Plant Disease • 2017 • 101:1692-1720 • https://doi.org/10.1094/PDIS-03-17-0355-FE

Cereal Cyst Nematodes: A Complex and Destructive Group of *Heterodera* Species

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Small grain cereals have served as the basis for staple foods, beverages, and animal feed for thousands of years (Breiman and Graur 1995; Gustafson et al. 2009; Newman and Newman 2008). Wheat, barley, oats, rye, triticale, rice, and others are rich in calories, proteins, carbohydrates, vitamins, and minerals. These cereals supply 20% of the calories consumed by people worldwide and are therefore a primary source of energy for humans and play a vital role in global food and nutrition security. Global production of small grains increased linearly from 1960 to 2005, and then began to decline (Alexandratos and Bruinsma 2012). Further decline in production is projected to continue through 2050 (OECD-FAO 2016) while global demand for these grains is projected to increase by 1% per annum (Alexandratos and Bruinsma 2012). Currently, wheat, barley, and oat production exceeds consumption in developed countries, while in developing countries the consumption rate is higher than production (OECD-FAO 2016). An increasing demand for meat and livestock products is likely to compound the demand for cereals in developing countries. Current production levels and trends will not be sufficient to fulfill the projected global demand generated by increased populations (Ray et al. 2013). For wheat, global production will need to be increased by 60% to fulfill the estimated demand in 2050 (Ackerman and Stanton 2008). Until recently, global wheat production increased mostly in response to development of improved cultivars and farming practices and technologies. Production is now limited by biotic and abiotic constraints, including diseases, nematodes, insect pests, weeds, and climate. Among these constraints, plant-parasitic nematodes alone are estimated to reduce production of all world crops by 10% (Whitehead 1998).

Cereal cyst nematodes (CCNs) are among the most important nematode pests that limit production of small grain cereals. Heavily invaded young plants are stunted and their lower leaves are often chlorotic, forming pale green patches in the field (Fig. 1A-C). Mature plants are also stunted (Fig. 1D-F), have a reduced number of tillers, and the roots are shallow and have a "bushy-knotted" appearance (Fig. 1G-J) (Nicol et al. 2011; Smiley and Nicol 2009). CCNs comprise a number of closely-related species and are found in most regions where cereals are produced (Fig. 2) (Dababat et al. 2014, 2015b, 2017; Nicol and Rivoal 2008; Smiley and Nicol 2009; Subbotin et al. 2010a).

Pathogen Taxonomy and Diversity

The genus *Heterodera* has long been divided into three groups (Schachtii, Goettingiana, and Avenae) based upon a set of fundamental differences in structural components of the cyst vulval cone (Fig. 3). More recently, the number of groups was expanded to seven and some

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Accepted for publication 16 June 2017.

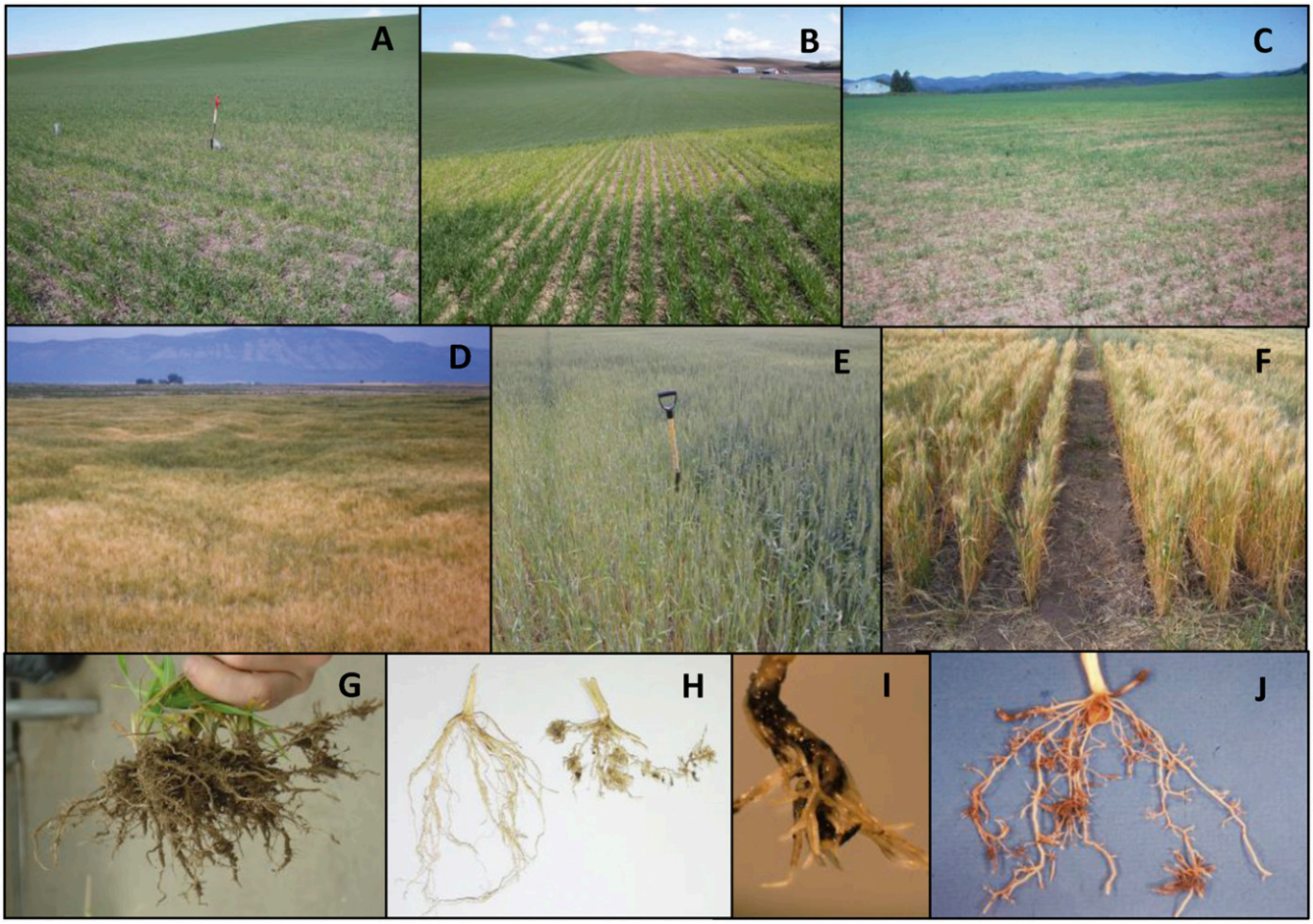

Fig. 1. Cereal cyst nematode injury on wheat (**A, B, D-J**) and oat (**C**) caused by *Heterodera avenae* (**A-D, F-J**) and *H. filipjevi* (**E**) in the Pacific Northwest, U.S.A.; all are without nematicide application except F, which shows growth response to nematicide application (right) compared with untreated soil (left). Images by Richard Smiley (A-F, H-J) and Yvonne Thompson (G).

