Nematicidal activity of *Solanum nigrum* and *S. sisymbriifolium* extracts against the root-lesion nematode *Pratylenchus goodeyi* and its effects on infection and gene expression

DOCTORAL THESIS

Margarida Cristina Camacho Pestana Correia
DOCTORATE IN BIOLOGICAL SCIENCES

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“In short, if all the matter in the universe except the nematodes were swept away, our world would still be dimly recognizable, and if, as disembodied spirits, we could then investigate it, we should find its mountains, hills, vales, rivers, lakes, and oceans represented by a film of nematodes.”

(Cobb, 1915)

To my Mother

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Contents

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List of abbreviations

Acet, acetone
AH, after hydrolysis
BH, before hydrolysis
BLAST, Basic Local Alignment Search Tool
CBS, Center for Biological sequence analysis
CRT, calreticulin
DMC, dichloromethane
EBI, European Bioinformatics Institute
ENG, beta-1,4-endoglucanase
ER, endoplasmatic reticulum
EtOH, etanol
FA, fatty acids
GC-MS, chromatography-mass spectrometry
LCAA, long-chain aliphatic alcohols;
NCBI, National Center for Biotechnology Information
ORF, open reading frame
Pg, *Pratylenchus goodeyi*
RACE, Rapid amplification of cDNA ends
Rp, uninfected banana roots from plants grown in a pot
RPg, banana roots infected with *P. goodeyi*
RT retention time
Rv, uninfected banana roots from *in vitro* culture
SMART, Simple Modular Architecture Research Tool
SN, *Solanum nigrum*
SS, *Solanum sisymbriifolium*
TMS, trimethylsilyl ethers and esters
TRAPδ, translocon associated protein delta subunit
UTR, untranslated region
UHPLC, ultra-high performance liquid chromatography

Outline of this thesis

Since nematicides used to control plant-parasitic nematodes are not effective enough to justify its application, besides being toxic to environment and animal life, search for plant extracts that could be efficient to control nematodes was the starting point of the present study. The main purpose of this doctoral thesis was to evaluate the nematicidal potential of two *Solanum* species (*S. nigrum* L. and *S. sisymbriifolium* Lam.) against the plant-parasitic nematode *Pratylenchus goodeyi* Sher & Allen 1953, an important parasite of banana roots in Madeira Island. Within this subject mobility and mortality was assessed and the possible effect analysed at behavioural and molecular level using effector genes related to parasitism or stress. In Chapter 1 a general introduction to the subject is given with the background on the main issues of the thesis. Chapter 2 describes all materials and methods used in this study. The nematicide properties of *S. nigrum* and *S. sisymbriifolium* as control agents against *P. goodeyi* were investigated through sequential extracts obtained from dried plants, and are reported in Chapter 3. In Chapter 4 the *in vitro* evaluation of the nematicide potential from *S. nigrum* and *S. sisymbriifolium* against *P. goodeyi* was made using sequential and aqueous extracts obtained from dried and fresh plants. The chemical profile concerning lipophilic and phenolic compounds present in *S. nigrum* and *S. sisymbriifolium* is given in Chapter 5. In the following chapters the molecular characterization and cloning of *calreticulin, beta-1,4-endoglucanase* (Chapter 6) and *translocon associated protein delta subunit* (Chapter 7) genes from *P. goodeyi* are reported. The genes relative expression and nematode behaviour under chemical stress are also assessed. Chapter 8 summarizes and discusses the data presented in foregoing chapters.

Abstract

The control of *Pratylenchus goodeyi* a common nematode parasite of banana crop in Madeira Island can benefit from searching for natural nematicides through plants extracts. With this aim we submitted *Solanum nigrum* and *S. sisymbriifolium* dried plants to a sequential extraction in the solvent sequence of dichloromethane, acetone, ethanol and water, and to an aqueous extraction of the fresh and dried plants. Analyses with the extracts at several concentrations were used to assess mobility and mortality on *P. goodeyi*. Results showed that the water extract and aqueous extracts from both plants at a concentration of 10 mg/mL affected nematode mobility and caused mortality but the acetone extract from *S. nigrum* was the most efficient, causing 100% mortality whereas dichloromethane had no effect on *P. goodeyi*. Determination of the lipophilic and phenolic compounds present in the two most effective *Solanum* extracts (acetone and water) and in dichloromethane extract revealed that some of these compounds had nematicidal activity. *S. nigrum* acetone extract (10 mg/mL) was used to find out the nematicidal potential following the effect at gene expression level and nematode behaviour. Genes coding for *calreticulin* and *beta-1,4-endoglucanase* related to parasitism and *translocon-associated protein* putatively connected to stress were obtained and its relative expression assessed in nematodes exposed to the extract. Results revealed that expression of *Pg-CRT* decreased showing to influence the infection, *Pg-ENG* remained steady and *Pg-TRAPδ* was induced over time exposure. Biological assays showed that *P. goodeyi* mobility and ability to infect the banana roots were affected as a decrease in the number of nematodes that reached the roots was obtained with the increased exposure time to the extract being implicated in the infection success. The information obtained from this thesis showed that *S. nigrum* has potential to be used for the development of a new control strategy against plant-parasitic nematodes.

Keywords: Nematicidal activity, *P. goodeyi*, *Solanum* extracts, gene expression, chemical compounds, infection, parasitism

Resumo

O controlo do nemátode *Pratylenchus goodeyi* parasita da bananeira, comum na Ilha da Madeira, pode beneficiar com a utilização de extratos naturais obtidos das plantas. Com este intuito duas plantas de S*olanum* (S. *nigrum* e *S. sisymbriifolium*) foram submetidas a uma extração sequencial utilizando a sequência de solventes: diclorometano; acetona; etanol e água e, ainda, a uma extração aquosa das plantas frescas ou secas. Foram efectuadas análises destes extratos, em várias concentrações, de modo a testar o efeito sobre a mobilidade e mortalidade de *P. goodeyi*. Os resultados mostraram que o extrato em água da sequência e os extratos aquosos das duas plantas na concentração de 10 mg/mL afetaram a mobilidade e causaram mortalidade do nemátode. O extrato de acetona de *S. nigrum* foi o mais eficaz causando 100% de mortalidade mas o extrato de diclorometano não afetou nem a mobilidade de *P. goodeyi* nem causou mortalidade. A determinação dos compostos lipofílicos e fenólicos presentes no extrato de diclorometano e nos extratos de acetona e água das plantas de *Solanum* revelou a presença de compostos com atividade nematicida conhecida. O extrato de acetona de *S. nigrum* (10 mg/mL) foi usado para avaliar o efeito nematicida ao nível da expressão de genes e no comportamento do nematode. Os genes *calreticulina* e *beta-1,4-endoglucanase* associados com o parasitismo e *translocon-associated protein* relacionado com o stress foram isolados e a expressão relativa avaliada em nematodes expostos ao extrato de acetona. Os resultados revelaram que a expressão de *Pg-CRT* diminuiu mostrando que pode influenciar a infeção e a de *Pg-ENG* manteve-se constante. Mas, a expressão de *Pg-TRAPδ* aumentou com o tempo de exposição. Ensaios biológicos posteriores demostraram que a mobilidade e a capacidade de *P. goodeyi* infetar a raiz de bananeira foram afetadas porque o número de nematodes que conseguiu atingir e penetrar na raiz diminuiu com a exposição ao extrato, influenciando o sucesso da infeção. Os resultados desta investigação indicaram que *S. nigrum* tem potencial para ser usada como uma nova estratégia de controlo dos nemátodes fitoparasitas.

Palavras-chave: Atividade nematicida, *P. goodeyi*, extratos de *Solanum*, expressão de genes, composição quimica, infeção, parasitismo

Chapter 1

General Introduction

1. General Introduction

The search for environmentally-friendly alternatives to control plant-parasitic nematodes that contribute to reduce the dependence on chemical nematicides is highly important not only to banana culture in Madeira agriculture, but also to other cultures worldwide. Recently, the demand for plants with nematicidal properties or that can be antagonistic to nematodes used as a soil amendment increased significantly (Ntalli & Caboni 2012)

Previous research (Pestana 2007) revealed that *Solanum nigrum* L. and *S. sisymbriifolium* Lam. plants are not hosts of *Pratylenchus goodeyi* Sher & Allen 1953. Besides that, its incorporation in the soil improved banana plantations, regarding to plant growth and reduction on the populations of root-lesion nematode which were affected due to the release of exudates with nematostatic or nematicides properties. Based on the hypothesis that these species have phytochemical compounds which influenced *P. goodeyi* populations we decided to investigate what effects they might cause on the nematodes and if they can be used to control plant-parasitic nematodes.

1.1 The banana culture

Banana plant was introduced in Madeira Island between the 16th and 17th centuries, maybe as a botanic curiosity (Silva & Meneses 1978). But, the real expansion and importance of banana culture for the development of agriculture in Madeira only occurred in the beginning of the 20th century. Because this culture is relatively easy to maintain, undemanding and conducted outdoors, it quickly spread on the Island assuming a great importance for the local economy (Ribeiro & Silva 1998).

Banana plantations from the species *Musa acuminata* Colla are carried out mainly in the southern part of the Island from sea level up to 300-400 m altitude and involve many human resources. This is the most important permanent crop with an estimated production of 16174 t, of which 12700 t was exported in 2013 (DREM 2014). Nevertheless, this culture has faced great difficulties due to external constraints related to the production of banana worldwide and to internal limitations such as the rough orography of the Island, the small size and difficult access to cultivated private properties. These factors contributed to the abandonment of cultivated fields and consequently to the quick decline of this culture. In order to continue the renewal of the local production of banana, it is urgent to find other options to conventional production based on chemical products, which can contribute to a more profitable

production, balanced to the consumer and safer. With these goals in mind, since 1996 the Government Services are fully committed to support the development and production of organic banana. The differences in the organic production are related to fertilization level and control of pests and diseases through the adoption of plant health preventive strategies such as installation of a drip irrigation system, weed control and promotion of biodiversity (Silva & Guerreiro 2010).

In Madeira Island some serious damages in banana plantations are caused by insects and mites like *Thrips exilicornis* Hood and *Tetranichus urticae* Koch which attack the fruit and feed on their skin, thus reducing fruit quality. The banana-weevil *Cosmopolites sordidus* Germar is another severe problem. The larvae bore into the plant underground stem, weakening the plant and its root system, causing its death in cases of heavy infestation (Aguiar 1999). The Panama disease or banana wilt caused by the fungus *Fusarium oxysporum* f. sp. *cubense* (E. F. Sm) Snyder and Hans. is also very common, although restricted to Funchal (Rodrigues & Sardinha 1999). Nematodes from the genera *Helicotylenchus*, *Pratylenchus*, *Rotylenchulus* and *Meloidogyne* are widespread, although prevailing the first two; mixtures of species from these genera often occur originating severe problems in banana plantations as toppling, decreasing the production and fruit quality (Pestana & Cravo 1999).

1.2 The root-lesion nematodes

The root-lesion nematodes *Pratylenchus* spp. are obligate root endoparasites of many crop plants, distributed worldwide are among those with the greatest impact on crops (Jones *et al*. 2013). There is a wide range of host plants for the most important root-lesion nematode species: *P. coffeae* (Zimmerman 1898) Filipjev & Schuurmans Stekhoven 1941; *P. neglectus* (Rensch 1924) Filipjev & Schuurmans Stekhoven 1941; *P. penetrans* (Cobb 1917) Filipjev & Schuurmans Stekhoven 1941; *P. thornei* Sher & Allen 1953 and *P. vulnus* Allen & Jensen 1951. However, among the root-lesion nematodes, associated with banana culture, the species *P. goodeyi* and *P. coffeae* are recognized as damaging pathogens, the former being the most harmful to banana (Gowen & Quénérhervé 1990). *P. goodeyi* was initially detected in banana roots in Grenada (Cobb 1919) and later in Canary Islands, (De Guiran & Vilardebo 1962), then in Kenya, Tanzania, Cameroon, Greece, and Madeira Island (Gichure & Ondieki 1977; Walker *et al*. 1983; Gowen & Quénérhervé 1990; Waudo *et al*. 1990; Sakwe & Geraert 1994; Vovlas *et al*. 1994; Prasad *et al*. 1995; Troccoli *et al*. 1996).

Typically of the genus, *P. goodeyi* is a slender nematode with roughly 0.5 mm

long and a diameter of 20 µm, an annulated lip region, a strong stylet with large basal knobs and an oesophagus that overlaps the intestine ventrally. Females of this species have a posterior vulva position at 73-75% and the males with paired slender spicules and usually with the bursa enveloping the tail (Fig. 1.1). The tail is conoid with a small irregular peg which is a distinguishing feature of this species (Machon & Hunt 1985; Loof 1991). Nonetheless, the identification of *P. goodeyi* based on morphological characters is complex because they are difficult to detect and also due to a high intraspecific variability. Furthermore, biochemical and molecular characters are not well established to this species (De Waele & Elsen 2002) and the identification is still being done on the basis of morphological characters.

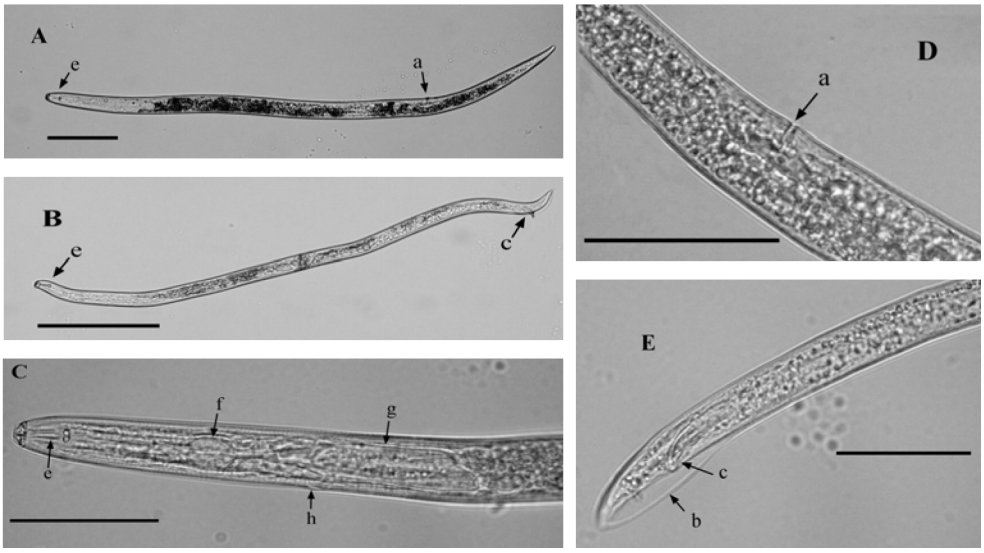

Figure 1.1 Morphological characters of *P. goodeyi*. Female (A) and male (B). Stylet with large basal knobs and oesophagus (C), vulva position in female (D), spicules and bursa enveloping the male tail (E). Vulva a, bursa b, spicules c, stylet e, median bulb f and oesophageal glands of the oesophagus g and excretory pore h. Scale bar (A and B) 80 µm; (C, D and E) 45 µm.

P. goodeyi can be found in roots, rhizomes, tubers and in the host pseudostem. After penetrating the roots, they multiply rapidly reaching 1000 to 3500 specimens per gram of root. All life stages are considered to be infective, capable to enter and leave the root tissues. The life cycle is completed within the root in 24-30 days at 24-25 ºC, thus several generations may develop during one growing season (Gowen & Quénérhervé 1990). *P. goodeyi* are also found in the rhizosphere where they can survive for some time and search for new roots to infect (Machon & Hunt 1985). Females produce eggs and the first moult occurs in the egg as a first-stage juvenile (J1). After hatching as second-stage juvenile (J2), these nematodes will try to localize roots from a susceptible host and following root penetration they complete the life cycle moulting to J3, J4 and adults (female or male). The feeding of migratory endoparasitic nematodes in the cortical tissue destroys the roots

and their functions become severely impaired. Roots infected by *P. goodeyi* have small brownish-red elongated lesions that tend to enlarge and coalesce, causing an extensive root necrosis (Gowen & Quénérhervé 1990; Loof 1991) (Fig. 1.2).

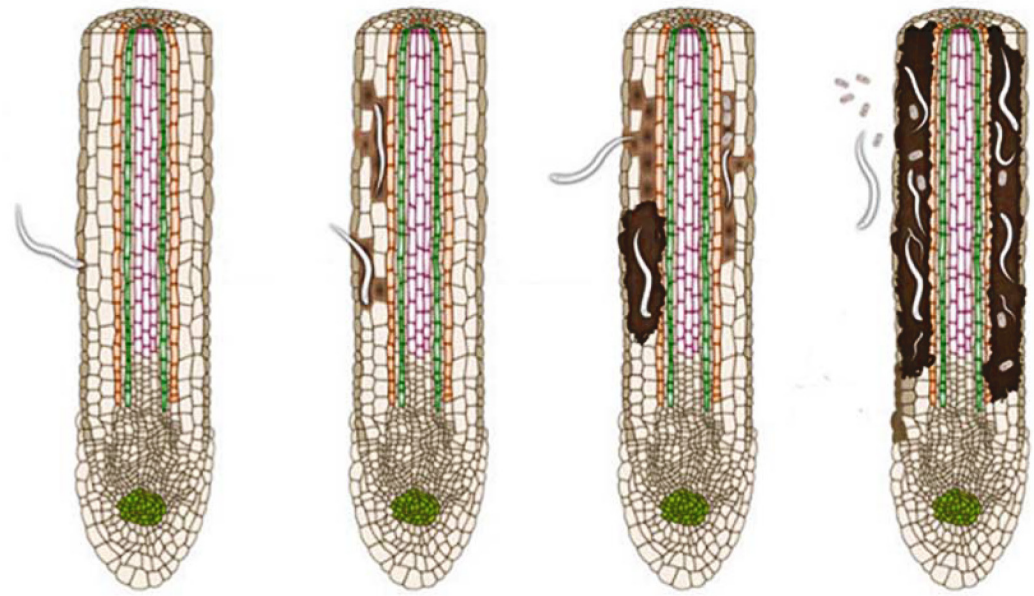

Figure 1.2 Drawing representing infection by the root-lesion nematode *P. goodeyi.* Nematode penetration, migration, feeding and root lesion development (Adapted from Jones & Fosu-Nyarko 2014).

Symptoms of damage are identical to those observed with other plant-parasitic nematodes as *Helicotylenchus multicinctus*, *P. coffeae* or *Radopholus similis* and may include: stunting of plants; lengthening of the vegetative cycle; reduction in leaf size and number; reduction in bunch weight, decrease in the plantation productive life and toppling. The interactions between *P. goodeyi* and other nematodes as *Helicotylenchus, Rotylenchulus* and *Meloidogyne* or *P. goodeyi* and other organisms as *F. oxysporum* or *C. sordidus* are common in Madeira potentiating symptoms and affecting severely the banana production (Pestana & Cravo 1999).

1.3 Plant nematode control

The control of diseases and pests is based on the prevention of loss of yield and quality from vulnerable crops and, at long-term, keeping microorganism populations at levels that do not damage crops. Ideally, the measures to control nematodes must preferably be preventive. When the plant is parasitized it is not easy to kill nematodes without destroying the plant and as it is nearly impossible to eradicate nematodes.

The management of nematode populations has relied on the use, by farmers all over the World, of expensive and toxic fumigant products (chemical nematicides) such as 1,3-dichloropropene, aldicarb, dazomet, emamectin, oxamyl, fenamiphos and metam-sodium among others (Whitehead 2002). These products are harmful to the environment, animals and humans and some of them have active substances which may affect hormones, causing a decrease in fertility, mutagenesis and carcinogenesis. Moreover, its use does not mean a suppression of nematodes and the cost of using them repeatedly is not economically viable for a sustainable agriculture. Nevertheless, chemical treatments are still used for the management of the plant-parasitic nematodes, maintaining the populations at low levels despite all the intrinsic hazards. In fact, the wide distribution and widespread application of chemicals on economically important crops made chemical nematicides detectable in ecosystems, aquifers and in other water systems of most agricultural areas (Ibrahim *et al*. 2006). These facts have led to the prohibition and restriction of application of such products in many countries. Legislation in European Union is continually being actualized in order to prevent and to prohibit the use of some chemical nematicides as aldicarb and 1,3-dichloropropene (EC Directive 2007/619/EC).

Further measures can be applied to control nematodes but they are effective only when integrated with one or more practices such as: i) organic correction that stimulates the proliferation of beneficial microorganisms or release toxins which accelerate the decomposition of soil promoting natural equilibrium (Badra *el al*. 1979; Bradow 1991); ii) biological control using natural enemies including fungi, bacteria and predacious nematodes; iii) culture rotations; iv) trap crops and v) aqueous plant extracts.

1.3.1 The nematicide potential of plants

Many studies have reported that plants produce compounds that can kill or keep away harmful organisms destroying their life cycles and acting as an attractive or repellent (Ibrahim *et al*. 2006). These compounds can be simply organized in two major groups, one containing primary metabolites as carbohydrates, lipids, amino acids and the other including secondary metabolites that are produced from the primary metabolites and are responsible for toxic effects (Valette *et al*. 1998).

Since plants are capable of producing a large variety of secondary metabolites with multiple applications, much research has been conducted to discover compounds in plant tissues that have an effect on nematodes (Chitwood 2002). Histochemical and ultrastructural studies suggest that some compounds are involved in the defence response of plants to nematodes (Valette *et al*. 1998). Several benefits

can result from the identification of those compounds which may be used as natural nematicides or as a model for the development of synthetic products with similar activity against nematodes and at the same time environmentally safer.

Extracts of plants containing volatile compounds, especially essential oils, have an anti nematode effect (Abd-Elgawad & Omer 1995; Ibrahim *et al*. 2006; Katooli *et al*. 2010; Oka *et al*. 2012). It was shown that the essential oils carvacrol, linalool and thymol have nematicide properties as they are toxic to *M. incognita* second-stage juveniles (Ibrahim *et al*. 2006). Some of these oils inhibit the activity of acetyl cholinesterase, but their mode of action is still unknown (Ryan & Byrne 1988; Zuckerman & Esnard 1994; Ibrahim *et al*. 2006).

Other volatile and non-volatile compounds may be also present in plant extracts (Brown & Morra 1997; Ibahim 2006) and some can be detected at a distance and display an attractive or repellent effect on nematodes being antagonist (Pickett & Stephenson 1980). One of the most studied and known antagonism between nematodes and plants is the plant *Tagetes*. *Tagetes patula* L. acts as a trap crop preventing the development of giant cells produced by root-knot nematodes, having a suppressive effect on nematode populations (Belcher & Hussey 1977). Therefore, plants of this genus have been used for some time as cover crops, crop rotation, green manure or plant extracts as a source for nematode antagonism (Chitwood 2002).

Since then, many other plants such as: *Artemisia vulgaris* L., *Artemisia annua* L., *Azadirachta indica* A. Juss, *Brassica napus* L., *Cannabis sativa* L., *Crotolaria juncea* L., *Eucalyptus citriodora* Hook., *Gliricidia maculata* H.B. & K., *Glycosmis pentaphylla* Retz., *Kalanchoe pinnata* (Lam.) Pers., *Moringa oleifera* Lam., *Myrtus communis L., Piper betle* L., *Ricinus communis* L., *Solanum sisymbriifolium* Lam., *Zanthoxylum alatum* Roxb. are being investigated to control plant-parasitic nematodes *Ditylenchus dipsaci, G. pallida, G. rostochiensis*, *Meloidogyne* spp., *R. similis* and *Tylenchulus semipenetrans* among others (Jasy & Koshy 1992; Scholte 2000a,b; Costa *et al*. 2003; El-Rokiek *et al*. 2011; Dias *et al*. 2012; Oka *et al*. 2012; D' Addabbo *et al*. 2013; Mukhtar *et al*. 2013) and this list of plants is still increasing.

Plants belonging to the genus *Solanum* are used widely in traditional medicine for different purposes. The chemical constituents of *Solanum* spp. have been described in many studies and its pharmacological and toxicological properties investigated (Kumar *et al*. 2001; Heo *et al*. 2004; Zhou *et al*. 2006; Jeong *et al*. 2007; Huang *et al*. 2010). To performe this study two species *S. nigrum* and *S. sisymbriifolium* were selected since our previous studies revealed that they are non-hosts for *P. goodeyi* and its incorporation into soil benefits banana plant growth (Pestana *et al*. 2009), justifying the need to deepen research for nematicidal properties.

1.4 Parasitism and genes from plant-parasitic nematodes

Plant-parasitic nematodes use the stylet to puncture the cell wall and reach the plasma membrane where they introduce secretory proteins synthesized by the nematode that play an important role during migration through root tissues and also to feed withdrawing the nutrients from the host cytoplasm (Davis *et al*. 2000). The major secretory organs are the chemoreceptors (amphids, phasmids and cephalic sensilla), the oesophageal glands, the excretory system and rectum that in some nematodes can produce a gelatinous matrix to protect the eggs from predators and dehydration (Eisenback 1985; Abad *et al*. 2003). The nematode cuticle also regulates selectively the flow of fluids through the body wall and may be a source of secretory compounds which are recognized by plants as signal molecules (Robertson *et al*. 2000; Lima *et al*. 2005).

The most studied nematode secretions are related to proteins produced by amphids and oesophageal glands. Several glycoproteins secreted by amphids, located in the cephalic region, were isolated and some of them are involved in the perception of environmental signals (Stewart *et al*. 1993a, 1993b; Abad *et al*. 2003). Oesophageal glands have specialized cells capable of producing secretions, expelled through the stylet, with different roles during parasitism (Hussey & Mims 1991; Abad *et al*. 2003), highlighting their importance in this process.

