
### [2] Status box

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<td>Draft new annex to ISPM 27:2006 (<em>Diagnostic protocols for regulated pests</em>)</td>
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2010-07: First draft presented to TPDP meeting  
2013-04: Submitted to Expert consultation system on draft diagnostic protocols on IPP  
2013-06: Draft presented to TPDP meeting  
2014-05: SC approved for member consultation (2014_eSC_May_11) |
| **Consultation on technical level** | The first draft of this protocol was written by (lead author and editorial team):  
- Antoinette Swart (Biosystematics Division ARC-PPRI, Republic of South Africa)  
- Eliseo Jorge Chaves (INTA, Argentina)  
- Renata C.V. Tenente (EMBRAPA, Brazil).  
In addition, Sergei Subbotin (Plant Pest Diagnostic Center, USA) was significantly involved in the development of this protocol.  
The draft, in whole or part, has also been commented upon by:  
- Johannes Hallmann (Julius Kühn-Institut, Münster, Germany)  
- Harvinder Bennypaul (Canadian Food Inspection, Canada)  
- Dr. Mikhail Pridannikov (The Center of Parasitology A.N. Severtsov Institute of Ecology and Evolution, Russia)  
- Dr. P. Castillo (Institute for Sustainable Agriculture (IAS), CSIC, Spain)  
In addition, the draft has also been subject to expert review and the following international experts submitted comments:  
- Thomas Prior (FERA, UK)  
- Hungarian NPPO |
| **Main discussion points during development of the diagnostic protocol** (to be updated during development as needed) | Merging of *D. dipsaci* and *D. destructor* protocols in one protocol  
Consolidate information on hosts and symptoms and place them in the pest information section. May classify hosts, important, or important crop but not important host  
Regarding extraction methods: add elements on recovery rate; indicate the basis for the methods  
Indicate only methods of relevance for diagnosis, e.g. scanning electron microscopy |
- Limit the list of synonyms to those that are not in the main publication on synonyms
- Keep important symptoms and indicate others through references
- Some measurements for nematodes characters use standardized names e.g. a, b, c'. These are known to all nematologists, so should stay as such in the protocol, but a reference to a glossary of nematology terms will be included.
- Characters specifically indicated on figures are cross-referenced in the text

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[3] **Endorsement**

This diagnostic protocol was adopted by the Commission on Phytosanitary Measures in XXXX [to be completed after adoption].


[5] Most nematode species within the large genus *Ditylenchus* Filipjev, 1936 are mycetophagous and have a worldwide distribution. However, the genus contains a few species that are of great importance as parasites of higher plants. 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 never occur together in the same plant (Andrássy and Farkas, 1988).

[6] **Ditylenchus dipsaci**

[7] *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 the *D. dipsaci* s.l. have suggested that it is a species 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, Troccoli, Palomares-Rius, De Luca, Liebanas, Landa, Subbotin and Castillo, 2011 (the “giant race” of *D. dipsaci* parasitizing *Vicia faba*); *D. weischeri* Chizhov, Borisov and Subbotin, 2010 (parasitizing *Circium 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 therefore covers *D. dipsaci* s.s. and presents *D. gigas* separately.

[8] *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* (broad bean), *Medicago sativa* (lucerne/alfalfa), *Allium cepa* (onion), *Trifolium* spp. (clovers), *Dipsacus* spp. (teasel) and *Cucumis melo* (melon) (Sikora *et al.*, 2005; Sousa *et al.*, 2003). 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. 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 infected seed (Palmisano *et al.*, 1971).

[9] Although *D. dipsaci* is seen as a parasite of higher plants, Viglierchio (1971) reported that a Californian population of *D. dipsaci* from *Allium sativum* (garlic) could reproduce on soil fungi (*Verticiculum* and *Cladosporium*) under laboratory conditions and Paesler (1957) stated that the nematode is of potential...
economic importance on *Agaricus bisporus* (mushroom).


[11] 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.

