ROOT-KNOT NEMATODES FROM ASPARAGUS AND ASSOCIATED BIOLOGICAL ANTAGONISTS IN PERU

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ABSTRACT

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A research study on the parasitic nematodes of asparagus and several associated antagonists was carried out in Northern Peru. Nematodes were identified by means of light microscopy and sequencing of the ITS1-2 regions. Nematophagous fungi were isolated from nematode-infested roots or soil, cultured *in vitro* and maintained in a culture collection for further characterization. The species recovered were mainly root-knot nematodes including *Meloidogyne incognita* and *M. ethiopica*. Nematophagous fungi identified through standard morphological methods as well as by ITS sequencing included *Drechslerella brochopaga, Lecanicillium psalliotae* and *Monacrosporium* sp. *Meloidogyne ethiopica* and the antagonistic fungi are new records for the country.

Key words: Asparagus, biological control, *Meloidogyne,* nematophagous fungi.

RESUMEN

Murga-Gutierrez S. N., M. Colagiero, L. C. Rosso, M. M. Finetti Sialer, and A. Ciancio. 2012. Nematodos agalladores y antagonistas biológicos asociados en cultivos de espárrago en el Norte del Perú. Nematropica 42:57-62.

Se estudiaron los nematodos parásitos de espárrago y algunos de sus antagonistas, en el norte de Perú. Los nematodos fueron identificados por medio de procedimientos estándares con microcopia optica y la secuencia de las regiones del ITS1-2. Los hongos nematófagos fueron aislados de raices infestadas o suelo, cultivados *in vitro* y mantenidos en una colección de cultivos para su caracterización. Los nematodos encontrados fueron principalmente las especies del nudo de la raíz incluyendose *Meloidogyne incognita* y *M. ethiopica*. Los hongos nematófagos identificados a través de métodos morfológicos estándares, así como por secuenciación del ITS, incluyen *Drechslerella brochopaga, Lecanicillium psalliotae y Monacrosporium* sp. *Meloidogyne ethiopica* y todas las especies de hongos antagonistas encontradas representan nuevos registros para el país.

Palabras clave: Asparagus, control biológico, *Meloidogyne*, hongos nematófagos.

INTRODUCTION

Asparagus (*Asparagus officinalis* L.), in comparison with other crops, has been considered as a species less prone to nematode parasitism or even capable of reducing nematode densities in the field (Esmenjaud *et al.*, 1990). However, cases of nematode parasitism are known from the literature and, in spite of the nematicidal activity of asparagusic acid, some root-knot nematode (RKN) populations and species were capable of parasitizing roots of this plant (Esmenjaud *et al.*, 1990; Dudash and Barker, 1992). Nematode reports from other *Asparagus* species also include parasitism of *A. macowanii* by a Florida population of *Meloidogyne incognita*, which induced root galling and reduced fresh stem weights in controlled conditions (Stamps *et al.*, 1995).

In Peru, severe infestations by indigenous populations of *Meloidogyne* spp. were detected and reported on asparagus cultivations in the coastal regions (De Guerra, 1982; Murga-Gutiérrez, 1997; Canto, 2000; E. Carbonell, pers. comm.). Field conditions of these areas are usually characterized by a desert climate with mild winters (10-15°C) and arable sandy fertile soils, mostly alluvial. In these areas concomitant attacks by RKN populations and *Fusarium* spp. on asparagus crops significantly reduce plants productivity (Fig. 1).

In Peru, asparagus is often grown using non-certified planting material, obtained locally from F1 crossings. This material is genetically variable and has poor

Fig. 1. Reduced growth of asparagus plants in a farm at California (Virú district, Peru), induced by root-knot nematode and *Fusarium* sp. (left side). First row shows healthy plants.

quality in terms of pest and disease resistance. The availability of elite germplasms is very limited and F1 crossings are the most common planting material locally available and marketed. Therefore, nematodes are usually controlled by the application of systemic nematicides such as oxamyl and no sustainable strategy is available to control RKN species, apart from organic matter applications.

The aim of the present study was to identify the *Meloidogyne* species attacking asparagus in Northern Peru, and the associated antagonists of the nematodes, to find biological control agents suitable for more sustainable control.

MATERIALS AND METHODS

Sampling was conducted during the austral winter season in six farms planted with asparagus var. UC-157 F1 or crosses. Six to nine samples of soil with roots of asparagus were collected per field, in the province of Viru (Dept. La Libertad, Peru) from farms specialized in export production. Crops were drip irrigated with water from canals or shallow depth wells, and fertilization usually included seasonal applications of organic matter or occasionally guano.

