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DP 8: *Ditylenchus dipsaci* and *Ditylenchus destructor*

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ISPM 27
Diagnostic protocols for regulated pests

DP 8: *Ditylenchus dipsaci* and *Ditylenchus destructor*

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CONTENTS

1. Pest Information

Species within the large genus *Ditylenchus* Filipjev, 1936 are distributed worldwide, and most species are mycetophagous. However, the genus contains a few species that are of great importance as pests of higher plants (Sturhan and Brzeski, 1991). It is worth mentioning that though there are certain plants (e.g. beets, lucerne, clover) that are affected by both *Ditylenchus dipsaci* and *Ditylenchus destructor*, the two species rarely occur together in the same plant (Andrássy and Farkas, 1988).

1.1 *Ditylenchus dipsaci*

D. dipsaci sensu lato (*s.l.*), or stem nematode, attacks more than 1 200 species of wild and cultivated plants. Many weeds and grasses are hosts for the nematode and may play an important role in its survival in the absence of cultivated plants. Morphological, biochemical, molecular and karyological analyses of different populations and races of *D. dipsaci s.l.* have suggested that it is a complex of at least 30 host races, with limited host ranges. Jeszke *et al*. (2013) divided this complex into two groups, the first containing diploid populations characterized by their "normal" size and named *D. dipsaci sensu stricto* (*s.s*.). This group comprises most of the populations recorded so far. The second group is polyploidal and currently comprises *Ditylenchus gigas* Vovlas *et al*., 2011 (the "giant race" of *D. dipsaci* parasitizing *Vicia faba* (broad bean)); *D. weischeri* Chizhov *et al*., 2010 (parasitizing *Cirsium arvense* (creeping thistle)); and three undescribed *Ditylenchus* spp. called D, E and F, which are associated with plant species of the Fabaceae, Asteraceae and Plantaginaceae, respectively (Jeszke *et al*., 2013). Of all these species only *D. dipsaci s.s*. and its morphologically larger variant *D. gigas* are plant pests of economic importance. This protocol includes information to distinguish between *D. dipsaci s.s.* and *D. gigas*.

D. dipsaci lives mostly as an endoparasite in aerial parts of plants (stems, leaves and flowers), but also attacks bulbs, tubers and rhizomes. This nematode is seed-borne in *V. faba*, *Medicago sativa* (lucerne/alfafa), *Allium cepa* (onion), *Trifolium* spp. (clovers), *Dipsacus* spp. (teasel) and *Cucumis melo* (melon) (Sousa *et al*., 2003; Sikora *et al*., 2005). Of great importance is the fact that the fourth stage juvenile can withstand desiccation for a long time, sometimes 20 years or more (Barker and Lucas, 1984). These nematodes clump together in a cryptobiotic state to form "nematode wool" when the plant tissue begins to dry (Figure 1). The wool can often be observed on the seeds in heavily infested pods and in dry plant debris (e.g. that which remains in the field after harvest). The presence of the infective fourth stage juveniles in seed and dry plant material is important in the passive dissemination of the nematode over long distances. The nematode in its desiccated state can survive passage through pigs and cattle on or in infected seed (Palmisano *et al.*, 1971).

Although *D. dipsaci* is seen as a pest of higher plants, Viglierchio (1971) reported that a Californian population of *D. dipsaci* from *Allium sativum* (garlic) could reproduce on soil fungi (*Verticilium* and *Cladosporium*) under laboratory conditions.

D. dipsaci is known to vector bacterial plant pathogens externally (i.e. *Clavibacter michiganensis* subsp. *insidiosus* (syn. *Clavibacter michiganensis* subsp. *insidiosum*, *Corynebacterium insidiosum*), causing alfalfa wilt).

According to EPPO (2013a), *D. dipsaci* is present in the following regions (interceptions excluded): Europe, Asia, Africa, North America, Central America and the Caribbean, South America and Oceania.

1.2 *Ditylenchus destructor*

D. destructor, or potato rot nematode, attacks almost exclusively the subterranean parts of plants (e.g. tubers, rhizomes and stem-like underground parts). It is a near-cosmopolitan species, common in temperate regions and responsible for severe losses in potato and hop production (EPPO, 2013a). The host range of the nematode is extensive, comprising more than 90 plant species, which include ornamental plants, crop plants and weeds. *Solanum tuberosum* (potato) is the principal host, the tubers developing wet or dry rot that will spread to other tubers in storage. Under certain conditions, wet rot organisms may damage the tubers extensively, but will also kill the nematodes. *D. destructor* can survive only when dry rot organisms invade the tuber. Rojankovski and Ciurea (1986) found 55 species of bacteria and fungi associated with *D. destructor* in *S. tuberosum* tubers, with *Fusarium* spp. being the most common.

Other common hosts are *Ipomoea batatas* (sweet potato), bulbous iris (hybrids and selections derived from *Iris xiphium* and *Iris xiphioides*), *Taraxacum officinale* (dandelion), *Humulus lupulus* (hop), *Tulipa* spp. (tulip), *Leopoldia comosa* (grape hyacinth), *Hyacinthus orientalis* (hyacinth), *Gladiolus* spp. (gladiolus), *Dahlia* spp. (dahlia), *Coronilla varia* and *Anthyllis vulneraria* (vetch), *Beta vulgaris* (sugar beet, fodder beet and beetroot), *Calendula officinalis* (marigold), *Daucus carota* (carrot), *Petroselinum crispum* (parsley) and *Trifolium* spp. (red, white and alsike clover) (Sturhan and Brzeski, 1991). In the absence of higher plants, *D. destructor* reproduces readily on the mycelia of about 70 species of fungi and it is known to destroy the hyphae of cultivated mushroom (Sturhan and Brzeski, 1991). The species is able to survive desiccation and low temperatures, but does not form nematode wool as does *D. dipsaci* (Kühn, 1857) Filipjev, 1936. This species, however, overwinters as eggs, which makes eggs more vital in *D. destructor* than in *D. dipsaci*. *D. destructor* in seed potatoes and flower bulbs is a regulated pest in many countries (Sturhan and Brzeski, 1991). *D. destructor* was reported on *Arachis hypogaea* (groundnut/peanut) in South Africa, but these records are now considered to be a separate species, *Ditylenchus africanus* Wendt, Swart, Vrain and Webster, 1995, which is morphologically and morphometrically close to *D. destructor*.

According to EPPO (2013a), *D. destructor* is present in the following regions (interceptions excluded): Europe, Asia, Southern Africa, North America, South America and Oceania.

2. Taxonomic Information

Name: *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936

Synonyms: Synonyms of the type species *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 are listed in Siddiqi (2000)

Taxonomic position: Nematoda, Secernentea, Diplogasteria, Tylenchida, Tylenchina, Tylenchoidea, Anguinidae

Common names: Stem nematode, stem and bulb eelworm (English) (Sturhan and Brzeski, 1991)

Note: *D. dipsaci* is now considered as a species complex composed of a great number of biological races and populations differing mainly in host preference. Consequently a total of 13 nominal species have been synonymized with *D. dipsaci* and up to 30 biological races have been differentiated, mainly distinguished by host range and generally named after their principal host plant.

Name: *Ditylenchus destructor* Thorne, 1945

Synonyms: None

Taxonomic position: Nematoda, Secernentea, Diplogasteria, Tylenchida, Tylenchina, Tylenchoidea, Anguinidae

Common names: Tuber-rot eelworm, potato rot nematode (English) (Sturhan and Brzeski,1991)

De Ley and Blaxter (2003) have constructed the most recent classification system, combining morphological observations, molecular findings and cladistic analysis.

3. Detection

D. dipsaci and *D. destructor* both have the following common symptoms that allow their detection: swelling, distortion, discoloration and stunting of the above-ground plant parts and necrosis or rotting of the bulbs and tubers (Thorne, 1945).

Ditylenchus dipsaci

D. dipsaci shows parasitic adaptation in its ability to invade solid parenchyma tissue following enzymatic lysis of the pectic or middle lamella layer between adjacent cell walls, leading to separation and rounding of the cells. This causes the typical glistening appearance or mealy texture of infested tissues, reminiscent of the flesh of an over-ripe apple (Southey, 1993).

According to Vovlas *et al.* (2011), *D. gigas* (giant stem and bulb nematode) infestation of *V. faba* causes swelling and deformation of stem tissue or lesions, which turn reddish brown then black. In severe infestations the seeds appear dark, distorted and smaller in size than uninfested seeds, and they have speckle-like spots on the surface. Hosts other than *V. faba* are *Lamium purpureum*, *Lamium album*, *Lamium amplexicaule*, *Ranunculus arvensis*, *Convolvulus arvensis* and *Avena sterilis.*

Ditylenchus destructor

D. destructor commonly infects the underground parts of plants (tubers and stolons of potato, rhizomes of mint, and roots of hop and lilac), causing discoloration and rotting of plant tissue. The above-ground parts are sometimes also infected, causing dwarfing, thickening and branching of the stem and dwarfing, curling and discoloration of the leaves (e.g. in potato) (Sturhan and Brzeski, 1991). More often, however, no symptoms of infection are found in the above-ground parts of plants.