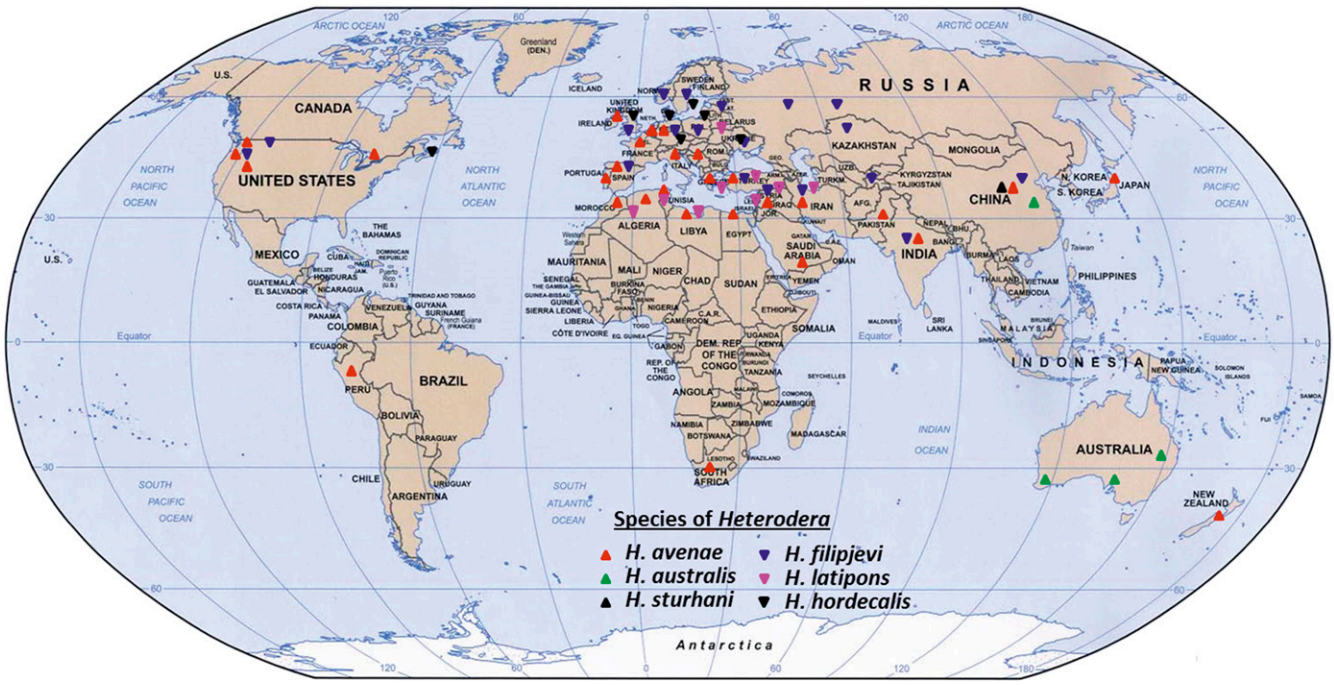

Fig. 2. Global distribution of six economically important species of cereal cyst nematodes.

species within the original groups were moved, based upon combined evidence from features in the vulval cone, number of incisures in the juvenile's lateral field (Fig. 4), and molecular analyses (Subbotin et al. 2010b).

Several nematodes of the genus *Heterodera* parasitize cereals and grasses. Extensive molecular and morphological studies of these nematodes have revealed the presence of several distinct species. Twelve species belong to the Avenae group (Table 1), which at present includes 10 species from the *H. avenae* species complex (*H. arenaria*, *H. avenae*, *H. aucklandica*, *H. australis*, *H. filipjevi*, *H. mani*, *H. pratensis*, *H. riparia*, *H. sturhani*, and *H. ustinovi*) as well as *H. latipons* and *H. hordecalis*. Three species were previously considered as members of the Avenae group but are not related to it (Subbotin et al. 2010b) and have been transferred to other cyst nematode groups; *Heterodera bifenestra* Cooper, 1955, *H. spinicauda* Wouts, Shoemaker, Sturhan & Burrows, 1995, and *H. turcomanica* Kirjanova & Shagalina, 1965.

From 12 species of the Avenae group, six species only parasitize grasses and six species are considered as agricultural pests of cereal crops (*H. australis*, *H. avenae*, *H. filipjevi*, *H. hordecalis*, *H. latipons*, and *H. sturhani*) and are the focus of this report. Thus, under the common name of "the cereal cyst nematode," at present there are five species with that common name and one with the common name of "the barley cyst nematode." The ability of nematodes to parasitise cereals might have appeared independently in several evolutionary lineages of the *H. avenae* species complex. Nematodes belonging to these lineages also show differences in their virulence and pathogenicity for cereal crops.

Although morphological characters can fail to differentiate species of the CCNs from each other or from sibling species parasitising grasses, molecular markers, such as those based on the internal transcribed spacer (ITS) region of the ribosomal RNA (rRNA) gene and, especially, the cytochrome oxidase I (*COI*) gene (Fig. 5), now provide reliable differentiation of this complex. Acceptance of the multiple species concept of the CCN raises an important question about reconsidering the present quarantine regulations and other measures (Subbotin 2015), which prevent dispersal to new areas of CCN species that are currently present only in geographically isolated regions (Fig. 2).

Names of features that are important for defining differences in cyst morphology are used in the following section but a description of those terms is beyond the scope of this paper. Features such as vulval underbridge, fenestra, semifenestrae, bullae, vulval slit, stylet, hyaline tail tip, and lateral field are well described in treatises by Baldwin and Mundo-Ocampo (1991) and Subbotin et al. (2010a) and are illustrated in Figures 3 and 4.

Further intraspecies diversity occurs as pathotypes (biological races) with different inheritable capacities for reproducing on specific genotypes of a host plant species, and as ecotypes with specific heritable adaptation to the different climates in which they evolved. The pathotype scheme for CCNs is based on the capacity of a local CCN population to reproduce on barley cultivars with different resistance genes as given in the International Cereal Cyst Nematode Test Assortment, which consists of 12 barley, six oat, and five wheat lines, proposed by Andersen and Andersen (1982). Gene designations for cultivars in the assortment were presented by Smiley et al. (2011b). Two distinct ecotypes of *H. avenae* were characterized by Rivoal (1986), who found that populations of *H. avenae* collected from northern or southern France had different thermal requirements for induction of juvenile emergence from cysts, and that those differences were retained when cysts of each population were moved to geographic regions with climates other than that in the area where the population evolved.

