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Nematology 16 (2014) 41-51

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

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Received: 2 April 2013; revised: 28 May 2013
Accepted for publication: 28 May 2013; available online: 18 July 2013

Summary – 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-nematicides. The present study was carried out to assess the potential nematicidal properties of two *Solanum* species (*Solanum sisymbriifolium* and *S. nigrum*) against *P. goodeyi*, using dichloromethane, acetone, ethanol and either cold or hot water extracts of the plants. Water extracts of both plants at a concentration of 10 mg ml^{-1} 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 mobility and cause mortality. The nematostatic and nematicidal potential of the extracts described herein merit further studies to find novel bio-nematicides against the root-lesion nematode.

Keywords – banana plantation, *Musa acuminata*, natural nematicide, plant extracts.

Nematodes of the genera *Helicotylenchus*, *Pratylenchus* and *Rotylenchulus* are very common on the island of Madeira. Mixtures of these nematodes can damage the banana culture and subsequently decrease yield and fruit quality. The root-lesion nematode *P. goodeyi* (Cobb) Sher & Allen, 1953 is considered the most damaging nematode for banana plantations in Canary Islands, Cyprus, Crete and Taiwan (Gowen & Quénéhervé, 1990; Stover & Simmonds, 1991; Pinochet *et al.*, 1995), as well as in Madeira (Troccoli *et al.*, 1996).

Pratylenchus 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 are strong (Speijer *et al.*, 1999; Stoffelen *et al.*, 1999; Gold *et al.*, 2004).

In order to control the nematode populations, farmers use chemical products that contribute to soil, groundwater and atmospheric contamination. Hence, it is imperative to change the agricultural practices associated with banana culture, making them less harmful to the environment and simultaneously enhance food safety and product quality. As plants synthesise 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, *Solanum sisymbriifolium* Lam. and *S. nigrum* L. While the first is a native of warm temperate climates of South America and nowadays is present as an invasive species worldwide (USDA, NRCS, 2007), the latter is a Eurasian species distributed all over the world (Edmonds, 1979; Frohne & Pfander, 1983; Valdés, 2012). *Solanum nigrum*

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DOI:10.1163/15685411-00002743

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 Madeira, 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, and with 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, 2008; Ji *et al.*, 2008; Cai *et al.*, 2010). *Solanum 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 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 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.

Materials and methods

Plant material

Solanum sisymbriifolium Pion seeds were provided by Vandijke Semo Seed and Services, and *S. nigrum* plants were obtained from natural habitats and kept in the laboratory for the production of fruits and seeds.

Seeds of both plants were germinated in sterile peat and the plants were maintained in a glasshouse 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). The dry plants were ground in a cutting mill (Arthur H. Thomas) and passed through sieves of 40 and 60 mesh (Retsch). The 40-60 mesh size fraction (425-250 μm) was used for the extractions. The water content in the samples was determined on a moisture balance (Gibertini-Eurotherm).

Water extractions

Two methods were used to obtain the water extracts: blender and reflux. In the blender method, *S. sisymbriifolium* and *S. nigrum* 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). 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 extraction. The plant material was refluxed during 1 h and the liquid fraction was obtained after filtration through a G4 filter.

All extracts were lyophilised, quantified and stored at −20°C in the dark until further analysis. Five determinations were performed and the results were expressed as a percentage of the extract in dry matter in relation to the original plant weight used in the extraction.

Sequential extraction

Dry *S. sisymbriifolium* and *S. nigrum* plants were subjected to a Soxhlet sequential extraction with dichloromethane, acetone and ethanol during 10 h in each solvent. Each extraction solution was dried by vacuum evaporation at 40°C until constant weight. Each solvent was completely evaporated and the solid residue recovered. The extract percentage was determined gravimetrically.

After ethanol extraction, the plant material was dried at 30°C. The dried material was refluxed for 1 h in water to obtain the remaining extracts in water. After filtration (G4 filter), the water extract was lyophilised and gravimetrically quantified. All extracts were stored as mentioned before.

All reagents used in this work, namely dichloromethane, acetone and ethanol, were chromatographic grade (Merck, Sigma-Aldrich or Fluka).

Pratylenchus goodeyi isolates

Twenty-eight samples of soil and banana roots were collected on the southern coast of Madeira. The root-lesion nematode isolates were multiplied on banana plants obtained from *in vitro* culture. The nematodes 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 identification of *P. goodeyi* isolates based on morphological characters was done in the Istituto per la Protezione delle Piante Sezione di Bari, Italy.

