root-knot Nematodes (R.N. Perry, M. Moens, J.L. Starr, eds.). CABI International, Wallingford, UK, 182-200.

Conceição, I. L. P. M. D., Cunha, M. J. M. D., Feio, G., Correia, M., Santos, M. C. V. D., Abrantes, I. M. D. O., & Santos, M. S. N. D. A. (2009). Root-knot nematodes, *Meloidogyne* spp., on potato in Portugal. Nematology, 11(2), 311–313.

Conceição, I. L., Dias, A. M. C., Abrantes, I., & Cunha, M. J. M. D. (2012). Hatching effect of root exudates from *Solanum sisymbriifolium* on *Meloidogyne* spp. Revista de Ciências Agrárias, 35(2), 274–281.

Cook, R. J. (2000). Advances in plant health management in the twentieth century. Annual Review of Phytopathology, 38, 95–116.

Copping, L. G., & Menn, J. J. (2000). Biopesticides: a review of their action, applications and efficacy. Pest Management Science, 56(8), 651–676.

Cordeiro, R. S. (2016). Valorização de resíduos de vagem de fava (*Vicia faba*) e de flor de cardo (*Cynara Cardunculus*): obtenção de extractos hidroalcoólicos e sua caracterização. Instituto Politécnico de Coimbra, 3-20.

Costa, J. M., & Heuvelink, E. (2005). Introduction: the tomato crop and industry. In: Tomatoes (E. Heuvelink, ed.). CABI Publishing, Wallingford, UK, 1–19.

Coyne, D. L., Fourie, H. H., & Moens, M. (2009). Current and Future Management Strategies in Resource-poor Farming. In: Root-knot Nematodes (R.N. Perry, M. Moens, J.L. Starr, eds.). CAB International, Wallingford, UK, 444-470.

Craigie, J. S. (2011). Seaweed extract stimuli in plant science and agriculture. Journal of Applied Phycology, 23(3), 371–393.

Crouch, I. J., & Van Staden, J. (1993). Commercial seaweed products as biostimulants in horticulture. Journal of Home & Consumer Horticulture, 1(1), 19–76.

Davis, T. A., Volesky, B., & Mucci, A. (2003). A review of the biochemistry of heavy metal biosorption by brown algae. Water Research, 37(18), 4311–4330.

Dawar, S., Younus, S. M., Tariq, M., & Zaki, M. J. (2007). Use of *Eucalyptus* sp., in the control of root infecting fungi on mung bean and chick-pea. Pakistan Journal Botany, 39(3), 975–979.

Degáspari, C. H., & Waszczynskyj, N. (2004). Antioxidant properties of phenolic compounds. Visão Académica, 5(1), 33–40.

Dimetry, N. Z. (2014). Different Plant Families as Bioresource for Pesticides. In: Advances in Plant Biopesticides (D. Singh, ed.). Springer, 1–20.

Dubey, N. K., Shukla, R., Kumar, A., Singh, P., & Prakash, B. (2010). Prospects of botanical pesticides in sustainable agriculture. Current Science, 98(4), 479–480.

Echeverrigaray, S., Zacaria, J., & Beltrão, R. (2010). Nematicidal activity of monoterpenoids against the root-knot nematode *Meloidogyne incognita*. Phytopathology, 100(2), 199–203.

Eilenberg, J., Hajek, A., & Lomer, C. (2001). Suggestions for unifying the terminology in biological control. BioControl, 46(4), 387–400.

Eisenback, J. D., & Triantaphyllou, H. H. (1991). Root-knot nematodes: *Meloidogyne* species and races. In: Manual of Agricultural Nematology (W. R. Nickle, ed.). Marcell Dekker: New York, 191-274.

El-Deen, A. H. N., & Issa, A. A. (2016). Nematicidal properties of some algal aqueous extracts against root-knot nematode, *Meloidogyne incognita in vitro*. Egyptian Journal of Agronematology, 15(1), 67–78.