[12] *Ditylenchus destructor*

[13] *Ditylenchus 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. Rojancovski and Ciurea (1986) found 55 species of bacteria and fungi associated with *D. destructor* in *S. tuberosum* tubers, with *Fusarium* spp. 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* (hyacinth), *Gladiolus* spp. (gladiolus), *Dahlia* spp. (dahlia), *Coronilla varia* and *Anthyllis vulneraria* (vetch), *Beta vulgaris* (sugar beet), *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 dessication and low temperatures, but does not form nematode wool as does *D. dipsaci* (Kühn, 1857) Filipjev, 1936. This species, however, overwinters in eggs, which makes eggs more vital in *D. destructor* than in *D. dipsaci*. *D. destructor* in seed potatoes and flower bulbs is on the list of quarantine pests of many countries and organizations (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*.

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

[16] 2. Taxonomic Information

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

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

[19] **Taxonomic position:** Nematoda, Secernentea, Diplogasteria, Tylenchida, Tylenchina, Tylenchoidea, Anguinidae

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

[21] **Note:** *Ditylenchus dipsaci* has come to be considered 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 used in recent years (Sturhan and Brzeski, 1991)

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

Common name: 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 roting of the bulbs and tubers.

*Ditylenchus dipsaci*

Common symptoms of *D. dipsaci* infestation are swelling, distortion, discoloration and stunting of above-ground plant parts, and necrosis and roting of bulbs and tubers. *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 that 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*, *Convulvulus arvensis* and *Avena sterilis*.

*Ditylenchus destructor*

*D. destructor* commonly infects the underground parts of plants (tubers and stolons of potato, bulbs of lilies, rhizomes of mint, and roots of hop and lilac), causing discoloration and roting 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. *D. africanus*, which infects groundnut in southern Africa, is morphologically very similar to *D. destructor*. It can, however, be separated from *D. destructor* by a combination of morphological and molecular characteristics, which are presented in sections 4.1, 4.2 and Tables 2 and 3. For *D. africanus* symptoms on groundnut, see McDonald et al. (2005).

### 3.1 Hosts and symptoms

#### 3.1.1 Ditylenchus dipsaci

(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 introduce themselves into 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. D. dipsaci may also infest seeds, from, for example, Phaseolus vulgaris, V. faba and Allium spp. 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 Secale 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 Triticum aestivum (wheat) 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 corkscrew. Internodes are shortened, 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 and 3). The base of young plants becomes swollen and leaves become distorted. Older infested 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, USA. 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.

Tulipa spp. (Southey, 1993): Symptoms of D. dipsaci attack on tulip, both on growing plants and 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 axis 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 *Phytophthora 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).

**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 *Brassica 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 4 and 5). 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 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 Asparagaceae, subfamily Scilloideae (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 hyacinth, Symptoms of infestation in Amaryllidaceae 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*, infestation causes severe crown rot, especially in autumn (Figure 6).

**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 smalilana*, 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* (sweet potato), *A. sativum*, *P. vulgaris* (snap bean, string bean or green bean), *Angelica sinensis* ("dong quai" or "female ginseng"), *Panax ginseng* (ginseng), *Taraxacum officinale*, *Begonia* spp. and bulbs of *Erytronom 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 7). 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 lying infected tissues dry and shrink (Brodie, 1998). In contrast, the skin of *Solanum 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.

**Beta vulgaris (sugar beet, fodder beet and beetroot):** 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 unsustainable 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 due to 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 (pers. comm., F.A. van der Vegte, 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) and the garlic cloves are cut into small pieces and put in a container (e.g. Petri dish) with tap water at room temperature. 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 extraction of nematodes from soil and plant material (bulbs, roots, potato peelings). A funnel with a piece of rubber tubing is attached to the stem and 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 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 microscope (Plegg 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 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.

The nematodes can be extracted from plant material by the Coolen and D’Herde (1972) method. 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 2 min. 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/cm³) is added to the tubes. The mixture is thoroughly stirred and centrifuged
at 1 750 r.p.m. for 4 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 course-meshed sieve (2 mm) 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 for two more times, but shortening the setting times to 20 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 for 7 min at 1 750 r.p.m. 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 for 3 min at 1 750 r.p.m. 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 nematodes may be lost. Hooper *et al*. (2005) describes different extraction methods adapted to take advantage of size, density and motility of nematodes.