Nematicidal activity

Each solid residue (10 mg) was dissolved in water (1 ml) and the aqueous solution containing water soluble compounds tested for activity against *P. goodeyi.* The bioassays were conducted using 1 ml of each extract solution in a syracuse watchglass containing ten adult *P. goodeyi*, and left in the dark at room temperature (25 ± 1°C) for 10 days. Each treatment was replicated five times and sterile distilled water was used as control. The effect of acetone extract on nematode mortality was assessed during a period of 24 h. The bioassay was repeated once as a biological replicate. The nematodes were observed daily using a binocular microscope and numbers of inactive or dead nematodes were recorded. Nematodes were considered dead when after being transferred to sterile water for 2 h and stimulated by prodding they remained inactive.

Statistical analysis

Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) 15.0 software for Windows. Registered mortality was converted into cumulative mortality and corrected by Abbott's (1925) formula prior to analysis. The values obtained for nematode mortality were analysed to verify if they have a normal distribution by normality tests of Kolmogorov-Smirnov and Shapiro-Wilk ($P > 0.05$). One-way analysis of variance (ANOVA) was used to find significant differences on the mortality of *P. goodeyi* using *S. sisymbriifolium* and *S. nigrum* extracts. In addition, Tukey's test was used for multiple comparisons of means. The statistical test was run independently for each treatment and plant species.

Results

Water extracts

The 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 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 material, whereas in *S. nigrum* the higher extract quantities were obtained when fresh plant material was used.

Sequential extracts

The water fraction yielded the highest amount of extract, whereas acetone had the smallest value (2%) for each plant species (Table 2). Components extracted from *S. nigrum* in dichloromethane and acetone were five- and 13-fold lower, respectively, than the components extracted in ethanol and water.

The content of extracts obtained in water sequential extraction was two- to three-fold higher than those in water 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.

Nematicidal activity of water extracts

Pratylenchus goodeyi progressively reduced mobility in water extracts obtained from fresh and dry plant of

Table 1. Extracts (% of dry matter) obtained from *Solanum sisymbriifolium* and *S. nigrum* blended in hot and cold water or by reflux.

Water extract	*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

Results are the mean of five replicates (mean ± S.D.).

Table 2. Extracts (% of dry matter) obtained from *Solanum sisymbriifolium* and *S. nigrum* dry plants using sequential extraction.

Sequential extract	*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

Results are the mean of five replicates (mean ± S.D.).

S. sisymbriifolium; up to the eighth 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 extracts being effective on nematode mortality (Fig. 1).

In the fresh and dry plant extracts of *S. nigrum*, lack of nematode mobility was observed as early as the second day and recovery of nematode mobility, tested on sterile water, diminished thereafter (Fig. 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 water extracts from fresh plant, all extracts being equally effective on nematode mortality (Fig. 3A). Nevertheless, significant differences were detected between hot and cold water extracts from dry *S. nigrum* plants (Fig. 3B). Dry cold water extracts were less effective on nematode mortality (Fig. 3B). 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.

Another relevant aspect observed in most of the water 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. 2A). These results indicate that water 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.

Nematicidal activity of sequential extracts

Pratylenchus goodeyi subjected to sequential extracts obtained from both plant species revealed significant differences in their activity (Fig. 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^{-1} (Fig. 4B). Acetone and water extracts were the most effective, with the acetone extract causing more than 95% cumulative mortality on the second day of exposure. The higher percentage of *P. goodeyi* mortality in the acetone and 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 a separation between the cuticle and internal content of the body, some rigidity, necrosis of tissues and partial disintegration of internal organs (Fig. 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 water extracts from single extraction (Fig. 2A). In