3.1 Hosts and symptoms

3.1.1 *Ditylenchus dipsaci*

According to Sturhan and Brzeski (1991), the principal hosts of *D. dipsaci* are Gramineae: *Avena sativa* (oat), *Secale cereale* (rye), *Zea mays* (maize), *Triticum aestivum* (wheat); Liliaceae: *A. cepa*, *A. sativum*, *Tulipa* spp.; Leguminosae: *M. sativa*, *Vicia* spp., *Pisum sativum*, *Trifolium* spp.; Solanaceae: *S. tuberosum*, *Nicotiana* spp.; Cruciferae: *Brassica campestris*; and Amarilidaceae: *Narcissus* spp. Other hosts include *D. carota*, *Fragaria* spp. (strawberry), *B. vulgaris*, *H. orientalis*, *Allium ampeloprasum* (leek), *Phlox drummondii*, *Phlox paniculata*, *Dianthus* spp. (carnation), *Apium graveolens* (celery), *Hydrangea* spp., *Lens culinaris* (lentil), *Brassica napus* (rape), *Petroselinum crispum* and *Helianthus annuus* (sunflower).

Various generations of *D. dipsaci* may be present in a host plant during a season, following each other. If affected parts of the plant die due to injuries by the pest, nematodes leave the host before it dies completely. When lacking host plants, the nematodes can enter non-host plants and feed there for a certain time, though they are unable to reproduce in non-host plants (Andrássy and Farkas, 1988). The most common symptoms of *D. dipsaci* infestation are stunted, chlorotic plants; thickened, stunted, gall-containing and distorted stems, petioles and flowers; and necrotic lesions in and rotting of bulbs and rhizomes, often appearing as brown rings when bulbs are sliced. *D. dipsaci* may also infest seeds, from, for example, *Phaseolus vulgaris* (snap bean, string bean or green bean), *V. faba, Allium* spp. and *M. sativa*. Small seeds generally show no visible symptoms of infestation but larger seeds may have a shrunken skin with discoloured spots.

3.1.1.1 Symptoms specific to Gramineae

Avena sativa* and *Secale cereale (McDonald and Nicol, 2005). Leaves become distorted, stems thicken, an abnormal number of tillers are produced, and the plant is short, bushy and stunted. In *S. cereale* cultivation, *D. dipsaci* occurs mainly in light soils poor in humus and naturally in areas where rye is regularly grown. The first signs of infestation can be observed in late autumn, but they are most conspicuous in spring. Several spots on plants with retarded growth in the rye field indicate damage by the pest. As infested *A. sativa* plants grow more slowly, they are conspicuous in the yellowing crop with their green colour. Affected *T. aestivum* has the same symptoms as other cereals and is attacked by *D. dipsaci* only in central and eastern Europe (Rivoal and Cook, 1993).

Zea mays is a poor host for *D. dipsaci* but invasion of the stem tissues of young plants produces necrosis in those tissues and causes the maize plants to die or fall over before harvest (Rivoal and

Cook, 1993). The leaves of the infested plants are crisp, and twisted like a corkscrew. Internodes are shortened and the bottom of the stem becomes hollow, while bigger plants break and lodge.

3.1.1.2 Symptoms specific to Liliaceae

***Allium cepa*, *Allium sativum* and *Allium cepa* var. *aggregatum* (shallot).** It is characteristic in most *Allium* spp. that leaves and bulbs become deformed on infestation with *D. dipsaci* (Figures 2, 3 and 4). The base of young plants becomes swollen and leaves become distorted. Older infected bulbs show swelling (bloat) of scales with open cracks often occurring at the root disc of the bulbs (Potter and Olthof, 1993). *A. cepa* attacked by *D. dipsaci* have a frosted appearance caused by the dissolution of cells that results from nematode feeding (Ferris and Ferris, 1998). Infested bulbs tend to rot readily in storage (Bridge and Hunt, 1986). The inner scales of the bulb are usually more severely attacked than the outer scales. As the season advances the bulbs become soft and when cut open show browning of the scales in concentric circles. Conversely, *D. dipsaci* does not induce deformation of leaves or swelling in *A. sativum*, but does cause leaf yellowing and death (Netscher and Sikora, 1990). Mollov *et al.* (2012) reported *D. dipsaci* for the first time from *A. sativum* in Minnesota, United States. The symptoms of the above-ground plant were stunting and chlorosis, while the symptoms of the bulbs were necrosis, underdevelopment and distortion. *Allium* spp. may have foliar spickels (i.e. blister-like swellings on the leaves). No symptoms of infestation are observed on infested *Allium* seeds.

***Tulipa* spp.** (Southey, 1993). Symptoms of *D. dipsaci* attack on tulip, both on growing plants and on bulbs, are quite different from those on *Narcissus* spp. In the field, infestation is best detected at flowering. The first sign is a pale or purplish lesion on one side of the stem immediately below the flower, which bends in the direction of the lesion. The lesion increases in size, the epidermis splits – revealing typical loose tissue beneath – and the damage spreads downwards and often upwards on to the petals. In more severe attacks, similar lesions extend down stems from leaf axils and growth may become distorted. Infestations start at the base of new bulbs, which arise as lateral offset buds from the base of the previous stems. The infection can be seen and felt on removal of the outer brown scales, as grey or brown soft patches on the outer fleshy scales. Infected bulbs do not show brown rings as they do in narcissus and hyacinth.

3.1.1.3 Symptoms specific to Leguminosae

Medicago sativa. *D. dipsaci* is the most important nematode pest of *M. sativa.* Infestation occurs readily in heavier soils and during times of high rainfall or in sprinkler-irrigated areas. "White flagging" associated with loss of leaf chlorophyll is often a feature of infested crops under conditions of moisture stress (Griffin, 1985). Infested fields often show irregular areas of sparse growth. Typical symptoms of nematode attack include basal swelling, dwarfing and twisting of stalks and leaves, shortening of internodes, and the formation of many axillary buds, producing an abnormal number of tillers to give the plant a bushy appearance (McDonald and Nicol, 2005). Infested plants sometimes do not grow tall enough for hay (Ferris and Ferris, 1998), and they often fail to produce flower spikes (McDonald and Nicol, 2005). *D. dipsaci* predisposes lucerne to *Phytophtora megasperma*. Damage by *D. dipsaci* is increased by the occurrence of other, saprophagous nematodes (*Rhabditis*, *Cephalobus* and *Panagrolaimus* species) on the diseased, broken plants, which also hasten the death of the plants (Andrássy and Farkas 1988). No symptoms of infestation are observed in infested *Medicago* seeds.

***Trifolium* spp.** (Cook and Yeates, 1993). Symptoms are quite similar to those described for *M. sativa*, except on red and white clovers. The pest invades red clover in particular in cool, rainy weather. Large, round areas of diseased plants appear in the field; plants are more diseased towards the inside of the area, frequently wilting in its centre. The bases of the plants are swollen like bulbs, and the leaves are crisp, shrivelled and with conspicuously thick veins. Flower initiations are swollen like galls, and a single flower gall may contain 5 000 nematodes (Courtney, 1962). Stems of white clover infected by *D. dipsaci* are short and swollen, buds are tufty, and the infested parts become brown in summer or autumn. Leaves are narrower than usual; however, their petioles are thicker and shorter. Flower buds are swollen at their bases (Andrássy and Farkas, 1988).

3.1.1.4 Symptoms specific to Solanaceae

Solanum tuberosum. *D. dipsaci* produces a funnel-shaped rot, which extends further into the tuber than the superficial rot caused by *D. destructor*. Stems and leaves are invaded by the nematode and this results in the typical stunting of the plant, accompanied by severe distortion of stems and petioles (Evans and Trudgill, 1992).

***Nicotiana* spp.** (Johnson, 1998). The infectious juveniles (fourth stage) enter the leaves and stems of tobacco seedlings during wet weather and induce small, yellow swellings (galls) that may extend 40 cm or more above the soil. As the number of galls increases, plant tissue begins to die prematurely. Lower leaves may fall off and upper leaves may turn yellow. Galls eventually rot, stopping growth of infected plants. Eventually, and especially in cool, damp weather and in heavy soils, the infected stems break and the plants fall over.

3.1.1.5 Symptoms specific to Cruciferae

Severe crown rot may develop in mature *B. campestris* infected with *D. dipsaci*.

3.1.1.6 Symptoms specific to Amarilidaceae

***Narcissus* spp.** (Southey, 1993). Typical symptoms are the presence of pale yellowish, blister-like swellings on the leaves (spickels) and concentric brown rings that can be seen when the bulbs are cut transversely (Figures 5 and 6). When bulbs are cut lengthwise, the necrosis is seen to have started at the neck, spreading downwards. Swellings are best seen before flowering when leaves are growing actively. In mild attacks, the swellings can be better felt between the finger and thumb than seen. *D. dipsaci* infection can be detected in dry bulbs with minimal bulb damage by cutting just below the neck. Careful examination in the early stages of infestation reveals glistening, spongy areas where cells have been separated. This is rapidly followed by brown necrosis.

3.1.1.7 Symptoms specific to other hosts

***Fragaria* spp.** *D. dipsaci* is the only species of *Ditylenchus* regarded as a pathogen of strawberry (Brown *et al.*, 1993). Damage is seen as small, distorted leaves, and short, thick and twisted petioles.

Family Asparagacae, subfamily Sciloideae (hyacinths) and other bulbs (Southey, 1993). Bulb symptoms are the same as in *Narcissus* spp., but distinct swellings are not usually seen on the plant leaves. The foliage may show pale yellow streaks, distortion and often slight swelling. Other liliaceous bulbs generally show the same symptoms as hyacinths. Symptoms of infestation in Amarylliaceae are similar to those in *Narcissus* spp.; for example, *Galanthus* spp. and *Nerine* spp. show swellings on their leaves and concentric, brown rings in bulbs.