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. destructor* and *D. dipsaci* to be identified with certainty by morphological examination alone. The morphological identification of *Ditylenchus* juveniles in a sample should only confirm their development. As mycophagous *Ditylenchus* spp. frequently contaminate decaying plant material, care must be taken in the identification of specimens in both plant and soil samples.

The identification of *D. dipsaci* and *D. destructor* should always 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 techniques 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.

Temporary preparation:

- 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, fixed in FAA (35% distilled water, 10% of 40% formalin, 5% glacial acetic acid, 50% of 95% alcohol) (Andrássy, 1984), 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) is recommended.

### 4.1.2 Morphological diagnostic characters

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

#### Table 1. Key to distinguish *Ditylenchus* spp. from other tylenchid and aphelenchid genera (adapted from Heyns, 1971 and Siddiqi, 2000)

<table>
<thead>
<tr>
<th>1</th>
<th>Outlet of dorsal oesophageal gland near base of stylet; median bulb roundish, ovoid or absent</th>
<th>Tylenchida - 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Outlet of dorsal oesophageal gland in median bulb; median bulb a prominent feature, usually oblong</td>
<td>Aphelenchida</td>
</tr>
<tr>
<td>2</td>
<td>Anterior part of oesophagus (procorpus) and median bulb not united into single unit; stylet never exceptionally long</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Procorpus gradually widened and fused with median bulb; stylet very long, its base often located in anterior part of median bulb</td>
<td>Other genera</td>
</tr>
<tr>
<td>3</td>
<td>Adult female vermiform</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Adult female saccate or pyriform sessile parasite on roots</td>
<td>Other genera</td>
</tr>
<tr>
<td>4</td>
<td>Valvular median bulb</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Median bulb without valve</td>
<td>Other genera</td>
</tr>
<tr>
<td>5</td>
<td>Oesophageal glands contained within basal bulb, not overlapping or slightly overlapping intestine; cephalic framework rarely conspicuous; stylet frail to moderately strong</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Oesophageal glands lobe-like, overlapping intestine; cephalic framework strong; spear massive</td>
<td>Other genera</td>
</tr>
<tr>
<td>6</td>
<td>Single prodelphic ovary; vulva posterior</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Ovaries two, amphidelphic; vulva slightly post-equatorial</td>
<td>Other genera</td>
</tr>
<tr>
<td>7</td>
<td>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</td>
<td><em>Ditylenchus</em></td>
</tr>
<tr>
<td></td>
<td>Female swollen; crustaformeria with more than 20 cells</td>
<td>Other genera</td>
</tr>
</tbody>
</table>
D. destructor, D. africanus, D. myceliophagus, D. gigas and D. dipsaci are morphologically and morphometrically similar, but can be differentiated from each other by the following (Table 2), providing that both male and female specimens can be measured and studied.

**Description of Ditylenchus dipsaci** (after Sturhan and Brzeski, 1991 and Wendt et al., 1995). Details and views are provided in Figure 8.

**Morphological diagnostic characters:** The number of lateral incisures (four) (Figure 8F), the comparatively long stylet, the length of the postvulval sac and the pointed tail (Figure 8D) are the distinguishing characters for this species (Andrássy, 2007). *D. dipsaci* can be distinguished from *D. gigas* by the shorter body (1.0–1.7 vs 1.5–1.7 mm) (Vovlas et al. 2011). When observed in the lateral view, the spicule is more arched in *D. dipsaci* than in *D. destructor* (Figure 8C). See Karssen and Willemse (2010) for more information on the spiculum and its use in the identification of *D. dipsaci* and *D. destructor*.

**General morphology:** Body straight or almost so when relaxed. Lateral field with four incisures. Head continuous with adjacent body (Figure 8B). 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 8A). 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 8D). Bursa envelopes three-quarters of the tail in males. Spicules 23–28 µm long. Tails of both sexes conical with a pointed tip.