European cereal cyst nematode – *H. avenae*. This species was first found as a parasite of cereals in 1874 in Germany. It is widespread throughout Europe, the Mediterranean region of Africa, Asia, and North America. *H. avenae* includes several pathotypes and ecotypes. Pathotypes of *H. avenae* belong to groups 1 (Ha11-Ha71) and 2 (Ha12) (Subbotin et al. 2010a). Ecotypic variability will be discussed later.

H. avenae is distinguished from several other species by morphology and morphometrics, except from *H. australis* (Subbotin et al. 2002), with which it shares most characters. Several biochemical and molecular methods distinguish the populations of CCN found in Australia: iso-electric focusing (IEF) (Gäbler et al. 2000; Rumpenhorst 1985; Sturhan and Rumpenhorst 1996), restriction fragment length polymorphism (RFLP), and sequences of the ITS rRNA gene, except from *H. arenaria* (Bekal et al. 1997; Rivoal et al. 2003; Subbotin et al. 1999, 2001, 2003). Two genotypes of the European CCN based on the ITS and *COI* gene sequences are now distinguished: types A and B. Type A is found widely across Europe and North America and type B is distributed in Asia and North Africa.

There are many reports and publications of cyst nematodes named as *H. avenae* in Russia, China, and Australia, but new evidence

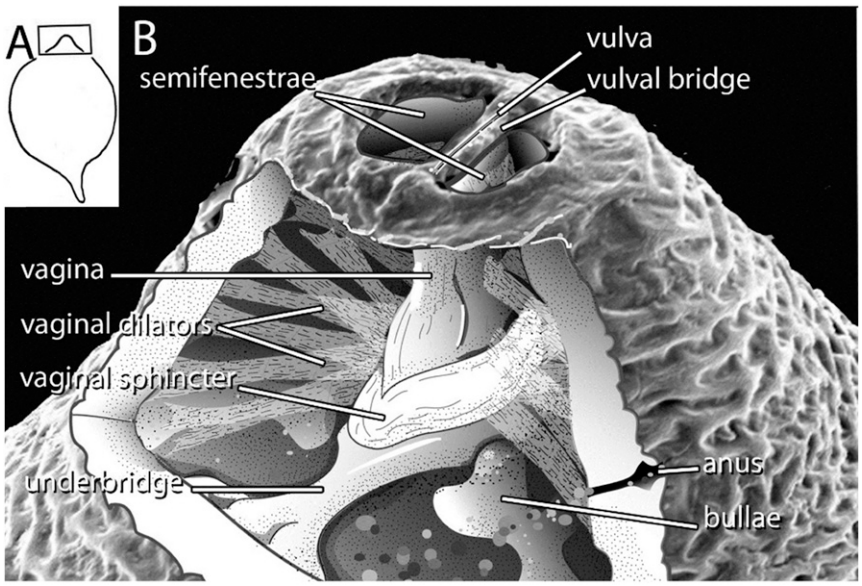

Fig. 3. Drawing depicting structures of the terminal region of cyst nematode females, including a box showing the location of the terminal region (**A**) that is expanded in the ventrolateral 3-dimensional drawing of internal structures (**B**); after Fig. 15 in Subbotin et al. 2010a. Some features that that may be exclusive to either female or cyst stages are combined to show a more complete range of possible structures. Several features of importance for distinguishing cysts of the Avenae group include cyst shape and length, fenestrae shape and length, underbridge presence and shape, bullae presence and arrangement, separation of vulval lips, and length and depth of the vulval slit.

suggests that these should be considered as belonging not to *H. avenae*, but to *H. filipjevi*, *H. sturhani*, or *H. australis*, respectively. However, the validity of taxonomic redesignations for the latter two species is not accepted by some researchers (D. L. Peng et al. 2016; Riley and McKay 2009; Vanstone et al. 2008). Therefore, in later sections of this paper we will use the species designation (*H. avenae*) as it was stated in the original papers from Australia (Australian population of '*H. avenae*') and China (Chinese population of '*H. avenae*'). Since the validity of *H. filipjevi* is not a subject of dispute, we have used that designation for reports of that specie even when older literature reported it under a name such as "Gotland strain" of *H. avenae*.

Australian cereal cyst nematode. The Australian population of '*H. avenae*' was thought to have been introduced to South Australia from Europe during the late 19th century (Brown 1984; McLeod 1992; Meagher 1977) and has long been studied as *H. avenae*. In 1930, it was described as a pest of cereals in South Australia (Davidson 1930) and by 1938 it was recognized as a pest in the Wimmera district of Victoria (Millikan 1938). The nematode was found in New South Wales (McLeod 1968), and more widely in Victoria and in Western Australia (Parkin and Goss 1968). This process was considered to represent long distance spread within Australia rather than new introductions (Riley and McKay 2009). It was suggested that this CCN was further distributed by wind-borne dust, farm machinery containing soil or soil residues, and even by transport of sheep between states (McLeod 1992).

The Australian CCN populations are considered as a different pathotype from European populations. This pathotype belongs to the Ha3 group, Ha13. The most interesting results have been obtained from biochemical and molecular studies. Rumpenhorst (1988) found that Australian populations, which had been morphologically identified as *H. avenae*, differed in their protein pattern compared with populations from Europe and Israel and that these should probably be considered as a separate species. Ferris et al. (1994) also showed that the 2-D PAGE of Australian isolates differed from those of a Swedish *H. avenae* isolate and isolates from the U.S. states of Oregon and Idaho. After detailed molecular (PCR-RAPD, PCR-ITS-RFLP, ITS-rRNA sequences), biochemical (IEF of proteins), and morphological analysis of second-stage juveniles (J2s) and cysts for several Australian, European, and Asian populations, Subbotin et al. (2002, 2003) showed that Australian populations of CCN have clear biochemical and molecular differences from all known European *H. avenae* populations and also from other species of the *H. avenae* complex. As a result, this nematode was described as a new species, *H. australis*, although there were no reliable morphological and morphometric characters distinguishing it from *H. avenae*. It has also been proposed that *H. australis* is a species native to Australia, although recent records of this species in China confirmed by PCR-ITS-RFLP (Fu et al. 2011) and ITS-rRNA gene sequence analysis suggest a wider distribution. Recent analysis of the *COI* gene sequences of the *H. avenae* species complex revealed that this gene can be used reliably to differentiate *H. australis* from all other species (Subbotin 2015). However, several scientists (Riley and McKay 2009; Vanstone et al. 2008) do not recognize this species as valid and consider that the CCN in Australia is an introduced species from Europe, although that conclusion may be at odds with available biochemical and molecular data (Subbotin et al. 2010b).