Elbadri, G. A., Lee, D. W., Park, J. C., Yu, H. Bin, & Choo, H. Y. (2008). Evaluation of various plant extracts for their nematicidal efficacies against juveniles of *Meloidogyne incognita*. Journal of Asia-Pacific Entomology, 11(2), 99–102.

European Commission. (2014). Questions and answers on the Commission Communication "Towards a Circular Economy" and the Waste Targets Review Accessed on 28 June 2017, in: http://europa.eu/rapid/press-release_MEMO-14-450_en.htm

FAO - Food and Agriculture Organization of the United Nations (2017a). FAOSTAT Database - Production quantities of Tomatoes by country. Accessed on 28 June 2017, in: http://www.fao.org/faostat/en/#data/QC/visualize.

FAO - Food and Agriculture Organization of the United Nations (2017b). Guidelines for the Integration of Sustainable Agriculture and Rural Development. The concept of SARD. Accessed on 28 June 2017, in: http://www.fao.org/docrep/w7541e/w7541e04.htm.

FAO - Food and Agriculture Organization of the United Nations (2017c). The future of food and agriculture – Trends and challenges. Rome. 46-55.

Febles, C. I., Arias, A., Hardisson, A., López, A. S., & Gil-Rodríguez, M. C. (1995). Antimicrobial activity of extracts from some Canary species of *Phaeophyta* and *Chlorophyta*. Phytotherapy Research, 9(5), 385–387.

Feeny, P. (1976). Plant apparency and chemical defense. In: Biochemical interaction between plants and insects (J. W. Wallace, R. L. Mansell, eds.). Springer, US, 1–40.

Ferraz, L., & Freitas, L. G. (2004). Use of antagonistic plants and natural products. Nematology–Advances and Perspectives, 2, 931–978.

Ferreira, de S. E. (2013). Vicilinas de leguminosas e seus efeitos no metabolismo lipídico de ratos dislipêmicos. Universidade Estadual Paulista “José de Mesquita Filho.”, Araraquara, SP, 17-21.

Freitas, L. G., Mitchell, D. J., & Dickson, D. W. (1997). Temperature effects on the attachment of *Pasteuria penetrans* endospores to *Meloidogyne arenaria* race 1. Journal of Nematology, 29(4), 547–555.

Galhano, C. I. C. (2005). Efeitos de plantas aráceas (*Colocasia esculenta* e *Xanthosoma sagittifolium*) sobre nemátodes-das-galhas-radiculares (*Meloidogyne javanica* e *Meloidogyne megadora*). Universidade de Coimbra, 286-306.

Gaur, H. S., & Perry, R. N. (1991). The use of soil solarization for control of plant parasitic nematodes. Nematological Abstracts, 60(4), 153–167.

Ghosh, R., Banerjee, K., & Mitra, A. (2011). Eco-Biochemical Studies of Common Seaweeds in the Lower Gangetic Delta. In: Handbook of Marine Macroalgae: Biotechnology and Applied Phycology (S.-K. Kim, ed.). John Wiley & Sons, Chichester, UK, 45–57.

González, L., Souto, X. C., & Reigosa, M. J. (1995). Allelopathic effects of *Acacia melanoxylon* R.Br. phyllodes during their decomposition. Forest Ecology and Management, 77(1), 53–63.

Gorham, J., & Lewey, S. A. (1984). Seasonal changes in the chemical composition of *Sargassum muticum*. Marine Biology, 80(1), 103–107.

Guiry, M. D., & Guiry, G. M. (2017a). AlgaeBase. Accessed on 07 July 2017, in: http://www.algaebase.org/search/species/detail/?species_id=vadcb4bdcb3cf7458&sk=0&from=results

Guiry, M. D., & Guiry, G. M. (2017b). AlgaeBase. Accessed on 07 July 2017, in: http://www.algaebase.org/search/species/detail/?species_id=cc0e1920bcaa73d8c&sk=0&from=results

Guiry, M. D., & Guiry, G. M. (2017c). AlgaeBase. Accessed on 05 July 2017, in: http://www.algaebase.org/search/species/detail/?species_id=U2543e35e22381632&sk=0&from=results

Gupta, S., & Dikshit, A. K. (2010). Biopesticides: an ecofriendly approach for pest control. Journal of Biopesticides, 3(1), 186–188.