The genes and products, which included proteins secreted by parasites to facilitate penetration, migration and to prevent plant defence responses are designated effectors. The search and identification of those effectors can be helpful to better understand nematode parasitism and to devise alternative nematode control measures (Atkinson *et al*. 2003; Davis *et al*. 2004; Fragoso *et al*. 2009; Gheysen & Mitchum 2011; Hewezi & Baum 2013; Jaouannet *et al*. 2013; Peng *et al*. 2013). Most candidate parasitism genes have been predicted using bioinformatic tools and many effectors have been identified among other plant-parasitic nematodes. The genes present in *Pratylenchus* spp. which encode effectors and other proteins with known function (Table 1.1) include plant cell wall degrading enzymes to help migration and infection; compounds produced by nematodes to protect themselves from plant defence responses such as compounds that metabolise reactive oxygen species (ROS) and several proteases that help feeding and host tissue degradation.

Table 1.1 Root-lesion nematodes genes and proteins related to infection and parasitism with known function.

Action/target	Nematode gene	Nematode	Function	Reference
Cell wall				
	Beta-1,4-endoglucanase	*P. penetrans* *P. coffeae* *P. vulnus* *P. goodeyi*	Hydrolysis of beta-1,4-glucan	Uehara *et al*. 2001 Kyndt *et al*. 2008 Rybarczyk-Mydlowska *et al*. 2012 this thesis
	Pectate lyase	*P. coffeae* *P. thornei*	Hydrolysis of alpha 1,4-linkages in pectate	Haegeman *et al*. 2011 Nicol *et al*. 2011
	Xylanase	*P. coffeae* *P. thornei*	Hydrolysis of xylan	Jones & Fuso-Nyarko 2014 ″
	Expansin-like proteins	*P. coffeae*	Cell wall softening or extension	Haegeman *et al*. 2011
	Beta-1,3-endoglucanase	*Pratylenchus* spp.	Hydrolysis of beta-1,3-glucan	Jones & Fuso-Nyarko 2014
	Polygalacturonase	*P. coffeae* *P. thornei*	Hydrolysis of alpha-1,4-o-galactosiduronic linkages	Jones & Fuso-Nyarko 2014 ″
	Arabinogalactan galactosidase/arabinase	*Pratylenchus* spp.	Hydrolysis of pectin	Jones & Fuso-Nyarko 2014
	Cellulose binding proteins	*P. thornei* *P. zeae*	Promote hydrolysis of crystalline cellulose	Jones & Fuso-Nyarko 2014 ″
Protection from host defences				
	Thioredoxin	*P. vulnus* *P. penetrans*	Detoxification of ROS	unpublished ″
	Peroxiredoxin	*P. thornei*	Detoxification of ROS	Nicol *et al*. 2012
	Superoxide dismutase	*P. coffeae* *P. thornei* *P. vulnus* *P. penetrans*	Detoxification of ROS	Jones & Fuso-Nyarko 2014 ″ unpublished ″
	Glutathione-S-transferase	*P. thornei*	Detoxification of ROS	Nicol *et al*. 2012
	Glutathione peroxidase	*P. thornei*	Detoxification of ROS	Nicol *et al*. 2012
	SPRYSEC-RBP-1	*P. thornei* *P. zeae*	Suppression of host defences	Jones & Fuso-Nyarko 2014 ″
	Sec-2/FAR	*P. coffeae* *P. vulnus*	Reduction in host defence responses	″ unpublished
Targeting regulation and signalling pathways				
	Annexin	*P. thornei*	Protection of plant cells against stress	Nicol *et al*. 2012
	SKP-1	*P. thornei*	Involved in ubiquitination, signal transduction	Nicol *et al*. 2012
	Ubiquitin extension protein	*P. thornei* *P. thornei*	Involved in ubiquitination	Nicol *et al*. 2012 Nicol *et al*. 2012
	Calreticulin	*P. zeae* *P. coffeae* *P. goodeyi*	Calcium spiking	Jones & Fuso-Nyarko 2014 ″ this thesis
	TRAP	*P. goodeyi*	Translocation of misfolded proteins	this thesis
Feeding				
	Aminopeptidase	*P. thornei* *P. zeae*	Protein digestion/degradation	Nicol *et al*. 2011 ″
	Proteases	*P. thornei*	Protein digestion/degradation	Nicol *et al*. 2012 ″

To understand the infection/parasitism mechanisms we select from the above list the effectors: calreticulin because it may be injected through the nematode stylet into plant tissues (Jaubert *et al*. 2005; Suchitra & Johi, 2005; Vieira *et al*. 2011) and has been identified as playing an important role in infection and parasitism (Jaouannet *et al*. 2012); beta-1,4-endoglucanase a plant cell wall degrading enzyme that seems to have a great importance to penetration, migration and degradation of host tissues (Haegeman *et al*. 2012) and is essential for the success of parasitism (Kyndt *et al*. 2008) and also the translocon-associated protein (TRAP) recently detected in *P. goodeyi* that is connected with the degradation of misfolded proteins as a response to stress (Nagasawa *et al*. 2007).

Chapter 2

Material and Methods

2. Material and Methods

2.1 Biological material

2.1.1 *Pratylenchus goodeyi* isolates

Samples of soil and banana roots were collected on the Southern coast of Madeira Island. The nematodes *Pratylenchus goodeyi* Sher & Allen 1953 were extracted from soil by centrifugal flotation using the sucrose method (Jenkins 1964; Abrantes *et al*. 1976) and from roots by the maceration and sieving method (Abrantes *et al*. 1976; Hooper 1986). The roots were cut into pieces of approximately 1 cm and ground in a blender with 100 mL of water for 20 s. The suspension was transferred to a small sieve, with a mesh of approximately 45 µm, placed in a Petri dish. After 48 h the suspension was poured into a sieve of 38 µm and washed with sterile distilled water. The material retained on the 38 µm sieve was transferred to a beaker, ressuspended in sterile water, placed on a Doncaster plate and observed in a stereomicroscope (Nikon SMZ-U). The identification of *P. goodeyi* isolates based on morphological characters was confirmed at the "Istituto per la Protezione delle Piante Sezione di Bari" Italy.

2.1.2 Nematode multiplication

Two methods were used for *in vitro* nematode multiplication: Inoculation on banana plants and carrots discs. On the first method nematode isolates were transferred to 10 mL of sterile water and quantified. Populations of root-lesion nematode *P. goodeyi* were then inoculated on banana plants (*Musa acuminata* Colla) from *in vitro* culture, 20 cm height, in pots containing sterilized soil. Using a glass stick three holes were made around the plant, which were covered with soil after nematode inoculation. These potted plants remained in the laboratory, being watered whenever necessary. In addition, multiplication of *P. goodeyi* was also performed on carrot discs as described by Nico *et al*. (1999) and maintained in the laboratory at 24 ºC to be used in molecular analysis.

2.1.3 Plant material

Solanum sisymbriifolium Lam. "Pion" seeds were provided by "Vandijke Semo Seed and Services", The Netherlands, whereas *S. nigrum* L. plants were collected from natural habitats at Lugar de Baixo, Ponta do Sol, Madeira Island and

kept in laboratory for the production of fruits and seeds.

Seeds of both plants were germinated in sterile peat and the plants were maintained in a greenhouse, under a 16 h photoperiod, day/night temperature of 35 ºC/18 ºC, respectively, and relative humidity of 70% until they reached 50-60 cm height. They were then collected and divided into two samples: one was weighed and frozen for further analysis (named fresh plant) and the other was placed in a ventilated drying chamber at 30 °C (named dry plant). Dried plants were ground in a cutting mill (Mod. 5KH35KG 254E, Arthur H. Thomas Co. Phila., PA., U.S.A.) and passed through sieves of 40 and 60 mesh (type AS200, Retsch, Germany). The 40-60 mesh size fraction (425-250 μm) was used to extract chemical compounds with water and organic solvents. Samples water content was determined on a moisture balance (Gibertini-Eurotherm).

2.2 Chemicals

All reagents used on plant extractions and chemical analysis were highly pure (p.a., 99% purity) and supplied by Sigma-Aldrich (Madrid, Spain): dichloromethane, trimethylchlorosilane, *N,O*-bis(trimethylsilyl)trifluoroacetamide, pyridine, stigmasterol (95%), octadecanoic acid, nonadecan-1-ol, tetracosane, acetone (≥ 99.5%) or Fluka Chemie (Madrid, Spain): ethanol absolute (≥ 99.8%).

2.3 Plant extractions

2.3.1 *Solanum* aqueous extractions

Two approaches were used to make the aqueous extractions: blender and reflux. In the blender approach, *S. nigrum* and *S. sisymbriifolium* were extracted in water at a ratio of 1:4 or 1:20 (w/v, material/water) of fresh plant or dry plant, respectively. The plant material was gently shaken in water, ground for 10 min in the blender and filtered using a G4 filter, pore size 10-16 μm (DURAN, Germany). This procedure was performed using water either at room temperature (cold water) or boiling (hot water). The same material/water ratio was used in the reflux approach. The plant material was refluxed for 1 h and the liquid fraction was obtained after filtration through a G4 filter.

All solid residues were lyophilized, quantified and stored at -20 ºC, in the dark, until further analysis.

2.3.2 Solanum sequential extraction

Milled dried material placed in handmade cartridges (4cm diameter × 10 cm length) was Soxhlet extracted in a sequential extraction of at least 10 hours each with the following solvent sequence: dichloromethane, acetone, ethanol and water. Each extraction was followed by solvent evaporation in a rotative evaporator (R-200 Büchi, Sigma-Aldrich, Spain) combined with a vacuum pump (V-500 Büchi Vac®, Sigma-Aldrich, Spain) and a bath (B-490 Büchi, Sigma-Aldrich, Spain) at a maximum temperature of 40 ºC. The extracts were collected and after drying under vacuum until constant weight, the percentage of extractives (compounds present at each extract fraction) was determined gravimetrically.

After ethanol extraction, the plant material that remained on the cartridges was washed with ethanol and dried at 30 °C. This material was refluxed for 1 hour to obtain the extraction in water. These solutions were filtered under vacuum (G4 porosity), lyophilized and quantified gravimetrically.

All solid residues from each fraction were stored in cold and dark conditions until chemical analysis or mortality assessment on *P. goodeyi*.

2.4 Chemical analysis

2.4.1 Samples preparation and selection

Dichloromethane, acetone and water solid residues from *S. nigrum* and *S. sisymbriifolium* were selected to carry out chemical analysis. The alkaline hydrolysis was performed as described by Oliveira *et al*. (2008).

2.4.2 Gas chromatography-mass spectrometry (GC-MS)

Dichloromethane solid residue (20 mg) from *S. nigrum* (SN) and *S. sisymbriifolium* (SS) was dissolved in 250 µL of pyridine to convert compounds with hydroxyl and carboxyl groups into trimethylsilyl ethers and esters (TMS) following the protocol described by Oliveira *et al*. (2005). The derivatised extracts were analysed by GC-MS at chromatographic conditions used previously (Oliveira *et al*. 2008; Vilela *et al*. 2014). Compounds were identified as TMS derivatives, comparing mass spectra with GC-MS spectral library (Wiley-NIST Mass Spectral Library 1999), retention times and fragmentation profiles with published data and by injection of standards (Oliveira *et al*. 2005, 2008; Vilela *et al*. 2014). GC-MS was calibrated for semi-quantitative analysis and the response factors calculated as described by Vilela *et al*. (2014). Bioactivities of the most representative lipophilic compounds were predicted by *in silico* tools available online: Dr.

Duke's Phytochemical and Ethobotanical database (http://www.ars-grin.gov/duke/) (Filimonov 1995) and the free Chemical structure database ChemSpider (http://chemspider.com).

2.4.3 Ultra-high performance liquid chromatography (UHPLC)

The analyses of acetone and water solid residue from both plants were performed by an UHPLC system consisted of a variable loop Accela autosampler (200 vial capacity set at 15 °C), an Accela 600 LC pump and an Accela 80 Hz PDA detector (Thermo Fisher Scientific, San Jose, CA, U.S.A). The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.48 mL/min, at 45 °C, using a Kinetex C_{18} (50 mm × 2.1 mm × 1.7 μm) column supplied by Phenomenex. The injection volume in the UHPLC system was 10 μL and the mobile phase consisted in water:acetonitrile (99:1,v/v) (A) and acetonitrile (B), both with 0.1% of formic acid. The following linear gradient was applied: 0-4 min: 2%B, 4-7 min: 2-4.5%B, 7-20 min: 4.5-12%B, 20-22 min: 12-12.8%B, 22-25 min: 12.8-30%B, 25-30 min: 30-100%B, 30-34 min: 100-2%B followed by re-equilibration of the column for 4 min before the next run. Double online detection was carried out in the diode array detector, at 280 and 340 nm, and UV spectra in a range of 200-600 nm were also recorded. Before the injection, each solid residue was dissolved in water or acetone HPLC grade, to obtain a final extract concentration between 10 and 30 mg/mL, and then filtered through a 0.2 μm PTFE syringe filter (Santos *et al*. 2013).

2.4.4 Mass spectrometry

A LCQ Fleet ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, U.S.A.) was used, equipped with an electrospray ionization source and operating in negative mode (Santos *et al*. 2013). The nitrogen sheath and auxiliary gas were 40 and 10 (arbitrary units), respectively. The spray voltage was 5 kV and the capillary temperature was 350 ºC. The capillary and tune lens voltages were set at -25 V and -125 V, respectively. CID-MS^n experiments were performed on mass-selected precursor ions in the range of *m/z* 100-2000. The isolation width of precursor ions was 1.0 mass units. The scan time was equal to 100 ms and the collision energy was optimized between 20 and 35 (arbitrary units), using helium as collision gas. The data acquisition was carried out by using Xcalibur® data system (ThermoFinnigan, San Jose, CA, U.S.A.) (Santos *et al*. 2013).

2.4.5 HPLC quantification

Calibration curves were obtained by UHPLC injection of gallic, caffeic and chlorogenic acids and catechin, quercetin, isorhamnetin and luteolin standard solutions in methanol, with five different concentrations each, between 2 and 86.4 μg/mL. Besides the linearity, the limits of detection (LOD) and quantification (LOQ) were also estimated using the S/N approach (n=5). The calibration curves and additional relevant analytical data are shown in Table 2.1. The quantification of individual compounds was accomplished with calibration data for the most similar standard, since for some of them no pure reference compounds were available (Santos *et al*. 2013).

Table 2.1 Calibration data used for the UHPLC–UV quantification of phenolic compounds in *Solanum nigrum* and *S. sisymbriifolium* acetone and water extracts.

Compound	λ (nm)	Conc. range (μg/mL)	Calibration curve[a]	R^2	LOD[b]	LOQ[b]
Gallic acid	280	5.2-83.2	y=159501x-184269	0.998	5.0	16.8
Caffeic acid	340	5.3-84.8	y=229699x-717985	0.996	6.8	22.5
Chlorogenic acid	340	5.1-81.6	y=102517x-201108	1.000	2.3	7.8
Catechin	280	5.0-80.0	y=28666x-45824	0.972	18.1	60.3
Quercetin	340	5.0-38.4	y=248648x-622681	0.995	3.7	12.5
Isorhamnetin	340	2.4-39.2	y=252963x-246614	0.996	3.1	10.2
Luteolin	340	2.6-41.6	y=773242x-325790	0.999	1.8	6.0

[a] y = peak area, x = concentration in μg/mL, LOD limit of detection, LOQ limit of quantification, [b] expressed in μg/mL.

Bioactivities of phenolic compounds were predicted *in silico* through the online available tools: Dr. Duke's Phytochemical and Ethobotanical database and free chemical structure database ChemSpider as described above (2.4.2).

2.5 Biological assays

2.5.1 Nematicidal activity

S. nigrum and *S. sisymbriifolium* solutions were prepared using the solid residue obtained from aqueous and sequential extractions at exact quantities of each fraction corresponding to the initial ratio of fresh and dry plant dissolved in water (basal concentration) and at the mean concentration of 10 mg/mL.

These solutions containing water soluble compounds from fresh plant or dry plant aqueous extractions were named aqueous extracts (2.3.1) and from sequential extraction dichloromethane, acetone, ethanol and water extracts (2.3.2) and their

activity were tested *in vitro* against *P. goodeyi.* The biological assays were conducted using l mL of each extract in a Syracuse containing 10 adults *P. goodeyi* and left in the dark at room temperature (25 ± 1 ºC) for 10 days. Each assay was replicated five times and sterile distilled water was used as control. Nematodes were observed daily using a binocular microscope and numbers of inactive or dead nematodes were recorded. Considering the efficiency of acetone extract nematode mortality was monitored in this extract for a period of 24 h. Nematodes were considered dead when after being transferred to sterile water for 2 h and stimulated by prodding they remained inactive. Registered mortality was converted into cumulative mortality corrected by Abbott's formula (Abbott 1925). These biological assays were performed twice.

2.5.2 Mobility and attraction

A solution of Pluronic gel (23% w/v; Pluronic F-127, Sigma) was prepared by stirring to dissolve the powder in sterile distilled water for 24 h at 4 ºC (Wang *et al*. 2009). The resulting gel was refrigerated at 4 ºC until used for nematode behaviour studies. A root tip (Rv), 1 cm long, from banana plants cultured *in vitro* was placed in the middle of a Syracuse (2 cm diameter) containing 1 mL of Pluronic gel. Ten *P. goodeyi*, mainly adults, were hand-picked and put on the edge of each Syracuse and the Pluronic gel allowed to solidify at room temperature (Wang *et al*. 2009). A Syracuse containing Pluronic gel, but without a root, was used as control to assess nematode movement. Nematode behaviour as mobility and attractivity toward the root was recorded after 2, 4, and 6 h by observing the movement and the number of nematodes that reached the root.

2.5.3 Infection

Drops (150 µL) of *S. nigrum* acetone extract (10 mg/mL) were placed on microscopic glass chambered test slides to perceive the nematicide effects of the water soluble compounds on the *P. goodeyi* mobility and ability to infect the roots of banana plants. Ten nematodes, J2, females and males, were placed on the chambers of the microscopic glass and exposed to the extract for 0, 6, 12 and 18 h. After exposure, nematodes were transferred to Syracuses containing a root tip embedded in the Pluronic gel as mentioned above (2.5.2). The Syracuses were incubated at room temperature and mobility and attractivity were determined as before. The percentage of infection was recorded and quantified (number of nematodes inside the roots/total number of nematodes inoculated x 100) after 1, 2 and 3 h (Mukhtar *et al*. 2013). Inactive or dead nematodes were registered at each time considering that nematodes were alive if they moved or appeared as a winding shape and dead

if they did not move when stimulated by prodding. The reduction in infection over control was calculated [(infection in control/infection in treated) - 1] (Mukhtar *et al*. 2013).

2.6 Molecular analysis

2.6.1 Biological samples

Roots from banana plants cultured *in vitro* (Rv), in sterilized soil (Rp) and infected with *P. goodeyi* (RPg) were washed with tap water, wiped and cut directly into a mortar containing liquid nitrogen, ground with a pestle and stored at -70 °C until the extraction of RNA.

Nematodes, mainly adults, obtained as previously described (2.1.1) from both infected roots and carrots discs were transferred manually to an Eppendorf tube containing sterilized distilled water in order to get *P. goodeyi* (Pg) samples of *ca*. 4000 individuals. These samples were quickly frozen in liquid nitrogen, ground with a micropestle and used immediately for the extraction of RNA.

2.6.2 RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of crushed uninfected banana roots (Rv and Rp) using the RNeasy Plant Mini Kit (Qiagen, U.S.A.) upon a minor modification. PVP40T at 1% and 0.4 volumes of 3 M potassium acetate (pH 6.5) were added to the RLC extraction buffer. Samples were incubated on ice for 15 min and centrifuged at 14000 *g* at 4 °C for 15 min. Then 0.5 volumes of ethanol were added to the supernatant. The RNA was treated with DNase (Qiagen, U.S.A.) to remove possible DNA contamination and cleaned according to the manufacturer's instructions.

The extraction of total RNA from nematodes (Pg) and from 5 mg of RPg samples were performed using the RNeasy Plus Micro Kit (Qiagen, U.S.A.) following the manufacturer's instructions and treated with DNAse.

All RNA extractions were confirmed on a 1% agarose gel buffered with TAE 1x containing ethidium bromide (0.5 µg/mL) and photographed under UV light (DigiGenius, Syngene, U.K.).

The single-stranded cDNA synthesis was performed in 20 µL using total RNA from nematode (10 µL) and roots (1 µg) samples and the Cloned AMV Reverse Transcriptase (Invitrogen, Spain) according to the manufacturer's instructions. The synthesized cDNA was diluted (5x) by adding 80 µL of ultrapure water.

2.6.3 cDNA amplification and cloning

Internal partial sequences of *calreticulin* (*CRT*) and *beta-1,4-endoglucanase* (*ENG*) cDNAs from *P. goodeyi* were obtained by amplification through degenerate oligonucleotides. CRTf1 and CRTr1 (Table 2.2) were designed from the conserved regions of MFGPIC and KWIHPEI, respectively, on amino acid sequences of calreticulin from different nematode species obtained through the National Center for Biotechnology Information (NCBI) database (*Heligmosomoides polygyrus*: CAL30086; *Caenorhabditis elegans*: CAA42159; *Necator americanus*: CAA07254; *Meloidogyne incognita*: AAL40720; *Onchocerca volvulus*: AAA59056). ENG1 and ENG2 oligonucleotides designed for the gene encoding *beta-1,4-endoglucanase* (Rosso *et al*. 1999) were used to amplify the correspondent internal partial sequence of the cDNA and a fragment coding for translocon-associated protein (TRAP) arose through serendipity.

Table 2.2 Primers used in this study for PCR amplification, RACE, RT-PCR, and expression analysis.

Gene	Primer name	Sequences 5'→ 3'	Cycles	T. annealing (ºC)
Calreticulin	CRTf1	ATGTTYGGICCIGAYATHTG	35	51
	CRTr1	ATYTCIGGRTGDATCCAYTT	35	51
	PgCRTf2	CGTTGGCTACAACGGGAAGAACC	35	65
	PgCRTr2	TCAGGGATGTGCTCGGGCTTCT	35	65
Beta-1,4-endoglucanase	ENG1	TAYGTIATHGTIGAYTGGCA	40	50
	ENG2	GTICCRTAYTCIGTIACRAA	40	50
	PgENGf3	CCACTGCCTACCCCGACAAAGC	35	67
	PgENGr4	CACGAAGGGGCTGCTTGTGGGA	35	67
Translocon-associated protein	PgTRAPf	ATGCCGTCGTAAATGGCCAGAGC	35	64
	PgTRAPr1	GTTGCTGACACCATTTTGGACGG	35	65
	PgTRAPr2	GTTGTTGTTTGAGGTGATAGC	35	64
Ubiquitin	UBI5	ATGCAGATYTTTGTGAAGAC	35	50
	UBI3	ACCACCACGRAGACGGAG	35	50
Rubisco	A5.2	GAGACCCTCTCCTACCTTCCT	35	50
	B8.1	TTCCACATGGTCCAGTASCGC	35	50

The PCR amplification performed in a 50 µL reaction mixture contained 5 µL cDNA, 1x $(NH_4)_2SO_4$ buffer (Fermentas, Lithuania), 2.5 mM $MgCl_2$, 1 U *Dream Taq* DNA polymerase (Fermentas, Lithuania), 0.2 mM dNTPs and 1 µM of each

oligonucleotide (Table 2.2). PCR products were gel purified through the QIAquick gel extraction kit (Qiagen, U.S.A.) and cloned into pJET 1.2/blunt plasmid vector using the CloneJET PCR Cloning Kit (Fermentas, Lithuania) and as host *Escherichia coli* DH5α competent cells.

After sequencing specific oligonucleotides PgCRTf2, PgCRTr2, PgENGf3, PgENGr4, PgTRAPf, PgTRAPr1 and PgTRAPr2 were designed through the Primer 3 program (http://primer3.wi.mit.edu/). The oligonucleotides PgCRTf2 and PgCRTr2 were used to clone, respectively, the 3' and the 5' ends of *CRT*. The 3' and 5' ends of *ENG* were generated using PgENGf3 and PgENGr4 specific primers, respectively and PgTRAPf and PgTRAPr1 were used to clone, respectively, the 3' and the 5' remaining cDNA sequences. Rapid amplification of cDNA ends (RACE) were conducted using adaptors, oligonucleotides, enzymes and procedures from the SMARTer™ RACE cDNA Amplification Kit (Clontech, U.S.A.). The amplified 5' and 3' cDNA fragments of the expected size were removed from the gel and purified as before (QIAquick gel extraction kit) or using the High Pure PCR Product Purification Kit (Boehringer Mannhein, Germany) following the instructions of their respective manufacturers. The 5' ends were cloned into pJET 1.2/blunt cloning vector (CloneJET PCR Cloning Kit) and the 3' cDNA ends into pGEM ®-T Easy vector (Promega, Spain) following the manufacturers' guidelines.

2.6.4 Plasmid extraction, sequencing and sequence analysis

Plasmid DNA from transformed colonies was extracted using the GeneJET Plasmid Miniprep kit (Fermentas, Lithuania) and eluted in 50 μL of elution buffer (10 mM Tris-HCl, pH 8.5). Plasmids were sequenced on both directions using commercial AB 3739XL capillary sequence (Macrogen Europe, Amsterdam, The Netherlands). DNA sequence data were analyzed through the NCBI web site (www.ncbi.nlm.nih.gov/) (Benson *et al*. 2013). The BLAST program was used to search for sequence homology in nucleotide and amino acids database (Altschul *et al*. 1997). The Expasy server (http://web.expasy.org/) through the available translate tool was used to translate the cDNA sequences and to determine the physicochemical parameters (ProtParam tool) (Gasteiger *et al*. 2005) of the deduced proteins: calreticulin, beta-1,4-endoglucanase and translocon-associated protein. Multiple protein sequence alignments were generated by ClustalW2.1 at the European Bioinformatics Institute (EBI) website (http://www.ebi.ac.uk.) using standard parameters and an alignment plot was created by The Sequence Manipulation Suite (http://bioinformatics.org/sms/). Predictions for the signal peptide were performed using SignalP-4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al*. 2011). Proteins motifs were identified using SMART (http://smart.embheidelberg.de/) and Conserved

Domain Database from NCBI. EBI Phobius (Käll *et al*. 2007) and Interproscan (Quevillon *et al*. 2005) were used to scan proteins signatures. The tool pTarget (http://golgi.unmc.edu) (Guda & Subramaniam 2005; Guda 2006) and PSORT (Prediction of Protein localization Sites) were used to confirm the possible location of the proteins (Nakai & Kanehisa 1992). The PredictProtein Server (Rost & Liu 2003) and the TMHMM (http://www.cbs.dtu.dk) were employed to obtain the topology prediction. The program ProtFun 2.2 from the Center for Biological Sequence Analysis (CBS) web site (http://www.cbs.dtu.dk) was used to find possible functions of the proteins (Jensen *et al*. 2002, 2003).