**Measurements** (criteria described in EPPO, 2013b): (Ex Oat, Avena sativa L., after Blake, 1962, in Hooper, 1972). \((n = 48 \div 52\): \(L = 1.3 \text{ mm} \pm 0.009; a = 62 \pm 5.6; b = 15 \pm 1.4; c = 14 \pm 2.1; V = 80 \pm 1.5. \(n = 23 \div 30\): \(L = 1.3 \text{ mm} \pm 0.017; a = 63 \pm 11.3; b = 15 \pm 1.7; c = 14 \pm 2.1; T = 72.

**Description of Ditylenchus destructor** (after Sturhan and Brzeski, 1991). Details and views are provided in Figure 9.

**Morphological diagnostic characters:** *D. destructor* is similar to *D. dipsaci*, but differs from that species by the lateral field showing six incisures (Figure 9F), the longer postvulval sac and the finely rounded tail terminus (Figure 9D). 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 PCR technology is sufficiently sensitive to resolve differences between closely related genera, Wendt et al. (1995) used restriction fragment length polymorphisms (RFLPs) generated by seven restriction enzymes on the internal transcribed spacer of ribosomal (r)DNA 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 9C).

**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, reduced to two on the neck and tail regions (Figure 9F). 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, occasionally specimens with stylets of 14 µm have been described. Stylet cone 45–50% of the 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 occasionally seen (Figure 9A). Excretory pore opposite oesophageal glands. Postvulval sac extending about three-quarters of the vulva–anus distance (Figure 9E). Eggs twice as long as wide (Adrássy, 2007). Lips of vulva thick, elevated (Figure 9B). Anterior ovary outstretched, sometimes reaching the oesophageal region. Postvulval part of uterine sac 40–98% of vulva–anus distance, not functioning as a spermtheca (Figure 9E). Male bursa surrounds 50–90% of the tail length. Spicules are 24–27 µm long. Testis outstretched approaching the base of esophagus. Tail of both sexes conical, three to five anal body widths long, usually ventrally curved, terminus rounded.

**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*.
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).


<table>
<thead>
<tr>
<th>Characters</th>
<th>D. destructor</th>
<th>D. africanus</th>
<th>D. myceliophagus</th>
<th>D. gigas</th>
<th>D. dipsaci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length female (mm)</td>
<td>0.8–1.9</td>
<td>0.7–1.1</td>
<td>0.6–1.4</td>
<td>1.6–2.2</td>
<td>1.0–1.3</td>
</tr>
<tr>
<td>Number of lateral lines</td>
<td>6</td>
<td>6–15</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Form of tail terminus</td>
<td>Rounded</td>
<td>Rounded</td>
<td>Rounded</td>
<td>Pointed to finely rounded</td>
<td>Pointed</td>
</tr>
<tr>
<td>c (body length/tail length) of female</td>
<td>−9–30</td>
<td>8.8–16.9</td>
<td>10.5–20.5–17</td>
<td>15.8–27.6</td>
<td>−14–18</td>
</tr>
<tr>
<td>Posterior bulb</td>
<td>Short, dorsally overlapping</td>
<td>Short, dorsally overlapping</td>
<td>Short, dorsally overlapping</td>
<td>Slightly overlapping</td>
<td>Not overlapping</td>
</tr>
<tr>
<td>Stylet length (µm) of female</td>
<td>10–14</td>
<td>8–10</td>
<td>7–8</td>
<td>10.5–13.0</td>
<td>10–12</td>
</tr>
<tr>
<td>PUS/vulva–anus length (%)</td>
<td>53–90</td>
<td>37–85</td>
<td>30–69</td>
<td>*About 50 %</td>
<td>40–70</td>
</tr>
<tr>
<td>Spiculum length (µm)</td>
<td>24–27</td>
<td>17–21</td>
<td>15–20</td>
<td>23.5–28</td>
<td>23–28</td>
</tr>
<tr>
<td>Bursa length (as % of tail length)</td>
<td>50–70</td>
<td>48–66</td>
<td>20–55</td>
<td>72–76</td>
<td>40–70</td>
</tr>
<tr>
<td>Host preference (helpful information in case of confusing morphological criteria)</td>
<td>Higher plants and mycelia of fungi</td>
<td>Groundnut and fungi</td>
<td>Mycelia of fungi</td>
<td>Higher plants</td>
<td>Higher plants and fungi</td>
</tr>
</tbody>
</table>

*Calculated from species description.