Haydock, P. P. J., Woods, S. R., Grove, I. G., & Hare, M. C. (2006). Chemical control of nematodes. In: Plant Nematology (R. N. Perry, M. Moens, eds.). CAB International, Wallingford, UK, 392-410.

Hou, W. W., Zhang, X. J., Shi, J. B., & Liu, Y. J. (2015). Genetic diversity analysis of faba bean (*Vicia faba* L.) germplasms using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Genetics and Molecular Research, 14(4), 13945–13953.

Hussey, R. S. (1985). Host-parasite relationships and associated physiological changes. In: An Advanced Treatise on *Meloidogyne* (J. N. Sasser & C. C. Carter, eds.). Dept. of Plant Pathology. North Carolina State University, 144–153.

Hussey, R. S., & Janssen, G. J. W. (2002). Root-knot nematodes: *Meloidogyne* species. In: Plant Resistance to Parasitic Nematodes (J.L. Starr, R. Cook, & J. Bridge, eds.). New York, USA, CABI Publishing, 43-70.

INE, Instituto Nacional De Estatística (2017). Estatísticas Agrícolas 2016, Lisboa, 13-30.

Jenkins, T., Blunden, G., Wu, Y., Hankins, S. D., & Gabrielsen, B. O. (1997). Are the reductions in nematode attack on plants treated with seaweed extracts the result of stimulation of the formaldehyde cycle? Acta Biologica Hungarica, 49(4), 421–427.

Kamble, K. J., Thakor, N. J., Sonawane, S. P., & Sawant, A. A. (2016). Review on Need of Utilization of Biopesticides in Agriculture for Safe Environment. International Journal of Engineering Technology Science and Research, 3(8), 6-13.

Karssen, G., & Moens, M. (2006). Root-knot nematodes. In: Plant Nematology (R. N. Perry, M. Moens, eds.). CAB International, Wallingford, UK, 59-90.

Khalil, M. S. (2014). Bright future with nematicidal phytochemicals. Biology and Medicine, 6(2), 1-3.

Khan, S. A., Abid, M., & Hussain, F. (2015). Nematicidal activity of seaweeds against *Meloidogyne javanica*. Pakistan Journal of Nematology, 33(2), 195–203.

Khan, Z., Park, S. D., Shin, S. Y., Bae, S. G., Yeon, I. K., & Seo, Y. J. (2005). Management of *Meloidogyne incognita* on tomato by root-dip treatment in culture filtrate of the blue-green alga, *Microcoleus vaginatus*. Bioresource Technology, 96(12), 1338–1341.

Kong, J.-O., Lee, S.-M., Moon, Y.-S., Lee, S.-G., & Ahn, Y.-J. (2006). Nematicidal activity of plant essential oils against *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae). Journal of Asia-Pacific Entomology, 9(2), 173–178.

Kulkarni, J., Kapse, N., & Kulkarni, D. K. (2009). Plant based pesticide for control of *Helicoverpa armigera* on *Cucumis sativus*. Asian Agri-History, 13(4), 327–332.

Kumar, P., Selvi, S. S., & Govindaraju, M. (2013). Seaweed-mediated biosynthesis of silver nanoparticles using *Gracilaria corticata* for its antifungal activity against *Candida* spp. Applied Nanoscience, 3(6), 495–500.

Kumar, S., & Singh, A. (2015). Biopesticides: present status and the future prospects. J Fertilizers and Pesticides, 6(2), 1-2.

Kutchan, T., & Dixon, R. A. (2005). Physiology and metabolism: Secondary metabolism: nature's chemical reservoir under deconvolution. Current Opinion in Plant Biology, 8(3), 227–229.