Beta vulgaris* and *Daucus carota (Cooke, 1993). *D. dipsaci* feeding results in the death of the growing point in seedlings (leading to the formation of multiple crowns); cotyledons and leaves may become twisted, swollen and distorted; and galls may develop on leaves or petioles of slightly older plants. Later in the season, feeding on the crown may cause a rot known as crown canker, crown rot or collar rot. This is first visible as raised, greyish pustules, usually among the leaf scars. Rotting then develops outwards and downwards, expanding across the shoulder of the plant, allowing the crown to become detached when pulled. In *D. carota*, additional symptoms may include straddled leaves and discoloration of the head of the main root. Symptoms mainly occur on the root and stem of the plant 2–4 cm below and above ground level. Severe infestation causes leaf death and crown rot, especially in autumn (Figure 7).

***Phlox paniculata* and other ornamental plants** (Southey, 1993). On phlox, infested shoots show typical thickening and brittleness of stems and shortening of internodes that have a tendency to split. Characteristic and unique to this host is the crinkling and reduction of laminae of the upper leaves, the uppermost of which may be reduced to attenuated filaments. Examples of plants recorded as hosts, with malformed growth, swelling and so forth, are species and cultivars of *Anemone, Calceolaria, Cheiranthus, Gypsophila, Helenium, Heuchera, Lychnis, Lysimachia* and *Penstemon* (Roberts, 1981).

Edwards (1937) reported stunting, leaf malformation, rotting and failure to flower in *Primula* spp. Woody plants are not often attacked, but *Hydrangea* may be infested with *D. dipsaci*, causing distortion of non-woody shoots, swelling of petioles and main veins, and pronounced crinkling of leaf laminae. The crinkled leaves are usually the first sign of infection. Another woody plant, *Yucca smaliana*, shows leaf distortion and blister-like swellings.

3.1.2 *Ditylenchus destructor*

According to Sturhan and Brzeski (1991), *D. destructor* parasitizes mainly tubers (e.g. potato and dahlia), bulbs (e.g. bulbous iris, tulips and gladioli) and root crops (e.g. sugar beet and carrot). It is able to destroy the hyphae of *Agaricus hortensis* (cultivated mushroom). Other hosts include *I. batatas*, *A. sativum*, *P. vulgaris*, *Angelica sinensis* ("dong quai" or "female ginseng"), *Panax ginseng (*ginseng)*, Taraxacum officinale*, *Begonia* spp. and bulbs of *Erytronium denscanis* (dog's tooth violet or doftooth violet).

***Solanum tuberosum* and *Dahlia* spp.** No symptoms are visible during the growth period. The nematodes enter potato tubers usually via the stolons. Most of the nematodes are located at the edge of the browning and undamaged parts. If a small sample from this part of the tuber is taken and placed in water, the mass of small nematodes is conspicuous even with a simple magnifying glass. The earliest symptoms of *D. destructor* infection are small, white, chalky or light-coloured spots that can be seen just below the skin of the tuber (Brodie, 1998). These spots later become larger and gradually darker (through grey, dark brown and black), and acquire a spongy texture (Figure 8). This is mostly a result of secondary invasion by bacteria, fungi and saprophytic nematodes (Brodie, 1998). On severely affected tubers there are typically slightly sunken areas with cracked, wrinkled, papery skin. The skin is not attacked but becomes thin and cracks as underlying infected tissues dry and shrink (Brodie, 1998). Finally, mummification of whole tubers may occur. Such fully damaged tubers float in water (Figure 9). In contrast, the skin of *S. tuberosum* infested with *D. dipsaci* is usually not cracked. The nematodes continue to reproduce inside the tubers after harvest and may build up to large numbers. Symptoms may be more visible after storage. Secondary infections of fungi, bacteria and free-living nematodes occur in general on infested tubers.

Beta vulgaris. Infestation results in dark, necrotic lesions on roots and rhizomes. Dallimore and Thorne (1951) reported symptoms similar to crown canker. In sugar beet, in addition to yield loss, sugar content will also be reduced.

Daucus carota. Infestation results in transverse cracks in the skin of the carrot with white patches in the cortical tissue. Secondary infections in these areas by fungi and bacteria may also result in decay. This damage is easily seen in a cross-section of the carrot. The nematode continues its destructive activity during winter storage and carrots become unsuitable for consumption.

***Iris* spp. and *Tulipa* spp.** (Southey, 1993). Infestation results in greyish linear marks that extend upwards from the basal plate on the outer fleshy scales. As infestation progresses, the damage spreads over and through the tissue of the bulb and leads to a secondary dry, fibrous rotting that results in collapse of the bulb. Ring-like brown spots are conspicuous when a cross-section is made of an infested bulb. Yellowing and dieback of the foliage are secondary symptoms caused by the damage to the bulb and eventual cessation of root functioning.

D. destructor infestation of ornamental *Liatris spicata* corms ("Gayflower", "Blazing Star" or "Button Snakeroot") in cold storage in South Africa showed a blackish rot with living nematodes at different stages in the tissue adjacent to the decaying areas (Van der Vegte and Daiber, 1983).

3.2 Nematode extraction

3.2.1 Extraction from bulbs and garlic

To extract the nematodes, the affected scales of bulbs (inner scales mainly) or garlic cloves are cut into small pieces and put in a container (e.g. Petri dish) with tap water at room temperature. To obtain a clear suspension the pieces may be placed on a sieve of 200–250 μm aperture covered with filter paper, as a support (Oostenbrink dish technique). After 1 h or more the nematodes can be observed with a stereomicroscope (at least 40× magnification).

3.2.2 Extraction from soil and plant material

The Baermann funnel method is a reference technique for the extraction of nematodes from soil and plant material (bulbs, roots, potato peelings and seeds). A funnel has a piece of rubber tubing attached to its stem that is closed by a spring or screw clip. The funnel is placed in a support and almost filled with tap water**.** Soil or plant tissue cut into small pieces is placed in a muslin or in tissue paper, which is folded to enclose the material and is gently submerged in the water in the funnel. Active nematodes pass through the cloth and sink to the bottom of the funnel stem. After some hours, or overnight, a small quantity of water containing the nematodes is run off and observed under a microscope (Flegg and Hooper, 1970).

In a variation of the technique the funnel is replaced by a dish. Lumps of soil are broken up and stones and plant debris removed. Soil (50 ml) is spread evenly on a circle of single-ply paper towel supported on a coarse-meshed plastic screen standing in a plastic container. Water is added to the container until the soil is thoroughly wet but not immersed. The container is covered with a large Petri dish top to reduce evaporation of water. This set-up is left for at least 24 h after which the soil is discarded and the nematode suspension is poured from the container into a dish for examination with the aid of a dissection microscope. The soil can be replaced by finely chopped plant tissue (Kleynhans, 1997).

The Seinhorst mistifier technique for bulbs and roots differs from the Baermann funnel method in that plant sap and toxic decomposition products are washed away. It should be used in preference to the Baermann funnel method for plants such as *Narcissus* spp. In this method a Baermann funnel or Oostenbrink dish is placed in a mist or fog of water to avoid the depletion of oxygen. The mist is produced by nozzles spraying water over the plant material or by nozzles spraying water upwards so that droplets fall softly back onto the plant material. Live nematodes leave the plant tissue and are washed into the funnel or dish where they sediment. The nematodes are collected every 24 to 48 h in a glass beaker by opening the screw clip on the funnel stem or by collecting the specimens on a 20–25 µm sieve. Extraction can be continued for up to four weeks. This technique is described by Hooper (1986).

Another method to extract *Ditylenchus* spp. from plant material was adapted from a description by Oliveira *et al.* (2013). Plant material is cut in 1 cm pieces and they are placed in 500 ml jars filled with tap water. Two holes are punched into the lids of these jars, one providing access to the tube of an aquarium pump and one acting as an outlet for air. The material is kept for 72 h under continuous aeration from the pump. The resulting suspension is poured through a 1 000 µm sieve to remove plant debris and then through a 38 µm sieve to extract the nematodes from the suspension. This method of aerating the suspension prevents the rotting of the plant material so there is a minimal increase of bacterial and fungal feeders and many of the nematodes stay alive. The agitation through the aeration of the suspension containing the plant material results in more nematodes being dislodged from the root tissue and therefore in a much more accurate estimate of the infestation of the plant material.

Nematodes can also be extracted from plant material by the method of Coolen and D'Herde (1972). The plant material is washed, cut into pieces of about 0.5 cm, and 5 g portions are macerated in 50 ml tap water in a domestic blender at the lowest mixing speed for 1 min. The disadvantage of this method is that large nematode specimens, such as *D. dipsaci* adults, can be cut to pieces in the blender. The suspension of nematodes and tissue fragments are washed through a 750 µm sieve placed on top of a 45 µm sieve. The residue on the 45 µm sieve is collected and poured into two 50 ml centrifuge tubes. About 1 ml kaolin is added to each tube, the mixture is thoroughly stirred and then it is centrifuged at 3 000 r.p.m. for 5 min. The supernatant is decanted and sucrose solution (density 1.13 g/cm^3) is added to the tubes. The mixture is thoroughly stirred and centrifuged at 1 750 r.p.m. for 1 min. The supernatant is washed through a 45 µm sieve, the residue is collected and the nematodes are studied.