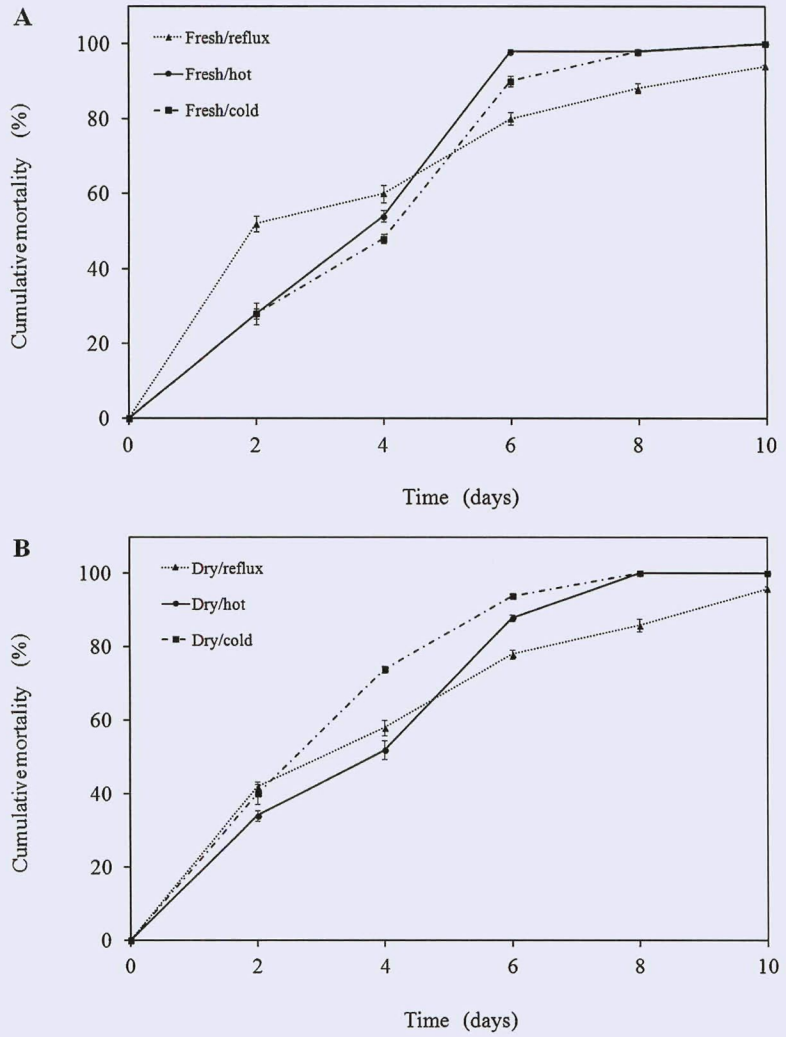

Fig. 1. Cumulative mortality of *Pratylenchus goodeyi* in cold, hot and reflux water extracts at 10 mg ml^{-1} concentration from fresh (A) and dry (B) *Solanum sisymbriifolium*. Each time point represents the average of five replicates and vertical bars represent standard deviation among replicates. The cumulative effect for water extracts is not significantly different ($P > 0.05$) according to the ANOVA.

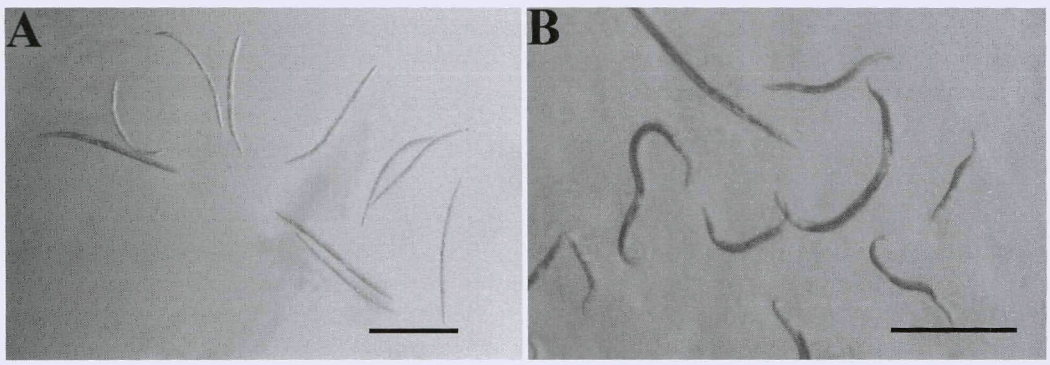

Fig. 2. Activity of *Pratylenchus goodeyi* after 2 days in water extracts from fresh *Solanum nigrum* at 10 mg ml^{-1} plant concentration (A) and in water as control (B). (Scale bars = 300 μm.)