Lann, K. L., Connan, S., & Stiger-Pouvreau, V. (2012). Phenology, TPC and size-fractioning phenolics variability in temperate Sargassaceae (Phaeophyceae, Fucales) from Western Brittany: Native versus introduced species. Marine Environmental Research, 80, 1–11.

Lann, K. L., Jégou, C., & Stiger-Pouvreau, V. (2008). Effect of different conditioning treatments on total phenolic content and antioxidant activities in two Sargassacean species: Comparison of the frondose *Sargassum muticum* (Yendo) Fensholt and the cylindrical *Bifurcaria bifurcata* R. Ross. Phycological Research, 56(4), 238–245.

Lee, C. Y. (1992). Enzymatic oxidation of phenolic compounds in fruits. In: Phenolic compounds in food and their effects on health I (C.-T. Ho, C. Y. Lee, and M.-T. Huang, eds.). American Chemical Society, Washington, 305–317.

Lehr, P. (2014). Global Markets for Biopesticides. In: BCC Research. Wellesley, USA, 2-10.

Leiro, J. M., Castro, R., Arranz, J. A., & Lamas, J. (2007). Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed *Ulva rigida* C. Agardh. International Immunopharmacology, 7(7), 879–888.

Leocádio, J. C. S. (2015). Invader vs invader: potential of *Acacia dealbata* against *Bursaphelencus xylophilus*. Instituto Politécnico de Coimbra, 22-35.

Li, J., Zou, C., Xu, J., Ji, X., Niu, X., Yang, J., Huang, X., & Zhang, K.-Q. (2015). Molecular mechanisms of nematode-nematophagous microbe interactions: basis for biological control of plant-parasitic nematodes. Annual Review of Phytopathology, 53, 67–95.

Lodeiro, P., Herrero, R., & Vicente, M. E. S. D. (2006a). Batch desorption studies and multiple sorption–regeneration cycles in a fixed-bed column for Cd (II) elimination by protonated *Sargassum muticum*. Journal of Hazardous Materials, 137(3), 1649–1655.

Lodeiro, P., Herrero, R., & Vicente, M. E. S. D. (2006b). The use of protonated *Sargassum muticum* as biosorbent for cadmium removal in a fixed-bed column. Journal of Hazardous Materials, 137(1), 244–253.

Lorenzo, P., González, L., & Reigosa, M. J. (2010). The genus *Acacia* as invader: the characteristic case of *Acacia dealbata* Link in Europe. Annals of Forest Science, 67(1), 101–111.

Lorenzo, P., Pazos-Malvido, E., González, L., & Reigosa, M. J. (2008). Allelopathic interference of invasive *Acacia dealbata*: physiological effects. Allelopathy Journal, 22(2), 452–462.

Lorenzo, P., Pereira, C. S., & Rodríguez-Echeverría, S. (2013). Differential impact on soil microbes of allelopathic compounds released by the invasive *Acacia dealbata* Link. Soil Biology and Biochemistry, 57, 156–163.

Maitre, D. C. L., Gaertner, M., Marchante, E., Ens, E., Holmes, P. M., Pauchard, A., O'Farrell, P. J., Rogers, A. M., Blanchard, R., & Blignaut, J. (2011). Impacts of invasive Australian *Acacia*s: implications for management and restoration. Diversity and Distributions, 17(5), 1015–1029.

Marchante, E., Kjøller, A., Struwe, S., & Freitas, H. (2008a). Invasive *Acacia longifolia* induce changes in the microbial catabolic diversity of sand dunes. Soil Biology and Biochemistry, 40(10), 2563–2568.

Marchante, E., Kjøller, A., Struwe, S., & Freitas, H. (2008b). Short-and long-term impacts of *Acacia longifolia* invasion on the belowground processes of a Mediterranean coastal dune ecosystem. Applied Soil Ecology, 40(2), 210–217.

Marchante, E., Kjøller, A., Struwe, S., & Freitas, H. (2009). Soil recovery after removal of the N_2-fixing invasive *Acacia longifolia*: consequences for ecosystem restoration. Biological Invasions, 11(4), 813–823.