2.6.5 RT-PCR analysis

RT-PCR analysis was performed with 5 µL of cDNA as template diluted 5x for nematode samples (Pg and RPg) and 10x for root samples (Rv and Rp) in a 50 µL reaction mixture as described above (2.6.3). cDNA amplifications were performed with the correspondent oligonucleotides (Table 2.2). PgCRTf2, PgCRTr2, PgENGf3, PgENGr4, PgTRAPf and PgTRAPr2 were used to amplify a fragment of *CRT*, *ENG* and *TRAPδ*, with 291, 315 and 320 bp respectively. UBI5 and UBI3 primers (Laplaze *et al*. 2000) were used to amplify a fragment of *ubiquitin* and as an internal control gene to normalize the amount of cDNA in semi-quantitative RT-PCR analysis. Also, A5.2 plus B8.1 primers designed from banana plant cDNA (Thomas-Hall *et al*. 2007) were used to amplify a fragment of *rubisco*. Amplicons were separated on a 1.5% agarose gel visualized under UV light as previously describe (2.6.2). The oligonucleotides used in this work were synthesized by "STAB VIDA, Lda, FCT/UNL" (Caparica, Portugal) except the *ubiquitin* pair that was prepared by Invitrogen (Life Technologies, Spain).

2.6.6 Expression analysis

Samples of *P. goodeyi* (*ca*. 6000) mainly adults were exposed for 0, 6, 12 and 18 h to the acetone extract of *S. nigrum* (10 mg/mL). Nematode RNA extraction and cDNA synthesis were done using the procedures described above (2.6.2). Primers PgCRTf2, PgCRTr2, PgENGf3, PgENGr4, PgTRAPf, PgTRAPr2, UBI5 and UBI3 were used to amplify internal fragments of *CRT*, *ENG*, *TRAPδ* and *ubiquitin* under the same PCR conditions reported before (2.6.3). The expression level of *CRT, ENG* and *TRAPδ* were determined by calculating the ratio of the gene intensity in relation to the *ubiquitin* intensity signal obtained from the same cDNA preparation through the IMAGE J program (Abràmoff *et al*. 2004).

2.7 Statistical analysis

Unless otherwise stated at least three replicates were performed and data from *S. nigrum* and *S. sisymbriifolium* sequential and aqueous extractions, *P. goodeyi* mortality, gene expression (*calreticulin*, *beta-1,4-endoglucanase* and *translocon-associated protein delta subunit*), infection and reduction of infection were subject to statistical analysis using the SPSS (Statistical Package for the Social Sciences) 15.0 software for Windows.

Data of the amount of extractives from each extract sequential fraction and from aqueous extractions were analysed to assess normal distribution by Kolmogorov-Smirnov and Shapiro-Wilk tests ($P > 0.05$) and one-way analysis of variance (ANOVA) was used to find significant differences between extracts. Significant differences were further analyzed by Tukey's multiple range test ($P < 0.05$). Registered mortality in *S. nigrum* and *S. sisymbriifolium* sequential extracts and aqueous extracts was converted into cumulative mortality and corrected by Abbott's formula (Abbott 1925) prior to analysis. The previous statistical tests were then carried out to analyzed normal distribution and to find significant differences on the mortality of *P. goodeyi* at sequential extracts and aqueous extracts, as well as expression of genes and the infection capacity of *P. goodeyi* after exposure to the *S. nigrum* acetone extract (10 mg/mL). Data from reduction in infection were transformed into (log x) to normalize before further statistical analyses and the previous statistical procedure was then repeated.

Chapter 3

Nematicidal activity of *Solanum sisymbriifolium* and *S. nigrum* extracts against the root-lesion nematode *Pratylenchus goodeyi*

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3. Nematicidal activity of *Solanum sisymbriifolium* and *S. nigrum* extracts against the root-lesion nematode *Pratylenchus goodeyi*

3.1 Abstract

The root-lesion nematode *Pratylenchus goodeyi* is a parasite of banana plants, frequently detected in Madeira Island (Portugal) affecting culture development and consequently the production, with economical damages. To identify the phytochemicals of *Solanum sisymbriifolium* and *S. nigrum* with nematicidal properties and determine the effect of those components on *P. goodeyi*, an extraction sequence of at least 10 hours each from dried plants was used. The chosen solvent sequence was: dichloromethane, acetone, ethanol and water. According to the results both plants have in their composition chemical components mainly found in water extracts, which affects the mobility and mortality of the root-lesion nematode. *S sisymbriifolium* and *S. nigrum* have potential to be used as a natural and environmentally friendly nematicide to control *P. goodeyi*.

3.2 Introduction

The root-lesion nematode *Pratylenchus goodeyi* Sher & Allen 1953 is very common in Madeira Island affecting banana culture. In order to control nematode populations farmers use phytopharmaceutical products, which also contribute to contaminate soil, groundwater and air. It is therefore of great importance to study alternative routes to those products by seeking less harmful chemicals to the environment and humans. Thus, some plants with nematicidal potential and its application have been analyzed (Musabyimana & Saxena 1999; Rahman & Somers 2005).

It is known that the incorporation of organic waste has a considerable impact on physical and biological properties of soil, promoting a favorable environment for the development of nematode antagonists (Badra *et al*. 1979; Bradow 1991; Bello *et al*. 2000). In some cases, it can be also ascertain toxicity to some nematodes. Since plants are capable of producing a large variety of secondary metabolites with multiple applications, much research has been conducted to find substances in plant tissues that may have an effect on nematodes (Mojtahedi *et al*. 1993; Walker 1997; Al-Rehiayani *et al*. 1999; Walker & Morey 1999; Kirkegaard *et al*. 2000; Costa

et al. 2003). Several benefits can result from the identification of phytochemicals involved in these interactions, which may be used as nematicidal or can serve as a model for the development of synthetic products with positive activity on nematodes or on the environment around them (Chitwood 2002).

Several chemical compounds present in *Solanum* species as steroidal glycosides and alkaloids among others have a broad spectrum of activity (Perez *et al*. 1998; Raju *et al*. 2003; Heo *et al*. 2004; Zhou *et al*. 2006; Jeong *et al*. 2007; Lin *et al*. 2007, 2008; Ji *et al*. 2008) and is therefore of great interest to develop studies for the application of this plant genus in different areas. Among this species *Solanum sisymbriifolium* Lam., which does not exist in Madeira Island, has been successfully used to control populations of potato-cyst nematodes, *Globodera* spp. (Scholte 2000a) whereas *S. nigrum* L. very common in Madeira is believed to have therapeutic properties against some types of tumors since some compounds showed cytotoxic effects in tumor cells (Zhou *et al*. 2006). Recent studies revealed that *S. sisymbriifolium* and *S. nigrum* are not good or non-hosts of *P. goodeyi* (Pestana *et al*. 2009). In addition, the incorporation of these plants into soil improved banana plant growth, directly through the release of exudates with nematicidal effect and indirectly by promoting the development of antagonists and making the rhizosphere unfavorable to the nematode.

In order to search for nematicidal substances plant extracts from *S. sisymbriifolium* and *S. nigrum* were evaluated against *P. goodeyi*.

3.3 Results

3.3.1 Fractions of S. sisymbriifolium and S. nigrum dry material

Figure 3.1 shows the amount of extractives (compounds present in each solid residue) from sequential Soxhlet extraction of *S. sisymbriifolium* and *S. nigrum* plants that was determined after a complete extraction in each solvent. The results clearly indicate a predominant amount of polar fractions. Water solid residue revealed the highest amount of extractives from either *S. sisymbriifolium* or *S. nigrum* followed by ethanol solid residue. The compounds extracted in dichloromethane and acetone were 6 to 8 fold lower, respectively, than the compounds extracted in ethanol and water.

Normal distribution of the amount of extractives from each sequential extract was confirmed ($P > 0.05$) through Kolmogorov-Smirnov and Shapiro-Wilk normality tests. There were significant differences by ANOVA for *S. sisymbriifolium* and Tukey test detected differences between extracts ($P < 0.05$).

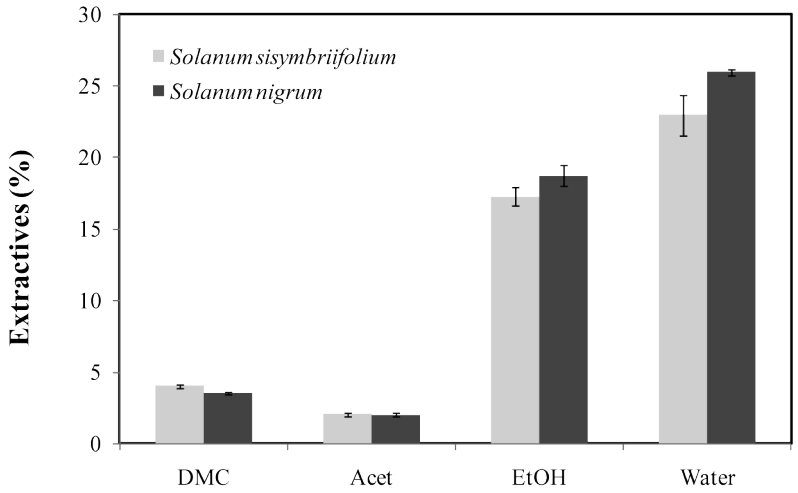

Figure 3.1 Percentage of extractives from dry *S. sisymbriifolium* and *S. nigrum* plants obtained from solvents sequence: dichloromethane (DMC), acetone (Acet), ethanol (EtOH) and water. Results are the mean of 5 replicates ± SD.

3.3.2 *P. goodeyi* mortality and mobility in *S. sisymbriifolium* and *S. nigrum* extracts

Bioassays of the *S. sisymbriifolium* and *S. nigrum* extracts solutions against the nematode *P. goodeyi* were made with the different extractives fractions in concentration corresponding to 25 g of fresh plant per 100 mL of water (basal concentration). The extracts revealed differences at the toxicity level. The mobility of *P. goodeyi* was not or little affected in dichloromethane and acetone extracts from both plants, slightly affected in ethanol extracts but very affected in water extracts (Fig. 3.2). Nematodes placed in water extracts revealed lack of mobility on the second day and the recuperation of their mobility tested on sterile water diminished onwards. Further studies would be necessary to determine this effect through time (hours) in order to evaluate the nematicidal potential of both plants.

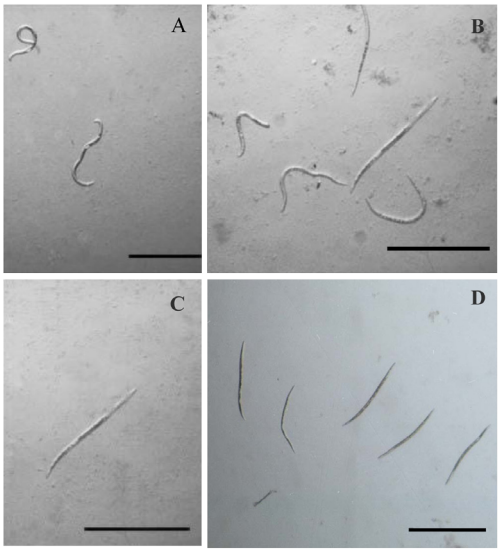

Figure 3.2 Mobility of *P. goodeyi* in dichloromethane (A), acetone (B), ethanol (C) and water extracts (D) obtained from *S. nigrum* plant sequential extraction. Scale bar = 500 µm.

The values obtained for *P. goodeyi* mortality, subjected to different extracts showed a normal distribution by Kolmogorov-Smirnov and Shapiro-Wilk ($P > 0.05$) normality tests. Tukey test showed significant differences in *P. goodeyi* mortality ($P < 0.05$) within the extracts, being water the most effective, reaching values of 99% for both plants. Dichloromethane and acetone extracts were statistically insignificant on the nematodes death ($P > 0.05$) (Fig. 3.3).

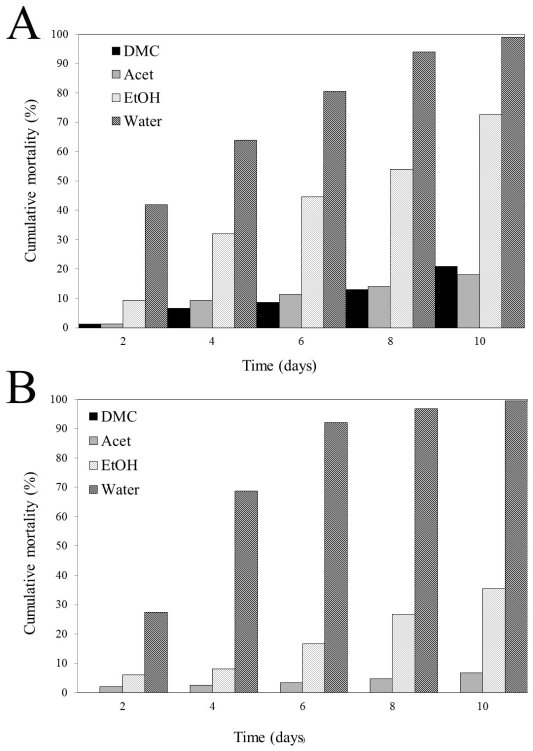

Figure 3.3 *P. goodeyi* cumulative mortality for 10 days in *S. sisymbriifolium* (A) and *S. nigrum* (B) solvents sequence extracts.

3.4 Discussion

Many plant products remain undiscovered although some are known to exert nematicidal activity (Ibrahim *et al*. 2006). In this study strong evidence for differences in the effect of the sequential extracts obtained from *S. sisymbriifolium* and *S. nigrum* were found. Dichloromethane is expected to extract lipids, volatile oils (Barbosa *et al*. 2005) and terpenoids (Tiwari *et al*. 2011). Analysis of the activity of this sequential extract against *P. goodeyi* revealed that, under the experimental conditions, the dichloromethane extract of both plants was less effective, than the other extracts, indicating that a lower quantity or quality of compounds was obtained. This result corroborates the presence of smaller amounts of extractives in the dichloromethane extract.

The acetone solvent should extract compounds such as: phenolics, flavonoids, saponins and pyrethroids among others (Sobrinho *et al*. 2010; Tiwari *et al*. 2011). But, the mobility of *P. goodeyi* was little affected by the acetone extract of *S. sisymbriifolium* and *S. nigrum*. However minor amounts of extractives were obtained in the acetone extract which may have influenced the results.

The ethanol extract is supposedly richer in glycosides, glycoalkaloids and some terpenoids (Tiwari *et al*. 2011) which have been reported to be effective on nematode activity (Chitwood 2002). In this extract, a slight effect on nematode mobility was observed, suggesting that such compounds were not extracted in quantity or were not as effective as nematicidal, when compared to the compounds extracted by water.

Water extracts from both plants had the strongest effects in the nematode mobility as motionless was observed after the first day of exposure, suggesting that compounds with nematicidal or nematostatic properties are present. Indeed, it was expected that compounds which act against nematodes such as: glycosides, alkaloids, saponins, tannins and terpenoids (Chitwood 2002) were extracted in water (Tiwari *et al*. 2011). Therefore, one or more of such compounds can be responsible for the immobilizing effect on the root-lesion nematode. But it is noteworthy that water extracts had the highest amounts of extractives compared to the other extracts. Considering these results and to clarify the influence of the amounts of extractives present in each extract additional analyses must be done with all the extracts at the same concentration.

Nevertheless, the results obtained in this study are very encouraging whereas they suggest that water extracts from both plants had nematicidal or nematostatic properties and can potentially be used towards *P. goodeyi*. In order to find out the phytochemicals or precursors of substances biosynthesized in response to plant/nematode interactions with potential to be explored as a natural nematicide, further analyses are needed to separate water extract into smaller fractions or into individual compounds and determine its effectiveness on *P. goodeyi*.

Chapter 4

In vitro evaluation of nematicidal properties of *Solanum sisymbriifolium* and *S. nigrum* extracts on *Pratylenchus goodeyi*

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4. *In vitro* evaluation of nematicidal properties of *Solanum sisymbriifolium* and *S. nigrum* extracts on *Pratylenchus goodeyi*

4.1 Abstract

The root-lesion nematode *Pratylenchus goodeyi* is among the most economically damaging parasites of banana plants. Nematode control can benefit from searching for novel bio-nematicide. The present study was carried out to assess the potential nematicidal properties of two *Solanum* species (*S. sisymbriifolium* and *S. nigrum*) against *P. goodeyi,* using the solvent sequence: dichloromethane, acetone, ethanol and water to obtain the sequential extracts and either cold or hot water the aqueous extracts of the two plants. Water and aqueous extracts of both plants at a concentration of 10 mg/mL greatly affected nematode movement and also caused mortality. The analysis of sequential extracts at the same experimental concentrations showed that although water extracts affect nematode mobility and mortality, the acetone extract from *S. nigrum* was the most efficient, causing 100% mortality after 23 h exposure. The results showed that *S. sisymbriifolium* and *S. nigrum* extracts contain chemical components that induce morphological changes in the body structure of the root-lesion nematode, affect the mobility and cause mortality. The nematostatic and nematicidal potential of the extracts described herein merit further studies to find novel bio-nematicide against the root-lesion nematode.

4.2 Introduction

Nematodes of the genera *Helicotylenchus*, *Pratylenchus* and *Rotylenchulus* are very common in Madeira Island. Mixtures of these nematodes can damage the banana culture and subsequently decrease yield and fruit quality. The root-lesion nematode *P. goodeyi* Sher & Allen 1953 is considered the most damaging nematode for banana plantations in Canary Islands, Cyprus, Crete and Taiwan (Stover & Simmonds 1987; Gowen & Quénérhervé 1990; Pinochet *et al*. 1995) as well as in Madeira Island (Troccoli *et al*. 1996).

P. goodeyi induces necrotic lesions in roots and pseudostems of banana plants. These nematodes feed, reproduce and migrate within the root tissue, causing necrosis and rotting, thus impairing the root system functions. A weakened root system affects banana plant establishment and anchoring to the soil, making it susceptible to toppling, especially when plants have a banana bunch in the final

formation phase or when winds blow stronger (Stoffelen *et al*. 1999; Speijer *et al*. 1999; Gold *et al*. 2004).

In order to control the nematode populations, farmers use chemical products that contribute to soil contamination, groundwater and atmosphere. Hence, it is imperative to change the agricultural practices associated to banana culture, making them less harmful to the environment and simultaneously enhancing food safety and product quality. As plants synthesised a large variety of secondary metabolites with multiple applications including the control of nematodes (Gommers 1973; Chitwood 2002; Osei *et al*. 2011; Kayani *et al*. 2012), they can be used either as natural nematicides or as a source of novel compounds.

In this work two *Solanum* species were selected, *S. sisymbriifolium* Lam. and *S. nigrum* L. While the first is a native of warm temperate climates of South America, and nowadays present as invasive worldwide (USDA NRCS 2007), the latter is an Eurasian species distributed all over the world (Edmonds 1979; Frohne & Pfander 1983; Valdés 2012). *S. nigrum* is very common in Madeira, where it blooms throughout the year and can be found in cultivated land, in walls and along the roads, appearing spontaneously in the banana plantations. Although *S. sisymbriifolium* is absent from the Madeira Island, it has been successfully used elsewhere to control populations of potato-cyst nematodes, *Globodera* spp., promoting the hatching of second-stage juveniles that invade its roots in large numbers but are unable to complete their life cycle (Scholte 2000a,b).

The chemical constituents of *Solanum* spp. have been described as steroidal glycosides, alkaloids and oligoglycosides, flavonoids and triperpenoids (Eltayeb *et al*. 1997; Ikeda *et al*. 2000; Raju *et al*. 2003; Heo *et al*. 2004; Cai *et al*. 2010). Several pharmacological and toxicological studies of these compounds have revealed their broad spectrum of activity as, for example, against human tumours, cancer chemopreventive, antihepatotoxic, antineoplastic, antiviral and antioxidant properties among others (Kumar *et al*. 2001; Heo *et al*. 2004; Zhou *et al*. 2006; Jeong *et al*. 2007; Lin *et al*. 2007; Ji *et al*. 2008; Lin *et al*. 2008; Cai *et al*. 2010). *S. nigrum* is widely used in popular medicine and it is also believed to have therapeutic properties against some types of tumours since some compounds showed cytotoxic effects in tumour cells (Zhou *et al*. 2006) and cancer preventive cells (Jeong *et al.* 2007).

Previous studies (Pestana *et al*. 2009) revealed that *S. sisymbriifolium* and *S. nigrum* are not suitable or non-hosts for *P. goodeyi* and the incorporation of these plants into soil benefits the growth of banana plants either by a direct action, through the release of exudates with nematicidal effect, and/or by an indirect contribution promoting the development of antagonists and making the rhizosphere

unfavourable to the nematode. In addition, it was found that mobility of *P. goodeyi* was affected by the components extracted in water at the quantities corresponding to the initial concentration of dry and fresh extracts from both plants. Furthermore, these extracts were effective in nematode mortality as after 10 days of exposure all nematodes were dead (Pestana *et al*. 2010). Therefore, this study aims to expand the knowledge of these plants by evaluating the nematicidal properties of the extracts from fresh and dried plants. These extracts were tested *in vitro* for their toxicity against *P. goodeyi* to disclose its effectiveness as a first attempt to identify constituents exhibiting nematicidal activity.

4.3 Results

4.3.1 Aqueous extracts

The aqueous extracts obtained through the blender method either with cold or hot water and through the reflux method, ranged from 7.53% for *S. sisymbriifolium* cold fresh plant to 12.42% for *S. nigrum* reflux fresh plant (Table 4.1). In general, extractions by reflux showed a higher content, while the cold water blender extraction was the least efficient in the extraction process. According to ANOVA, no differences ($P>0.05$) were observed between the use of fresh and dry plant. However, in *S. sisymbriifolium* the extract content was slightly higher in dry plant whereas in *S. nigrum* the higher extract quantities were obtained when the fresh plant material was used.

Table 4.1 Extractives (% of dry matter) obtained from *Solanum sisymbriifolium* and *S. nigrum* blended in hot and cold water or by reflux. Results are the mean of five replicates (mean ± SD).

Aqueous extracts	*S. sisymbriifolium*		*S. nigrum*	
	Fresh plant	Dry plant	Fresh plant	Dry plant
Cold	7.53 ± 0.24	8.78 ± 0.41	9.09 ± 0.11	7.65 ± 1.26
Hot	7.78 ± 0.15	11.20 ± 1.32	11.75 ± 0.40	9.26 ± 0.48
Reflux	8.83 ± 0.20	11.81 ± 0.09	12.42 ± 0.27	10.02 ± 0.94

4.3.2 Sequential extracts

The water extract fraction yielded the highest amount of extractives whereas acetone had the smallest value (2.0%) for each plant species (Table 4.2). Compounds extracted from *S. nigrum* in dichloromethane and acetone were, respectively, 5 and 13 fold lower than the compounds extracted in ethanol and water.

Table 4.2 Extractives (% of dry matter) obtained from *Solanum sisymbriifolium* and *S. nigrum* dry plants using sequential extraction. Results are the mean of five replicates (mean ± SD).

Sequential extracts	*S. sisymbriifolium*	*S. nigrum*
Dichloromethane	4.02 ± 0.09	3.52 ± 0.09
Acetone	2.05 ± 0.12	2.04 ± 0.12
Ethanol	17.28 ± 0.74	18.73 ± 0.63
Water	22.97 ± 0.23	25.97 ± 1.42

The content of extractives obtained in water sequential extraction was 2 to 3 fold higher than those in aqueous extraction from the dry and fresh plants. This discrepancy might be due to the previous solvent used (dichloromethane, acetone and ethanol) before the final water extraction, which could either increase the destruction of plant cells or promote the accessibility/solubility of chemical components.

4.3.3 Nematicidal activity of aqueous extracts

The *P. goodeyi* progressively reduced mobility in aqueous extracts (10 mg/mL) obtained from fresh and dry plant of *S. sisymbriifolium*, up to the 8th day of exposure few differences were observed between the two extracts. Cumulative mortality analysis showed that for *S. sisymbriifolium* the differences between fresh and dry plant were not significant, both aqueous extracts being effective on nematode mortality (Fig. 4.1).

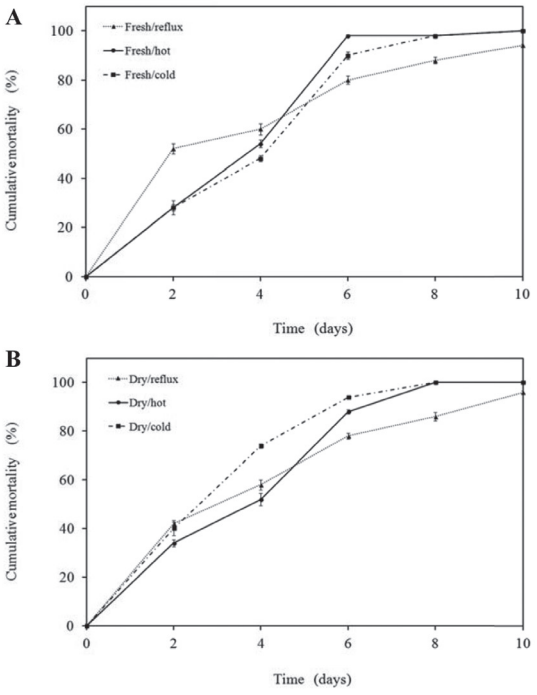

Figure 4.1 Cumulative mortality of *P. goodeyi* in cold, hot and reflux aqueous extracts at 10 mg/mL concentration from fresh (A) and dry (B) *S. sisymbriifolium*. Each time point represents the average of five replicates and vertical bars represent standard deviation among replicates. The cumulative effect for aqueous extracts is not significantly different (P >0.05) according to the ANOVA.