The testing of dried legumes and other pulse crops for the presence of *D. dipsaci* is a two-step procedure involving (1) soaking of a quantity of seed in aerated water overnight, and (2) extracting a portion of the soaked seed under mist for three days. The presence of nematodes in the soaking water and mist extract are determined by sieving aqueous fractions from each of the two steps followed by microscopic observation for identification. The process takes about seven days, but can be shortened to three days by eliminating step (2) (i.e. extraction under mist). The modified procedure consists of soaking the pulses overnight in aerated water, followed by sieving and microscopic observation for identification.

For extraction of nematodes from soil, the following method (after Kleynhans, 1997) can be used. Soil (250 ml) is washed through a coarse-meshed (2 mm) sieve into a 5 litre bucket. Tap water is added to make a volume of 5 litres. The suspension is stirred, then allowed to settle for 30 s before being poured through a 45 µm sieve. This procedure is repeated with the soil in the bucket two times, but shortening the setting times to 20 s and then 10 s. The residue is transferred from the 45 µm sieve to 50 ml centrifuge tubes. If the solution in the tubes is very sandy, 5 ml kaolin can be added to the tubes (and thoroughly mixed) to assist in the settling of the nematodes. The tubes are centrifuged at 1 750 r.p.m. for 7 min. The supernatant is decanted from each tube and discarded. A sugar solution (450 g/litre water) is added to the tubes and this sugar and soil mixture is thoroughly shaken before centrifuging again at 1 750 r.p.m. for 3 min. The supernatant is poured through a 45 µm sieve and the residue, with nematodes in it, is collected in a beaker for examination. This is a basic technique and depending on the skill of the technician and type of soil, up to 40% of the nematodes may be lost. Other methods that may be used for the extraction of nematodes from soil include the Flegg-modified Cobb technique and the Oostenbrink elutriator method (EPPO, 2013c). Hooper *et al*. (2005) describes different extraction methods adapted to take advantage of size, density and motility of nematodes.

4. Identification

Identification of *Ditylenchus* spp. by morphological means is restricted to adult specimens and preferably both male and female nematodes of a species are examined under a high-power microscope. Good-quality slide preparations should allow adult *D. dipsaci* and *D. destructor* to be identified with certainty by morphological examination alone. The morphological identification of *Ditylenchus* juveniles in a sample should be used only to confirm the presence of the species in the sample. As mycophagous *Ditylenchus* spp. frequently contaminate decaying plant material, care must be taken in the identification of specimens in both plant and soil samples.

4.1 Morphological identification

The identification of *D. dipsaci* and *D. destructor* should preferably be based on morphological methods. Molecular methods developed for identifying these species can be used for low infestation levels or when only juveniles are present. Molecular methods can be applied to damaged and atypical adults, and all life stages, including the juvenile stages, for which morphological identification to species is not possible.

4.1.1 Preparation of specimens

Temporary preparations for quick identification or study of features best seen in unfixed specimens are prepared as follows (Kleynhans, 1997):

- Live specimens are transferred to a small drop of water on a glass slide.
- The slide is briefly heated over a spirit flame, checking frequently for nematode movement. Heating should be stopped as soon as the specimens stop twitching.
- A coverslip is applied and sealed around the edge with nail varnish. When the varnish has dried, the slide with specimens is ready for study.

For light microscopy, live nematodes are extracted from soil or plant material, killed by gentle heat (65–70 °C), fixed in FAA (35% distilled water, 10% of 40% formalin, 5% glacial acetic acid, 50% of 95% alcohol) (Andrássy, 1984), transferred into glycerol (Hooper *et al*., 2005) and mounted in anhydrous glycerine between coverslip slides as described by Seinhorst (1959) and Goodey (1963).

For light microscopy identification work, magnification of 500× to 1 000× (oil immersion lens) in combination with differential interference contrast microscopy is recommended.

4.1.2 Morphological diagnostic characters

Keys for diagnosis for *Ditylenchus* species can be found in Viscardi and Brzeski (1993) and Brzeski (1998). A key to distinguish *Ditylenchus* spp. from other tylenchid and aphelenchid genera is presented in Table 1 below.

Table 1. Key to distinguish *Ditylenchus* spp. from other tylenchid and aphelenchid genera

1	Outlet of dorsal pharynx gland near base of stylet; median bulb roundish, ovoid or absent	Tylenchida – 2
	Outlet of dorsal pharynx gland in median bulb; median bulb a prominent feature, usually oblong	Aphelenchida
2	Anterior part of oesophagus (procorpus) and median bulb not united into single unit; stylet never exceptionally long	3
	Procorpus gradually widened and fused with median bulb; stylet very long, its base often located in anterior part of median bulb	Other genera
3	Adult female vermiform	4
	Adult female saccate or pyriform sessile parasite on roots	Other genera
4	Valvular median bulb	5
	Median bulb without valve[1]	Other genera
5	Pharynx glands contained within basal bulb, not overlapping or slightly overlapping intestine; cephalic framework rarely conspicuous; stylet weak to moderately strong	6
	Pharynx glands lobe-like, overlapping intestine; cephalic framework strong; stylet massive	Other genera
6	Single prodelphic ovary; vulva posterior	7
	Ovaries two, amphidelphic; vulva slightly post-equatorial	Other genera
7	Female not swollen; crustaformeria in female in form of quadricollumella with four rows of four cells each; bursa in males enveloping one-third or more of tail	*Ditylenchus*
	Female swollen; crustaformeria with more than 20 cells	Other genera

Source: Adapted from Heyns (1971) and Siddiqi (2000).

[1] A few non-plant-parasitic species of *Ditylenchus* do not have a valvular median bulb.

D. africanus, D. destructor, D. dipsaci, D. gigas and *D. myceliophagus* are morphologically and morphometrically similar, but can be differentiated from each other by the following (Table 2), providing both male and female specimens can be measured and studied.

4.1.2.1 Description of **Ditylenchus dipsaci**

After Sturhan and Brzeski (1991), Wendt *et al.* (1995) and Brzeski (1998). Details and views are provided in Figure 10.

Measurements (criteria described in EPPO (2013b)). (*Ex* Oat, *Avena sativa* L., after Blake, 1962, in Hooper, 1972.) (n = 48♀♀): L = 1.3 mm ± 0.009; a = 62 ± 5.6; b = 15 ± 1.4; c = 14 ± 2.1; V = 80 ± 1.5. (n = 23♂♂): L = 1.3 mm ± 0.017; a = 63 ± 11.3; b = 15 ± 1.7; c = 14 ± 2.1; T = 72.

General morphology. Body straight or almost so when relaxed. Lateral field with four incisures. Head continuous with adjacent body (Figure 10B). Stylet 10–13 µm long in females, 10–12 µm in males. Stylet cone about half of stylet length, knobs rounded and well developed. Median bulb muscular, with thickenings of lumen walls 4–5 µm long (Figure 10A). Basal bulb offset or overlapping intestine for a few micrometres. Excretory pore opposite posterior part of isthmus or glandular bulb. Postvulval part of uterine sac occupying about half to slightly more of vulva–anus distance (Figure 10D). Bursa envelops three-quarters of the tail in males. Spicules 23–28 µm long. Tails of both sexes conical with a pointed tip.

Morphological diagnostic characters. The number of lateral incisures (four) (Figure 10F), the comparatively long stylet, the length of the postvulval sac and the pointed tail (Figure 10D) are the distinguishing characters for this species (Andrássy, 2007). *D. dipsaci* can be distinguished from *D. gigas* by the shorter body of females (1.0–1.7 vs 1.6–2.2 mm) and the longer vulva–anus distance (202–266 vs 132–188 µm) (Vovlas *et al.*, 2011). When observed in the lateral view, the spicule is more arched in *D. dipsaci* than in *D. destructor* (Figure 10C). See Karssen and Willemsen (2010) for more information on the spiculum and its use in the identification of *D. dipsaci* and *D. destructor*. It must be noted that the seed of *V. faba* contains mainly larvae of the fourth stage.

4.1.2.2 Description of **Ditylenchus destructor**

After Sturhan and Brzeski (1991) and Brzeski (1998). Details and views are provided in Figure 11.

Measurements (after Goodey, 1952, from various higher plant hosts). (n = 237♀♀): L = 1.07 (0.69–1.89) mm; a = 32 (18–49); b = 7 (4–12); c = 17 (9–30); V = 80 (73–90). (n = 231♂♂): L = 0.96 (0.76–1.35) mm; a = 35 (24–50); b = 7 (4–11); c = 14 (11–21); T = 65 (40–84).

General morphology. Adults of *D. destructor* are minute, worm-like animals, 0.8–1.4 mm long, 23–47 µm wide and slightly ventrally arcuate. Considerable morphometric variation occurs in adults according to their host and age. Males and females are similar in general appearance. Lateral field with six incisures (Figure 11F), reduced to two on the neck and tail regions. Cuticular and head annulation fine, head often narrower than adjacent body, about four head annules discerned by scanning electron microscopy (Wendt *et al.*, 1995). Stylet 10–12 µm long, specimens with stylets of 14 µm have been described occasionally. Stylet cone 45–50% of stylet length, knobs distinct, rounded and sloping backwards. Median bulb muscular, with thickenings of lumen walls (or valve) about 3 µm long. Posterior bulb overlaps intestine for a short distance on the dorsal body side, although specimens with an offset glandular bulb are seen occasionally (Figure 11A). Excretory pore opposite oesophageal glands. Postvulval sac extending about three-quarters of the vulva–anus distance (Figure 11E). Eggs twice as long as wide (Andrássy, 2007). Lips of vulva thick, elevated (Figure 11B). Anterior ovary outstretched, sometimes reaching the oesophageal region. Postvulval part of uterine sac 40–98% of vulva–anus distance, not functioning as a spermatheca (Figure 11E). Male bursa surrounds 50–90% of the tail length. Spicules 24–27 µm long. The spiculum shape of *D. dipsaci* differs from *D. destructor* in having a ventral tumulus in the calomus area (Figure 12) (Karssen and Willemsen, 2010). Testis outstretched approaching the base of oesophagus. Tail of both sexes conical, three to five anal body widths long, usually ventrally curved, terminus rounded.