Molecular techniques were used for *Meloidogyne* spp. identification. Second-stage juveniles (J2) of RKN populations were extracted from soil samples using Cobb's sieving and decanting technique or from egg masses on asparagus roots. The J2 picked from the resulting suspensions were placed individually or in groups of five in vials for DNA extraction. Single females or egg masses were also excised from roots under a stereo microscope from tomato plants grown in 50 ml plastic tubes filled with infested soil and maintained at room temperature.

The nematode DNA extraction followed a protocol based on grinding nematodes with glass beads (400 µm diam., Sigma Aldrich, USA) for 10 sec. in Tris-HCl buffer (pH 8, 10 mM) to release the total nucleic acids prior to PCR amplification (Atibalenja *et al.*, 2004). The primers and molecular diagnosis key for *Meloidogyne* J2 described by Adam *et al.* (2007) were used to discriminate between seven of the economically important and most common *Meloidogyne* species in tropical areas. When the sequences were not sufficient to identify the corresponding RKN species due to a high nucleotide similarity, the DNA was amplified using primers specific to *M. incognita* (Adam *et al.*, 2007).

Amplified products were examined on 1.5% agarose gels and the fragments were purified using a commercial kit (QIAGEN, Germany) for extraction from the agarose, and then ligated to the commercial vector pGEM-T Easy (Promega, USA). The vector and the amplified products were allowed to ligate for 16 h at 4°C. Subsequently, the DNA (in a 5 µl final volume) was used to transform *E. coli* TG1 competent cells, grown in Luria-Bertani solid rich medium supplemented with ampicillin, 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 15 g l-1 of bacteriological agar, 50 mg ml^{-1} ampicillin, adding 50 mg m^{l-1} 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal), to select the recombinant white colonies. The plasmids were digested with restriction enzyme EcoRI (Promega, USA), identifying recombinant clones that yielded the expected size bands. The recombinant plasmids were then sequenced through available commercial services (Eurofins MWG Synthesis GmbH, Germany; Macrogen, S. Korea).

For the phylogenetic analysis of the RKN sequences produced, an alignment was constructed using the closest BLAST hits for sequence JF330242 (701 bp), selecting the entries with 97% or higher identity and a 90% or higher query coverage. The alignment was performed using Clustal_X (Thompson *et al.*, 1997) implemented on the software BioEdit (Hall, 1999). Species redundancies and entries not identified at the species level were also eliminated. The phylogenetic analysis was performed on a subset of 37 aligned sequences using the Neighbor Joining method (Saitou and Nei, 1987) based on the Jukes-Cantor model (Jukes and Cantor, 1969), with the program MEGA5 (Tamura *et al.*, 2011). The percentage of replicated trees in which the associated taxa clustered together was calculated with bootstraps resampled 500 times (Felsenstein, 1985). All positions containing gaps and missing data were eliminated, for a total of 498 positions in the final dataset.

For the fungal isolation, approx. 10 g of asparagus roots were cut with a sterile blade and then spread in pieces on the surface of 1.5% water agar (WA) in 10 cm diam. Petri dishes and incubated in the dark at 26°C for 3-4 weeks. The plates were routinely examined for presence of nematode trapping or parasitic fungi, as well as of other invertebrates and predators under a stereoscope or a light transmission microscope at 50-125×. For single conidial isolation of fungi, a small

number of propagules from conidiophores emerging from the WA surface or root fragments were extracted and smeared on the surface of a sterile medium (potato dextrose agar, PDA) in a Petri dish. The smears were examined after 2-3 days incubation at 25 ± 1°C and a small, marginal mycelial fragment was dissected from the smallest colonies for culture on PDA.