Over the last 30 years, population densities of Australian CCN populations have declined strongly as a result of deployment of resistant cultivars, and populations in New South Wales and Western Australia have so far not developed to more than a localized problem (Riley and McKay 2009).

Filipjev cereal cyst nematode – *H. filipjevi*. This species was first found in 1964 in cereal fields near Dushanbe, Tajikistan. It was first identified as *H. avenae* (Kirjanova and Krall 1971) and was only later described as a new species by Madzhidov (1981) on the presence of a characteristic vulval underbridge in the cysts and differences in morphometrics of juveniles and cysts from other species. Madzhidov (1991) reported the presence of *H. filipjevi* from many regions of Tajikistan. Subbotin et al. (1996) compared several populations of cereal

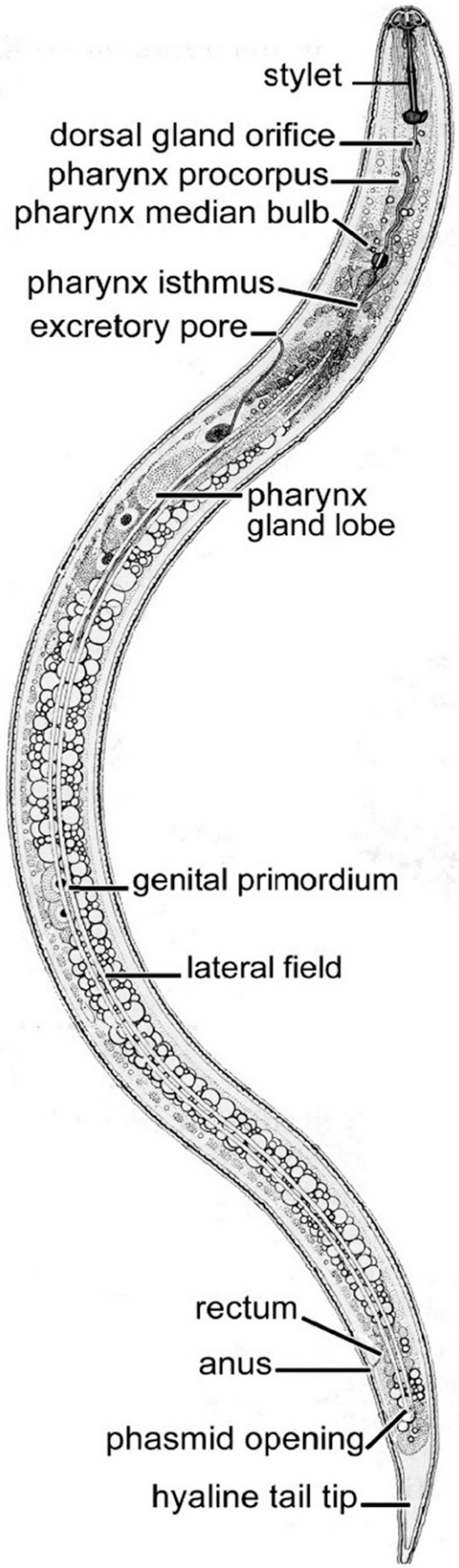

Fig. 4. Second-stage juvenile of *Heterodera schachtii*; after Fig. 5 in Subbotin et al. 2010a. Several features of importance for distinguishing species of the Avenae group include body length, body diameter at midbody, stylet length, shape of stylet knobs, number of incisures in the lateral field, tail length, length of the hyaline part of the tail, and shape of the tail terminus.

cyst nematodes from Russia, Ukraine, and Germany with *H. filipjevi* collected in Tajikistan with the help of Dr. A. Madzhidov in respect to morphology, morphometrics, and IEF of proteins. It was concluded that *H. filipjevi* is clearly distinct by protein patterns from *H. avenae* and all samples of *H. avenae* studied from the former U.S.S.R. should now be considered to be *H. filipjevi.* Sturhan and Rumpenhorst (1996) compared this sample from Tajikistan morphologically and biochemically with other *H. avenae* and related populations from various geographical origins and found that the British "pathotype 3" and the "Gotland strain", previously identified as *H. mani* infecting cereals in Germany and Spain, and concluded that they were indeed *H. filipjevi*. Presently, *H. filipjevi* is found in many countries of Europe and Asia (Dababat et al. 2015b, 2017; Riley et al. 2009), and in three northwestern states of the U.S. (Smiley 2016). Hosts include wheat, rye, barley, corn, and many grass species. At least two pathotypes (Ha22 and Ha33) from the Ha3 group can be differentiated using the International Cereal Cyst Nematode Test Assortment (Subbotin et al. 2010a). The species differs from other CCNs of the *H. avenae* complex by the presence of an underbridge and from *H. ustinovi* by the shorter tail and shorter hyaline region lengths for the J2s. PCR-ITS-RFLP profiles, especially as generated by *Pst*I, sequences of ITS rRNA, and *COI* genes clearly differentiated *H. filipjevi* from other members of the *Avenae* group. In several regions, *H. filipjevi* occurs in a mixture with *H. avenae* in cereal fields.

Chinese or sturhan cereal cyst nematode. The Chinese population of '*H. avenae*' was identified as a distinct species during the last decade, when special attention was devoted to studies of CCNs in China (Riley and Qi 2015). In 1989, CCN was first reported in wheat fields of Hubei Province, China (P. S. Chen et al. 1991). Subsequent surveys revealed that this nematode is widely distributed in provinces, autonomous regions, and cities in China, including Hubei, Shanxi, Henan, Hebei, Beijing, Inner Mongolia, Qinghai, Anhui, Shandong, Shaanxi, Gansu, Jiangsu, Tianjin, Tibet, and Xinjiang

Table 1. Composition of the Avenae group of the genus *Heterodera*[a]

Species and authority[b]	Common name[c]	*H. avenae* species complex[d]	Parasite of: Cereals and grasses	Parasite of: Grasses only	Multiple pathotypes described
H. avenae Wollenweber, 1924	European CCN	x	x		x
H. australis Subbotin, Sturhan, Rumpenhorst & Moens, 2002	Australian CCN	x	x		
H. filipjevi (Madzhidov, 1981) Stelter, 1984	Filipjev's CCN	x	x		x
H. hordecalis Andersson, 1975	Barley CN		x		x
H. latipons Franklin, 1969	Mediterranean CCN		x		
H. sturhani Subbotin, 2015[e]	Chinese CCN	x	x		x
H. arenaria Cooper, 1955	Marram CN	x		x	
H. aucklandica Wouts & Sturhan, 1995	Auckland CN	x		x	
H. mani Mathews, 1971	Ryegrass CN	x		x	
H. pratensis Gäbler, Sturhan, Subbotin & Rumpenhorst, 2000	Meadow CN	x		x	
H. riparia (Kazachenko, 1993) Subbotin, Sturhan, Rumpenhorst & Moens, 2003	Riverbank CN	x		x	
H. ustinovi Kirjanova, 1969	Ustinov's CN	x		x	