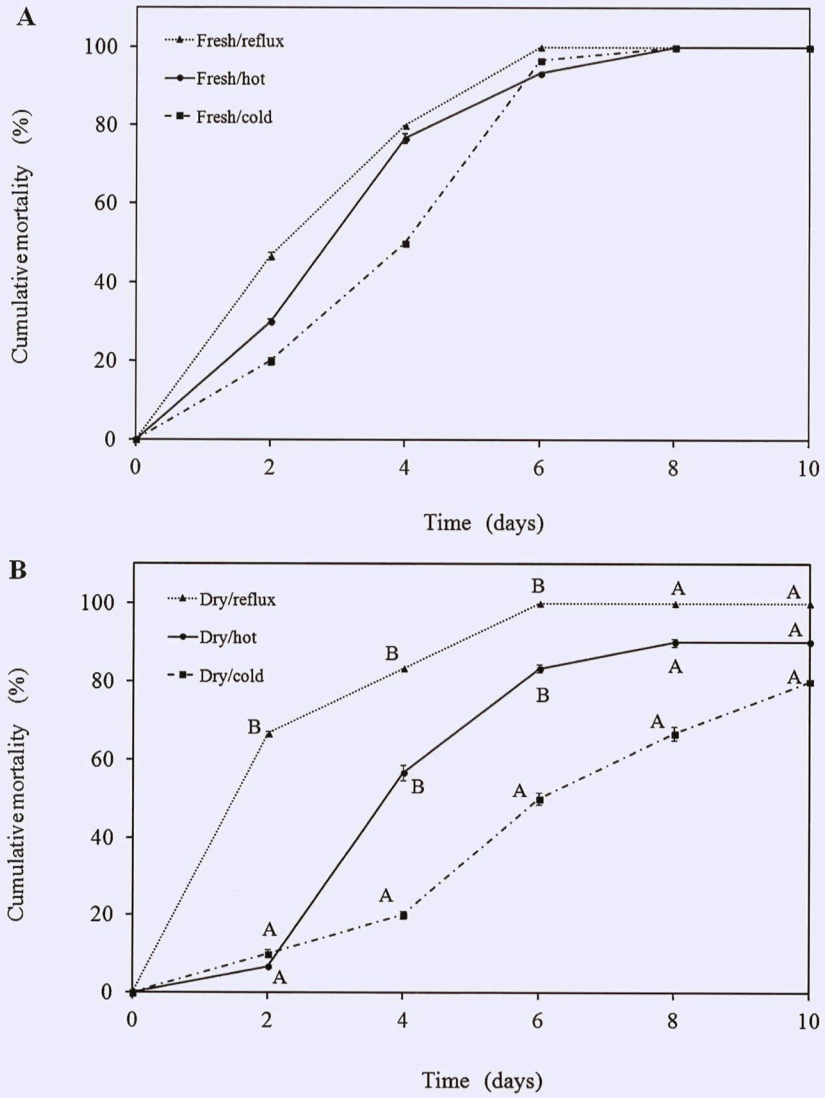

Fig. 3. Cumulative mortality of *Pratylenchus goodeyi* in cold, hot and reflux water extracts, at 10 mg ml^{-1} concentration, from fresh (A) and dry (B) *Solanum nigrum*. Each time point represents the average of five replicates and vertical bars represent standard deviation among replicates. The same letter at each time indicates that values are not different ($P > 0.05$) according to Tukey's multiple comparison test.

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 after the first hour of exposure. The mortality of *P. goodeyi* was more pronounced after 15 h of exposure (*ca* 50%), and at the end of the experiment all nematodes had died (Fig. 6).