Morphological diagnostic characters. D. destructor is similar to *D. dipsaci*, but differs from that species by the lateral field showing six incisures (Figure 11F), the longer postvulval sac and the finely rounded tail terminus (Figure 11D). Morphologically *D. destructor* differs from *D. africanus* mainly in the stylet length, which may overlap slightly, and the spicule length, which implies that males must be present in the population. As polymerase chain reaction (PCR) technology is sufficiently sensitive to resolve differences between closely related genera, Wendt *et al*. (1995) used restriction fragment length polymorphisms (RFLPs) to separate *D. destructor* from *D. africanus*. When observed in the lateral view, the spicule is less arched in *D. dipsaci* than in *D. destructor* (Figure 11C).

Remarks. The above characters may vary and it is almost impossible to identify a single specimen to species level. It is recommended that at least one male and one female specimen are examined. Lateral incisures in the male may, for instance, occasionally be reduced to four near the tail, forming a pattern similar to that of *D. dipsaci*.

Table 2. Comparative diagnostic characteristics of *Ditylenchus africanus*, *Ditylenchus destructor*, *Ditylenchus dipsaci*, *Ditylenchus gigas* and *Ditylenchus myceliophagus*

Characters	***D. destructor* (after Hooper, 1973)**	***D. africanus* (after Wendt *et al.*, 1995)**	***D. myceliophagus* (after Hesling, 1974)**	***D. gigas* (after Vovlas *et al.*, 2011)**	***D. dipsaci* (after Hooper, 1972)**
Body length female (mm)	0.8–1.9	0.7–1.1	0.6–1.4	1.6–2.2	1.0–1.7
Number of lateral lines	6	6–15	6	4	4
Form of tail terminus	Rounded	Rounded	Rounded	Pointed to finely rounded	Pointed
c (body length/tail length) of female	14–20	8.8–16.9	8.2–17	15.7–27.6	11–20
Posterior bulb	Short, dorsally overlapping	Short, dorsally overlapping	Short, dorsally overlapping	Slightly overlapping	Not overlapping
Stylet length (µm) of female	10–14	8–10	7–8	10.5–13.0	10–12
PUS/vulva–anus length (%)[1]	53–90	37–85	30–69	About 50[2]	40–70
Spiculum length (µm)	24–27	17–21	15–20	23.5–28	23–28
Bursa length (as % of tail length)	50–70	48–66	20–55	72–76	40–70
Host preference[3]	Higher plants and mycelia of fungi	Groundnuts and fungi	Mycelia of fungi	Higher plants	Higher plants and fungi

[1] PUS, the postvulval part of the uterine sac.

[2] Calculated from species description.

[3] Helpful in case of confusing morphological criteria.

4.2 Molecular identification

When necessary, a molecular identification of the species *D. dipsaci* or *D. destructor* can be conducted, especially when confounding species may occur (e.g. *D. myceliophagus*, *D. africanus* or *D. gigas*) and cannot be distinguished conclusively from the target species morphologically.

In this case, the solution containing the nematode individuals should preferably be stored in cold conditions (i.e. refrigerated) for not more than few days before the DNA is extracted.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.1 *Ditylenchus dipsaci*

Various molecular approaches have been developed for *D. dipsaci* identification.

Southern hybridization (Wendt *et al*., 1993) and electrophoresis (Tenente and Evans, 1997; Palazova and Baicheva, 2002) were used to investigate the concept of races within *D. dipsaci* species and the genetic diversity among *Ditylenchus* species.

Molecular approaches have also been thoroughly investigated for specific identification, mostly by PCR or PCR-RFLP, and for population variation detection by sequence analysis (Leal-Bertioli *et al*., 2000; Zouhar *et al*., 2002).

Six molecular tests (PCR, PCR-RFLP) have been published that can be used in the identification of *D. dipsaci*; these are described in sections 4.2.4 to 4.2.9. The specificity of each test is included in the description, as is the nematode genus and species against which each test has been evaluated.

The molecular analysis of ribosomal (r)DNA sequences, including different regions (the internal transcribed spacer (ITS)1-5.8S-ITS2 region, the D2–D3 fragment of the *s8S* gene, the small 18S subunit, the partial mitochondrial gene for *cytochrome c oxidase I* (mitochondrial (mt)DNA) and *hsp90* gene sequences (nuclear (n)DNA)), clearly distinguishes *D. gigas* from *D. dipsaci s.s.* (Vovlas *et al*., 2011).

4.2.2 *Ditylenchus destructor*

Molecular diagnosis of *D. destructor* is based on PCR-RFLP or sequencing of the ITS region of the rRNA gene.

Wendt *et al.* (1993) showed that PCR-RFLP of the ITS region allowed *D. destructor* parasitizing potato to be distinguished from two races of *D. dipsaci* and from *D. myceliophagus.* They published the diagnostic RFLP profiles for these three species. *D. africanus* can be distinguished from *D. destructor* by a combination of the following characters: RFLP generated by seven restriction enzymes on the ITS region of rDNA.

Ji *et al*. (2006) obtained RFLP profiles for several populations of *D. destructor* from sweet potato and revealed some differences in their RFLP profiles.

Powers *et al.* (2001) first sequenced the ITS1 region for *D. dipsaci*, but more than 50 sequence accessions of rRNA fragments obtained from *D. destructor* collected from different localities and host plants are presently available in the GenBank database.

4.2.3 DNA extraction

Several juveniles or adults are transferred to a microtube and DNA is extracted from them. DNA extraction is described by Webster *et al*. (1990).

4.2.4 ITS-rRNA PCR-RFLP test for *D. dipsaci* and *D. destructor*

This test was developed by Wendt *et al.* (1993).

Methodology

The ITS rRNA universal primers (as described in Vrain *et al*. (1992)) used in this test are:
18S: 5′-TTG ATT ACG TCC CTG CCC TTT-3′
26S: 5′-TTT CAC TCG CCG TTA CTA AGG-3′

The amplicons are 900 base pairs (bp) for both *D. dipsaci* and *D. myceliophagus,* and 1 200 bp for *D. destructor*.

Amplification is obtained following the manufacturer's recommendations for PCR kits containing Taq DNA polymerase, nucleotides and reaction buffer.

The PCR cycling parameters[1] consist of a first cycle of 1.5 min at 96 °C, 30 s at 50 °C and 4 min at 72 °C; 40 cycles of 45 s at 96 °C, 30 s at 50 °C and 4 min at 72 °C; and a final cycle of 45 s at 96 °C, 30 s at 50 °C and 10 min at 72 °C. After DNA amplification, 2–5 µl of the product is run on a 1% agarose gel. The remainder is stored at –20 °C and used for RFLP. Several restriction enzymes are useful for distinguishing *D. destructor* and *D. dipsaci* from other *Ditylenchus* species; for example, *Hae*III, *Hpa*II, *Hin*fI and *Rsa*I (Wendt *et al*., 1993). The lengths of the restriction fragments generated by these diagnostic enzymes are given in Table 3.

Table 3. Approximate length (bp) of RFLP fragments of the ITS-rRNA for *Ditylenchus* species generated by four restriction enzymes

Enzyme	*D. destructor*	*D. myceliophagus*	*D. dipsaci*	*D. gigas*[1]	*D. africanus*
Unrestricted PCR product	1 200	900	900	900	1 000
*Hae*III	450, 170	450, 200	900	800, 200	650, 540
*Hpa*II	1 000	900	320, 200, 180	600, 200	950
*Hin*fI	780, 180	630, 310	440, 350, 150	350, 150	450, 340, 150, 130, 100
*Rsa*I	600, 250, 170	900	450, 250, 140	490, 450	690, 450

Source: Wendt *et al.* (1993, 1995).

bp, base pairs; ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; rRNA, ribosomal RNA.

1 Named in the original paper as *D. dipsaci* giant race.

4.2.5 SCAR PCR test for *D. dipsaci*

This sequence characterized amplified region (SCAR) PCR test developed by Esquibet *et al*. (2003) was designed as a species-specific test for *D. dipsaci* with differentiation between normal and giant

[1] The PCR cycling parameters are those described in the original article (Wendt *et al.*, 1993). Improvement of thermocyclers and reagents for PCR may lead to revision of these cycling parameters.

races. It was evaluated against *D. myceliophagus* (one population), *D. dipsaci* normal race (11 populations from different hosts and locations) and *D. dipsaci* giant race, described as *D. gigas* by Vovlas *et al*. (2011) (11 populations from different locations isolated from *V. faba*).