In the fresh and dry plant aqueous extracts (10 mg/mL) of *S. nigrum*, lack of nematode mobility was observed as early as the second day and the recovery of nematode mobility, tested on sterile water, diminished thereafter (Fig. 4.2), but in the dry cold extract a less pronounced reduction of mobility was found. In *S. nigrum*, no significant differences in *P. goodeyi* mortality were found by Tukey multiple comparison test ($P > 0.05$) for aqueous extracts from fresh plant, all extracts being equally effective on nematode mortality (Fig. 4.3 A). Nevertheless, significant differences were detected between hot and cold aqueous extracts from dry *S. nigrum* plants (Fig. 4.3 B). Dry cold aqueous extracts were less effective on nematode mortality (Fig. 4.3 B). Thus, it is apparent that the potential nematicidal compounds of *S. nigrum* are more accessible either in fresh plants or when dried material is submitted to hot extractions.

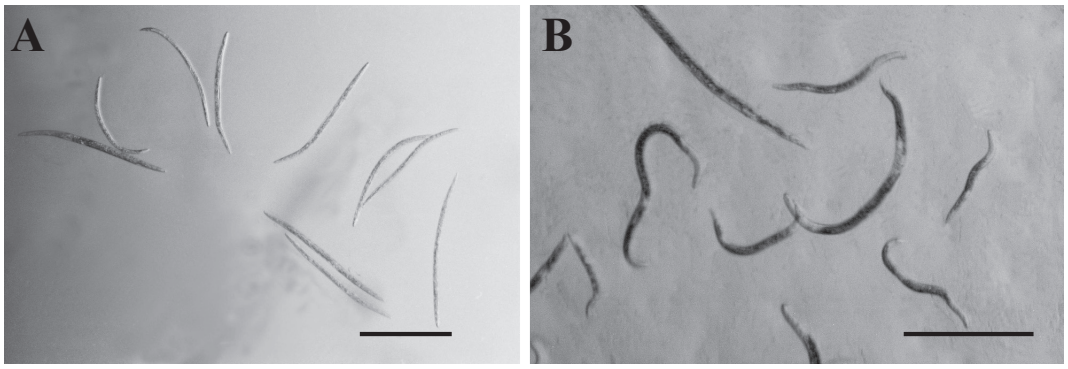

Figure 4.2 Nematode activity after two days in aqueous extracts from fresh *S. nigrum* at 10 mg/mL plant concentration (A) and in water as control (B). Scale bars = 300 µm.

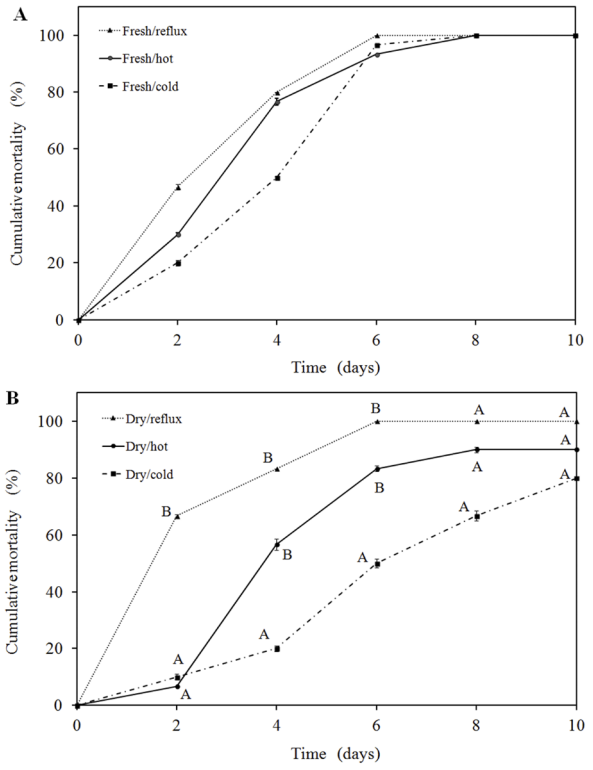

Figure 4.3 Cumulative mortality of *P. goodeyi* in cold, hot and reflux aqueous extracts, at 10 mg/mL concentration, from fresh (A) and dry (B) *S. nigrum*. Each time point represents the average of five replicates and vertical bars represent standard deviation among replicates. The same letter at each time indicates that values are not different ($P > 0.05$) according to Tukey's multiple comparison test.

Another relevant aspect observed in most of the aqueous extracts from both plants, is that the majority of dead nematodes had a mostly straight or only slightly bent body shape with few showing a sigmoid shape (Fig. 4.2 A). These results indicate that aqueous extracts from *Solanum* plants may contain one or more compounds that affect nematodes and differences observed in the nematicidal activity from these extracts are possibly due to their composition and extraction method.

4.3.4 Nematicidal activity of sequential extracts

P. goodeyi subjected to sequential extracts obtained from both plant species revealed significant differences in their activity (Fig. 4.4). Nematode mobility was little affected in dichloromethane and ethanol extracts. A clear drop in nematode activity was observed in acetone and water extracts.

The effect of dichloromethane extracts on nematode mortality was not statistically significant. Moreover, the *S. nigrum* dichloromethane extract did not seem to be toxic as little loss of nematode mobility and low mortality was recorded in this extract at the concentration of 10 mg/mL (Fig. 4.4 B). Acetone and water extracts were the most effective, with the acetone extract causing more than 95% of cumulative mortality on the second day of exposure. The higher percentage of *P. goodeyi* mortality in the acetone and in the water extract, after the solvent sequence, might suggest that either the active nematicidal components were not separated in the initial solvent or negligible amounts were present in less polar extracts.

After the first hours of exposure to acetone extracts, nematode movements became slower and morphological changes in the body structure were detected such as the separation between cuticle and internal content of the body, some rigidity, necrosis of tissues and partial disintegration of internal organs (Fig. 4.5). Exposure to the acetone and water extracts resulting from sequential extraction led to more than 85% of nematodes dying with a mostly straight or slightly bent body shape, with few (< 15%) showing a sigmoid shape, as checked in aqueous extracts from single extraction (Fig. 4.2 A).

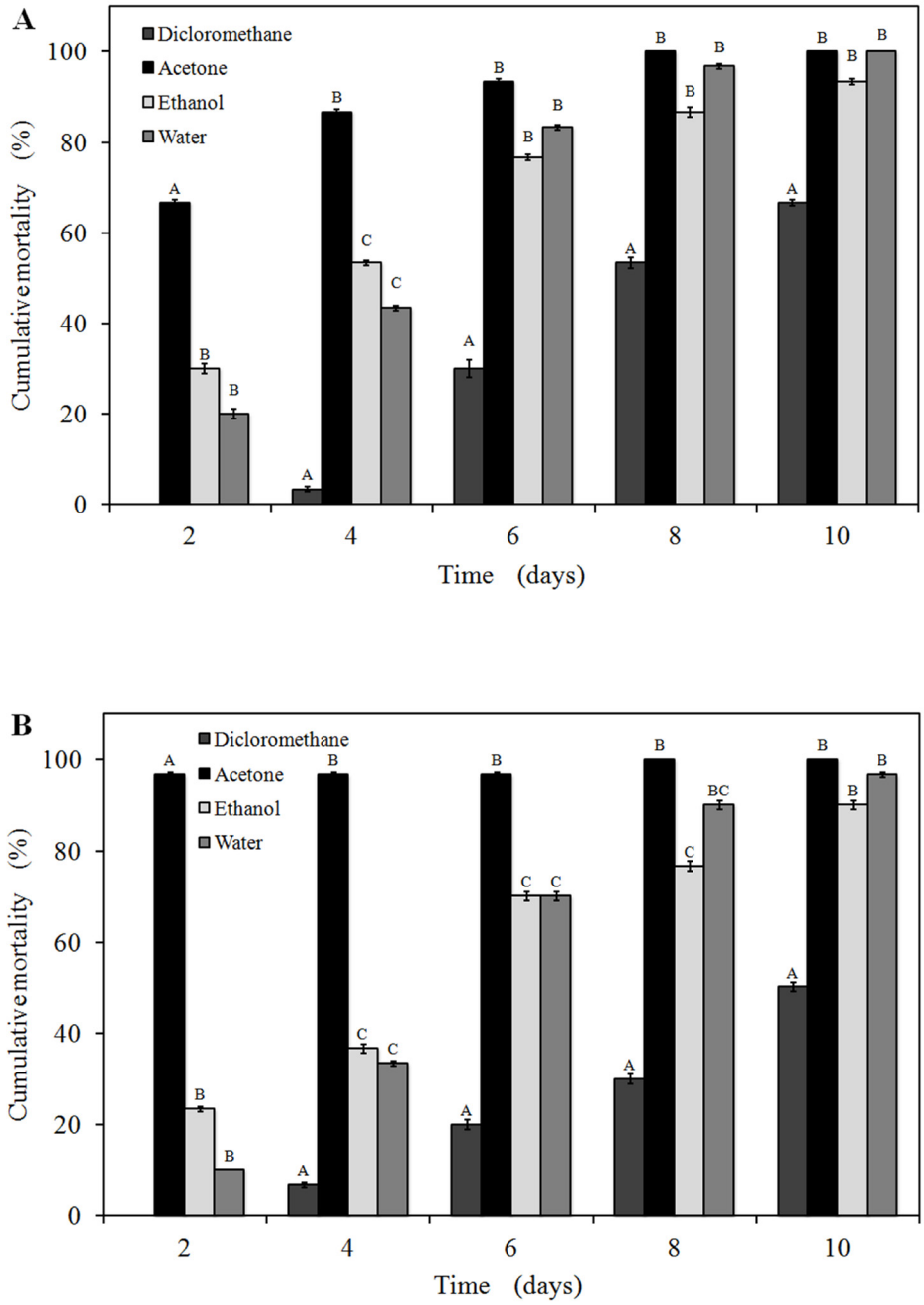

Figure 4.4 Cumulative mortality of *P. goodeyi* in dichloromethane (DMC), acetone (Acet), ethanol (EtOH) and water extracts, at 10 mg/mL concentration, from *S. sisymbriifolium* (A) and *S. nigrum* (B). Results are five replicates means and vertical bars represent standard deviation among replicates. The same letter at each time indicates that they are not different ($P > 0.05$) according to Tukey's multiple comparison test.

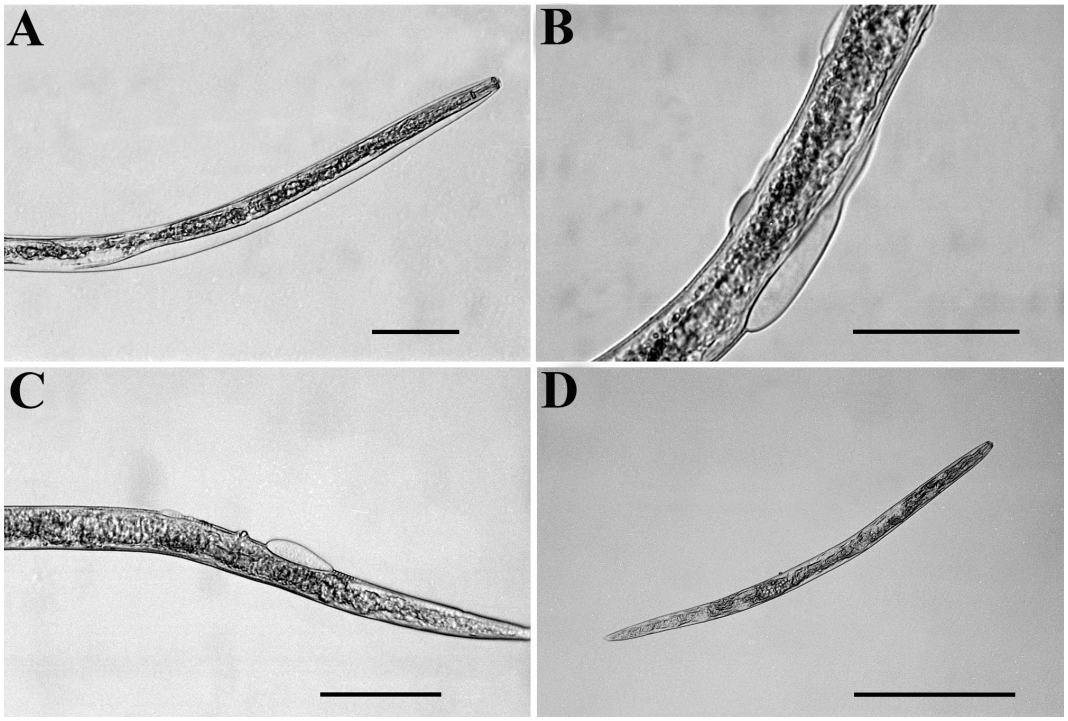

Figure 4.5 Morphological changes of *P. goodeyi* in *S. nigrum* acetone extract (10mg/mL). Separation between cuticle and internal content of the body (A, B and C). Condensation, rigidity and necrosis of tissues (D). Scale bar (A and C) 50 µm; (B) 40 µm and (D) 200 µm.

In dichloromethane and ethanol extracts nematodes were found with straight, bent and sigmoid body shape but also some with a curled body shape, most common in the ethanol extract. The efficiency of acetone extract from *S. nigrum* on nematode mortality recorded over a 24 h period revealed that nematode immobility was evident since the first hour of exposure. The mortality of *P. goodeyi* was more pronounced after 15 h of exposure (*c.a.* 50%) and at the end of the experiment all nematodes had died (Fig. 4.6).

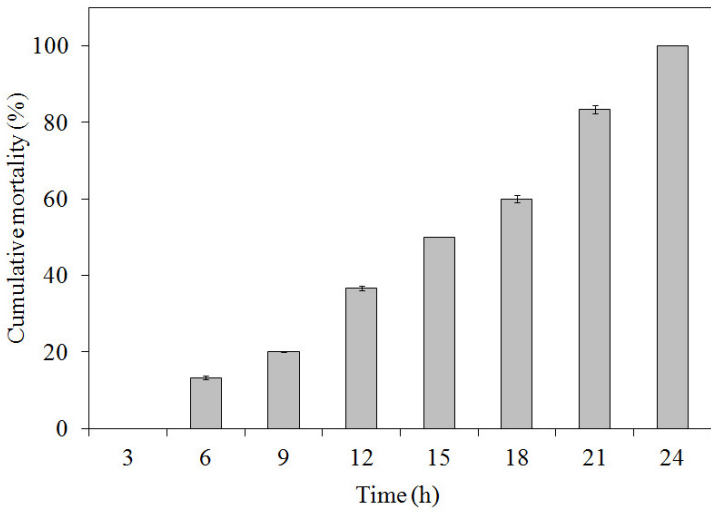

Figure 4.6 Mortality of *P. goodeyi* over 24 h incubation in *S. nigrum* acetone extract (10 mg/mL). Each time point represents the average of five replicates and vertical bars represent standard deviation among replicates.

4.4 Discussion

Strong evidence for the role of some of the components present in *S. sisymbriifolium* and *S. nigrum* plants against *P. goodeyi* were found. Plant extracts from both *Solanum* plants affect *P. goodeyi* mobility and cause mortality suggesting that compounds with nematostatic or nematicidal properties are present. In fact, it is well known that plants of the family Solanaceae are sources of secondary metabolites which act as bioactive compounds such as alkaloids, flavonoids and saponins that have a broad spectrum of activity (Evans *et al*. 1984; Eltayeb *et al*. 1997; Zhou *et al*. 2006; Ono *et al*. 2009; Cai *et al*. 2010).

The aqueous extracts obtained from dry and fresh plants demonstrated the same efficiency on *P. goodeyi* mortality as the water extracts from the sequential solvent extraction. Total lack of mobility and 100% of mortality after exposure to aqueous and water extracts at basal concentration (Pestana *et al*. 2010) and at the concentration of 10 mg/mL for *Solanum* plants were reported and these results were consistent with those obtained by Haseeb & Butool (1996) for *S. nigrum*. It was expected that several nitrogen-rich compounds, glycosides, alkaloids, saponins, tannins and terpenoids (Tiwari *et al*. 2011) would be extracted in water and many of these compounds act against nematodes (Chitwood 2002). Therefore, it is likely that one or more of such compounds could be changing the nematode activity and may be the cause of the observed mortality.

The acetone solvent should extract phenolics, flavonoids, saponins and some pyrethroids compounds (Sobrinho *et al*. 2010; Tiwari *et al*. 2011). In fact, the mobility of *P. goodeyi* was affected mainly by the *S. nigrum* acetone extract, from the first day of exposure, a feature that can be also observed through the morphological changes that were found in the bodies of nematodes subjected to this extract (Fig. 4.5). These results showed that comparatively to the other solvents, the acetone extract, at a 10 mg/mL concentration in relation to the basal concentration (data not shown), may have nematicidal compounds that act synergistically on nematode mortality.

In water and acetone sequential extracts of both *Solanum* plants, a large percentage of nematodes died with a body shape mostly straight or slightly bent, with very few showing a sigmoid shape and none curled as had been obtained in the fresh and dry plant aqueous extracts (Fig. 4.2 A). The changes in the body shape of nematodes might be assigned to the toxic effect of pyrethroids, well-known insecticides that affect directly the central nervous system (Santos *et al*. 2007). According to Wiratno *et al*. (2009) the pyrethroid-like action resulted in dead nematodes that never had curly shapes but were mostly bent or straight and very

few sigmoid, and this effect is consistent with our results. Furthermore, among the groups of compounds that may be found in both the acetone and the water extracts, it is well known that alkaloids affect the central nervous system causing paralysis and that saponins generally cause vacuolation and disintegration of the integument and alteration of membrane permeability. So, it can be assumed that the mobility behavioural and morphological changes observed are related to the toxic effect of the extracts assayed.

As reported previously (Pestana *et al*. 2009) *S. sisymbriifolium* and *S. nigrum* do not host *P. goodeyi* but the incorporation of these plants into the soil benefited banana plant growth and decreased the nematode reproduction factor. Hypothetically either a direct action occurred through the release of exudates with nematicidal effect, or an indirect contribution through the promotion of antagonists that make rhizosphere unfavourable to the nematode. Altogether, the results showed the nematicidal potential of these *Solanum* plants and especially *S. nigrum*, easily accessible to farmers, can be incorporated into the soil or used between banana plants.

In conclusion, the water and acetone extracts of *S. sisymbriifolium* and *S. nigrum* show in their composition components that possess nematostatic or nematicidal activity against *P. goodeyi* which are irreversible. It is clear that *S. nigrum* has the greatest effect on nematode mobility and mortality. For this reason we believe that further studies are worthwhile to identify and characterize the active component(s) to be used as environmentally friendly nematicide for plant parasitic nematodes.

Chapter 5

Comparative analysis of lipophilic and phenolic compounds from *Solanum nigrum* and *S. sisymbriifolium*

Margarida Pestana, Isabel de O. Abrantes, Carla Vilela, Sónia Santos, Manuela Gouveia, Lúcia Oliveira, Armando Silvestre, Nereida Cordeiro

5. Comparative analysis of lipophilic and phenolic compounds from *Solanum nigrum* and *S. sisymbriifolium*

5.1 Abstract

Solanum nigrum and *S. sisymbriifolium* revealed nematicidal activity against root-lesion nematode *Pratylenchus goodeyi* parasite of banana roots. In previous studies a sequential extraction of both plants with dichloromethane, acetone, ethanol and water was made and the effect of the water soluble extractives from each fraction tested on nematodes. In the present study the lipophilic composition of the dichloromethane extract from *S. nigrum* and *S. sisymbriifolium* with 3.52% and 4.02% of dry matter respectively, were analyzed by gas chromatography-mass spectrometry (GC-MS). Also, the phenolic compounds present in the acetone extract (2.04 % and 2.05% of dry matter) and water extract (25.97% and 22.97% of dry matter) of *S. nigrum* and *S. sisymbriifolium* respectively, were studied by ultra-high performance liquid chromatography-mass spectrometry techniques (UHPLC-MS). Fatty acids were the most abundant lipophilic compounds in the dichloromethane extract representing 79% and 80% of the total amount of the lipophilic composition in *S. nigrum* and *S. sisymbriifolium* respectively. Long-chain aliphatic alcohols and sterols were also present in both *Solanum* plants but in smaller amounts (6-9%). The total phenolic compounds detected in the acetone extract were lower in *S. nigrum* (5.04 mg/g) compared to *S. sisymbriifolium* (15.54 mg/g). The most abundant phenolic compounds in this extract were quinic acid (3.91 mg/g and 4.76 mg/g) in *S. nigrum* and *S. sisymbriifolium* respectively. The water extracts of both *Solanum* species presented higher amounts of phenolics (21.29 mg/g and 23.91 mg/g respectively), with quinic acid (4.63 mg/g and 3.53 mg/g), caffeoylquinic acid (4.77 mg/g and 13.75 mg/g) and chlorogenic acid (2.03 mg/g and 6.64 mg/g) as the main phenolic compounds in *S. nigrum* and *S. sisymbriifolium,* respectively. *In silico* analyses showed that some lipophilic compounds detected in the dichloromethane extract of both plants have activity against nematodes. Phenolic compounds analysis of the acetone extract of *S. nigrum,* which was the most efficient causing high nematode mortality, revealed the presence of compounds with negligible nematicide activity. These observations indicate that more chemical analyses of the extracts are needed to find and identify the compounds with nematicide potential.

5.2 Introduction

Plants from the genus *Solanum* (Solanaceae) are well known for their multiple applications for different purposes. Many chemical constituents have been described from *Solanum* spp. as steroid glycosides, alkaloids, oligoglycosides, flavonoids and triperpenoides (Eltayeb *et al*. 1997; Ikeda *et al*. 2000, Raju *et al.* 2003; Heo *et al*. 2004; Cai *et al*. 2010). Studies carried out with those compounds revealed activity and cytotoxicity against tumours, cancer chemopreventive, antihepatotoxic, antineoplastic, antiviral and antioxidant properties (Kumar *et al*. 2001; Heo *et al*. 2004; Zhou *et al*. 2006; Jeong *et al*. 2007; Huang *et al*. 2010). Due to these facts two *Solanum* species, *S. nigrum* L. and *S. sisymbriifolium* Lam. were chosen to evaluate their action against the root-lesion nematode *Pratylenchus goodeyi* Sher & Allen 1953 parasite of banana roots. Recent research revealed that these species do not act as a host for *P. goodeyi* and can control nematode populations upon incorporation into the soil (Pestana *et al*. 2009). Indeed, pharmachological properties from *S. nigrum* were reported as having antimicrobial, nematicidal and molluscicidal effects among others (Atanu *et al*. 2011), whereas *S. sisymbriifolium* used as a trap crop was proved to efficiently control potato-cyst nematodes *Globodera* spp. (Scholte 2000a, 2000b) and root-knot nematodes *Meloidogyne* spp. (Dias *et al*. 2012). So, a sequential extraction with the solvents dichloromethane, acetone, ethanol and water of both plants were made and the water soluble extractives of each fraction tested on *P. goodeyi* (Pestana *et al*. 2010). Previous studies showed that *S. nigrum* acetone and water extracts directly affected morphological features, mobility and caused 100% of mortality on *P. goodeyi* (Pestana *et al*. 2010, 2014a).

Among the bioactive constituents that may be found in plants, lipophilic and phenolic compounds are well known phytochemicals synthesized in response to ecological and/or physiological stress such as pathogen infection or insect attack (Chitwood 2002; Khoddami *et al*. 2013). Many fatty acids and phenolic compounds act as antioxidants, reducing inflammations and incidence of cancers and diabetes (Oliveira *et al*. 2008; Dai & Mumper 2010; Zhang *et al*. 2011). Indeed, sterols and steryl glucosides, simple phenols, coumarins, lignins, lignans, tannins, phenolic acids, flavonoids are being investigated to develop phytochemical strategies for nematode control (Chitwood 2002).

Given the nematicidal potential of *S. nigrum* and *S. sisymbriifolium* the detailed study of the chemical composition of both plants may represent an important contribution for the development of new control measures of plant-parasitic nematodes. For this purpose, here we report the comparative analysis of the lipophilic and phenolic chemical profiles of *S. nigrum* and *S. sisymbriifolium*

species, through gas chromatography-mass spectrometry (GC-MS), ultra-high performance liquid chromatography (UHPLC) and mass spectrometry (MS) techniques in order to assess the existence of potential nematicidal compounds.

5.3 Results

5.3.1 Lipophilic composition

Lipophilic composition can be determinated through GC-MS, which has become established as a useful technique for the separation and detection of chemical compounds from plant extracts. This technique was used to study the dichloromethane extracts from *S. nigrum* and *S. sisymbriifolium* obtained through a sequential extraction (2.3.2). The yield of the dichloromethane extractives from *S. nigrum* and *S. sisymbriifolium* was similar, with values of 3.52 ± 0.09 and 4.02 ± 0.09 % of dry matter, respectively. Evaluation of nematicidal proprieties of the both dichloromethane extracts revealed that *P. goodeyi* mobility was not affected in these extracts solutions at basal concentration (Pestana *et al*. 2010). After using the extract solutions at concentration 10mg/mL results for mobility and mortality have not changed (Pestana *et al*. 2014).

The qualitative composition of the dichloromethane extracts from *S. nigrum* and *S. sisymbriifolium* were almost similar as fatty acids, long-chain aliphatic alcohols (fatty alcohols) and sterols, were the families of the lipophilic compounds mainly represented in both plants. At least thirty different lipophilic compounds were identified in these extracts based on their characteristic retention time (RT) and fragmentation pattern. Fig. 5.1 shows a typical chromatogram of *S. nigrum* derivatized extracts before alkaline hydrolysis.

Considering that dichloromethane extracts usually present a high content of esterified structures, they were analysed before and after alkaline hydrolysis, in order to verify the existence of these structures in the lipophilic extract. Table 5.1 list the identified families and individual components and their corresponding abundances in the dichloromethane extracts of *S. nigrum* and *S. sisymbriifolium*, before and after alkaline hydrolysis based on their characteristic retention time (RT) and fragmentation pattern.