[a] The genus *Heterodera* is currently divided into seven groups of species on the basis of fundamental differences in structural components of the cyst vulval cone, number of incisures in the juvenile's lateral field, and molecular analyses (Subbotin et al. 2010b). The Avenae group consists of 12 species.
[b] The species authority in nematological literature includes the year of publication. These are not references cited in this paper.
[c] Common names are as designated in Subbotin (2015) and Subbotin et al. (2010b); CCN = cereal cyst nematode and CN = cyst nematode.
[d] Two species in the Avenae group, *H. hordecalis* and *H. latipons*, are morphologically and molecularly distinct from the 10 species in the *H. avenae* species complex (Subbotin et al. 2010b).
[e] *H. sturhani* was designated as the "*Heterodera* '*avenae*' from China" in Subbotin et al. (2010b). Those populations were then redescribed as a distinct species (Subbotin 2015).

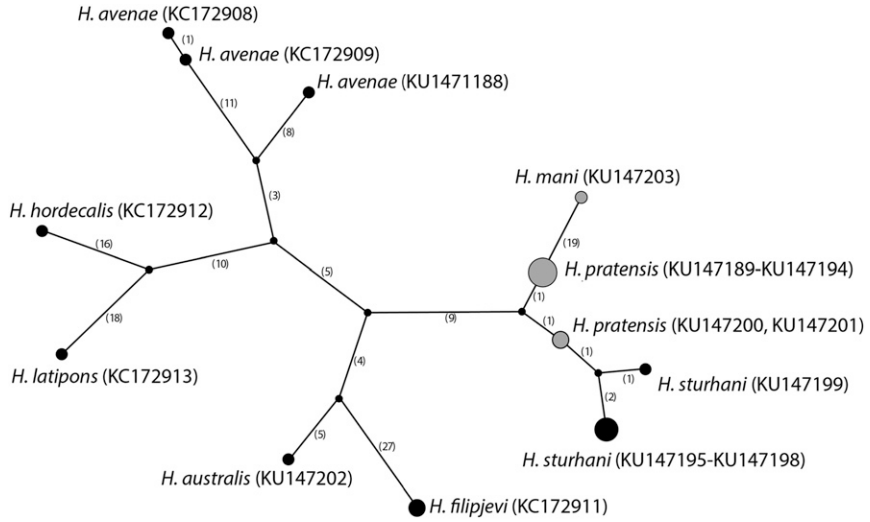

Fig. 5. Statistical parsimony network showing the phylogenetic relationships between COI haplotypes (nucleotide variants in segments of DNA) of several species from the Avenae group. Small black circles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype. Number of changes are indicated in brackets.

(Cui et al. 2015; D. L. Peng et al. 2009). Molecular and morphological analysis of samples of the CCNs in China revealed the presence of three species: *H. avenae*, *H. filipjevi*, and *H. australis* (Fu et al. 2011; H. L. Li et al. 2010; D. L. Peng et al. 2010). However, Subbotin et al. (2003, 2010b) noticed that nematode samples identified as *H. avenae* from China appeared to form a distinct group within the *H. avenae* complex. Sturhan and Rumpenhorst (1996) had also shown that '*H. avenae*' from Fanshan, China, had a different IEF protein profile from those of European *H. avenae* and other species. Further study has demonstrated that these nematodes were close morphologically and genetically to *H. pratensis* and were described as a new species, *H. sturhani* (Subbotin 2015). *H. sturhani* differs from *H. avenae* by having smaller average cyst sizes and and smaller average fenestral length. Some Chinese scientists believe that differences between *H. avenae* in China and *H. avenae* in other countries is insufficient to warrant the redescription of those populations as *H. sturhani* (D. L. Peng et al. 2016). Several studies demonstrated that these populations belong to new pathotypes, which are distinct from European ones (Cui et al. 2015; D. L. Peng and R. Cook 1996; Yuan et al. 2010; J. W. Zheng et al. 1997b). The *COI* gene sequences and the PCR-COI-RFLP profile generated by *Bcu*I differentiate *H. sturhani* from *H. pratensis*, *H. avenae*, and all other species of the *H. avenae* complex (Subbotin 2015). Although molecular results show close relationships of *H. sturhani* and *H. pratensis*, they are different in plant-host range. *H. sturhani* parasitizes wheat, barley, wild and cultivated oat, *Festuca arundinacea*, *Datylis glomerata*, *Phalaris tuberosa*, and others, whereas *H. pratensis* is presently known only as a parasite of grasses (Subbotin et al. 2010b).

Mediterranean cereal cyst nematode – *H. latipons*. This nematode was detected in the Mediterranean region early in the 1960s on the roots of stunted wheat plants and described as a new species based on morphological characteristics of the population in Israel (Franklin 1969). Presently, the species is found from several Asian, North African, and European countries, including Russia (Subbotin et al. 2010b; Subbotin, unpublished data). The highest frequency of reports are from western Asia. Host plants include wheat, barley, oat, rye, and several species of *Phalaris* and *Elytrigia repens*. Morphologically, *H. latipons* closely resembles *H. hordecalis*. These nematodes share similar vulval plate structure with circular semifenestrae separated by wide vulval bridge. The underbridge shows a pronounced thickening in the middle. The most important differentiating character between *H. latipons* and *H. hordecalis* is the vulval slit, which in *H. latipons* is much shorter (5 to 11 vs. 15 to 25.5 µm). Moreover, the J2 stylet knobs are anteriorly more concave in *H. hordecalis* than in *H. latipons* (Subbotin et al. 2010b). The PCR-ITS-RFLP, ITS rRNA, and *COI* gene sequences clearly distinguish *H. latipons* from other species (Subbotin et al. 2010b; Tanha Maafi et al. 2003). The sequence divergence of the ITS-rRNA gene is rather high and can reach 1.8% between some European and Asian populations. Two pathotypes of *H. latipons* appear to have been distinguished in Israel (Oka et al. 2009). In several regions, *H. latipons* occurs in a mixture with *H. avenae* in cereal fields. An unconfirmed report of *H. latipons* being present in North America, on Prince Edward Island in Canada, is now thought to be a misidentification of *H. hordecalis* (Sullivan and Mackesy 2010).