PUS, postvulval part of uterine sac.

4.2 Molecular identification of D. dipsaci and D. destructor

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.
[105] **Ditylenchus dipsaci**

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

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

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

[109] Six molecular assays (PCR, PCR-RFLP) have been published that can be used in the identification of *D. dipsaci*; these are described in sections 4.2.2 to 4.2.7. The specificity of each assay is included in the description, as is the nematode genus and species against which each assay has been evaluated.

[110] The molecular analysis of rDNA sequences including 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).

[111] **Ditylenchus destructor**

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

[113] 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.

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

[115] Powers et al. (2001) first sequenced the ITS1 region for *D. dipsaci*. More than 50 sequence accesions of rRNA fragments obtained from *D. destructor* collected from different localities and host plants are presently available in the GenBank.

[116] **4.2.1 DNA extraction**

Several juveniles or adults are transferred into a microtube and used for extraction of DNA. DNA extraction is described by Webster et al. (1990); other DNA extraction methods are provided in each test described below.

[118] **4.2.2 ITS-rRNA PCR-RFLP assay for *D. dipsaci* and *D. destructor* (Wendt et al., 1993)**

**Methodology**

The ITS rRNA universal primers (as described in Vrain et al., 1992) used in this assay 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 was obtained following the manufacturer’s recommendations for PCR kits containing Taq DNA polymerase, nucleotides and reaction buffer.

The PCR cycling parameters 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 identifying *D. destructor* and *D. dipsaci* from other *Ditylenchus* species; for example, *HaeIII*, *HpaII*, *Hinfi* and *Rsal* (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

<table>
<thead>
<tr>
<th>Enzyme</th>
<th><em>D. destructor</em></th>
<th><em>D. myceliophagus</em></th>
<th><em>D. dipsaci</em> (giant race of <em>D. dipsaci</em>)</th>
<th><em>D. africanus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Unrestricted PCR product</td>
<td>1 200</td>
<td>900</td>
<td>900</td>
<td>900</td>
</tr>
<tr>
<td><em>HaeIII</em></td>
<td>450, 170</td>
<td>450, 200</td>
<td>900</td>
<td>800, 200</td>
</tr>
<tr>
<td><em>HpaII</em></td>
<td>1 000</td>
<td>900</td>
<td>320, 200, 180</td>
<td>600, 200</td>
</tr>
<tr>
<td><em>Hinfi</em></td>
<td>780, 180</td>
<td>630, 310</td>
<td>440, 350, 150</td>
<td>350, 150</td>
</tr>
<tr>
<td><em>Rsal</em></td>
<td>600, 250, 170</td>
<td>900</td>
<td>450, 250, 140</td>
<td>490, 450</td>
</tr>
</tbody>
</table>


4.2.3 SCAR PCR assay for *D. dipsaci* (Esquibet et al., 2003)

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these define the original level of sensitivity, specificity and reproducibility achieved. The use of names of chemicals 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.

This assay developed by Esquibet et al. (2003) was designed as a species-specific assay for *D. dipsaci* with differentiation between normal and giant races. It was evaluated against *D. myceliophagus* (one population), *D. dipsaci* normal race (eleven populations from different hosts and locations) and *D. dipsaci* giant race, described as *D. gigas* by Vovlas et al. (2011) (eleven populations from *V. faba* and from different locations).

**Methodology**

The *D. dipsaci* specific primers used are:

* D. dipsaci* (normal race):
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.

4.2.4 18S and ITS1-specific PCR assay for *D. dipsaci* (Subbotin et al., 2005)

This assay developed by Subbotin et al. (2005) was designed as a species-specific assay for *D. dipsaci* s.s. (normal race only). It was evaluated against *D. destructor* (1 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 CCA 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: 1X from 10X 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) 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.

4.2.5 5.8S rDNA-specific PCR assay for *D. dipsaci* (Marek et al., 2005)

This assay developed by Marek et al. (2005) was designed as a species-specific assay 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 MgCl₂, 200 µM each dNTP, 10 pmol each primer (PF1-PR1 primer set) and 1.5 U Taq DNA polymerase (Fermentas). The PCR assay was developed on a 96-well Peltier type thermocycler (PTC200, MJ Research), 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.