The total amount of the lipophilic compounds was higher in *S. nigrum* (9.2 g/kg of dry matter) compared to *S. sisymbriifolium* (5.5 g/kg of dry matter). Fatty acids were the most represented chemical family, around 80% in the total amount of the lipophilic composition (Fig. 5.2).

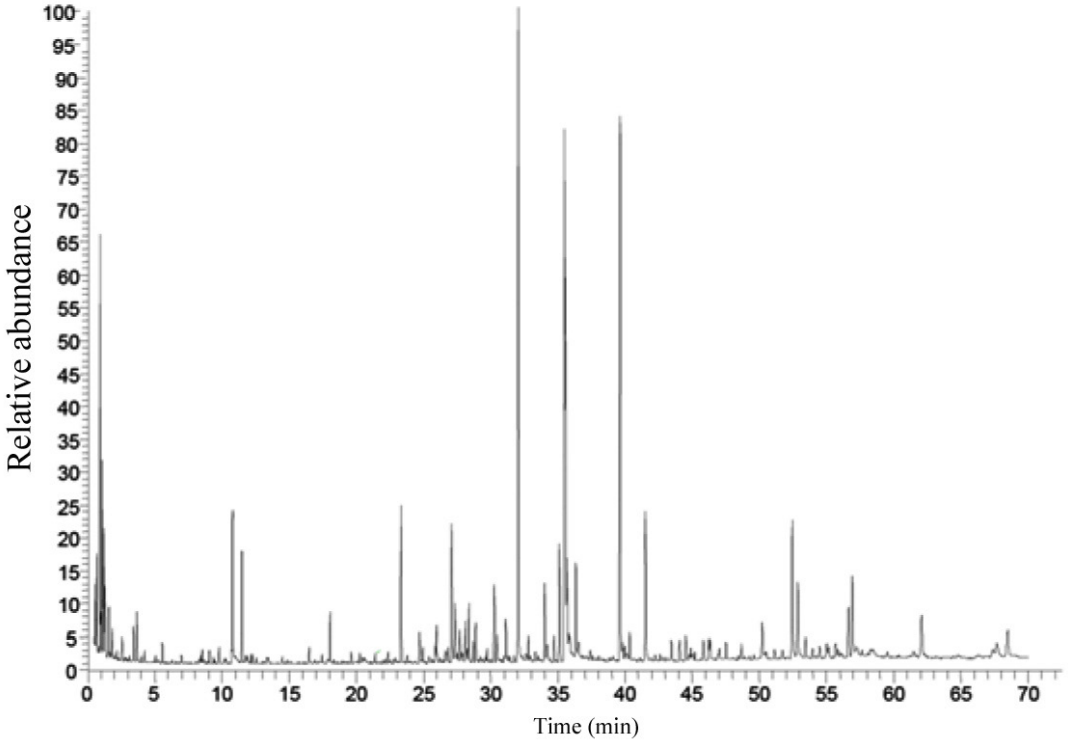

Figure 5.1 GC-MS chromatogram of the derivatized dichloromethane extract from the *S. nigrum* before alkaline hydrolysis (BH).

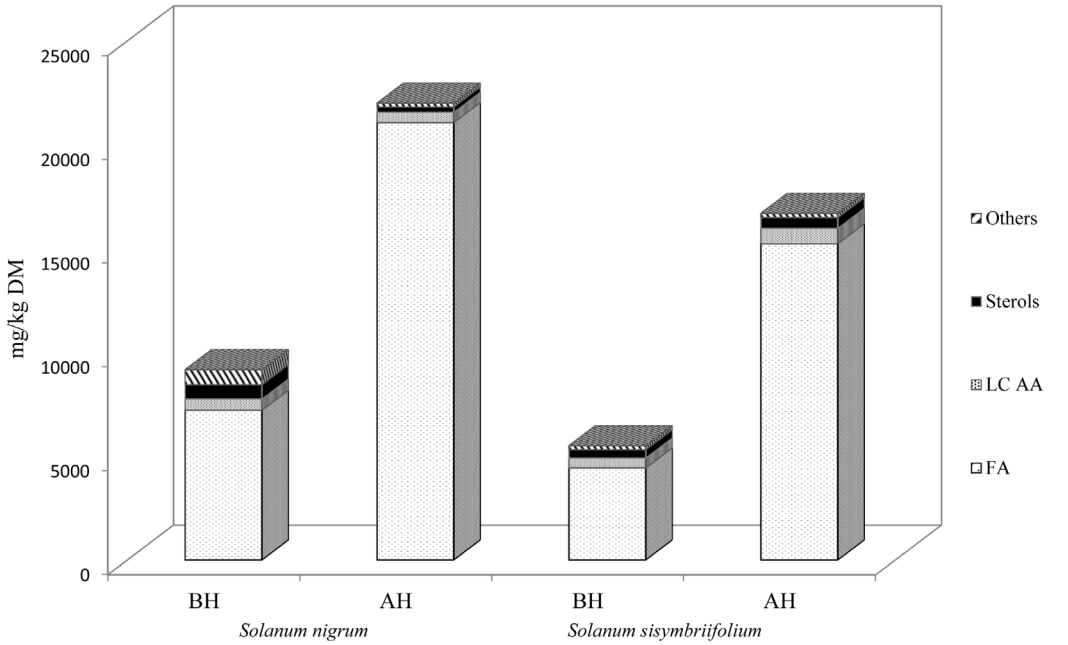

Figure 5.2 Major families of lipophilic components identified by GC-MS in the *Solanum* dichloromethane extracts. Fatty acids FA, long-chain aliphatic alcohols LCAA, before hydrolysis BH, after hydrolysis AH.

Table 5.1 Lipophilic composition of *Solanum nigrum* and *S. sisymbriifolium* before (BH) and after (AH) hydrolysis expressed in mg/Kg of dry matter (DM).

		Solanum nigrum		*Solanum sisymbriifolium*	
	RT	**BH**	**AH**	**BH**	**AH**
Fatty acids		**7205,69**	**21062,93**	**4427,54**	**15228,31**
Saturated		3689,60	6930,30	2150,63	6870,20
Dodecanoic acid	24,69	18,46	25,94	11,78	56,85
Tetradecanoic acid	29,84	269,33	173,54	131,71	245,88
Pentadecanoic acid	32,24	54,72	78,44	36,58	113,84
Hexadecanoic acid	34,61	2533,83	4804,11	1253,79	4336,29
Heptadecanoic acid	36,74	60,85	105,50	71,17	123,02
Octadecanoic acid	38,89	386,49	1035,64	375,19	1165,36
Eicosanoic acid	42,87	74,06	290,06	64,79	251,18
Docosanoic acid	46,57	79,42	174,02	68,02	214,46
Tricosanoic acid	48,34	98,74	60,52	46,02	73,8
Tetracosanoic acid	50,03	72,24	107,57	45,01	158,6
Pentacosanoic acid	51,78	13,54	20,62	15,62	30,28
Hexacosanoic acid	53,63	27,91	54,35	30,96	100,64
Unsaturated		3516,09	14132,63	2276,91	8358,11
Hexadeca-9-enoic (cis+trans)	33,93	52,40	182,04	48,71	125,35
Octadeca-9,12-dienoic acid	38,14	1647,75	9497,31	949,88	3959,13
Octadeca-9,12,15-trienoic acid	38,21	1435,73	3042,13	1140,55	3878,82
Octadec-9-enoic acid	38,28	380,21	1411,15	137,77	394,81
Long-chain aliphatic alcohols		**543,88**	**491,85**	**481,14**	**739,63**
Tetradecan-1-ol	27,92	31,60	21,92	18,48	33,99
Pentadecan-1-ol	30,41	ND	ND	ND	13,81
Hexadecan-1-ol	32,76	288,80	177,53	151,26	216,54
Octadecan-1-ol	37,21	106,55	105,29	71,05	134,95
Eicosan-1-ol	41,31	16,57	20,60	14,63	34,57
Docosan-1-ol	45,10	28,95	60,39	69,68	165,5
Octacosan-1-ol	55,94	71,41	106,12	156,05	140,25
Sterols		**688,55**	**255,95**	**396,17**	**515,61**
Campesterol	57,52	253,58	38,81	60,22	68,76
Stigmasterol	58,17	66,17	42,56	95,93	127,89
β-sitosterol	59,44	368,80	174,57	240,02	318,96
Others		**723,62**	**186,32**	**194,92**	**207,42**
Vanillic acid	27,44	39,44	19,48	40,89	46,85
Ferulic acid	35,20	20,71	61,77	27,38	70,21
γ-tocopherol	52,70	195,11	32,58	ND	ND
α-tocopherol	55,37	468,37	72,49	126,65	90,36
Total detected		**9161,74**	**21997,05**	**5499,77**	**16690,96**

ND, not detected

Long-chain aliphatic alcohols and sterols were present in both plants but in smaller amounts (below 9%). Apart from the above-referred families, minor amounts of aromatic and tocopherol compounds were also identified.

A large increase in the total amount of extractives (2.4 and 3.0 fold in *S. nigrum* and *S. sisymbriifolium* respectively) was detected by GC-MS, after hydrolysis particularly, among the fatty acids (Fig. 5.2). These increases indicate the presence of significant amounts of esterified structures in the original extracts.

Before alkaline hydrolysis, saturated fatty acids, such as hexadecanoic, octadecanoic and tetradecanoic acids were the most abundant components of fatty acid family in both plants, with 51% and 49% of the total fatty acids amount in *S. nigrum* and *S. sisymbriifolium* respectively (Fig. 5.3). Detailed analysis showed that saturated fatty acids relative composition in the dichloromethane extract of *S. nigrum* was higher before hydrolysis (51%) than after hydrolysis (33%) in relation to the total fatty acids amounts. In *S. sisymbriifolium* extract the composition of saturated fatty acids was similar before and after hydrolysis (49% and 45%). This can be related to the extract of *S. nigrum* unsaturated fatty acids increase from 49% before hydrolysis to 67% after hydrolysis, while in *S. sisymbriifolium* this amount was similar (51% and 55%). The hexadecanoic acid was the most abundant saturated fatty acid either in *S. nigrum* or *S. sisymbriifolium,* ranging from 23 to 35% of the total fatty acids in *S. nigrum* and about 28% in *S. sisymbriifolium* after and before hydrolysis, respectively.

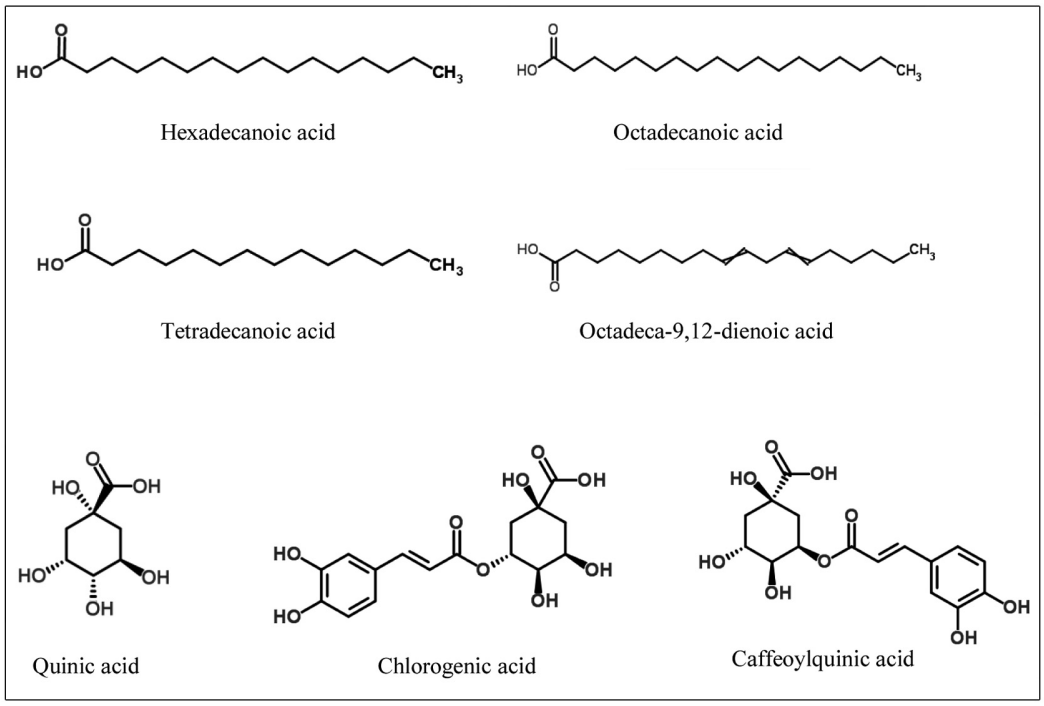

Figure 5.3 Chemical structures of the main lipophilic and phenolic compounds identified in *S. nigrum* and *S. sisymbriifolium* dichloromethane, acetone and water extracts.

Unsaturated fatty acids were also present in the lipophilic extract representing 49 and 51% of the total fatty acids analysed in *S. nigrum* and *S. sisymbriifolium* respectively (Table 5.1). Octadeca-9,12-dienoic (Fig. 5.3) and octadeca-9,12,15-trienoic acids were the most abundant compounds of this group, representing 43% of the total fatty acids amount in the *S. nigrum* extract and around 90% of the total unsaturated fatty acids for both species (Table 5.1). After alkaline hydrolysis, an increase in the amount of unsaturated fatty acids (particularly, in octadeca-9,12-dienoic acid) was observed in both plants.

Fatty alcohols represent only a small fraction (2 to 9%) of the total amount of lipophilic extractives analysed by GC-MS. The most abundant fatty alcohols found are the hexadecan-1-ol, representing 53% and 31% of the total amount of long chain aliphatic alcohols in the extract of *S. nigrum* and *S. sisymbriifolium,* respectively. Sterols identified in *Solanum* species represent *c.a.* 7% of the lipophilic components with β-sitosterol and campesterol the major sterols present in the extracts. Other compounds such as tocopherols and aromatic compounds were detected in small amounts (194 to 723.62 mg/kg; 3.4 to 7.9%) in both plants.

Searches in databases of the lipophilic compounds found in the dichloromethane extract of *S. nigrum* and *S. sisymbriifolium* showed that the saturated fatty acids hexadecanoic acid have antioxidant, anti-inflammatory and nematicidal activity and tetradecanoic acid possess repellent, larvicidal and nematicidal activity. Also, the unsaturated fatty acid octadeca-9,12-dienoic exhibit nematicide properties (Fig. 5.3).

5.3.2 Phenolic composition

Phenolic compounds can be determined by ultra-high performance liquid chromatography in combination with ultraviolet detection (UHPLC-UV) which separate and quantify the components present in complex liquid mixtures. This analytical technique was used to study the phenolic composition of acetone and water extracts from *S. nigrum* and *S. sisymbriifolium* obtained through a sequential extraction (2.3.2). The content of extractives was 2.04 ± 0.12 and 2.05 ± 0.12% of dry matter in acetone extracts and 25.97 ± 1.42 and 22.97 ± 0.23% of dry matter in water extracts of *S. nigrum* and *S. sisymbriifolium,* respectively. *In vitro* evaluation of nematicidal activity of the acetone and water extracts at basal concentration (concentration at exact quantity corresponding to the initial ratio of dry plant) from *S. nigrum* and *S. sisymbriifolium* revealed that *P. goodeyi* mobility was not affected by the acetone extracts but, was greatly affected by water extracts from both plants causing also mortality (Pestana *et al*. 2010). After using the concentration 10mg/mL for each extract results have not changed except for acetone extract of *S. nigrum*

since total immobility was observed after the first hours of exposure and 100% mortality was obtained at 23 h exposure (Pestana *et al*. 2014).

The analysis of the chromatograms of acetone and water extracts from *S. nigrum* and *S. sisymbriifolium* (Fig. 5.4) showed differences in the chemical composition of the two plants.

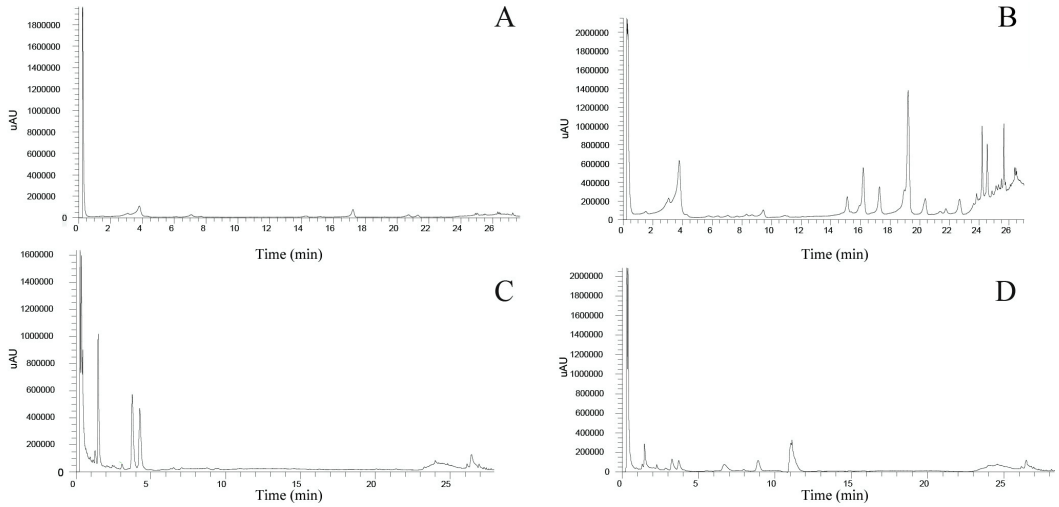

Figure 5.4 UHPLC-UV chromatograms of the acetone extract from *S. nigrum* (A) and *S sisymbriifolium* (B) at 340 nm. Water extract from *S. nigrum* (C) and *S. sisymbriifolium* (D) at 280 nm.

At least twenty different phenolic compounds could be identified by comparison of spectra and retention times. All major peaks detected up to 25 min in the acetone and water extracts from the two *Solanum* species belonging to different phenolic compounds are summarized in Table 5.2.

The abundance of phenolic compounds is listed in Table 5.3. Comparison of chemical profiles of the two species showed that the total phenolic compounds detected was higher in water extracts of *S. sisymbriifolium* and *S. nigrum* (23.91 and 21.29 mg/g respectively). The acetone extract from *S. sisymbriifolium* had more total phenolic compounds (15.54 mg/g) than *S. nigrum* (5.06 mg/g) and was the extract with more diversity of phenolic compounds obtained mainly at 340 nm.

Detailed analysis showed that the acetone extract of *S. nigrum* had quinic acid representing 77% of the total phenolic detected, whereas *S. sisymbriifolium* had 31%. Caffeoylquinic acid was present in *S. nigrum* and *S. sisymbriifolium* corresponding to 16% and 26% of the total phenolic detected in acetone extracts. Also, chlorogenic acid was obtained in both *S. nigrum* and *S. sisymbriifolium* acetone extracts but in smaller amounts (7% and 9% respectively) (Fig. 5.3). Phenolic compounds such as the flavonoids (luteolin and quercetin) were detected in minor amounts in the acetone extract of *S. sisymbriifolium* and were absent in *S. nigrum.*

Table 5.2 Phenolic compounds identified in *Solanum nigrum* and *S. sisymbriifolium* acetone and water extracts and corresponding MS^n fragmentation profile.

Nº	RT (min)	Compound	λ (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	MS^4 (*m/z*)
1	0.32	Quinic acid	252	191	173,		
2	0.40	Gallic acid	270	169	125		
3	1.41	Caffeoylquinic acid isomer	242, 301(sh),322	353	191, 179, 135	93	
4	3.21	Chlorogenic acid	243, 301(sh), 324	353	191, 179	93	Co
5	3.66	Caffeoylquinic acid isomer	243, 301(sh), 324	353	191, 179, 173, 135	93	
6	6.64	Caffeoylmalate	244, 302(sh), 325	295	227, 179, 133		
7	8.86	Epicatechin	229, 279	289	245, 243, 221, 215, 175		
8	11.08	Quercetin-glucosyl-rhamnosyl-hexoside isomer	227, 255, 359	771	753, 609, 607, 505, 489, 343, 325, 301, 300, 271, 255		
9	15.11	Quercetin-rutinoside	231(sh), 255, 348	609	301	283, 273, 239, 179, 151	
10	15.95	Quercetin-hexoside	231(sh), 255, 348	463	301	283, 273, 239, 179, 151	
11	16.19	Quercetin-rutinoside	231(sh), 255, 349	609	301	273, 229, 179, 151	
12	17.28	Luteolin-rhamnoside-hexoside	230(sh), 265, 340	593	285	267, 257, 229, 213, 197, 169, 151	
13	18.95	Luteolin-hexoside	231(sh), 265, 335	447	285	267, 257, 229, 213, 197, 169, 151	
14	19.21	Quercetin-rutinoside	234(sh), 265, 340	609	301	273, 229, 179, 151	
15	19.24	Luteolin-rhamnoside-hexoside		593	285	267, 257, 229, 213, 197, 169, 151	
16	20.35	Isorhamnetin-hexoside	232, 306	477	315, 300, 285, 271, 243	300	271, 255
17	21.39	Isorhamnetin-hexoside	232, 314	477	315, 300	300	271, 255
18	23.63	Quercetin	233, 319	301	273, 179, 151		
19	24.55	Isorhamnetin	233, 254(sh), 337	315	300	271, 255, 243, 151	
20	25.14	Luteolin	234, 309	285	217, 169, 151		

RT, retention time

In *S. nigrum* water extract the most abundant phenolic compounds were: epicatechin, representing 28% of the total phenolics, not detected in the other extracts; quinic acid and caffeoylquinic acid with both 22% of total phenolics and chlorogenic acid with 9%. Quinic acid (15% of total phenolics), caffeoylquinic acid (57% of total phenolics) and chlorogenic acid (28% of total phenolics) were the phenolic mainly obtained in the water extract of *S. sisymbriifolium*. In addition, *S. nigrum* water extract had caffeolylmalate (6% of the total phenolics), quercetin-glucosyl-rhamnosyl-hexoside (12% of the total phenolics) that were also absent in the remaining extracts (Table 5.3).

Searches on databases revealed that quinic, caffeoylquinic and chlorogenic acids are considered as having antioxidant activity but with no proven nematicide effect against plant-parasitic nematodes. Instead, luteolin, quercetin and epicatechin compounds have many biological activities, including acting as a pesticide but were present in minor amounts.

Table 5.3 Abundance of phenolic compounds identified in *Solanum nigrum* and *S. sisymbriifolium* acetone and water extracts.

N.	Compound	λ (nm)	Acetone extracts		H2O extracts	
			S. nigrum (mg/g)	*S. sisymbriifolium* (mg/g)	*S. nigrum* (mg/g)	*S. sisymbriifolium* (mg/g)
1	Quinic acid[a]	280	3.91[(1+2)]	4.76[(1+2) h]	4.63	3.53
2	Gallic acid[a]	280				
3	Caffeoylquinic acid isomer[b]	340			2.66	7.08
4	Chlorogenic acid[b]	340	0.34	1.48	2.03	6.64
5	Caffeoylquinic acid isomer[b]	340	0.81	4.11	2.11	6.67
6	Caffeoylmalate[c]	280			1.33	-
7	Epicatechin[d]	280			5.94	-
8	Quercetin-glucosyl-rhamnosyl-hexoside isomer[e]	340			2.60	-
9	Quercetin-rutinoside[e]	340		0.39		
10	Quercetin-hexoside[e]	340		0.23		
11	Quercetin-rutinoside[e]	340		0.88		
12	Luteolin-rhamnoside-hexoside[f]	340		0.17		
13	Luteolin-hexoside[f]	340		0.13		
14	Quercetin-rutinoside[e]	340		2.28[(14+15) h]		
15	Luteolin-rhamnoside-hexoside	340				
16	Isorhamnetin-hexoside[g]	340		0.32		
17	Isorhamnetin-hexoside[g]	340		0.07		
18	Quercetin[e]	340		0.33		
19	Isorhamnetin[g]	340		0.36		
20	Luteolin[f]	340		0.03		
Total detected			5.06	15.54	21.29	23.91

Calibrations curves used: [a] gallic acid, [b] chlorogenic acid, [c] caffeic acid, [d] catechin, [e] quercetin, [f] luteolin, [g] isorhamnetin, [h] Sum of the phenolic content by partial overlapping.

5.4 Discussion

Plants of the genus *Solanum* are known to possess nematicidal activity against a wide range of plant-parasitic nematodes (Haseeb & Butool 1996; Scholte 2000a, 2000b; Dias *et al*. 2012). Previous studies revealed that extracts of *S. nigrum* and *S. sisymbriifolium* showed activity against the root-lesion nematode *P. goodeyi* (Pestana 2007; Pestana *et al*. 2010). Accordingly, the present study compared the chemical profile of the lipophilic and phenolic compounds that were found in *S. nigrum* and *S. sisymbriifolium* in order to identify potential nematicidal compounds.

Lipophilic compounds of dichloromethane extracts from *S. nigrum* and *S. sisymbriifolium* were composed mainly by fatty acids relatively to the total amount of compounds detected. After hydrolysis the total fatty acids amount available increased considerably in both plants indicating that esterified structures were present in the original extracts. Among the most representative saturated fatty acids, hexadecanoic acid had many activities such as an antioxidant and anti-inflammatory effect and possesses nematicidal activity. Indeed, studies showed that hexadecanoic acid caused significantly higher mortality in second-stage juvenile of *Meloidogyne incognita* (Zhang *et al*. 2012). Tetradecanoic acid had larvicidal and repellent activity against Culicidae as *Aedes aegypti* and *Culex quiquefasciatus* (Sivakumar *et al*. 2011) and its application showed nematicidal properties against *M. incognita* juveniles (Debprasad *et al*. 2000). Also, the unsaturated fatty acid octadeca-9,12-dienoic acid which had increased after alkaline hydrolysis mainly in *S. nigrum*, revealed nematicidal activity against the free-living nematode *Caenorhabditis elegans* and seems to be the nematicidal agent of several nematophagus fungi (Stadler *et al*. 1993). Despite the higher amounts of lipophilic compounds found mainly in *S. nigrum*, our previous studies showed no effects of the dichloromethane extract from both plants either in mobility or mortality of *P. goodeyi* even after using the concentration 10 mg/mL (Pestana *et al*. 2010, 2014a). These results may be related to the yield of the dichloromethane extractives that was obtained at the beginning of the experiment or to the fact that lipophilic compounds are poorly soluble in water since in our previous studies only the compounds water soluble were tested against *P. goodeyi*.