For DNA extraction, mycelia of single spore isolates were grown on PDA for 1 week or in culture flasks with a liquid medium (Malt extract broth, Oxoid, UK) and were recovered on filter paper (Whatman). Equal amounts of sterile glass beads (425-600 µm in diam.) and 700 µl of extraction buffer (50 mM Tris HCl pH 7.2; 50 mM EDTA; 3% SDS; 1% 2-mercaptoethanol) were added to approx. 75-100 µg of fungal biomass with 300 µl sterile distilled water. The mycelial fragments and conidia were then crushed with a sterile micropestle for approximately 1 to 2 min and incubated for 60 min at 65 °C. After centrifugation at 15776 rpm for 10 min, the supernatant was collected, 500 µl of chloroform was added to the suspension, the suspension vortexed and centrifuged at 13443 rpm for 15 min. This procedure was repeated twice. The supernatant was then collected and 1 ml of precipitation buffer (CTAB 14 mM and NaCl 40 mM, pH 8) was added, the samples were incubated at room temperature for 60 min, followed by a centrifugation (5 min at 15776 rpm). The resulting pellet was then dissolved in 350 µl NaCl 1.2 M and 350 µl chloroform. After centrifugation (10 min at 15776 rpm), the supernatant was collected and 150 µl of isopropanol was added. After 20 min at room temperature and a further centrifugation (10 min at 13000 rpm), the pellets with DNA were washed in 200 µl of 70% ethanol, dried under vacuum, and re-suspended in 50 µl sterile-distilled water.

For PCR amplification of the internal transcribed spacer (ITS) ribosomal region, the fungi specific primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') regions were used (White *et al.*, 1990). The 25 µl of PCR mixture contained 2.5 µl 10X PCR buffer, 1 U of taq polymerase, 100 µM of each dNTPs, 500 nM of each primer and 1 µl of the template (50 ng). PCR was performed in a thermal cycler (BioRad, USA) with 1 initial cycle at 95°C for 7 min; 35 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, followed by a final extension cycle at 72°C for 10 min. The PCR products were purified with the QIA quick gel extraction kit (QIAGEN, Hilden, Germany) as recommended by the manufacturer. DNA templates were cloned and sequenced as previously described. The ITS region sequences obtained were checked to confirm the species identification on the NCBI sequence databases using the BLAST algorithm (Altschul *et al.*, 1990).

RESULTS AND DISCUSSION

Following the key provided for tropical nematode species (Adam *et al.*, 2007) and cross checking this information with the ITS sequence data, the RKN species found on asparagus in the area sampled were identified as *Meloidogyne incognita* and *M. ethiopica*, with other putative records for *M. arenaria* and *M. javanica*, due to insufficient discrimination among the BLAST hits, and highest frequency for *M. incognita.* The identification of Peruvian populations was confirmed by the close similarity of sequences produced with those of other South America populations available in GenBank (Tigano *et al.*, 2006), as shown by the Neighbor Joining (NJ) phylogenetic analysis (Fig. 2). The species distribution and the NCBI accession numbers for the sequences produced are shown in Table 1 and Fig. 2. When the sequences were not sufficient to identify the RKN species due to a high nucleotidic similarity, *M. incognita* was identified by means of the DNA amplified with specific primers that yielded bands of the expected lengths (Fig. 3).

Nematophagous fungi, identified by light microscopy and BLAST analysis of ribosomal sequences produced, were found in 61% of plates containing asparagus roots and soil. Species included the trapping fungi *Drechslerella brochopaga* and a *Monacrosporium* sp. Endoparasitic species were represented only by *Lecanicillium psalliotae* (Table 2). Occasionally,

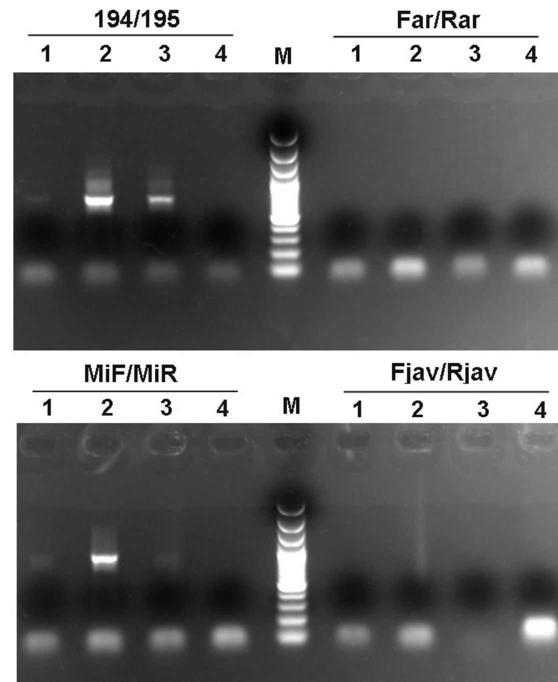

Fig. 3. Agarose gels showing the *M. incognita* amplification products obtained with the diagnostic primer combinations 194/195 and MiF-MiR, from 20 juveniles (J2) collected at San Carlos Alto (Chao district, sample 2). No amplification product was obtained using other samples collected in the same location (sample 1), from 40 J2 collected at San José (Virú district, sample 3) and from negative control (amplification mix only, 4). Ladder (M) scale = 100 bp.