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,

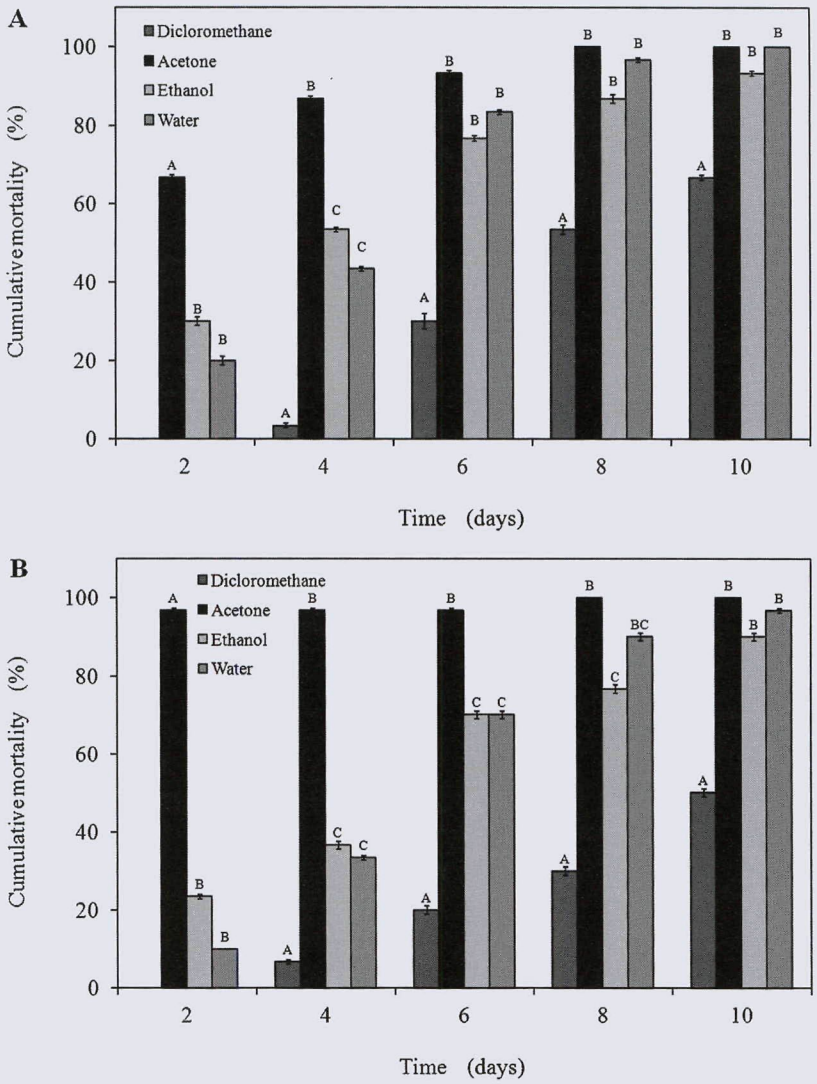

Fig. 4. Cumulative mortality of *Pratylenchus goodeyi* in dicloromethane, acetone, ethanol and water extracts at 10 mg ml^{-1} concentration, from *Solanum sisymbriifolium* (A) and *S. nigrum* (B). Results are five replicates means and vertical bars represent standard deviation among replicates. The same letter at each time indicates that they are not different ($P > 0.05$) according to Tukey's multiple comparison test.

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 water extracts obtained from dry and fresh plants demonstrated the same efficacy on *P. goodeyi* mortality as the water extracts from the sequential solvent extraction. Total lack of mobility and 100% of mortality after exposure to water extracts at initial concentration and at the concentration of 10 mg ml^{-1} for *Solanum* plants was previously reported (Pestana *et al.*, 2011), and these results were consistent with those obtained by Haseeb & Butoll (1996) for *S. nigrum*. It is expected that several nitrogen-rich compounds, glycosides, alkaloids, saponins, tannins and terpenoids (Tiwari *et al.*, 2011) are 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.