Barley cyst nematode – *H. hordecalis*. This species was found in a barley field in Halland Province, Sweden, in 1967 and later was described by Andersson (1975) as a new species. *H. hordecalis* is presently reported from several European and central and western Asian countries, mainly from grasslands, forests, and coastal areas, preferably from sandy soil, and also from cereal fields. Host plants also include rye, wheat, *Ammophila arenaria*, *Leymus arenarius*, *Bromus inermis*, and other grasses. The species is most similar to *H. latipons* and differs from all other species by a long vulval slit. PCR-ITS-RFLP, sequences of ITS rRNA, and *COI* genes clearly differentiated *H. hordecalis* from other members of the *Avenae* group (Subbotin et al. 2010b). Several pathotypes of *H. hordecalis* have been distinguished in Sweden (Ireholm 1994).

Marram grass cyst nematode – *H. arenaria*. This species was first recorded from marram grass, *Ammophila arenaria*, by Triffitt (1929). Cooper (1955) included a marram root eelworm, *H. major* var. *arenaria*, in his key to British species of *Heterodera*. *H. arenaria* was subsequently raised to species level by Kirjanova and Krall (1971) and redescribed by Robinson et al. (1996). The species is distributed along the coast of the North Sea in England, Scotland, Germany, Denmark, France, and the Netherlands, and is also found from coastal dunes in Italy (Subbotin et al. 2010b). The list of host plants of this species also includes *Agropyron pungens* and *Elymus farctus.* From most species of the *H. avenae* complex, this species differs by longer mean body and mean tail lengths of J2s. The analysis studies did not reveal any differences in the ITS-rRNA gene sequences between *H. arenaria* and *H. avenae* type A (Clapp et al. 2000; Subbotin et al. 2001, 2003). However, *H. arenaria* differs from other species by IEF (Gäbler et al. 2000) and sequences of *COI* gene (Subbotin et al. unpublished).

Auckland cyst nematode – *H. aucklandica*. This nematode was first isolated in 1975 in turf samples from Auckland, New Zealand. It was found on the native grass *Microlaena stipoides* and was described as a distinct species by Wouts and Sturhan (1995). In Europe, *H. aucklandica* was found in natural grasslands on a stream bank at Zarren, Belgium (Subbotin et al. 1999) and on a river bank at St. Albans, U.K. (Subbotin et al. 2003). Auckland cyst nematode differs from *H. avenae*, *H. australis*, *H. pratensis*, *H. sturhani*, and *H. riparia* by a longer mean tail length in the J2s (Subbotin et al. 2003; Wouts and Sturhan 1995). It can also be differentiated from other species of the *H. avenae* complex by IEF (Gäbler et al. 2000; Sturhan and Rumpenhorst 1996), PCR-ITS-RFLP, and sequence of the ITS-rRNA gene (Subbotin et al. 1999, 2001, 2003). Restriction of PCR-ITS-rDNA products by several enzymes (*Cfo*I, *Hin*fI, *Pst*I, and *Taq*I) distinguishes *H. aucklandica* from other species of the *H. avenae* complex (Subbotin et al. 2003). The *COI* gene sequences also clearly differentiate this species from all others (Subbotin et al. unpublished).

Meadow cyst nematode - *H. pratensis*. This species was collected from grasslands from a number of localities in Europe (Rumpenhorst 1994; Sturhan and Rumpenhorst 1996; Subbotin et al. 1996, 1999). The nematode showed some minor morphological and morphometric differences and a different profile of IEF of proteins and PCR-RFLP from *H. avenae* and some other species and later was described as *H. pratensis* by Gäbler et al. (2000). Presently, *H. pratensis* is known from several locations with grassy vegetation in Germany, the European part of Russia, Estonia (Gäbler et al. 2000), the Netherlands (Subbotin et al. 2003), Belgium (Subbotin 2015), and Iran (Tanha Maafi et al. 2003; Subbotin et al. unpublished). Host plants include *Poa annua*, *Festuca pratensis*, *Dactylis glomerata*, *Lolium perenne*, *Elymus repens*,

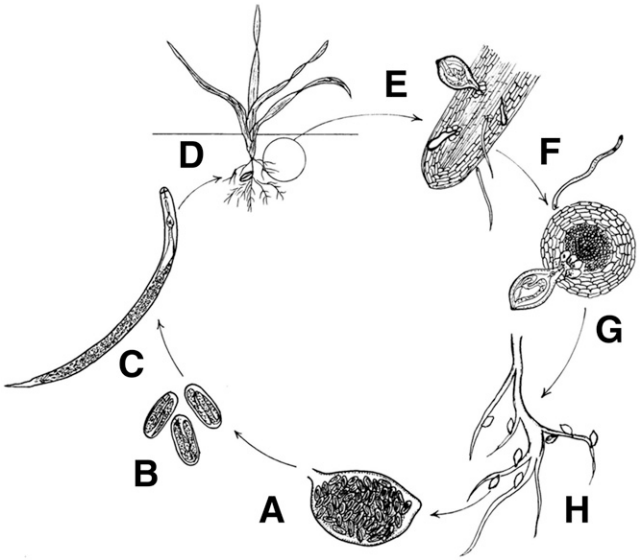

Fig. 6. An illustrated drawing of the life cycle of a cereal cyst nematode; cyst filled with eggs overwintering in soil (**A**), egg containing second stage juvenile (J2) (**B**), J2 free in soil (**C**), emerged plant roots host J2 (**D**), young roots attacked by J2 developing to various stages (**E**), male free in soil (**F**), mature female and formation of syncytium (**G**), mature female and cyst still attached to root (**H**).

Agrostis stolonifera, and *Cynosurus cristatus.* Attempts to rear the species on barley and wheat failed (Gäbler et al. 2000; Subbotin et al. 2010b). Morphologically and molecularly, *H. pratensis* is very similar with *H. sturhani* and can be distinguished by the *COI* gene sequence from this and all other species (Subbotin 2015).

Riverbank cyst nematode – *H. riparia*. This nematode was described by Kazachenko (1993) from roots of false wheat *Elytrigia repens* and *Phragmites australis* along the coast of the Olga Bay, Okhot Sea, Kamchatka, Russia. It most resembles *H. pratensis* and *H. sturhani* in morphometrics; however, its relationships with these species are unclear. Biochemical and molecular studies are still lacking (Subbotin et al. 2003, 2010b).

Rye grass cyst nematode – *H. mani*. This nematode was described as *H. mani* by Mathews (1971) from an infested grass in Northern Ireland. The species is presently reported from pastures and grasslands in several European countries, South Africa, and the U.S.A. (California and Washington). Host plants include *Lolium perenne*, *Dactylis glomerata*, *Festuca pratensis*, and several other grass species, but not *Agrostis* spp., wheat, oats, or barley. *H. mani* differs from other species of the *H. avenae* complex by the J2s having strongly developed and deeply concave stylet knobs as well as a combination of morphometric and morphological characters (Subbotin et al. 2010b).