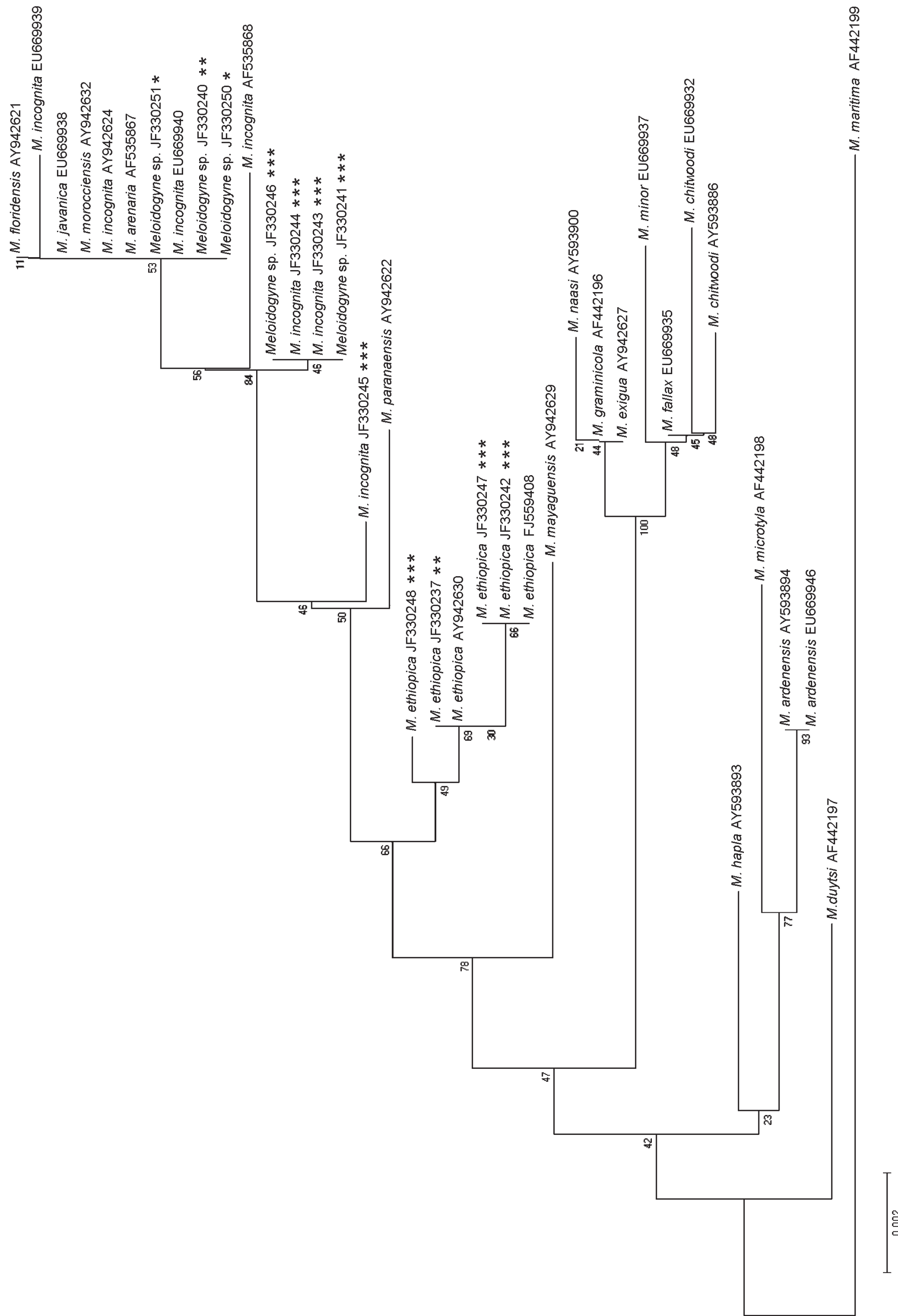

Fig. 2. Phylogenetic anaylsis of sequences obtained from *Meloidogyne ethiopica* and *M. incognita* from the province of Virú (Dept. La Libertad, Peru), using a 498 nt alignment of a fragment of the 18S regions and related NCBI accessions, as resulting from closest BLAST hits. The bootstrap consensus tree was constructed with the Neighbor Joining method based on the Jukes-Cantor model. Numbers on branches show the percentage of trees in which the associated taxa clustered together in the bootstrap test (500 replicates). The evolutionary distances used to infer the phylogenetic tree are expressed as number of base substitutions per site. Populations from the following Peruvian localities: California (Virú district), San José (Virú district) and San Carlos Alto (Chao district) are marked with one, two and three asterisks, respectively.