Methodology

The *D. dipsaci*-specific primers used are:

D. dipsaci (normal race):

H05: 5′-TCA AGG TAA TCT TTT TCC CCA CT-3′

H06: 5′-CAACTG CTA ATG CGT GCT CT-3′

D. dipsaci (giant race, described as *D. gigas* by Vovlas *et al.* (2011)):

D09: 5′-CAA AGT GTT TGA TCG ACT GGA-3′

D10: 5′-CAT CCC AAA ACA AAG AAA GG-3′

The amplicon is approximately 242 bp for *D. dipsaci* (normal race) and 198 bp for *D. dipsaci* (giant race). For both primer sets, no amplification is observed with non-target species and non-target race (Esquibet *et al*., 2003).

The 10 µl PCR mixture is composed of: 1.5 mM $MgCl_2$, 250 µM each dNTP, 690 nM each primer for duplex PCR (H05-H06) or (D09-D10) or 500 nM each primer for multiplex PCR (H05-H06-D09-D10) and 0.5 U Taq DNA polymerase. The cycling parameters are: initial denaturation 3 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 59 °C and 1 min at 72 °C; and final elongation of 10 min at 72 °C. The PCR products are analysed by agarose gel electrophoresis.

4.2.6 18S and ITS1-specific PCR test for *D. dipsaci*

This test developed by Subbotin *et al*. (2005) was designed as a species-specific test for *D. dipsaci s.s.* (normal race only). It was evaluated against *D. destructor* (one population), *D. dipsaci* normal race (18 populations from different hosts and locations) and *Ditylenchus* sp. (12 populations from different hosts and locations).

Methodology

The *D. dipsaci*-specific primers used are:

rDNA2: 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (Vrain *et al*., 1992)

DitNF1: 5′-TTA TGA CAA ATT CAT GGC GG-3′

The amplicon is approximately 263 bp for *D. dipsaci s.s.* (giant race, later called *D. gigas*, not included). No amplification is observed with non-target species.

The 25 µl PCR mixture is composed of: 1× from 10× PCR buffer including 15 mM $MgCl_2$, 0.2 mM each dNTP, 60 nM each primer and 1 U Taq DNA polymerase. The PCR is performed in a 96-well Peltier type thermocycler (PTC100, MJ Research[2]) with the following cycling parameters: initial 4 min at 94 °C; 35 cycles of 15 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C; and final elongation of 10 min at 72 °C. The PCR products are analysed by agarose gel electrophoresis.

[2] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

4.2.7 5.8S rDNA-specific PCR test for *D. dipsaci*

This test developed by Marek *et al*. (2005) was designed as a species-specific test for *D. dipsaci*. It was evaluated against *D. dipsaci* (three European populations from different hosts) and non-target genus populations (*Globodera pallida, Bursaphelenchus xylophilus, Rhabditis* spp.).

Methodology

Two specific primer sets were developed for *D. dipsaci* identification, but the most sensitive (10 pg of target DNA detected) is:

PF1: 5′-AAC GGC TCT GTT GGC TTC TAT-3′

PR1: 5′-ATT TAC GAC CCT GAG CCA GAT-3′

The amplicon with this primer set is approximately 327 bp for *D. dipsaci*.

The 25 µl PCR mixture is composed of: 1× Taq buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmol each primer (PF1-PR1 primer set) and 1.5 U Taq DNA polymerase (Fermentas[2]). The PCR test was developed on a 96-well Peltier type thermocycler (PTC200, MJ Resarch[2]), with the following cycling parameters: 3 min at 94 °C; 30 cycles of 2 min at 94 °C, 30 s at 62 °C and 2 min at 72 °C; and final elongation of 10 min at 72 °C. The PCR products are analysed by agarose gel electrophoresis.

4.2.8 5.8S rDNA and ITS-specific PCR test for *D. dipsaci*

This test developed by Kerkoud *et al*. (2007) was designed as a species-specific test for *D. dipsaci*. It was evaluated against *D. dipsaci* (ten populations from different hosts and locations), *D. africanus, D. destructor, D. myceliophagus, Aphelenchoides ritzemabosi* (one population for each species) and *Ditylenchus* sp. (according to the paper and now described as *D. gigas*) (ten populations from different locations isolated from *V. faba*).

Methodology

Two specific primer sets are used, one for the identification of *D. dipsaci* alone and one for the identification *of D. gigas* and *D. dipsaci*. The use of both primer sets allows separation of *D. gigas* from *D. dipsaci*. The primers are:

First primer set:

DdpS1: 5′-TGG CTG CGT TGA AGA GAA CT-3′

rDNA2: 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (Vrain *et al*., 1992)

The amplicon is approximately 517 bp for *D. dipsaci*. No amplification is observed with non-target species, including *D. gigas*.

Second primer set:

DdpS2: 5′-CGA TCA ACC AAA ACA CTA GGA ATT-3′

rDNA2: 5′-TTT CAC TCG CCG TTA CTA AGG-3′ (Vrain *et al*., 1992)

The amplicon is approximately 707 bp for *D. dipsaci* and *D. gigas.*

The 20 µl PCR mixture is composed of: 1.5 mM amplification buffer with final $MgCl_2$ concentration of 5 mM, 200 µM each dNTP, 0.5 µM each primer (in the simplex PCR with DdpS1-rDNA2 or DdpS2-rDNA2; in the duplex PCR, the final concentration of DdpS1 primer is 0.5 µM whereas it is 1 µM for DdpS2 and rDNA2) and 1 U Taq DNA polymerase (MP Biomedicals[2]). The PCR was developed on a 96-well Peltier type thermocycler (GeneAmp 9600 PCR System, Perkin Elmer[2]), with the following cycling parameters: 1 min at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C; and final elongation of 10 min at 72 °C. The PCR products are analysed by agarose gel electrophoresis.

4.2.9 SCAR PCR test for *D. dipsaci*

This SCAR PCR developed by Zouhar *et al*. (2007) was designed as a species-specific test for *D. dipsaci*. It was evaluated against only *D. dipsaci* (ten European populations from different hosts).

Methodology

Two specific primer sets were designed for *D. dipsaci* identification:
First primer set:
DIT_2 forward: 5′-GCA ATG CAC AGG TGG ATA AAG-3′
DIT_2 reverse: 5′-CTG TCT GTG ATT TCA CGG TAG AC-3′

The amplicon with this primer set is approximately 325 bp for *D. dipsaci*.

Second primer set:
DIT_5 forward: 5′-GAA AAC CAA AGA GGC CGT AAC-3′
DIT_5 reverse: 5′-ACC TGA TTC TGT ACG GTG CAA-3′

The amplicon with this primer set is approximately 245 bp for *D. dipsaci*.

The 25 µl PCR mixture is composed of: 1× PCR buffer (Fermentas[2]), 1.5 mM $MgCl_2$, 200 µM each dNTP, 10 pmol each primer (either DIT_2 or DIT_5 primer set), 1.5 U Taq DNA polymerase (Fermentas[2]) and 50 ng DNA as template. The PCR is performed in a 96-well Peltier type thermocycler (PTC200, MJ Research[2]), with the following cycling parameters: 3 min at 94 °C; 30 cycles of 1 min at 94 °C, 30 s at 60 °C and 1 min at 72 °C; and final elongation of 10 min at 72 °C. The PCR products are analysed by agarose gel electrophoresis.

4.2.10 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the nucleic acid of the target pest or target nucleic acid. A positive nucleic acid control, a negative amplification control and a negative extraction control are the minimum controls that should be used.

Positive nucleic acid control. This control is used to monitor the efficiency of the amplification (apart from the extraction). Pre-prepared (stored) nucleic acid of the target nematode may be used.

Negative amplification control (no template control).This control is necessary for conventional PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction. This control comprises nucleic acid extraction and subsequent amplification of extraction buffer only. Multiple controls are recommended to be included when large numbers of positive samples are expected.

4.2.11 Interpretation of results from conventional PCR

The pathogen-specific PCR will be considered valid only if both these criteria are met:
- the positive control produces the correct size amplicon for the target nematode species
- no amplicons of the correct size for the target nematode species are produced in the negative extraction control and the negative amplification control.

5. Records

Records and evidence should be retained as described in ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be adversely affected by the diagnosis, the records and evidence (in particular preserved or slide-mounted specimens, photographs of distinctive morphological features, DNA extracts and photographs of gels, as appropriate), should be kept for at least one year.

6. Contacts Points for Further Information

Further information on this protocol can be obtained from:

Biosystematics Division, ARC-PPRI, Private Bag X134, Queenswood, 0121 Republic of South Africa (Antoinette Swart; e-mail: SwartA@arc.agric.za).

Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832-1448, United States (Sergei Subbotin; e-mail: subbotin@ucr.edu).

Charlottetown Laboratory – Potato Diseases, Canadian Food Inspection Agency, 93 Mount Edward Rd, Charlottetown PEI, C1A 5T1, Canada (Harvinder Bennypaul; e-mail: bennypaulhs@inspection.gc.ca).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

This protocol was drafted by Antoinette Swart (Nematology Unit, Biosystematics Division, ARC-PPRI, Republic of South Africa), Eliseo Jorge Chaves (INTA-Estación Experimental de Balcarce, Laboratorio de Nematología, Argentina) and Renata C.V. Tenente (EMBRAPA, Recursos Genéticos e Biotecnología, , Brazil).

The description of the molecular techniques was done by Sergei Subbotin (Plant Pest Diagnostic Center, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, CA 95832-1448, United States).