Ustinov cyst nematode – *H. ustinovi*. This nematode was described from the roots of *Agrostis capillaries* from the Beskids, eastern part of the Eastern Carpathians, Ukraine (Kirjanova 1969). Because of the inadequate description, the taxonomic status of *H. ustinovi* has long been controversial. Sturhan and Krall (2002) re-examined paratypes and studied original material collected from places close to the type locality and concluded that *H. iri* described by Mathews (1971) from Northern Ireland was a junior synonym of *H. ustinovi.* Presently, the species has been found from grasslands of several European countries and several states in the U.S.A. (Subbotin et al. 2010b). It parasitizes several species of *Agrostis* and *Glyceria fluitans.* *H. ustinovi* is distinguished from other species of this complex by longer tail and hyaline region lengths of J2s (Mathews 1971), IEF of proteins (Gäbler et al. 2000), RFLP and sequence of the ITS rRNA gene (Subbotin et al. 1999, 2001, 2003), and sequence of *COI* gene (Subbotin et al., unpublished).

Life Cycle, Disease Process, and Symptoms

CCNs coevolved with their host plants, resulting in a coincidence of nematode and host life cycles that promoted survival and reproduction of the nematode. Reviews of the life cycle of CCNs have been published (Baldwin and Mundo-Ocampo 1991; Hajihasani et al. 2010a; Rivoal 1986; Rivoal and Cook 1993; Schölz and Sikora 2004; Sikora 1988; Subbotin et al. 2010a; Turner and Subbotin 2013; Wu et al. 2014).

Life cycle. The ontogeny of all species of cyst nematodes is similar. CCNs are sexually dimorphic species. The life cycle starts from the egg stage and ends with additional eggs being produced by a gravid female (Fig. 6). The eggs of all species of CCNs are cylindrical, similar in size, and without visible marking on their surface. Once fertilization takes place, the eggs pass through embryogenesis, with the four cells resulting from the first cleavage aligning in tandem. The final stage of embryogenesis, morphogenesis, produces a first-stage juvenile (J1). The J1 is retained in the egg and molts into the second stage juvenile (J2), also within the egg (Fig. 7A). Depending on the cyst nematode species and environmental conditions, the J2 may either hatch from the egg or enter into a dormant state inside

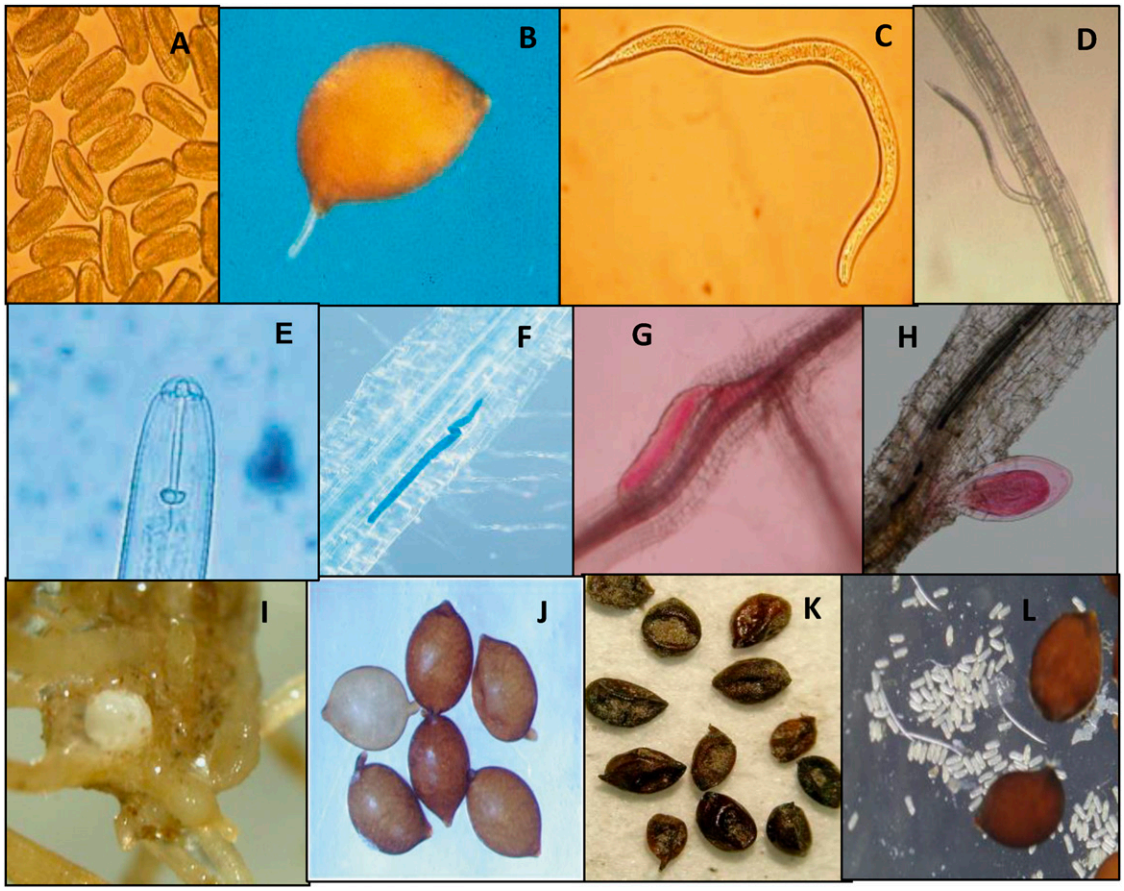

Fig. 7. Life stages of cyst nematodes; eggs showing outlines of the J2 folded within the egg (**A**), second-stage juvenile (J2) emerging through the fenestra of a cyst (**B**), J2 extracted from in soil (**C**), J2 probing a root (**D**), J2 stylet used to penetrate root cells (**E**), J2 migrating through root cortex (**F**), third-stage juvenile 10 days after invading root (**G**), fourth-stage juvenile feeding from syncitium (**H**), swollen egg-filled white female lodged in root tissue (**I**), young encysted females dislodged from roots (**J**), old cysts extracted from soil (**K**), and cysts crushed to expose eggs and J2s (**L**). Images by Guiping Yan (A, C, I), John Lewis (B), Hugh Wallwork (D, E, F), Hai Yan Wu (G, H), Shree Pariyar and Abdelfattah Dababat (J), Richard Smiley (K), and Najoua Namouchi-Kachouri (L).

the cyst (Koenning and Sipes 1998). The J2 is the dormant stage of the life cycle of cyst nematodes.