As expected, abundance of phenolic compounds was higher in water extracts relatively to acetone extracts. Quinic, caffeoylquinic and chlorogenic acids were the most representative phenolics in both acetone and water extracts of *S. nigrum* and *S. sisymbriifolium* and they are long recognized as powerful antioxidants. Quinic acid was the most abundant and it is considered as having protective effects against cell damage (Soh *et al*. 2003). Caffeoylquinic acid is a potent antioxidant being

synthesized in response to oxidative stress (Mondolot *et al*. 2006). Chlorogenic acid is involved in plant responses to pathogens and oxidative cell injury but showed negligible nematicidal activity against root-knot nematode although being highly active on *Globodera rostochiensis* and *Xiphinema index* (D'Addabbo *et al*. 2013). The polyphenol epicatechin detected only in the water extract of *S. nigrum* had some bioactivity, decreasing the mobility of *Heterodera glycines* juveniles (Masler 2013). Nevertheless, the minor amount of compounds detected in acetone extract of *S. nigrum* do not reflected the higher nematicidal effect obtained after using the concentration 10 mg/mL (Pestana *et al*. 2014a). These findings support that nematicidal activity is probably related to the action of other compounds not yet identified or to the simultaneously action of two or more lipophilic and phenolic compounds found, justifying further research of the chemical composition of both plants in order to determine the compounds that may be responsible for decreasing nematode mobility and mortality.

Chapter 6

Effect of chemical stress imposed by *Solanum nigrum* in *calreticulin* and *beta-1,4- endoglucanase* genes and in infectivity of *Pratylenchus goodeyi*

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6. Effect of chemical stress imposed by *Solanum nigrum* in *calreticulin* and *beta-1,4- endoglucanase* genes and in infectivity of *Pratylenchus goodeyi*

6.1 Abstract

Isolation and expression of effector genes encoding proteins secreted by plant-parasitic nematodes into a host can be helpful in improving the understanding of parasitic interactions. In this study, calreticulin, a highly conserved Ca^{2+}-binding and multifunctional protein, and beta-1,4-endoglucanase, a cell wall-degrading enzyme, both known to be secreted from oesophageal gland cells and injected through the nematode stylet into host tissue, were analysed. Full-length cDNAs from *calreticulin* (*CRT*) and *beta-1,4-endoglucanase* (*ENG*) with an estimated size of 1549 and 1342 bp, respectively, were isolated from the root-lesion nematode *Pratylenchus goodeyi* (Pg) by RT-PCR and RACE techniques. *Pg-CRT* and *Pg-ENG* cDNAs were characterized *in silico*, and their expression assessed by semi-quantitative PCR in nematodes exposed to a chemical stress provided by a *Solanum nigrum* acetone extract showing nematicidal activity. It was demonstrated that the plant extract down-regulated the levels of *Pg-CRT* mRNA, whereas the transcripts of *Pg-ENG* mRNA held steady. This extract also affected nematode behaviour towards the roots since the number of nematodes that reached and penetrated the roots diminished when the exposure time rose. These observations indicate that the nematicidal compounds present in the plant acetone extract were effective as a signal to influence the infection success of *P. goodeyi in vitro* and it might be tested against other phytoparasitic nematodes.

6.2 Introduction

Survival of plant-parasitic nematodes relies on the ability to locate and to infect the roots of hosts. It is known that these processes are facilitated by effector genes, such as *calreticulin* (*CRT*) and *beta-1,4-endoglucanase* (*ENG*), whose products are secreted by parasites to facilitate penetration, migration and to counter plant defence responses (Rosso *et al*. 1999; Abad *et al*. 2003; Jaubert *et al*. 2005; Jaouannet *et al*. 2012).

The *CRT* gene encodes a multifunctional protein expressed in the cells of higher organisms. It is highly conserved in plants and animals as it shares an overall identity of 70% (Jaubert *et al*. 2005). Calreticulin protein is mainly present in the

lumen of the endoplasmic reticulum (ER) where it acts as a chaperone, involved in Ca^{2+} homeostasis and in the folding of newly synthesized glycoproteins via the calreticulin/calnexin cycle (Michalak *et al*. 1999; Johnson *et al*. 2001; Park *et al*. 2001; Michalak *et al*. 2002). But, calreticulin located in the nuclear envelope, in the cytoplasm or in the cell surface is also involved in the regulation of multiple functions that have been summarized by Johnson *et al*. (2001). This gene has been found in the plant-parasitic nematodes *Meloidogyne incognita* (Jaubert *et al*. 2005), *Bursaphelenchus xylophilus* (Li *et al*. 2011), *Ditylenchus destructor* (Peng *et al*. 2013) and in the free-living nematodes *Caenorhabditis* spp. (Park *et al*. 2001) among other nematodes.

Recently, a correlation between calreticulin and parasitism in the endoparasitic sedentary nematode *M. incognita* was established. By immunolocalization, calreticulin was detected in the secretions from juvenile (J2) *M. incognita*. It is present in the oesophageal subventral glands of J2 and in dorsal gland of sedentary females (Jaubert *et al*. 2005). By *in situ* hybridization calreticulin was detected in dorsal gland of *D. destructor* (Peng *et al*. 2013). In addition, it was found that this protein is injected into plant tissues through the nematode stylet (Jaubert *et al*. 2005). So far, two possible roles have been suggested for calreticulin in parasitism. Either calreticulin acts as a chaperone for secreted proteins in the oesophageal glands and has, hence no direct role in the nematode-plant interaction or it is involved in the infection process through the modulation of plant responses and thus plays a direct role in the success of infection (Jaubert *et al*. 2005, Jaouannet *et al*. 2013).

The *ENG* was the first effector gene whose product was proven to be able to degrade beta-1,4-glycosidic bonds of cellulose, the major component of the plant cell wall, and the first to be localized by immunocytology on the host plant tissue along with nematode migration through the root, confirming its importance on parasite invasion, migration and degradation of host tissues (Haegeman *et al*. 2012; Hewezi & Baum 2013; Kyndt *et al*. 2013). This gene appears to be widely present in plant-parasitic nematodes and has been detected in nematodes with diverse parasitic habits such as *M. incognita*; *Globodera rostochiensis*, *Heterodera glycines*; *B. xylophilus*, *Rotylenchulus reniformis*, *D. africanus*, *P. coffeae, P. penetrans* and *P. vulnus* (Smant *et al*. 1998; Rosso *et al*. 1999; Uehara *et al*. 2001; Kyndt *et al*. 2008, Wubben *et al*. 2010; Fanelli *et al*. 2014).

Pratylenchus goodeyi Sher & Allen 1953 is a migratory endoparasitic nematode harmful to banana crops that is widespread in Madeira Island where it causes severe problems (Pestana *et al*. 2009). To control nematode populations farmers use chemical nematicides but, plants such as *S. nigrum* L. which is not a host for *P. goodeyi* (Pestana *et al*. 2009) may be an alternative to chemical control

as it contains many compounds with antimicrobial, nematicidal and molluscicidal properties (Atanu *et al*. 2011). It is thought that these compounds are responsible for the paralysis, morphological changes and mortality found on *P. goodeyi* in previous studies (Pestana *et al*. 2014a).

Here, we confirm that the chemical stress provided by the acetone extract of *S. nigrum* (2.3.2) affects the expression of genes that can be associated to infection and parasitism as well as nematode behavioural aspects such as the ability to find the roots of the host, mobility and capacity of penetration.

6.3 Results

6.3.1 Cloning of *calreticulin* gene

Degenerate primers designed from highly conserved domains of published nematode calreticulin sequences, were first used to amplify an internal specific PCR fragment of 481 bp from *P. goodeyi* cDNA (2.6.3). After cloning, the sequence analysis of this fragment revealed its homology to already known calreticulin from different plant-parasitic nematode species with an identity of 84% to *M. incognita* (GenBank: AAL40720), 83% to *Radopholus similis* (GenBank: ACY01917) and 78% to *D. destructor* (GenBank: ACV33082). Then, gene specific primers were designed to assemble the full-length cDNA sequence of *Pg-CRT* through RACE. The PCR product of 5' end was a fragment of 790 bp and the 3' end a fragment of 1050 bp, which overlap 341 bp and 431 bp respectively, with the internal sequence of *Pg-CRT* cDNA. Thus, the full-length cDNA of *Pg-CRT* was 1549 bp in size (GenBank accession number KF993343), containing a 62 bp 5' untranslated region (5' UTR), an open reading frame (ORF) of 1239 bp and a 248 bp 3' untranslated region (3' UTR) including a stop codon (TAA), a polyadenylation consensus signal (AATAAA) and two other conserved motifs ATTTA and TTGTT, the latter specific to nematodes (Hajarnavis & Durbin 2006). The ORF was predicted to encode a protein of 413 amino acids (Fig. 6.1).

Figure 6.1 Full-length cDNA and deduced amino acid sequence of *P. goodeyi calreticulin* (GeneBank: KF993343). The nucleotide coding sequence is in capital letters and asterisk indicates the stop codon. Regions 5' UTR and 3' UTR are in lowercase. The predicted n-terminal region of the signal peptide is in bold, the hydrophobic core in italics and the c-terminal region is dashed underlined. A vertical arrow indicates the cleavage site for signal peptidase. The canonical polyadenylation signal and conserved motifs are underlined. The sequences of specific oligonucleotides used for semi-quantitative RT-PCR are indicated by horizontal arrows (PgCRTf2 and PgCRTr2, respectively).

6.3.2 Characteristics of the calreticulin protein

The deduced amino acid sequence of Pg-CRT was analyzed by BLAST program and the results revealed that the putative protein encoded by this cDNA was similar to other calreticulin from plant-parasitic nematodes (Fig. 6.2). The highest identity was observed towards calreticulin from *M. incognita* (84%) and *R. similis* (83%).

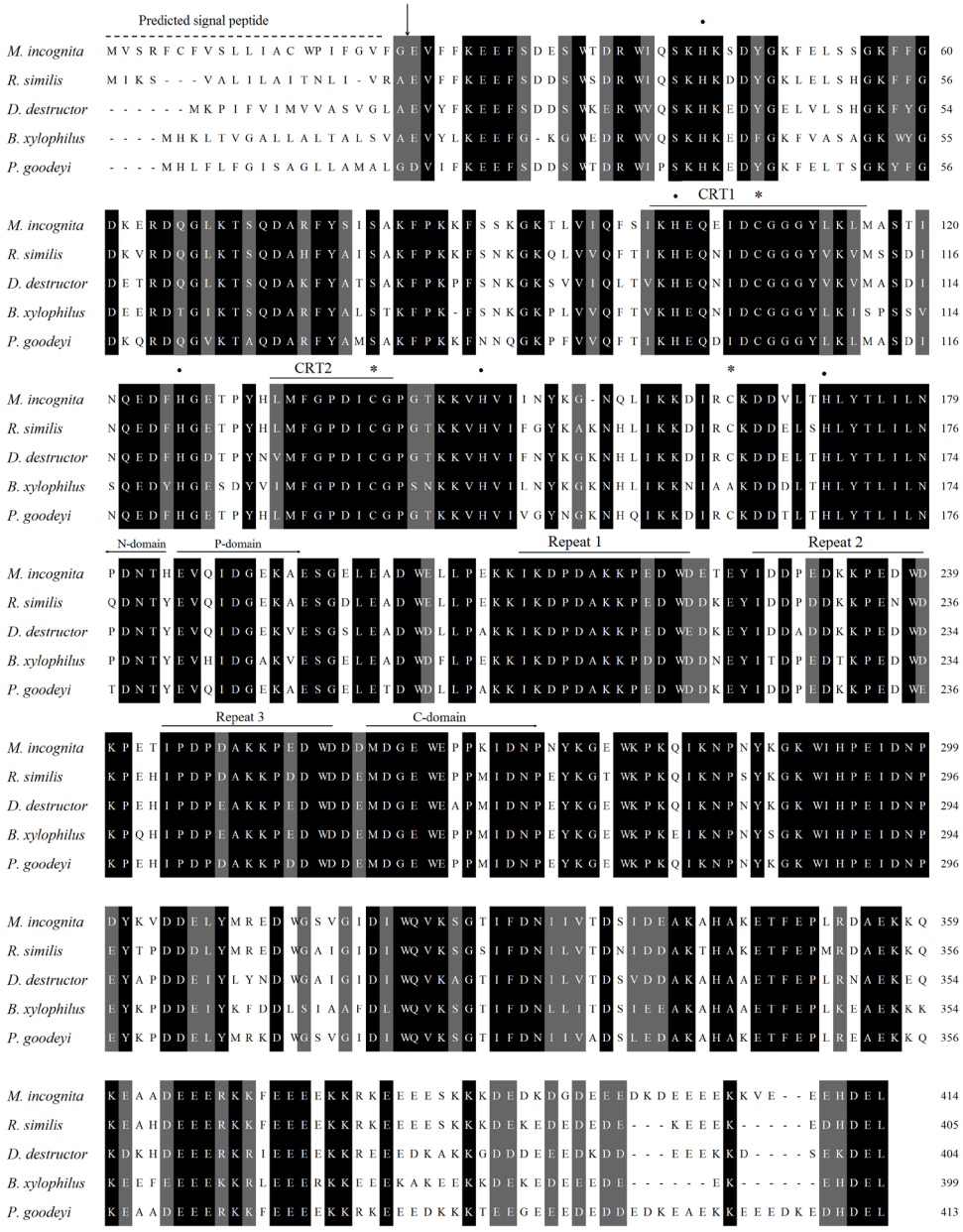

Figure 6.2 Comparison of deduced amino acid sequence of *P. goodeyi* calreticulin with similar protein sequences from plant parasitic nematodes: *R. similis* (GenBank: ACY01917); *M. incognita* (GenBank: AAL40720); *D. destructor* (GenBank: ACV33082) and *B. xylophilus* (GenBank: ADD82420). Identical or less conserved residues are shaded or grey. The predicted signal peptide is indicated by a dashed line. The N-domain, P-domain and C-domain are showed by a black arrow. At the N-domain asterisks indicate the amino acid cysteine and bullets the histidine residue. CRT1 and CRT2 family signature motifs and repeated motifs signatures (Repeat 1, 2 and 3) are indicated.

The predicted molecular mass of the calreticulin was approximately 46,39 KDa and the theoretical isoelectric point of 4.63. The SignalP software analysis indicated that Pg-CRT comprises a signal peptide of 18 amino acids with the following features: a cleavage site for signal peptidase between amino acids G^{18} and D^{19}; a n-terminal region at position 1 through 2 (MH); a hydrophobic core (h-region) in the middle of the signal peptide from amino acids 3 to 13; and a c-terminal region (AMALG) from position 14 to 18 (Fig. 6.2). It also showed a significant correspondence with the calreticulin protein family between amino acids 20-331, with the three representative structural domains N-terminal, prolin-rich and C-terminal. This correspondence was further evidenced by the presence of two signature motifs: CRT 1 (KHEQDIDCGGGYLKLM) and CRT 2 (LMFGPDICG). In addition, three calreticulin family repeated motifs (IKDPDAKKPEDWD), (IDDPEDKKPEDWE) and (IPDPDAKKPDDWD), three cysteine residues (Cys^{104}, Cys^{136}, Cys^{162}) and five histidine residues (H^{40}, H^{98}, H^{122}, H^{144}, H^{169}) were also found in the deduced amino acid sequence of Pg-CRT. Located at the C-terminal domain is the tetrapeptide HDEL, a putative ER targeting sequence (Fig. 6.2).

6.3.3 Cloning of *beta-1,4-endoglucanase* gene

Degenerate primers of *ENG* were used to amplify an internal PCR fragment of 404 bp from *P. goodeyi* cDNA (2.6.3). This partial sequence exhibited high homology to beta-1,4-endoglucanase from *R. reniformis* (81%, GenBank: ADM72857) and to a putative gland protein from *H. glycines* (80%, GenBank: AAM50039). Specific primers were designed and PCR products of 799 bp and 861 bp were obtained for 5' and 3' ends, respectively. The 1342 bp full-length cDNA sequence of *ENG* from *P. goodeyi* (*Pg-ENG*) (GenBank accession number KM005101) is composed of 87 bp 5' UTR, 232 bp 3' UTR including a stop codon (TAA), containing conserved motifs (three conserved motifs ATTTA and two ATAAA) and a 1023 bp ORF encoding 341 amino acids (Fig. 6.3).

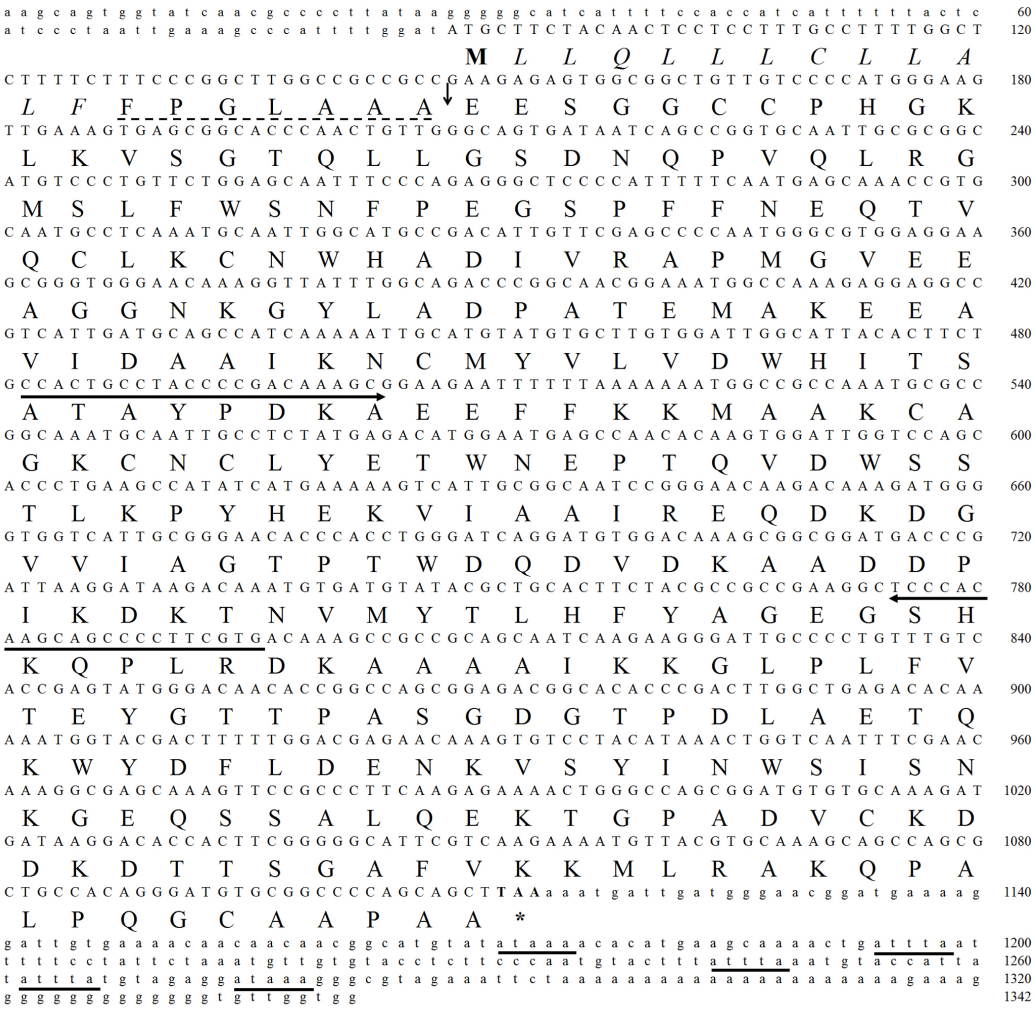

Figure 6.3 *Beta-1,4-endoglucanase* of *P. goodeyi* full-length cDNA and deduced amino acid sequence (GenBank: KM005101). The nucleotide coding sequence is in capital letters and asterisk indicates the stop codon. Regions 5' UTR and 3' UTR are in lowercase. The predicted n-terminal region of the signal peptide is in bold, the hydrophobic core in italics and the c-terminal region is dashed underlined. A vertical arrow indicates the cleavage site for signal peptidase. The canonical polyadenylation signal and conserved motifs are underlined. The sequences of specific oligonucleotides used for semi-quantitative RT-PCR are indicated by horizontal arrows (PgENGf3 and PgENGr4, respectively).

6.3.4 Characteristics of the beta-1,4-endoglucanase protein

The deduced beta-1,4-endoglucanase protein showed an estimated molecular weight of 37,27 KDa and a theoretical isoelectric point of 5.19. A signal peptide for secretion was predicted by the SignalP software, with a cleavage site between amino acids A^{20} and E^{21} (Fig. 6.3). The deduced amino acid sequence of Pg-ENG was analyzed by BLAST program and the results revealed similarities with other plant-parasitic nematode beta-1,4-endoglucanases. The highest identity was observed with the ENG1 from semi-endoparasitic nematode *R. reniformis* (70%,

GenBank: ADM72857) and with the putative gland protein ENG3 from soybean cyst nematode *H. glycines* (56%, GenBank: AAC33860). But, curiously among the Pratylenchidae, Pg-ENG shared an identity with *R. similis* ENG 1A (48%, GenBank: ABV54446,) and ENG 2 (50%, GenBank: ABV54448), with *P. penetrans* ENG 1 (49%, GenBank: BAB68522,) and ENG 2 (46%, GenBank: BAB68523) (Fig. 6.4).

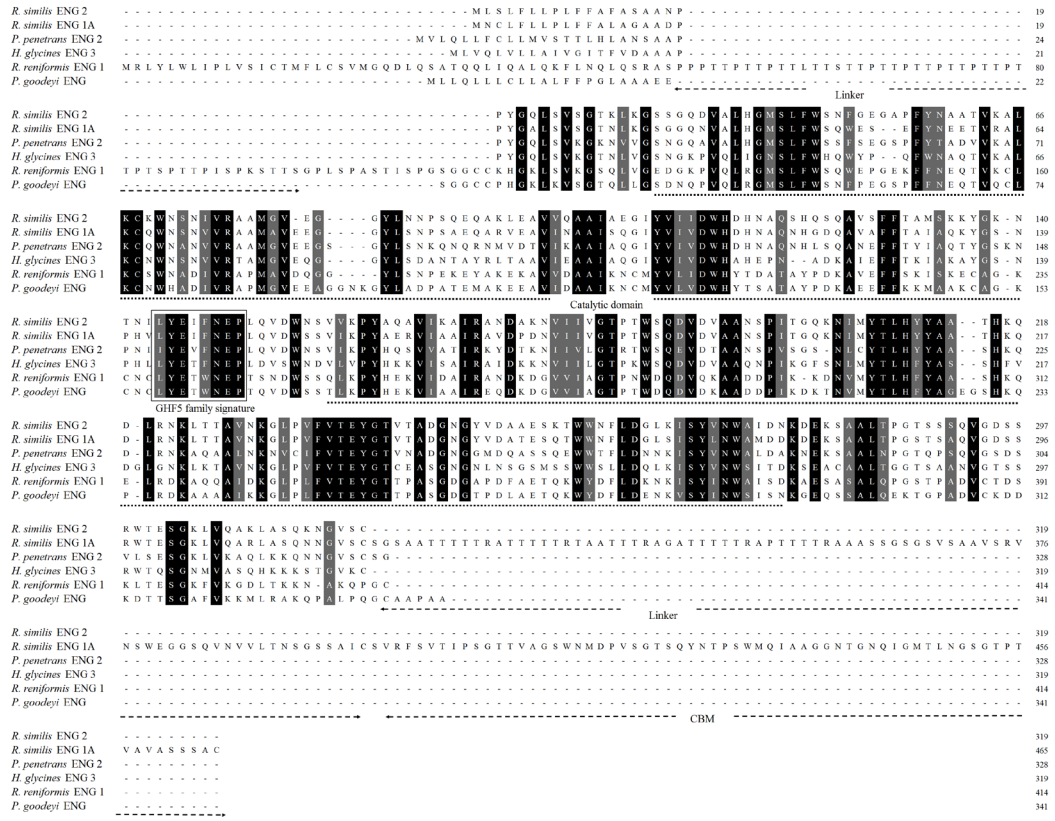

Figure 6.4 Protein alignment of beta-1,4-endoglucanases from *R. similis* ENG 2 (GenBank: ABV54448) and ENG1A (GenBank: ABV54446), *P. penetrans* ENG2 (GenBank: BAB68523), *H. glycines* ENG3 (GenBank: AAC33860), *R. reniformis* ENG1 (GenBank: ADM72857) and *P. goodeyi* ENG (GenBank: KM005101). Identical or less conserved residues are shaded or grey. The locations of the catalytic domain, GHF5 signature, linker and CBM are indicated.

Besides the signal peptide, Pg-ENG comprises a catalytic domain from amino acid 42 to 292 which includes a glycosyl hydrolase 5 (GHF5) family signature (pfam00150). On the contrary, a linker sequence represented by proline and serine/ threonine amino acids that is present at the N-terminal protein sequence of *R. reniformis* and a linker threonine-rich with an additional carbohydrate-binding module (CBM) present at the C-terminal of *R. similis* are both absent in the Pg-ENG sequence (Fig. 6.4). These linker and CBM sequences seem to be common in ENG1 plant-parasitic nematodes endoglucanases despite being extremely variable.