The following nematologists improved the protocol by their comments:

- Harvinder Bennypaul (Canadian Food Inspection Agency, Canada)
- Johannes Hallmann (Julius Kühn-Institut, Germany)
- Mikhail Pridannikov (Center of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russia)
- P. Castillo (Instituto Agricultura Sostenible, Consejo Superior de Investigaciones Cientificas, Spain).

8. References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

Figure 1. *Vicia faba* seed infected by *Ditylenchus dipsaci* (with nematode wool showing).
Photo courtesy G. Caubel, Nemapix (1999).

Figure 2. *Allium sativum* infected by *Ditylenchus dipsaci*.
Photo courtesy G. Caubel, Nemapix (1999).

Figure 3. Young *Allium cepa* plants infected by *Ditylenchus dipsaci*.
Photo courtesy E. Hennig, State Plant Health and Seed Inspection Service, Torun, Poland.

Figure 4. Garlic bulb infected by *Ditylenchus dipsaci.*
Photo courtesy G. Caubel, Nemapix (2002).

Figure 5. *Narcissus* spp. infected by *Ditylenchus dipsaci.*
Photo courtesy G. Caubel, Nemapix (1999).

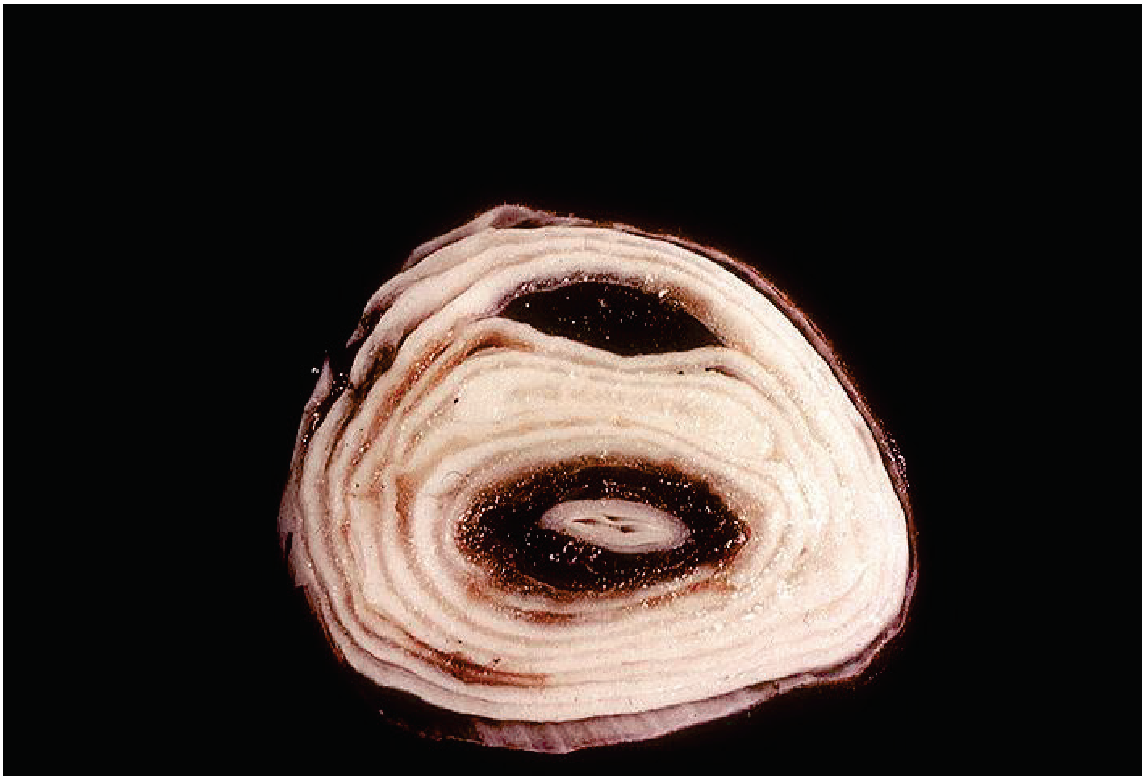

Figure 6. Cross-section of *Narcissus* sp. bulb infected by *Ditylenchus dipsaci.*
Photo courtesy C.W. Laughlin, Nemapix (2002).

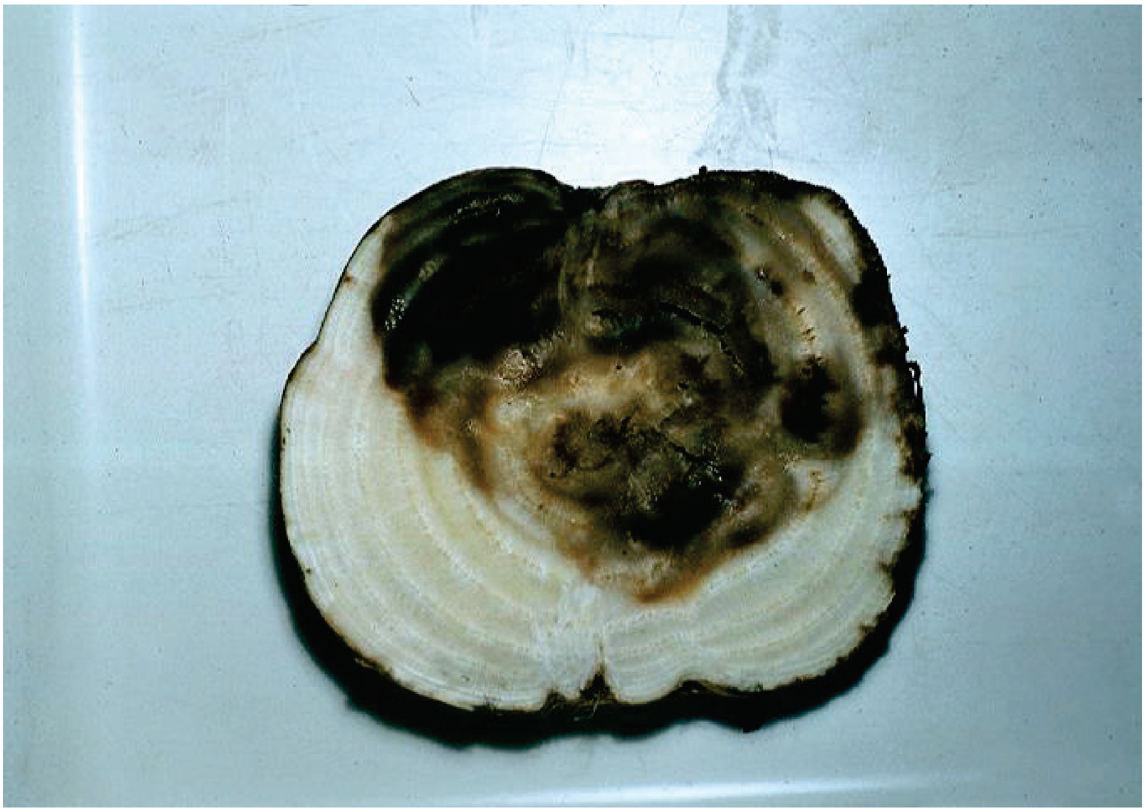

Figure 7. Cross-section of sugar beet infected by *Ditylenchus dipsaci.*
Photo courtesy C. Hogger, Nemapix (1999).

Figure 8. Cross-section of potato infected by *Ditylenchus destructor* compared with non-infected potato.
Photo courtesy S. Ayoub, Nemapix (2000).

Figure 9. Potatoes of various levels of infestation by *Ditylenchus destructor.*
Photo courtesy H. Andersen.