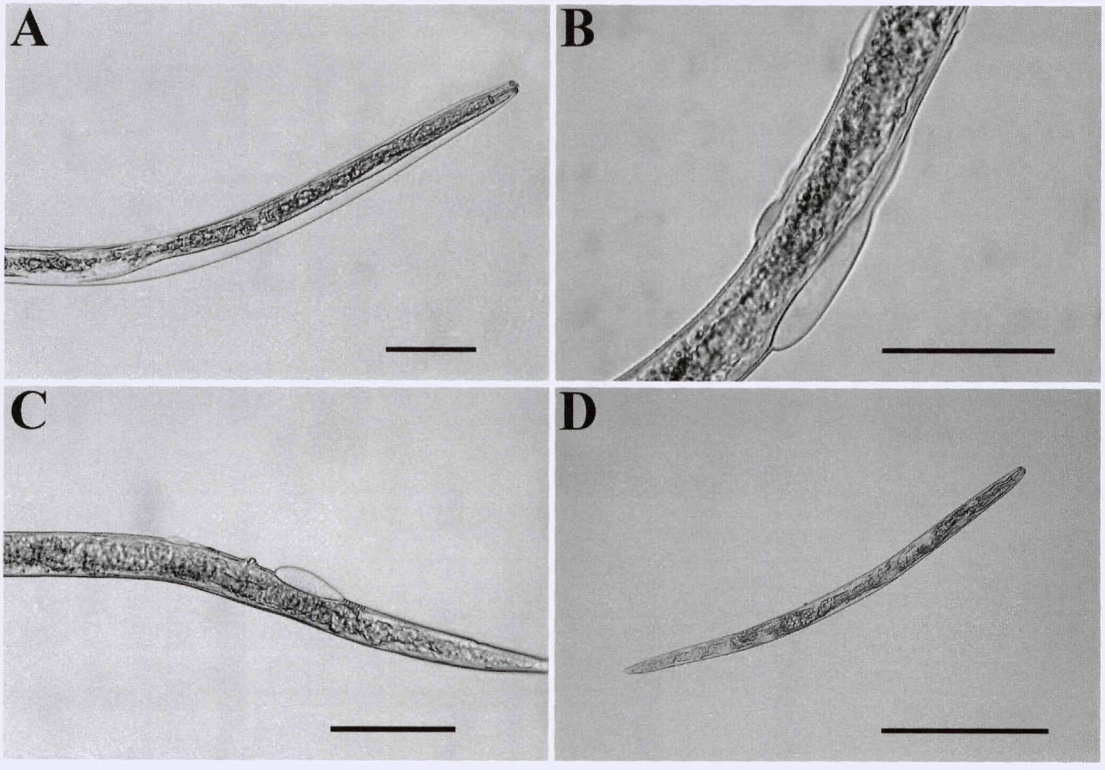

Fig. 5. Morphological changes of *Pratylenchus goodeyi* in *Solanum nigrum* acetone extract. Separation is evident between the cuticle and internal content of the body (A, B and C). Condensation, rigidity and necrosis of tissues occurs (D). (Scale bars: A, C = 50 μm; B = 40 μm; D = 200 μm.)

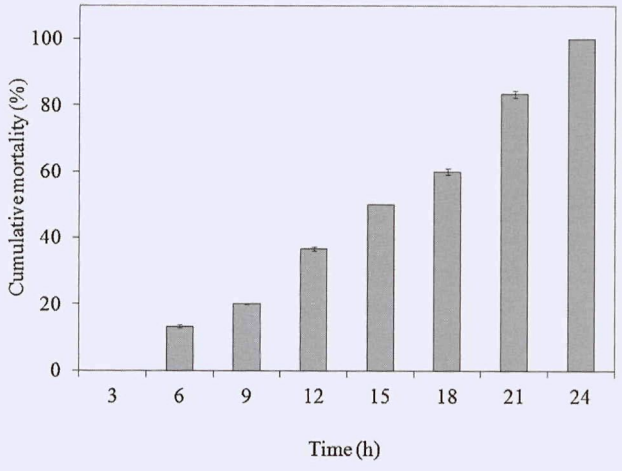

Fig. 6. Mortality of *Pratylenchus goodeyi* over 24-h incubation in *Solanum nigrum* acetone extract (10 mg ml^{-1}). Each time point represents the average of five replicates and vertical bars represent standard deviation among replicates.

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. These results showed that, compared with the other solvents, the acetone extract, at a concentration of 10 mg ml^{-1} in relation to the initial 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 was obtained in the fresh and dry plant extracts. The changes in the body shape of nematodes might be assigned to the toxic effect of pyrethroids, well-known insecticides that directly affect 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. Therefore, 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. It was hypothesised that a direct action through the release of exudates with nematicidal effect or an indirect contribution through the promotion of antagonists would make the rhizosphere unfavourable to the nematode. Altogether, the results showed the nematicidal potential of these *Solanum* plants and especially that *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* are shown to have in their composition components that possess nematostatic or nematicidal activity against *P. goodeyi* and these effects 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 characterise the active component(s) to be used as environmentally friendly nematicides for plant-parasitic nematodes.

Acknowledgements

M.P. thanks CITMA for the award of a Doctoral grant (Project No. 001080/2010/132). We thank N. Vovlas from L'Istituto per la Protezione delle Piante Sezione di Bari, Italy, for the identification of *P. goodeyi* and S. Costa from the Department of Botany, University of Coimbra, Portugal, for a critical review of the manuscript.

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