4.2.6 5.8S rDNA and ITS-specific PCR assay for *D. dipsaci* (Kerkoud *et al.*, 2007)

This assay developed by Kerkoud *et al.* (2007) was designed as a species-specific assay 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 *V. faba* and from different locations).

Methodology

Two specific primer sets are used, one for the identification *D. dipsaci* alone and one for the identification of *D. gigas* and *D. dipsaci*. The use of both primer sets allows to separate *D. gigas* from *D. dipsaci*, and they 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 MgCl₂ concentration of 5 mM, 200 µM each dNTP, 0.5 µM each primer (in case of simplex PCR with DdpS1-rDNA2 or DdpS2-rDNA2; in case of duplex PCR, the final concentration of DdpS1 primer is 0.5µM whereas it is 1µM for DdpS2 and rDNA2) and 1 U Taq DNA polymerase (MP Biomedicals). The PCR was developed on a 96-well Peltier type thermocycler (GeneAmp 9600 PCR System, Perkin Elmer), 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.

4.2.7 SCAR PCR assay for *D. dipsaci* identification (Zouhar et al., 2007)

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

**Methodology**

Two specific primer sets were designed for *D. dipsaci* identification and they are:

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 AC-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), 1.5 mM MgCl₂, 200 µM each dNTP, 10 pmol each primer (either DIT_2 or DIT_5 primer set), 1.5 U Taq DNA polymerase (Fermentas) and 50 ng DNA as template. The PCR is performed in a 96-well Peltier type thermocycler (PTC200, MJ Research), 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.

4.2.8 Controls for molecular tests

For the test result obtained to be considered reliable the following controls should be considered for each series of nucleic acid isolation and amplification of the nucleic acid of the target pest or target nucleic acid. As a minimum, the positive nucleic acid control, negative amplification control and negative extraction control, as described below, should be used.
Positive nucleic acid control

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

Negative amplification control (no template control)

This is a necessary control for conventional PCR to rule out false positives due to contamination during the 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 requires nucleic acid extraction and subsequent amplification of extraction buffer only. It is recommended that multiple controls are included when large numbers of positives are expected.

4.2.9 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 section 2.5 of ISPM 27:2006.

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

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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 to develop Diagnostic Protocols (TPDP).

7. Acknowledgements

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- Johannes Hallmann (Julius Kühn-Institut, Germany)
- Mikhail Pridannikov (Center of Parasitology, A.N. Severtsov Institute of Ecology and Evolution, Russia)
- P. Castillo (Institute for Sustainable Agriculture (IAS), Spain)

8. References


9. Figures

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

Figure 2. *Allium sativum* infected by *Ditylenchus dipsaci*.

Photo G. Caubel, Nemapix (1999).
Figure 3. Narcissus bulb infected by *Ditylenchus dipsaci*.

Photo G. Caubel, Nemapix (2002).
Figure 4. Narcissus spp. infected by Ditylenchus dipsaci.

Photo G. Caubel, Nemapix (1999).
Figure 5. Cross-section of Narcissus bulb infected by *Ditylenchus dipsaci*.

Photo C.W. Laughlin, Nemapix (2002).
Figure 6. Cross-section of sugar beet infected by *Ditylenchus dipsaci*.

Photo C. Hogger, Nemapix (1999).

Figure 7. Potato infected with *Ditylenchus destructor* compared with non-infected potato.

[308] Figure 8. *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 (after Sturhan and Brzeski, 1991). (A), female, oesophageal region; (B), head of female; (C), male, spicule region; (D), posterior region of female; (E), part
of female reproductive system; (F), lateral field at midbody. Each unit marking on bars, 10 µm.

Figure 9. *Ditylenchus destructor* Thorne, 1945 (after Sturhan and Brzeski, 1991). (A), female, oesophageal region; (B), head of female; (C), male, spicule region; (D), tail tips of two females; (E), posterior region of female; (F), lateral field at midbody. Each unit marking on bars, 10 µm.
Footnote 1: The PCR cycling conditions 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.