Marchante, H., Freitas, H., & Hoffmann, J. H. (2010). Seed ecology of an invasive alien species, *Acacia longifolia* (*Faba*ceae), in Portuguese dune ecosystems. American Journal of Botany, 97(11), 1–11.

Marchante, H., Freitas, H., & Hoffmann, J. H. (2011). Post-clearing recovery of coastal dunes invaded by *Acacia longifolia*: is duration of invasion relevant for management success? Journal of Applied Ecology, 48(5), 1295–1304.

Marchante, H., Marchante, E., Freitas, H., & Hoffmann, J. H. (2015). Temporal changes in the impacts on plant communities of an invasive alien tree, *Acacia longifolia*. Plant Ecology, 216(11), 1481–1498.

Marchante, H., Morais, M. C., Gamela, A., & Marchante, E. (2017). Using a WebMapping platform to engage volunteers to collect data on invasive plants distribution. Transactions in GIS, 21(2), 238–252.

Martins, F., Costa, M., & Galhano, C. I. C. (2015). On the way for a new bionematicide. Agriculture and Food, 3, 130–136.

Mathews, J. A., & Tan, H. (2011). Progress toward a circular economy in China. Journal of Industrial Ecology, 15(3), 435–457.

Mazumder, S., Ghosal, P. K., Pujol, C. A., Carlucci, M. J., Damonte, E. B., & Ray, B. (2002). Isolation, chemical investigation and antiviral activity of polysaccharides from *Gracilaria corticata* (*Gracilaria*ceae, Rhodophyta). International Journal of Biological Macromolecules, 31(1), 87–95.

McSorley, R. (1998). Alternative practices for managing plant-parasitic nematodes. American Journal of Alternative Agriculture, 13(3), 98–104.

Melakeberhan, H., Xu, A., Kravchenko, A., Mennan, S., & Riga, E. (2006). Potential use of arugula (*Eruca sativa* L.) as a trap crop for *Meloidogyne hapla*. Nematology, 8(5), 793–799.

Melander, B., & Jørgensen, M. H. (2005). Soil steaming to reduce intrarow weed seedling emergence. Weed Research, 45(3), 202–211.

Mezghani, S., Csupor, D., Bourguiba, I., Hohmann, J., Amri, M., & Bouaziz, M. (2016). Characterization of phenolic compounds of *Ulva rigida* (Chlorophycae) and its antioxidant activity. European Journal of Medicinal Plants, 12(1), 1-9.

Milledge, J. J., Nielsen, B. V., & Bailey, D. (2016). High-value products from macroalgae: the potential uses of the invasive brown seaweed, *Sargassum muticum*. Reviews in Environmental Science and Biotechnology, 15(1), 67–88.

Moens, M., Perry, R. N., & Starr, J. L. (2009). *Meloidogyne* species – a diverse group of novel and important plant parasites. In: Root-knot Nematodes (R.N. Perry, M. Moens, J.L. Starr, eds.). CAB International, Wallingford, UK, 1-17.

Moshe, A., Pfannstiel, J., Brotman, Y., Kolot, M., Sobol, I., Czosnek, H., & Gorovits, R. (2012). Stress responses to *Tomato yellow leaf curl virus* (TYLCV) infection of resistant and susceptible tomato plants are different. Metabolomics, 1, 1–13.

Natário, M. M., Braga, A. M., & Rei, C. (2016). A valorização dos recursos endógenos no desenvolvimentos dos Territórios Rurais. Pluris, 1-12.

Naustdalslid, J. (2014). Circular economy in China – the environmental dimension of the harmonious society. International Journal of Sustainable Development and World Ecology, 21(4), 303–313.

Ness, D. (2008). Sustainable urban infrastructure in China: Towards a Factor 10 improvement in resource productivity through integrated infrastructure systems. International Journal of Sustainable Development and World Ecology, 15(4), 288–301.