6.3.5 Expression of *Pg-CRT* and *Pg-ENG*

Semi-quantitative RT-PCR analysis (2.6.5) revealed that *Pg-CRT* and *Pg-ENG* were expressed in nematodes but not in the roots of banana plants (Fig. 6.5). The expression patterns of *Pg-CRT* and *Pg-ENG* genes were assessed in nematodes exposed to the acetone extract of *S. nigrum* up to 18 h (2.6.6). Under this chemical stress the expression of *Pg-CRT* was down-regulated whereas the expression of *Pg-ENG* remained stable (Fig. 6.6 A). Significant lower transcript levels of *Pg-CRT* were detected when nematodes were under chemical stress for 18 h (Fig. 6.6 B).

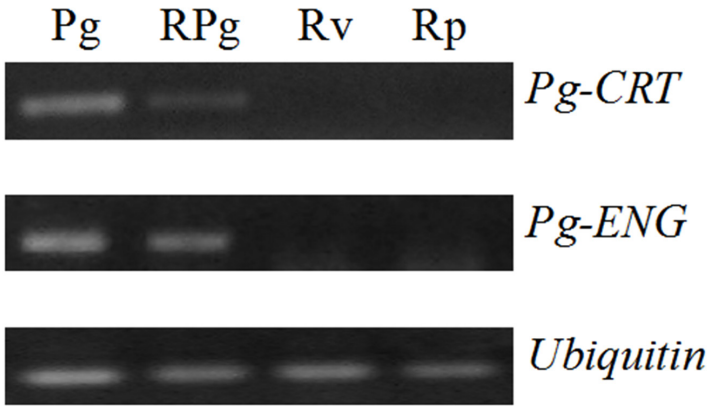

Figure 6.5 Transcripts of the *calreticulin, beta-1,4-endoglucanase* and *ubiquitin* by specific RT-PCR analysis from the cDNA samples of *P. goodeyi* (Pg), banana roots infected with *P. goodeyi* (RPg) and uninfected banana roots from *in vitro* culture (Rv) and from plants grown in a pot with sterilized soil (Rp).

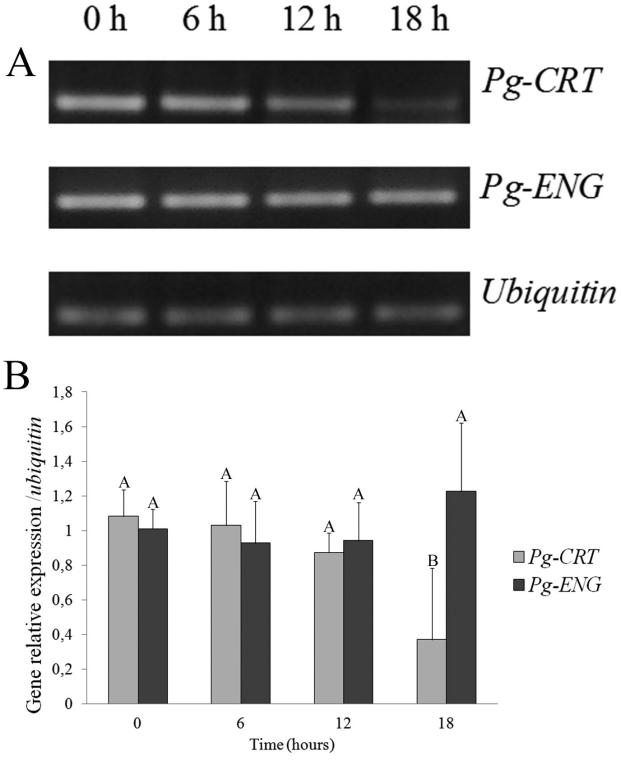

Figure 6.6 Time-course expression levels of *P. goodeyi*: *calreticulin, beta-1,4-endoglucanase* and *ubiquitin* under chemical stress. Relative abundance of transcripts by semi-quantitative RT-PCR (A). Relative amount of *calreticulin, beta-1,4-endoglucanase* normalized by *ubiquitin* transcripts (B). Data are the means ± SE for three independent experiments. Different letters indicate $P < 0.05$ for Tukey's test.

6.3.6 Biological assays

The behaviour of *P. goodeyi* was examined in Pluronic gel either with or without a banana root tip. In the control experiment, nematodes showed more free movement and were scattered throughout the Pluronic gel. In the presence of a banana root, the nematode attraction towards the root was time-dependent. After 4 h, more than 50% of the nematodes went away from the border of the Syracuse and 2 h later, 70% were near the root surface or inside the root (Fig. 6.7). The chemical stress imposed by the acetone extract of *S. nigrum* (10 mg/mL) affected nematode mobility since their movements became slower with time exposure and ceased in 50% of the nematodes after 18 h. This extract influenced attraction and infection of *P. goodeyi* regardless of being juveniles, females or males.

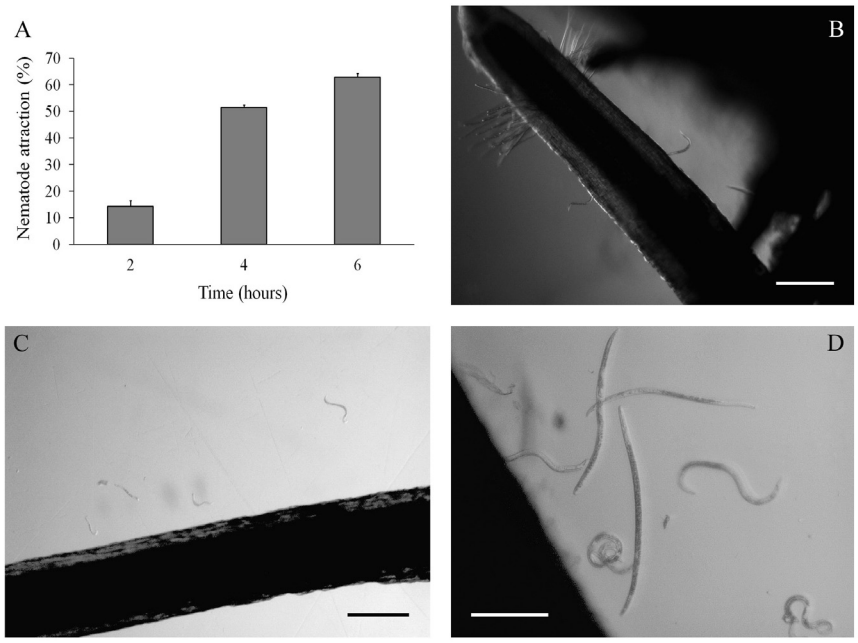

Figure 6.7 Nematode attraction to root of banana plants (Rv) in Pluronic gel (A). Data are the mean values ± SE of three independent experiments. Attraction after 2h (B), 4h (C) and 6 h (D). Scale bar (B and C) 350 µm, (D) 150 µm.

Clearly, a decrease in the number of nematodes that reached and penetrated the roots was observed with the increased exposure time. In the control (0 h), after 1 h of incubation 75% of J2 (Fig. 6.8 A), 52% of females (Fig. 6.8 B) and 49% of males (Fig. 6.8 C) have penetrated into the roots. This ability to infect was inhibited with the increasing time exposure to *S. nigrum* acetone extract since after 18 h less than 20% of nematodes has reached or entered the roots and the infectivity reduction was highest at 18 h (Fig. 6.8). Statistical analysis revealed that the differences between the life stages were not significant at the $P < 0.05$ level as all nematodes were equally affected by exposure to plant extract (Fig. 6.8).

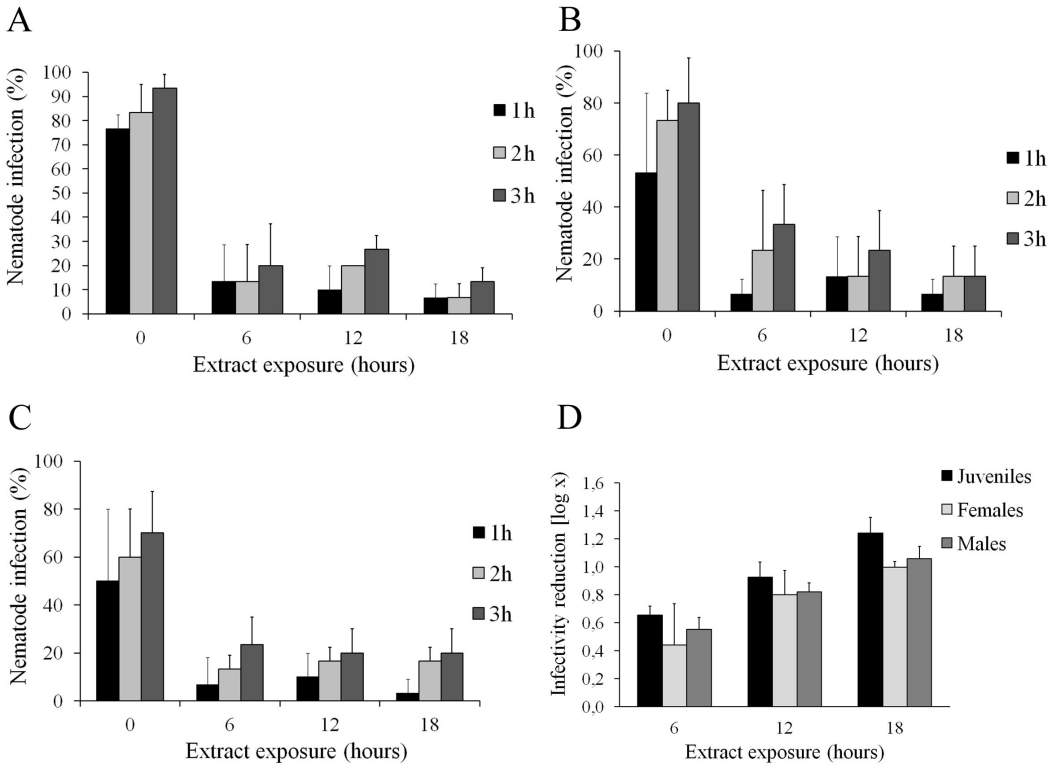

Figure 6.8 Infection of banana root with *P. goodeyi*: J2 (A); females (B); males (C) and the infectivity reduction (D) after exposure for 0, 6, 12 and 18 h to the acetone extract of *Solanum nigrum*. Data are the means values ± SE of three independent experiments.

6.4 Discussion

Transcript sequences from the root lesion-nematode *P. goodeyi* encoding calreticulin and beta-1,4-endoglucanase proteins both known to be related to parasitism were identified in this study. The Pg-CRT predicted protein showed high identity to calreticulin from plant-parasitic nematodes by the presence of calreticulin typical signature motifs in addition to the three representative structural domains (N-terminal, prolin-rich and C-terminal) (Michalak *et al*. 1999). The N-terminal domain includes a predicted signal peptide, three cysteine residues of which the latter two (Cys^{136}, Cys^{162}) can form a disulphide bridge important for the correct folding of the N-terminal region of calreticulin (Martin *et al*. 2006). Moreover, this domain contains five histidine residues, well conserved among plant-parasitic nematodes, that are involved in Zn^{2+} binding to calreticulin, being essential for the chaperone activity of the protein. Interestingly, Guo *et al*. (2003) found that a single histidine amino acid mutation changed the protein conformation and subsequently its folding. These molecular features of Pg-CRT, the lack of a transmembrane domain and the overall similarity are consistent with the results of calreticulin from the plant-parasitic nematodes *M. incognita* and *B. xylophilus* (Jaubert *et al*. 2005; Li *et al*. 2011) and similar to the calreticulin known to act as chaperone. Nevertheless,

when *P. goodeyi* are under stress an increasing expression of *Pg-CRT* will be expected to support its activity as chaperone (Park *et al*. 2001). Surprisingly, the expression profile of *Pg-CRT,* in contrast to what was expected, showed a significant decreased pattern of transcripts in nematodes after 18 h of exposure to the acetone extract of *S. nigrum*. This result contradicts studies by Jaubert *et al*. (2005) showing the synthesis of calreticulin occurring throughout nematode migration and infection, with an increased *CRT* expression. A possible explanation for our findings is that a blocking of *Pg-CRT* occurs by the action of *S. nigrum* acetone extract that affects the progression of infection. So, the role of calreticulin to suppress plant defences in the beginning of the infection process seems more important than its activity as chaperone (Jaouannet *et al*. 2013). This hypothesis is consistent with the results of Jaouannet *et al*. (2013) that after knock-down *CRT* gene obtained a reduction in the ability of J2 from *M. incognita* to infect roots, undoubtedly showing the importance of *CRT* for the infection process.

The deduced protein sequence of Pg-ENG revealed a signal peptide and a catalytic domain in addition to a GHF5 cellulase family specific signature but no linker and CBM module were found. The catalytic domain of Pg-ENG was more similar to the ENG1catalytic domain from *R. reniformis* (70%), despite lacking the linker present therein. The lower similarity observed among the Pratylenchidae beta-1,4-endoglucanase proteins known so far at databases and Pg-ENG is related to the variation in the catalytic domain. In addition, whereas some Pratylenchidae endoglucanases have a linker and a CBM module sequences, others only have a linker but no CBM (Haegeman *et al*. 2008; Wubben *et al*. 2010). Apparently, a selective advantage seems to promote the cellulase gene duplication in the nematode genome (Danchin *et al*. 2010). So, it is expected that new cellulase sequences even from *P. goodeyi* will soon be available. Moreover, studies revealed that the expression of *ENG* decreased when juveniles of cyst and root-knot nematodes became sedentary (Rosso *et al*. 1999), reflecting its importance during the early stages of the nematode life cycle (Ithal *et al*. 2007). Instead, significant changes in expression of *Pg-ENG* were not found. We assume that is because *P. goodeyi* do not develop a long-term feeding site as do the sedentary nematodes (Jones & Fosu-Nyarko 2014) and so need to continuously produce celullases to help the migration throughout host tissues (Rosso *et al*. 1999). Furthermore, as the expression of *Pg-ENG* seems to be unaffected in nematodes exposed to *S. nigrum* acetone extracts, before they entered the root, it will be an interesting challenge to confirm if a reduction or increase in *Pg-ENG* expression occurs after root infection by migratory endoparasitic nematodes, since the continuous cell wall degradation is important to all stages of the nematode life cycle.

Likewise, our results clearly show that the *S. nigrum* acetone extract affected the activity of all nematodes after 18 h of exposure to chemical stress, because their movements became slower and attraction towards the root decreased with time exposure, influencing the infection progression. This result is consistently the case in all nematode stages as they invade and infect the host tissues and confirms the potential nematicidal effect of water soluble compounds present in *S. nigrum* acetone extract (Haseeb & Butool 1996; Pestana *et al*. 2010).

Comparing our results with the studies from Muto *et al*. (2006), which have identified saponins in *S. nigrum* extracts with antimicrobial effect, it is tempting to propose that these compounds are responsible for the changes in nematode behaviour, paralysis and death (Pestana *et al*. 2014a, 2014b) and also for blocking the expression of *Pg-CRT*. These facts seem to prove that it is possible to manipulate the infection process by the inactivation of a nematode effector gene/protein (calreticulin), to avoid the root invasion (Jaouannet *et al*. 2013) and thus to prevent infection. Nevertheless, it will be necessary to isolate saponins from the acetone extract of *S. nigrum* and assess their affects on plant-parasitic nematodes. Indeed, detailed analyses of the compounds are currently being investigated and *S. nigrum* extract is being used against other nematodes as *B. xylophilus*.

In conclusion the cloning and molecular characterization of *Pg-CRT* and *Pg-ENG* full length-cDNAs that code for calreticulin and beta-1,4-endoglucanase proteins, respectively, were provided in this study. Using an acetone extract of *S. nigrum*, it was demonstrated the influence of chemical stress in the decreased expression of *Pg-CRT*, which seems to be blocked by compounds, hypothetically saponins, present in the extract. However, no effect was observed in the expression of *Pg-ENG*. The acetone extract also affects nematode mobility and infectivity with implications for the success of infection. Our findings provide useful information for the functional analysis of calreticulin and extend our understanding of some biological functions such as infection/parasitism when nematodes were subjected to chemical stress. Further research is needed to investigate whether *Pg-CRT* has potential as a new target in the control of *P. goodeyi* by blocking its expression.

Chapter 7

Molecular cloning and characterization of cDNA encoding a Translocon-Associated Protein (TRAP δ) from the root-lesion nematode *Pratylenchus goodeyi*

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7. Molecular cloning and characterization of cDNA encoding a *Translocon-Associated Protein (TRAP δ)* from the root-lesion nematode *Pratylenchus goodeyi*

7.1 Abstract

The translocon-associated protein (TRAP) complex comprises four subunits (α, β, γ, δ) and is located in the endoplasmic reticulum membrane at translocation sites. The TRAP complex is required for the efficient translocation of substrates and to correct or eliminate misfolded proteins. In this study, we described the cloning and characterization of a cDNA encoding a TRAP from the phytoparasitic nematode *Pratylenchus goodeyi* (Pg). The full-length cDNA had an estimated size of 690 bp and encodes a 177 amino acid peptide. The deduced protein after sequence analysis codes for TRAPδ subunit homologous to TRAPδ from other nematodes. The Pg-TRAPδ had a signal peptide indicating a possible involvement in the transport and binding of other proteins at the endoplasmic reticulum membrane. The increase in relative expression of *Pg-TRAPδ*, assessed by semi-quantitative PCR, was induced over time in nematodes exposed to a nematicide acetone extract of *Solanum nigrum*, suggesting that this gene product might be influenced by response mechanisms to stress in *P. goodeyi*. This is the first report of the cloning and characterization of *TRAP* cDNA from plant endoparasitic nematodes.

7.2 Introduction

Migratory endoparasitic nematodes damage plants and affect the development of crops as result of the mechanical migration in the root tissue and with the stylet acting as a feeding structure removing nutrients from the host cell cytoplasm (Gowen & Quénérhervé 1990). These actions may lead to changes on root cells that could cause the induction of necrosis, stunted growth, reduction in brunch weight and toppling. The root-lesion nematode *Pratylenchus goodeyi* Sher & Allen 1953 is considered an important migratory endoparasitic nematode of banana roots conditioning the production of banana worldwide (Gowen & Quénéhervé 1990; Pinochet *et al*. 1995; Stover & Simmonds 1987).

The molecular mechanism of the interaction between endoparasitic nematodes and host plants is poorly understood. The identification of genes from nematodes and their hosts have been studied extensively, being host resistance genes and genes

associated with the plant-nematode interaction the most investigated (Atkinson *et al*. 2003; Davis *et al*. 2000; Fragoso *et al*. 2009; Hulbert *et al*. 2001). However, molecular studies with *P. goodeyi* are restricted to species characterization (Waeyenberge *et al*. 2000) and phylogenetic analysis using ribosomal sequences (De Luca *et al*. 2011; Meldal *et al*. 2007). During a study to uncover the genes of *P. goodeyi* putatively related to parasitism an unexpected amplicon was obtained using a set of degenerate oligonucleotides that subsequently was found to be a partial sequence of a gene encoding a translocon-associated protein (TRAP).

TRAP is a heterotetrameric complex composed of α, β, γ and δ subunits (Hartmann *et al*. 1993; Ménétret *et al*. 2005). The γ subunit spans the endoplasmic reticulum (ER) membrane four times and the other subunits contain a single ER transmembrane region, being the main part of the subunits and the N-terminus localized in the ER lumen. This complex is closely associated with the heterotrimeric Sec61 complex but it is not considered to be part of the translocon core complex, although it modulates its function by an unknown mechanism.

It is widely accepted that secretory and membrane proteins are transported across or inserted into the ER membrane through the translocon. As a functional component of the translocon TRAP complex are involved in the translocation of substrates depending on signal sequences according to the substrate specificity (Fons *et al*. 2003). The way that TRAP is required for translocation appears to be inversely proportional to the efficiency of the signal sequence. TRAP can interact with the mature domain of the nascent polypeptide chain, in the lumen side of the translocon, to help stabilize and correctly orient the newly inserted nascent chain into the translocation channel. The absence of TRAP can result in a considerable efficiency decrease of the translocation of proteins (Mesbah *et al*. 2006). A stabilizing role for TRAP would be consistent with the observation that the dependence of TRAP is higher for signals that are relatively inefficient (Hegde & Kang 2008). When proteins are not well folded, or do not have the correct conformation, they accumulate in the ER and some mechanisms to correct or eliminate these proteins are activated. The TRAP complex seems to be involved in these responses, particularly in those associated with degradation that occurs in the ER (Nagasawa *et al*. 2007).

Despite what is already known about TRAP, i.e. its location in Mammalia and possible modes of action in eukaryotes, no data is available for Pratylenchidae phytoparasitic nematodes. Since TRAP is associated with the degradation of misfolded proteins and initiation and efficient translocation of proteins as a response to environmental changes, the study of the mechanisms linked with genes or proteins that are stimulated, released or whose expression is altered when the nematode is under stress is of great importance.

Therefore, in this study, we characterized a cDNA coding for the TRAPδ in *P. goodeyi* and investigated whether the expression of this gene is induced during nematode exposure to a putative nematicide acetone extract obtained from *Solanum nigrum* L. (2.3.2). The knowledge acquired in this study might be useful to shed light into the molecular mechanism of the plant-nematode interactions and to develop new strategies against plant parasitic nematodes.

7.3 Results

7.3.1 Cloning and sequence analysis of *Pg-TRAP* cDNA

A fragment of 267 bp was amplified by RT-PCR from *P. goodeyi* total RNA. Sequence analysis revealed that only degenerate oligonucleotide ENG1 was present at the 5' end of the sequence, which was homologous to genes encoding a family of proteins in eukaryotes named translocon-associated protein (TRAP). This partial sequence exhibited 76% similarity and 53% identity to *Brugia malayi* (EDP32123) TRAP. Based on this cDNA sequence, specific primers were designed to amplify the 5' and 3' flanking sequences through RACE (2.6.3). The fragments obtained by rapid ends amplification 5' RACE and 3' RACE were 308 bp and 454 bp, respectively. After cloning and sequencing, the alignment of 5' *Pg-TRAP* and 3' *Pg-TRAP* ends with the partial *Pg-TRAP* overlapped by 215 bp and 202 bp, respectively. Nucleotide sequence analysis of this cDNA revealed that it corresponds to the sequences of genes which encode TRAP family and was identical to the TRAP delta subunit (Pg-TRAPδ) (GenBank accession number KF359552). The full-length sequence of the cDNA consisted of 690 bp and contained an open reading frame of 531 bp with G+C and A+T content of 42 and 58%, respectively. This ORF flanked by a 37 bp 5' untranslated region (5' UTR) and a 122 bp 3' untranslated region (3' UTR) both with G+C and A+T content of 24 and 76%, respectively, encodes a peptide of 177 amino acids (Fig. 7.1). The sequence analysis exhibited in the 3' UTR the presence of two polyadenylation AATAAA processing signals. Additionally, two ATTTA and two TTGTT sequences were also found around the canonical polyadenylation signals (Fig. 7.1).

Figure 7.1 Complete coding sequence and deduced amino acid sequence of *P. goodeyi* translocon-associated protein (Pg-TRAPδ) (KF359552). The nucleotide coding sequence is in capital letters and asterisk indicates the stop codon. Regions 5' UTR and 3' UTR are in lowercase. The predicted n-terminal region of the signal peptide is in italics, the hydrophobic core in bold and the c-terminal region is double underline. A vertical arrow indicates the cleavage site for signal peptidase. The amino acid alanine at N-terminus and the disulphide bridge (dashed line) are indicated. Amino acids from transmembranar region are in bold italics. The possible polyadenylation signals are underlined. The sequences of the specific oligonucleotides used for semi-quantitative RT-PCR are indicated by horizontal arrows (PgTRAPf and PgTRAPr2, respectively).

7.3.2 Protein Pg-TRAPδ characterization

Comparison of the deduced amino acid sequence of Pg-TRAPδ with the GenBank data sequences revealed that the putative protein encoded by this cDNA shares similarities with those from *Ascaris suum* (47%), *Brugia malayi (*46%), *Loa loa* (46%) and *Caenorhabditis elegans* (43%) (Fig. 7.2).

In silico analysis of the Pg-TRAP amino acid sequence predicted a molecular weight of 19,95 KDa and a theoretical pI (isoelectric point) of 6.28. The instability index (56.68) suggests that Pg-TRAP is an unstable protein. A signal peptide was present with the following features: a cleavage site for signal peptidase between amino acids G^{22} and A^{23}; a n-terminal region at position 1 through 5 (MRCHI); a hydrophobic core (h-region) in the middle of the signal from amino acids 6 to 17; and a c-terminal region (ATVSG) from position 18 to 22 (Fig. 7.1). The mature protein had, in its N-terminus, the amino acid alanine (A) and, at positions 3 and 34, the amino acid cysteine (C) that might form a disulphide bridge. Our results revealed that the protein span one time the ER membrane. A major proportion, corresponding to the N-terminus, is at the lumen side of the ER, a transmembrane segment (VSSEIVFLLVGAAALYYGY) is attached to the membrane (amino acid

149 through 167) which is close to the C-terminus of the mature protein localized in the cytosol (Fig. 7.1 and Fig. 7.2). The hydrophobic residues leucine, valine and alanine over occurred in the transmembrane segment.

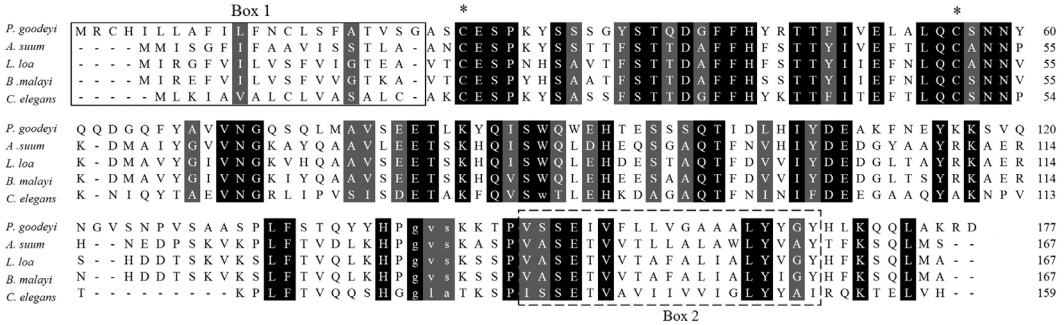

Figure 7.2 Comparison of deduced amino acid sequence of *P. goodeyi* translocon-associated protein (Pg-TRAP) (KF359552) with similar protein sequences from nematodes listed in the GenBank database under the following accession numbers: *A. suum* (ADY45498), *L. loa* (EFO28252), *B. malayi* (EDP32123), and *C. elegans* (NP_499554). Identical residues are highlighted in black and highly conserved are in grey. The asterisks indicate the amino acid cysteines that establish the disulphide bridge. Box1 and Box2 emphasize the signal peptide and transmembranar region, respectively.