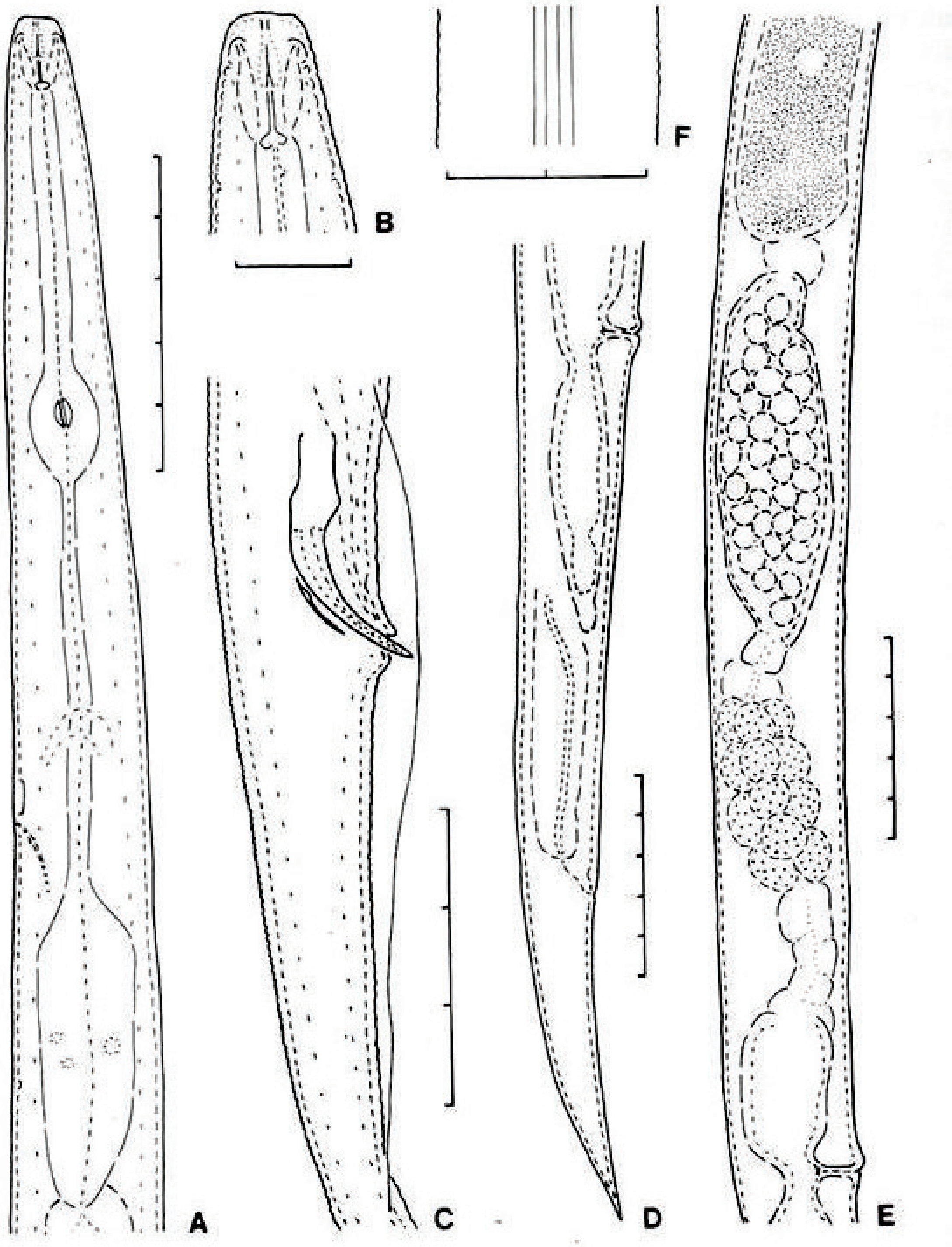

Figure 10 *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 (after Sturhan and Brzeski, 1991). (A) female, oesophageal region; (B) head of female; (C) male, spicule region; (D) female, posterior region; (E) part of female reproductive system; and (F) lateral field at midbody. Each unit marking on scale bars = 10 µm.

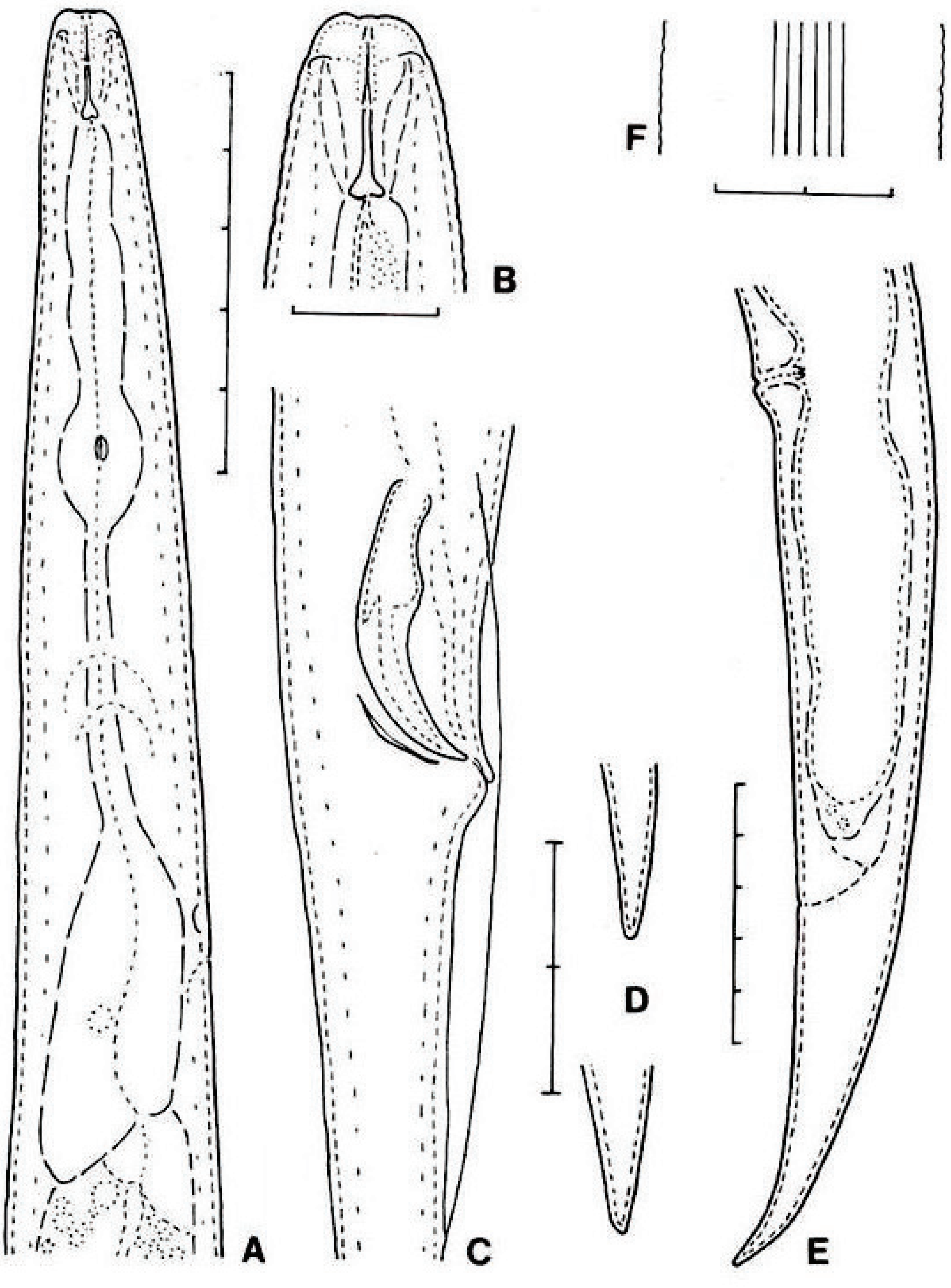

Figure 11. *Ditylenchus destructor* Thorne, 1945 (after Sturhan and Brzeski, 1991). (A) female, oesophageal region; (B) female, head; (C) male, spicule region; (D) tail tips of two females; (E) female, posterior region; and (F) lateral field at midbody. Each unit marking on scale bars = 10 µm.

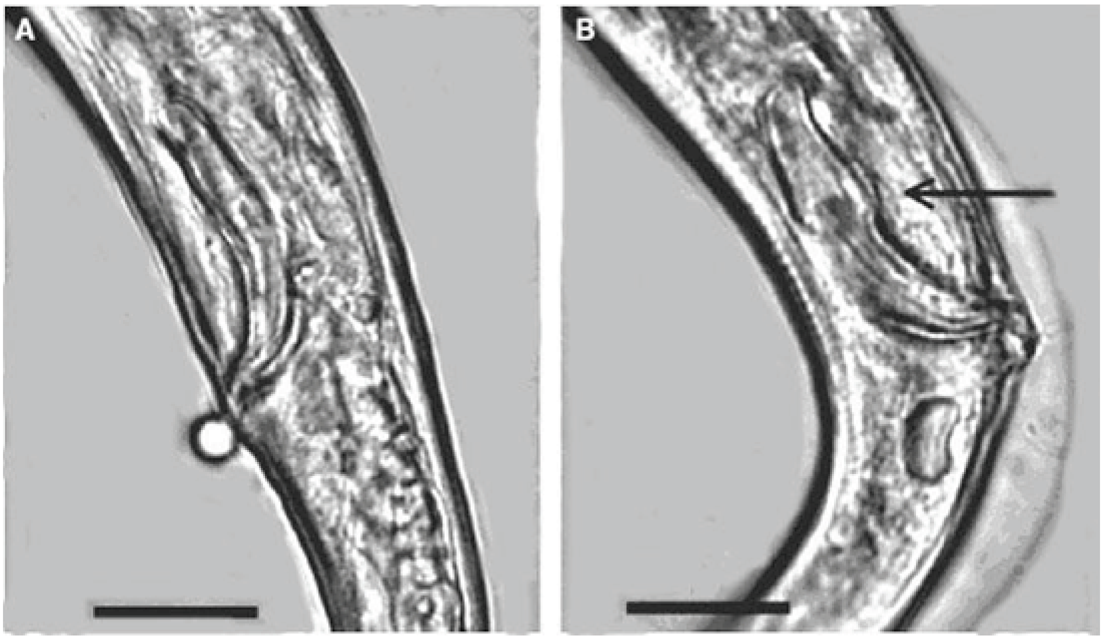

Figure 12. *Ditylenchus* spiculum: (A) *D. dipsaci* and (B) *D. destructor.* Arrow = tumulus. Scale bars = 12 μm. *Photo courtesy Karssen and Willemsen (2010).*

Publication history

This is not an official part of the standard

2006-04 CPM-1 (2006) added topic to work programme (Nematodes, 2006-008).

2004-11 SC added subject: *Ditylenchus destructor* / *D. dipsaci* (2004-017).

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2013-04 Expert consultation.

2013-06 Draft presented to TPDP meeting.

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2014-07 Member consultation.

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ISPM 27. **Annex 8**. *Ditylenchus dipsaci* and *Ditylenchus destructor* (2015). Rome, IPPC, FAO.

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

International Plant Protection Convention (IPPC)

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