Ntalli, N. G., Ferrari, F., Giannakou, I., & Menkissoglu-Spiroudi, U. (2010). Phytochemistry and nematicidal activity of the essential oils from 8 Greek Lamiaceae aromatic plants and 13 terpene components. Journal of Agricultural and Food Chemistry, 58(13), 7856–7863.

Nyczepir, A. P., & Thomas, S. H. (2009). Current and future management strategies in intensive crop production systems. In: Root-knot Nematodes (R.N. Perry, M. Moens, J.L. Starr, eds.). CAB International, Wallingford, UK, 412-443.

OECD, Organization for Economic Co-operation and Development (2015). Biological Pesticides. Accessed on 31 June 2017, in:http://www.oecd.org/chemicalsafety/pesticides-biocides/biological-pesticides.htm.

Oka, Y., Koltai, H., Bar-Eyal, M., Mor, M., Sharon, E., Chet, I., & Spiegel, Y. (2000a). New strategies for the control of plant-parasitic nematodes. Pest Management Science, 56(11), 983–988.

Oka, Y., Nacar, S., Putievsky, E., Ravid, U., Yaniv, Z., & Spiegel, Y. (2000b). Nematicidal activity of essential oils and their components against the root-knot nematode. Phytopathology, 90, 710–715.

Pandey, R., Kalra, A., Tandon, S., Mehrotra, N., Singh, H. N., & Kumar, S. (2000). Essential oils as potent sources of nematicidal compounds. Journal of Phytopathology, 148(7–8), 501–502.

Plouguerne, E., Hellio, C., Deslandes, E., Véron, B., & Stiger-Pouvreau, V. (2008). Anti-microfouling activities in extracts of two invasive algae: *Grateloupia turuturu* and *Sargassum muticum*. Botanica Marina, 51(3), 202–208.

Popp, J., Pető, K., & Nagy, J. (2013). Pesticide productivity and food security. A review. Agronomy for Sustainable Development, 33(1), 243–255.

Prakash, A., Rao, J., Berliner, J., Pokhare, S. S., Adak, T., & Saikia, K. (2014). Botanical pesticides for the management of plant nematode and mite pests. In: Advances in Plant Biopesticides (D. Singh, ed.). Springer, India, 89–118.

Pullman, G. S., DeVay, J. E., & Garber, R. H. (1981). Soil solarization and thermal death: a logarithmic relationship between time and temperature for four soilborne plant pathogens. *Phytopathology*, *71*(9), 959–964.

Püntener, W. (1981). Manual for field trials in plant protection. Ciba-Geigy, Basle, Switzerland, 2th ed., 205.

Pyšek, P., Jarošík, V., Hulme, P. E., Pergl, J., Hejda, M., Schaffner, U., & Vilà, M. (2012). A global assessment of invasive plant impacts on resident species, communities and ecosystems: the interaction of impact measures, invading species' traits and environment. Global Change Biology, 18(5), 1725–1737.

Raiola, A., Rigano, M. M., Calafiore, R., Frusciante, L., & Barone, A. (2014). Enhancing the health-promoting effects of tomato fruit for biofortified food. Mediators of Inflammation, 1–16.

Rao, A. V., & Rao, L. G. (2007). Carotenoids and human health. Pharmacological Research, 55(3), 207–216.

Riegel, C., & Noe, J. P. (2000). Chicken litter soil amendment effects on soilborne microbes and *Meloidogyne incognita* on cotton. Plant Disease, 84(12), 1275–1281.

Rizvi, M. A., & Shameel, M. (2006). *In vitro* nematicidal activities of seaweed extracts from Karachi coast. Pakistan Journal of Botany, 38(4), 1245–1248.

Ruiz-Suárez, N., Boada, L. D., Henríquez-Hernández, L. A., González-Moreo, F., Suárez-Pérez, A., Camacho, M., Zumbado, M., Almeida-González, M., del Mar

Travieso-Aja, M., & Luzardo, O. P. (2015). Continued implication of the banned pesticides carbofuran and aldicarb in the poisoning of domestic and wild animals of the Canary Islands (Spain). Science of the Total Environment, 505, 1093–1099.