7.3.3 Gene expression analysis

The amplification of *Pg-TRAPδ* by the specific oligonucleotides (PgTRAPf and PgTRAPr2) originated amplicons with 320 bp in the cDNA nematode samples (Pg and RPg) that were absent in the cDNA from uninfected roots (Rv and Rp) (Fig. 7.3). This result confirms the specificity of the oligonucleotides for gene expression analysis of *P. goodeyi*. The *ubiquitin* gene was used to normalize and to confirm the quantities of cDNA, since its transcription level is not altered by elevated temperature, ethanol or over time. In fact, the amounts of transcripts after normalization were similar in all cDNA samples as shown by the *ubiquitin* expression. Transcripts encoding *rubisco* were detected only in Rv and Rp samples and, as expected absent in cDNAs from samples having Pg. Moreover, cDNA samples from infected banana roots (RPg) were not amplified which confirmed that the genetic material present was only from animal origin (Fig. 7.3).

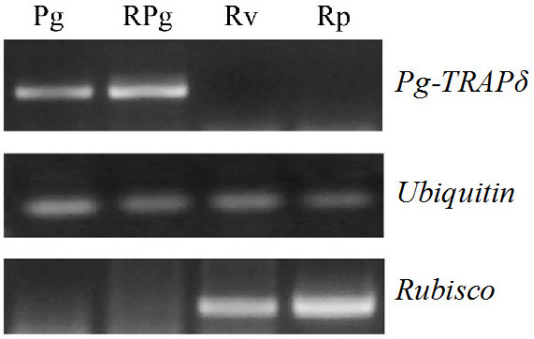

Figure 7.3 Transcripts of the *TRAPδ*, *rubisco* and *ubiquitin* by specific RT-PCR analysis from the cDNA samples of *P. goodeyi* (Pg), banana roots infected with *P. goodeyi* (RPg), banana roots from *in vitro* culture (Rv) and roots from banana plants grown in a pot with sterilized soil (Rp).

7.3.4 Expression pattern of Pg-TRAP in response to putative nematicides

In order to understand if this gene can be affected by a nematicide solution, semi-quantitative RT-PCR analysis (2.6.6) were performed with cDNA from nematodes exposed to water soluble compounds, from the acetone extract of *S. nigrum* (2.3.2), that have nematostatic or nematicide properties. After 18 h of exposure more than 50% of the nematodes were dead and changes in body shape were registered (Fig. 7.4). The expression of *Pg-TRAPδ* was induced over time and the expression of *ubiquitin* remains unchanged as expected, for the same experimental conditions (Fig. 7.5).

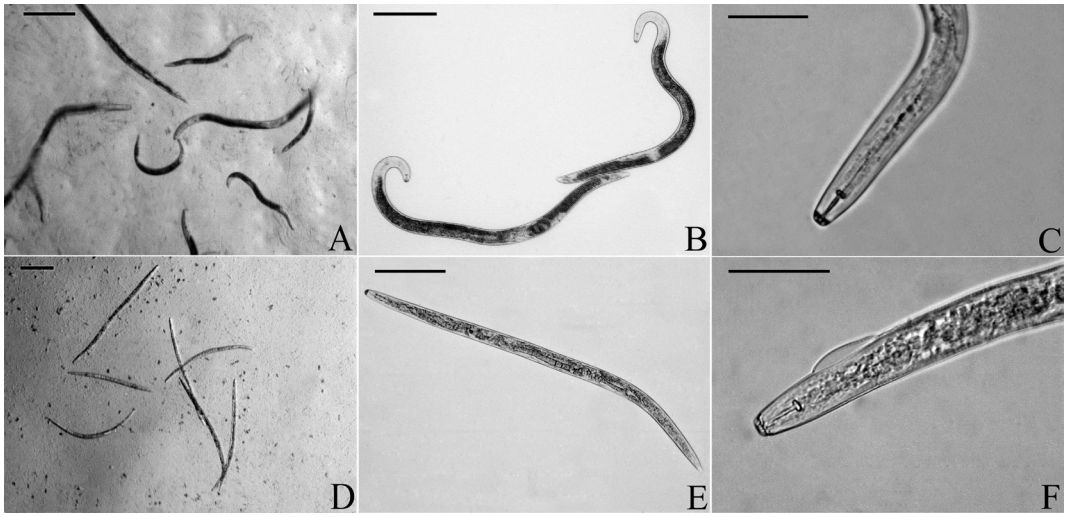

Figure 7.4 Mobility and morphological changes of *P. goodeyi*. Wavy movements (A), common aspect of body content (B), detail of the cephalic region (C) of nematodes in water. Paralysis (D), condensation and rigidity of the tissues (E) and separation between cuticle and internal content of the body (F) after 18 h exposure to acetone extract of *S. nigrum*. Scale bar (A and D) 160 µm, (B and E) 100 µm, (C and F) 30 µm.

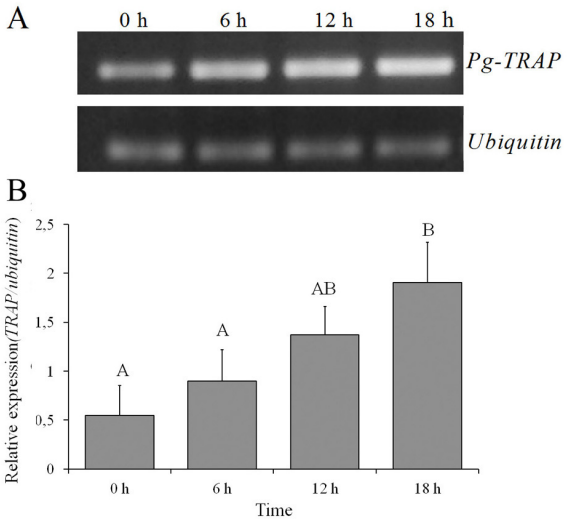

Figure 7.5 Semi-quantitative RT-PCR analysis of *Pg-TRAPδ* expression during exposure for 0, 6, 12 and 18 h to the acetone extract of *S. nigrum*. The cycle number for RT-PCR was selected to allow linear amplification of the cDNAs under study (A). Relative abundance of *Pg-TRAPδ* and *ubiquitin* transcripts (B). Relative intensity of *Pg-TRAPδ* transcripts indicated as a ratio of the *TRAPδ/ubiquitin* was determined and compared using the Software ImageJ program. Data are the means ± SE for three independent experiments. Different letters indicate $P < 0.05$ for Tukey's test.

7.4 Discussion

This study describes the cloning and characterization of the cDNA encoding the translocon-associated protein delta subunit from *P. goodeyi*. So far, this is the first report of a complete coding sequence from *P. goodeyi* gene and the first report of *TRAP delta subunit* cDNA among plant parasitic nematodes.

Analysis of the nucleotide sequence revealed a full-length cDNA containing a 531 bp open reading frame and a short 5' UTR common in nematodes, being related to the efficiency of some aspects of nematode molecular biology as trans-splicing (Hajarnavis & Durbin 2006). The *Pg-TRAP* 3' UTR was also short having a high A+T content similar to their counterparts *A. suum, B. malayi* and *L. loa*. The presence of two AATAAA polyadenylation signals may be a distinct feature as well as the increased prevalence of TTGTT and ATTTA sequences, before and after the canonical polyadenylation signal. These signals may be involved in the 3' end cleavage and in the regulation of stability or translation at mRNA level (De Luca *et al*. 1996; Hajarnavis & Durbin 2006). The ATG in the *P. goodeyi* cDNA is preceded by a consensus sequence for translation, particularly in what concerns the presence of a purine (adenine) in position -3, the most highly conserved nucleotide in all eukaryotic mRNAs (Kozak 1989).

The comparison of the deduced amino acid sequence from Pg-TRAPδ with TRAP homologous from nematodes obtained in the GenBank showed common features such as a signal peptide at N-terminus of the protein, which shares a conserved signal peptidase cleavage site at the c-region with an alanine residue immediately before or after the cleavage site (Hiss & Schneider 2009). The mature protein Pg-TRAPδ has at the same positions, in the N-terminus cysteine residues that establishes a disulphide bridge and shares the predicted membrane topology with other TRAPδ (Hartmann *et al*. 1993; Ménétret *et al*. 2008). Moreover, the prevalence of hydrophobic residues in the transmembrane region of Pg-TRAPδ match with the amino acid preferences predicted for the sequences that are integrated into the membranes and with the general mature protein topology (Martínez-Gil *et al*. 2011).

The presence of a signal peptide in Pg-TRAPδ indicates that this subunit is possibly involved in the translocation of substrates (Fons *et al*. 2003; Kapp *et al*. 2000). It can affect interactions with the translocon or regulate the efficiency of the proteins translocated through the ER (Mesbah 2006). Nagasawa *et al*. (2007) found that all four TRAP subunits were induced simultaneously by ER stress and that the TRAP complex binds preferentially to misfolded proteins. The TRAPδ subunit is known to form aggregates with some neuronal and with misfolded components

facilitating the initiation of protein translocation in a substrate-specific manner (Miyazaki *et al*. 2004). Apparently, the TRAP complex might be involved in the unfolded protein response pathway to maintain cellular homeostasis when cells are exposed to unfavorable conditions. Following this hypothesis, experiments are being conducted to get a better insight into the function of TRAP complex.

In fact, after submitting nematodes to a nematicide acetone extract of *S. nigrum* (2.3.2), the changes observed in the nematode activity and in the body shape might be assigned to certain compounds such as saponins, among others. Saponins have a lytic effect making the plasma membrane and intracellular membranes permeable to proteins, it also change the structure of membrane channels (Launikonis & Stephenson 1997; Wassler *et al*. 1987), affecting the ER function and causing spontaneous paralysis and death detected in the nematodes. Taking into account the changes observed in the bodies of *P. goodeyi* and the accumulation pattern of *Pg-TRAPδ* transcripts in nematodes exposed to the *S. nigrum* extract, we suggest a synergistic effect of water soluble compounds on membrane and membrane channels that leads to the up regulation of *Pg-TRAPδ*. Accordingly, the product of *Pg-TRAPδ* must be important for nematodes to handle with unfavorable conditions as stress. Hence, the transcriptional induction of *Pg-TRAPδ* might be involved in the response mechanism to the ER stress to cope with the cell necessities. Nevertheless, silencing the gene with antisense probes might be useful to clarify the exact function of Pg-TRAPδ.

With this study we have provided the cloning and molecular characterization of *TRAPδ* from *P. goodeyi* that code for delta subunit of the TRAP complex. The expression of this gene was up-regulated in nematodes exposed to a nematostatic/ nematicide extract of *S. nigrum*, which suggests that the product of the *Pg-TRAPδ* gene is up regulated as a response mechanism to stress. This background information might be useful to provide a new target to investigate and to elucidate the functional status of this gene.

Chapter 8

Discussion and Conclusions

8. Discussion and Conclusions

8.1 General Discussion

Plant products can be exploited as natural nematicides due to their biodegradable nature, availability and lack of direct effect on the non-target organisms that share the same environment. In this thesis the main issue was to evaluate the nematicidal potential of the water soluble compounds from two *Solanum* species (*S. nigrum* and *S. sisymbriifolium*) against the root-lesion nematode *P. goodeyi*. Therefore, aqueous extractions from fresh and dry plants, in cold or hot water, and a sequential extraction from dried plants through the solvents dichloromethane, acetone, ethanol and water were made.

Generally, aqueous extractions with hot water had higher amounts of extractives, but fresh *S. nigrum* plant had the highest quantity which was a good indicative for the use of this fresh plant directly by farmers as a preventive measure to control nematodes. Sequential extractions in water and ethanol revealed higher quantities of extractives whereas in dichloromethane and acetone they were 6 to 8 fold lower, indicating that chemical compounds predominate in the polar fractions. It is noteworthy that the content of extractives obtained in water following sequential extraction was 2 to 3 fold higher than those found in aqueous extractions revealing that previous solvent used in the sequential extraction increased the accessibility or solubility of chemical compounds. Each extract was dissolved in water at two concentrations (basal concentration corresponding to the initial ratio of fresh or dry plant and a mean concentration of 10 mg/mL) (2.5.1) and the effect of these solutions containing the water soluble compounds was analysed through nematode activity. *In vitro* evaluation of nematicidal activity of the extracts at basal concentration showed that aqueous extracts from both plants reduced nematode mobility. In the hot extractions of fresh and dry plant aqueous extract of *S. nigrum* lack of *P. goodeyi* mobility was observed as early as the second day. The dichloromethane and acetone extracts from both plants had no effect on nematodes, whereas the extracts of ethanol and water slightly affected or reduced, respectively, the nematode mobility. Nevertheless, it was interesting to verify that aqueous extracts of *S. nigrum* demonstrated the same efficiency on *P. goodeyi* mortality as the water extract from the sequential extraction. Indeed total lack of mobility and 100% of mortality was achieved after nematode exposure to these extracts, similarly to what was reported by Haseeb & Butool (1996) for *S. nigrum*. The assays performed with each extracts solutions at concentration 10mg/mL revealed that the effect of the extracts remained

unchanged except for the acetone extract of *S. nigrum*, which induced a total lack of mobility and morphological changes on nematode body structures following 100% mortality after 23 h exposure.

Given the nematicidal potential of *S. nigrum* and *S. sisymbriifolium* the study of the chemical composition of both plants may represent an important contribution for the development of new control measures of plant-parasitic nematodes. For this purpose, the chemical determination of the lipophilic compounds extracted by dichloromethane and phenolic compounds present in the acetone and water extracts were made by GC-MS and UHPLC-MS, respectively. The chemical profile of both dichloromethane extracts from *S. nigrum* and S. *sisymbriifolium* was similar and mainly composed by fatty acids, long-chain aliphatic alcohols and sterols. Such composition is expected since this solvent extract mostly lipids, volatile oils and terpenoids (Barbosa *et al*. 2005; Tiwari *et al*. 2011). The prevailing saturated fatty acids were the hexadecanoic and tetradecanoic acids, which have nematicidal activity against *M. incognita* J2 (Debprasad *et al*. 2000; Zhang *et al*. 2012). In addition, the unsaturated fatty acid octadeca-9,12-dienoic was also reported as having nematicidal properties (Stadler *et al*. 1993). Nevertheless, the dichloromethane extracts of *S. nigrum* and *S. sisymbriifolium* were the least effective against *P. goodeyi*, either in what concerns mobility or mortality. This fact can be related to the smaller amount of extractives obtained in the dichloromethane extract or, more probably, with the prevalence of compounds that are insoluble in water since at the concentration 10mg/mL the toxicity level remained lower.

Chemical analysis of the acetone and water extracts from both *Solanum* plants showed that water extract had higher amounts of phenolic compounds in relation to the acetone extract, especially the water extract of *S. sisymbriifolium.* Moreover, the acetone extract of *S. nigrum* had the smallest amount and diversity of phenolic compounds. The most abundant phenolic detected in the acetone and water extracts of the two plants were quinic, caffeoylquinic and chlorogenic acids which are long recognized as powerful antioxidants (Soh *et al*. 2003; Mondolot *et al*. 2006), yet have negligible nematicidal activity against plant-parasitic nematodes (D'Addabbo *et al*. 2013). These results are in agreement with the lowest amount of extractives obtained in the acetone extract after the sequential extraction, but contradict what was expected since this solvent should extract many compounds such as phenolics, flavenoids and saponins among others (Sobrinho *et al*. 2019; Tiwari *et al*. 2011). Also, the chemical profile obtained does not reflect the total immobility and mortality achieved after 23h of exposure to the acetone extract of *S. nigrum* at concentration 10 mg/mL. Nonetheless, evidence of nematicidal proprieties were confirmed when additional assays with the acetone and water extracts from sequential extraction

and aqueous extracts of *S. nigrum* were made against other nematodes such as *Helicotylenchus multicinctus*, *Meloidogyne incognita*, *Rotylenchulus reniformis*, detected in banana roots, and the pinewood nematode *Bursaphelenchus xylophilus*. These outcomes are very encouraging as they indicate that *S. nigrum* can potentially be used against *P. goodeyi* as well as to control other plant-parasitic nematodes. Additionally, it supports that the nematicidal activity is probably related to the action of compounds not yet identified or with the compounds that act synergistically on nematode mortality thus, justifying further research on the chemical composition of the extracts from *S. nigrum*. Indeed, separation procedure consisting of different techniques from sequential extraction with other solvent sequence to thin-layer chromatography (TLC), nuclear magnetic resonance and mass spectrometry (NMR-MS) and HPLC techniques correctly optimized might allow examination of compounds which were not obtained at experimental conditions used as saponins among other compounds (Oleszek 2002).

Many extracts from several plants were tested and proved to be effective against plant-parasitic nematodes (Kim *et al*. 2008; Wiratno *et al*. 2009; Barbosa *et al*. 2010; Andrés *et al*. 2012; Ntalli *et al*. 2012; Pavaraj *et al*. 2012; Akyazi 2014), but their effects must be validated in order to select the most promising extracts. In this sense, further analyses were carried out to verify the effect of the acetone extract of *S. nigrum* at molecular and behaviour levels on *P. goodeyi*. The cloning, sequencing and characterization of cDNAs from *calreticulin* and *beta-1,4-endoglucanase*, which code for proteins related to parasitism and, also from *translocon-associated protein delta subunit,* which is putatively related to stress were made with success, for the first time on *P. goodeyi*.

The expression of *Pg-CRT*, *Pg-ENG* and *Pg-TRAPδ* were analysed in nematodes exposed to the acetone extract of *S. nigrum.* These assays revealed that the *Pg-CRT* transcripts were significantly lower after 18 h of nematode exposure contradicting the expected increased expression of *calreticulin* to support the activity as chaperone (Park *et al*. 2001) or the increased expression following the synthesis of calreticulin during nematode migration and infection (Jaubert *et al*. 2005). Based on these findings, it was hypothesized that a kind of a block of *Pg-CRT* occurs due to the action of the *S. nigrum* extract. Moreover, Jaouannet *et al*. (2013) obtained a reduction in the ability of the second-stage juveniles (J2) of *M. incognita* to infect roots, after knock down *calreticulin* gene, confirming the supposition that this gene has an important role in the infection process. Indeed, the analysis of the effect from acetone extract of *S. nigrum* directly on *P. goodeyi* behaviour clearly showed that the activity of nematodes was affected, as their movements became slower and the attraction to root decreased with the time exposure. Remarkably, after 18 h of

being exposed to the acetone extract, nematode activity decreased and spontaneous paralysis occurred influencing the infection progression. This result seems to corroborate the hypothesis that *Pg-CRT* expression was block before root infection by the acetone extract of *S. nigrum* and is consistent with results of Arguel *et al*. (2012) that after knock-down *Mi-CRT* in infective juveniles of *M. incognita* through small interfering RNAs (siRNA) obtained a reduction of nematode virulence.

Evidences of a decreased expression of *beta-1,4-endoglucanase*, which is important on nematode invasion, migration and degradation of host tissues (Haegeman *et al*. 2012; Hewezi & Baum 2013; Kyndt *et al*. 2013) was found when cyst and root-knot nematodes J2 became sedentary, confirming its importance in the initial stages of infection (Rosso *et al*. 1999; Ithal *et al*. 2007). Instead, our study revealed no significant changes in expression of *Pg-ENG*. This may be because *P. goodeyi* do not develop a long-term feeding site with the host, as the sedentary nematodes, and need to produce celullases continuously to help the invasion and migration throughout host tissues (Rosso *et al*. 1999). Indeed, Fanelli *et al*. (2014) after silencing *beta-1,4-endoglucanase* from *P. vulnus* obtained a reduction in the ability of these nematodes to invade and migrate in the roots. Therefore, it will be interesting to confirm if a reduction or increase in *Pg-ENG* expression occurs after root infection.

Accumulation of *Pg-TRAPδ* transcripts on nematodes exposed to the acetone extract of *S. nigrum* was detected in this investigation. These finding are in agreement with the results reported by Nagasawa *et al*. (2007) which observed an increase of the gene expression after the induction of translocon-associated protein subunits by stress, proving that *Pg-TRAPδ* is important for nematodes to handle with chemical stress in this case caused by the acetone extract.

These results lead us to point out two things: firstly, the extract of *S. nigrum* indeed causes stress on *P. goodeyi*; initially influencing nematode mobility and behaviour and then morphology and mortality; secondly, the extract also influences genes since the expression of *Pg-CRT* effector gene which is involved in infection and parasitism was down regulated and thus the extract can be used as an alternative strategy to reduce nematodes populations in the field. Taking into consideration our findings in relation to both *Solanum* plants and the occurrence of *S. nigrum* in Madeira Island, being easily accessible to farmers, in our opinion it would be interesting to use *S. nigrum* extracts as an opportunity to limit nematode damage on multiple crops. But, to develop a new strategy to control plant-parasitic nematodes questions may arise, such as how to make a sufficiently stable aqueous formulation of *S. nigrum* i) being specific enough to not cause damage in other organisms? ii) and being applied in soil and/or by pulverization with an effect sufficiently durable?

Future research should be intensified in order to find the answers to these questions and to proceed with the isolation and identification of the nematicidal compounds. Molecular tools such as small interfering RNAs (siRNA) to target genes expressed during parasitism and silencing genes (Arguel *et al*. 2012) or RNA interference (RNAi) technology (Danchin *et al*. 2013) must be used to study the gene functions and to identify the possible strategies for nematode control and the genes sequences identified in this work might undoubtedly contribute to these purposes. Additional experimental studies to extend the application of such products to other nematodes and other cultures are also essential in order to develop an environmentally safer new commercial nematicide based in a natural product.

8.2 Conclusions

From the present study, it is concluded that the water and aqueous extracts from dry and fresh plants of *S. nigrum* and *S. sisymbriifolium* have in their composition nematicidal components that affected *P. goodeyi* mobility and causes mortality. Considering the results obtained with *S. nigrum* further studies will be necessary to deepen our knowledge about the potential use of this plant as new strategy to control nematodes.

Lipophilic compounds detected in the dichloromethane extract of *S. nigrum* and *S. sisymbriifolium* were mainly the fatty acids: hexadecanoic; tetradecanoic and octadeca-9,12-dienoic acids, which are recognized as having some nematicidal activity. Also, the phenolic compounds present in the acetone and water extracts of *S. nigrum* and *S. sisymbriifolium* differ slightly between the two species and were mainly quinic, caffeoylquinic and chlorogenic acids which possess little nematicidal activity. The differences found between composition of these extracts cannot be considered sufficient to explain the registered mobility and mortality of *P. goodeyi* in water extracts. This result might indicate that the extracts have compounds that were not identified through the techniques used or a synergistic effect of two or more compounds occurred. Therefore, the application of other techniques from sequential extraction with a different solvent sequence to TLC, NMR-MS and HPLC techniques optimized to allow examination of other compounds must be done to clarify these results.

Acetone extract (10 mg/mL) of *S. nigrum* had the highest effect on mobility and mortality of *P. goodeyi* despite having the lowest amount of extractives and identical phenolic composition in comparison to others extracts. Further chemical analyses are worthwhile to identify the compounds that can be responsible for the nematicidal activity.

The coding sequences of *calreticulin, beta-1,4-endoglucanase* and *translocon-associated protein δ subunit* were successfully cloned and characterized for the first time in the root-lesion nematode *P. goodeyi.* These genes encode calreticulin and beta-1,4-endoglucanase proteins with 413 and 341 amino acids respectively which are related to infection/parasitism and translocon-associated protein δ subunit with 177 amino acids which is associated with stress.

The expression of *Pg-CRT* decreased in nematodes exposed to the acetone extract of *S. nigrum*, contradicting the expected increase in the expression. This fact can be explained if some kind of a block of *Pg-CRT* expression occurs due to the action of the acetone extract, indicating that this gene has an important role in the infection process. Therefore, the manipulation of the infection seems to be achievable by the inactivation of a nematode effector gene such as *Pg-CRT,* which have potential as new target in the *P. goodeyi* control. More assays are needed to corroborate this hypothesis.

No effect was observed in the expression of *Pg-ENG*. This result is not consistent with the predicted decreased expression found in juveniles from cyst and root-knot nematodes, when they became sedentary, and might indicate that in migratory endoparasitic nematodes, which do not develop a long-term feeding site the need to continuously produce celullases is determinant to help the invasion and migration throughout host tissues and therefore important for the success on infection.

The transcripts of *Pg-TRAPδ* increased in nematodes exposed to acetone extract, suggesting that the product of the *Pg-TRAPδ* gene is up-regulated as a response mechanism to stress. Therefore, a correlation between chemical stress provided by *S. nigrum* acetone extract and gene expression was established.

P. goodeyi behaviour previously exposed to the acetone extract of *S. nigrum* was greatly affected. Nematode mobility decreased and the number of nematodes that was capable to find and penetrate banana roots diminished with the time exposure having implications for the success of infection. Therefore, a relation between the *S. nigrum* acetone extract, nematode mobility, root invasion, infection and the expression of *Pg-CRT* gene was established since they all decrease by the action of the extract.

This research provided new and useful information on *calreticulin*, *beta-1,4-endoglucanase* and *translocon-associated protein* genes and extended our understanding of some biological functions such as infection/parasitism, when nematodes were subject to chemical stress. This background information might be useful to provide a new target to investigate and to elucidate the functional status of effector genes.

The application of *S. nigrum* extracts are of great interest because this plant can be found among banana plants, being relatively abundant and its effects on *P. goodeyi* were validated at molecular and behaviour levels.

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