Harposporium spp. were also observed parasitizing free living nematodes, whereas a *Hirsutella* sp. was found parasitic on soil mites. *Fusarium proliferatum,* a specific root parasite of asparagus, was also isolated from a farm at Río Seco (Virú, acc. JF748760).

Esmenjaud *et al.* (1990) reported parasitism of asparagus by populations of *M. incognita, M. javanica, M. arenaria* and *M. hapla,* although no difference was found in crown weights between RKN inoculated and uninoculated plants. These observations were carried out on different host genotypes, and *M. incognita* was the most severe species. Nematode reproduction rates were lower on asparagus than on tomato, but populations did not appear stressed, as deduced by the low numbers of males present (Esmenjaud *et al.*, 1990). In contrast, damage observed in infested fields in Peru was occasionally severe, and possibly related to the species or populations of RKN present. Plant genotype and *Fusarium* sp. may also contribute to the greater damage in the areas sampled.

Table 1. Root-knot nematode species, *Meloidogyne* spp., on asparagus crops in the province of Virú (Dept. La Libertad, Peru) and NCBI accession numbers of the sequences produced from juvenile stages.

Species	Location (district)	Accessions
M. incognita (Kofoid and. White) Chitw.	San José (Virú)	JF330238
	San Carlos Alto (Chao)	JF330241, JF330243, JF330244, JF330245, JF330246
	Pur Pur (Virú)	-
M. ethiopica Whitehead, 1968	San José (Virú)	JF330237
	San Carlos Alto (Chao)	JF330242, JF330247, JF330248
Meloidogyne sp.	California (Virú)	-

Table 2. Nematophagous fungi found in the rhizosphere of asparagus plants in the province of Virú (Dept. La Libertad, Peru) and NCBI accession numbers of the sequences produced.

Species	Location (district)	Accessions
Drechslerella brochopaga (Drechsler) Scholler, Hagedorn & Rubner	Pur Pur (Virú)	JF748752 JF748757JF748758
	Río Seco (Virú)	JF748753JF748754 JF748755
	Puerto Morín (Virú)	-
	San Carlos Alto (Chao)	JF748756
Lecanicillium psalliotae (Treschow) Zare & Gams	Pur Pur (Virú)	JF748759
Monacrosporium sp.	California (Virú)	-

Except for *M. incognita* (Canto, 2000; E. Carbonell, pers. comm.), other records of *Meloidogyne* on asparagus in Peru were not determined at the species level (De Guerra, 1982; Murga-Gutiérrez, 1997). Other unidentified RKN populations from this study were phylogenetically close to *M. incognita* and distant from *M. fallax* (Fig. 2), the latter being previously reported as capable of attacking asparagus (Goossens, 1995).

Meloidogyne ethiopica represents a new record for Peru. This nematode deserves further research concerning its pathogenicity and epidemiology on asparagus, which may be a new host for this pest. In Chile and other countries in South America, *M. ethiopica* is recognized as a serious parasite of grapevine and other crops, and is considered a pest of quarantine importance (Carneiro *et al.*, 2007).

The nematophagous fungi recovered in the sampled area included the trapping species *D. brochopaga* as well as the egg parasite *L. psalliotae*. The former is a widespread predatory species producing constricting rings. In controlled assays, however, the efficacy of *D. brochopaga* was low, due to the low densities reached in soil microcosm (Jaffee and Strong, 2005). *Lecanicillium psalliotae* also has a worldwide distribution and is known as parasitic on cyst and RKN species (Gams, 1988; Gan *et al.*, 2007; Sukarno *et al.*, 2009). In the American region it was isolated from eggs of *M. mayaguensis* (= *M. enterolobii*) from Brazil (Arevalo *et al.*, 2009) and *Meloidogyne* sp. from Cuba (Hidalgo-Diaz *et al.*, 2000). This fungus is easily cultured and produces large numbers of conidia per g of substrate (Arevalo *et al.*, 2009). Considering its natural occurrence in Peruvian soils, it appears to be the best potential candidate for biological management of RKN on asparagus.

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