Ruqqia, K., Sultana, V., Ara, J., Ehteshamul-Haque, S., & Athar, M. (2015). Hypolipidaemic potential of seaweeds in normal, triton-induced and high-fat diet-induced hyperlipidaemic rats. Journal of Applied Phycology, 27(1), 571–579.

Sabina, H., Tasneem, S., Samreen, K. Y., Choudhary, M. I., & Aliya, R. (2005). Antileishmanial activity in the crude extract of various seaweed from the coast of Karachi, Pakistan. Pakistan Journal of Botany, 37(1), 163–168.

Sarwar, M. (2015). Information on Activities Regarding Biochemical Pesticides: An Ecological Friendly Plant Protection against Insects. International Journal of Engineering and Advanced Research Technology, 1(2), 27–31.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D. R., & Dean, D. H. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiology and Molecular Biology Reviews, 62(3), 775–806.

Schwarz, D., Thompson, A. J., & Kläring, H.-P. (2014). Guidelines to use tomato in experiments with a controlled environment. Frontiers in Plant Science, 5, 625.

Sijmons, P. C., Atkinson, H. J., & Wyss, U. (1994). Parasitic strategies of root nematodes and associated host cell responses. Annual Review of Phytopathology, 32(1), 235–259.

Sikora, R. A., Bridge, J., & Starr, J. L. (2005). Management practices: an overview of integrated nematode management technologies. In: Plant parasitic nematodes in subtropical and tropical agriculture (M. Luc, R. A. Sikora, J. Bridge, eds.). CAB International, Wallingford, 751–760.

Sikora, R. A., & Fernandez, E. (2005). Nematode parasites of vegetables. In: Plant parasitic nematodes in subtropical and tropical agriculture (M. Luc, R. A. Sikora, J. Bridge, eds.). CAB International, Wallingford, 319-392.

Simberloff, D., Martin, J.-L., Genovesi, P., Maris, V., Wardle, D. A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., & Pascal, M. (2013). Impacts of biological invasions: what's what and the way forward. Trends in Ecology & Evolution, 28, 58–66.

Smith, S. (2016). Managing Tomato Diseases in Arkansas. Agriculture and Natural Resources, University of Arkansas System. Accessed on 15 June 2017, in: https://www.uaex.edu/publications/pdf/FSA-7568.pdf.

Souza, B. W. S., Cerqueira, M. A., Bourbon, A. I., Pinheiro, A. C., Martins, J. T., Teixeira, J. A., Coimbra, M. A., & Vicente, A. A. (2012). Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed *Gracilaria birdiae*. Food Hydrocolloids, 27(2), 287–292.

Spoehr, H. A., & Milner, H. W. (1949). The chemical composition of Chlorella; effect of environmental conditions. Plant Physiology, 24(1), 120-150.

Stamp, N. (2003). Out of the quagmire of plant defense hypotheses. The Quarterly Review of Biology, 78(1), 23–55.

Taboada, C., Millán, R., & Míguez, I. (2010). Composition, nutritional aspects and effect on serum parameters of marine algae *Ulva rigida*. Journal of the Science of Food and Agriculture, 90(3), 445–449.

Thakore, Y. (2006). The biopesticide market for global agricultural use. Industrial Biotechnology, 2(3), 194–208.

Thirumaran, G., Arumugam, M., Arumugam, R., & Anantharaman, P. (2009). Effect of seaweed liquid fertilizer on growth and pigment concentration of *Abelmoschus esculentus* (l) medikus. American-Eurasian Journal of Agronomy, 2(2), 57–66.

Thomas, C., & Cottage, A. (2006). Genetic engineering for resistance. In: Plant Nematology (R. N. Perry, M. Moens, eds.). CAB International, Wallingford, UK, 255-272.

Timper, P., Davis, R. F., & Tillman, P. G. (2006). Reproduction of *Meloidogyne incognita* on winter cover crops used in cotton production. Journal of Nematology, 38(1), 83–89.

Topal, N., & Bozoğlu, H. (2016). Determination of L-Dopa (L-3, 4-dihydroxyphenylalanine) content of some faba bean (*Vicia faba* L.) Genotypes. Journal of Agricultural Sciences, 22(2), 145–151.

Trigui, M., Gasmi, L., Zouari, I., & Tounsi, S. (2013). Seasonal variation in phenolic composition, antibacterial and antioxidant activities of *Ulva rigida* (Chlorophyta)

and assessment of antiacetylcholinesterase potential. Journal of Applied Phycology, 25(1), 319–328.

Tuney, İ., Cadirci, B. H., Ünal, D., & Sukatar, A. (2006). Antimicrobial activities of the extracts of marine algae from the coast of Urla (Izmir, Turkey). Turkish Journal of Biology, 30(3), 171–175.

UN, United Nations (2015). World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. Department of Economic and Social Affairs. New York, USA, 1-2

UN, United Nations (2016a). Agenda 2030 de Desenvolvimento Sustentável. Accessed on 30 July 2017, in: http://www.instituto-camoes.pt/images/ods_2edicao_web_pages.pdf

UN, United Nations (2016b). Sustainable Development. Accessed on 30 July 2017, in: https://sustainabledevelopment.un.org/sdg2

UNEP, United Nations Environment Programme (2010). Ridding the world of POPs. A Guide for the Stockholm Convention on Persistent Organic Pollutants. The Secretariat of the Stockholm Convention and UNEP's Information Unit for Conventions, 1-10.

US EPA, United States Environmental Protection Agency (2016). What are Biopesticides? Accessed on 30 June 2017, in: https://www.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides.

US EPA, United States Environmental Protection Agency (2017). Biopesticides. Accessed on 30 June 2017, in: https://www.epa.gov/pesticides/biopesticides.

Vanholme, B., Meutter, J. D., Tytgat, T., Montagu, M. V., Coomans, A., & Gheysen, G. (2004). Secretions of plant-parasitic nematodes: a molecular update. Gene, 332, 13–27.

Viaene, N. M., & Abawi, G. S. (1998). Management of *Meloidogyne hapla* on lettuce in organic soil with sudangrass as a cover crop. Plant Disease, 82(8), 945–952.

Wang, K.-H., & McSorley, R. (2008). Exposure time to lethal temperatures for *Meloidogyne incognita* suppression and its implication for soil solarization. Journal of Nematology, 40(1), 7–12.

Wang, S., Ye, X., Chen, J., & Rao, P. (2012). A novel chitinase isolated from *Vicia faba* and its antifungal activity. Food Research International, 45(1), 116–122.

Wright, D. J. (1981). Nematicides: mode of action and new approaches to chemical control. In: Plant parasitic nematodes (B. M. Zuckerman, R. A. Rohde, eds.). Academic Press, New York, 421–449.

Wu, Y., Jenkins, T., Blunden, G., Whapham, C., & Hankins, S. D. (1997). The role of betaines in alkaline extracts of *Ascophyllum nodosum* in the reduction of *Meloidogyne javanica* and *M. incognita* infestations of tomato plants. Fundamental and Applied Nematology, 20(2), 99–102.

Yildiz, G., Celikler, S., Vatan, O., & Dere, S. (2012). Determination of the anti-oxidative capacity and bioactive compounds in green seaweed *Ulva rigida* C. Agardh. International Journal of Food Properties, 15(6), 1182–1189.

Zaki, M. H., Moran, D., & Harris, D. (1982). Pesticides in groundwater: the aldicarb story in Suffolk County, NY. American Journal of Public Health, 72(12), 1391–1395.

Zar, J. H. (1996). Biostatistical analysis, third edition. Prentice Hall International, New Jersey, USA, 328-360.

Zhao, B.-G. (1999). Nematicidal activity of quinolizidine alkaloids and the functional group pairs in their molecular structure. Journal of Chemical Ecology, 25(10), 2205–2214.