The hatching process is generally divided into three steps; changes in eggshell permeability, activation of the J2, and hatching of the J2 from the egg. The J2 emerges from the egg and migrates out of the cyst, either through the fenestration of the vulval plate (Fig. 7B) or the opening made at the neck after the cyst separates from the root. After emerging from the cyst, the J2 becomes free living in the soil (Fig. 7C). Generally, the emerged J2 seeks a host root and once it reaches a root it explores the surface by pressing its lips on to the surface to detect an optimal point for invasion (Fig. 7D). The J2 invades the root by penetrating the root tip using its strong stylet (Fig. 7E). The root tip region is very active metabolically at the region of vascular cell differentiation and elongation. The other sites where lateral roots emerge can also be priority sites for invasion by J2s (Wyss and Zunke 1986). After penetration, the J2 migrates through cortical cells (Fig. 7F) and reaches the differentiating vascular cylinder where it selects a competent cell to initiate an induced feeding site, the syncytium. Syncytium development is induced when pharyngeal gland secretions are injected through the stylet. The syncytium provides a food resource for the developing nematode.

After establishing the syncytium and beginning the feeding process, the J2 immediately becomes swollen and loses its mobility. Then it undergoes three additional molts to complete its life cycle. The genital primordium is developed and elongated during growth and molting to the third stage juvenile (J3; Fig. 7G). The sex of juveniles becomes distinguishable in J3s. Juveniles with a single unbranched genitalia develop as males and those with branched genital primordium become females. Generally the feeding of J3s that will become males continues until the end of the J3 stage. At the fourth molt the juvenile is still wrapped in the third-stage cuticle. The adult male is vermiform and motile, and develops within the cuticle of the J4. Adult males emerge from the J4 cuticle, exit the root, and seek adult females. Males are attracted to females through sex pheromones exuded by the females.

The J3-stage female continues to feed and becomes swollen inside the root. It changes to the fourth stage (J4) and develops into an adult lemon-shaped female after a final molt (Fig. 7H). As the mature female becomes swollen, the posterior end of its body ruptures the root cortex, exposing the vulva to the rhizosphere for mating by the male. At this time, the white color of mature swollen females becomes visible on the surface of the roots without the aid of magnification; they appear similar in size to the head of a pin (Fig. 7I). Soon after mating, eggs begin to develop within the female body and the embryos start to develop. The number of eggs inside white females varies among CCNs, depending on the species, host plant variety, soil type, and environmental factors. As many as 600 eggs have been reported, although the more typical number is in the range of 100 to 400.

All eggs remain inside the body of CCNs. This differs from some other cyst nematode species such as the beet cyst nematode, *H. schachtii*, and soybean cyst nematode, *H. glycines*, in which eggs are laid also in an egg-sac at the posterior end of the body. After eggs are produced, the CCN female dies and the cuticle becomes tanned to form a tough, protective cyst wall. The cysts vary in color from cream to light to dark brown (Fig. 7J and K). The cyst wall protects the eggs from desiccation and predation by soil organisms. Eggs inside cysts (Fig. 7L) may remain viable for many years. Phenolic compounds found in the cyst wall might be responsible for the tanning of the female's cuticle, as was found in *Globodera rostochiensis* (Vlachopoulos and Smith 1993).

CCNs have only one generation per year but the time required to complete the life cycle varies with environmental conditions. Generally, the *H. avenae* life cycle is between 3 and 4 months under optimum temperature conditions. The life cycle of the Chinese population of '*H. avenae*' in Shandong Province was completed on winter wheat in 99 and 83 days during the 2010 and 2011 growing seasons, respectively (Wu et al. 2014). In the Slovak Republic, the duration of the *H. avenae* life cycle on spring oat was 56 days, while on winter wheat it took 84 and 63 days in 1981 and 1982, respectively (Sabová et al. 1985a, b).

Response to root diffusates. Hatching of *H. avenae* and *H. latipons* does not depend on diffusates from host roots and these species hatch in a large numbers in the absence of a stimulus (Al Abed et al. 2009; Perry 2002; Perry and Gaur 1996; Schölz and Sikora 2004; J. W. Zheng et al. 1997a). The cyst nematodes are classified into three categories on the basis of hatching rate in water; low, moderate, and high. *H. avenae* and *H. latipons* are considered to exhibit a high water hatching ability (Schölz and Sikora 2004; Turner and Subbotin 2013). Most studies on the effects of root diffusates on hatching of juveniles of *H. avenae* suggest that root exudates do not increase the emergence of J2s from eggs. Banyer and Fisher (1971b) and Fisher and Hancock (1991) found little or no evidence that wheat root exudates were effective to overcome the suppression of hatch induced by a temperature of 20°C after 8 weeks at 7°C on a population of '*H. avenae*' from South Australia. Hatching rates of the Chinese population of '*H. avenae*' in Shanxi Province showed that leachates of five plants, including wheat, barley, oats, maize, and tomato, did not stimulate the hatching of cysts pretreated at 5°C (J. W. Zheng et al. 1997a). In contrast, the English population of *H. avenae* has responded to root exudates of host plants. In outdoor and indoor pot tests, hatching of *H. avenae* was affected by the cereal species grown, providing evidence that root exudates stimulated hatching of the English population at prevailing soil temperatures (5 to 15°C) during spring (Kerry and Jenkinson 1976; T. D. Williams and J. Beane 1980).

The general absence of a hatching stimulus for CCNs is in contrast to many other species of cyst nematodes that hatch in large numbers in the presence of host root diffusates. This is best exemplified by the potato cyst nematodes, *G. rostochiensis* and *G. pallida*. These species are almost completely dependent on host root diffusates that contain hatch-stimulating chemicals to induce hatching of J2s. However, about 10% of the J2s can hatch without a stimulation from the host plant, an ability that is probably genetically controlled (Evans 1979).

Survival. CCNs are an exception among the cyst nematodes that possess narrow host ranges. Generally, cyst nematode species with a limited host range are responsive to host root exudates, resulting in greater longevity in the absence of a host (Winslow and Willis 1972). While CCNs have narrow host ranges within the Poaceae, hatching does not normally depend on host root diffusates. Kerry and Jenkinson (1976) showed that 73% of hatching of *H. avenae* in England occurred in fallow soil, of which 26% was during the autumn and 47% in the spring. A hatch of 85% was measured in the presence of host plants such as winter and spring barley, wheat, and oats. The fact that eggs hatch successfully in the absence of host plants indicates that the persistance of eggs and J2s inside the cysts is compromised and these nematodes have a reduced ability to survive long periods in the absence of a host plant. The advantage of this specificity provides the basis for implementing management strategies involving crop rotations.

Lack of dependency of CCNs on root diffusates for hatching results in a diminished survival during fallow, crop rotation, and planting of resistant cultivars. Andersson (1982) reported a 70 to 80% annual decline of *H. avenae* population in the absence of a favorable host. Maximum annual decrease of populations in southern France was 42% on a nonhost crop (Rivoal and Cook 1993). Moreover, Rivoal (1986) observed 57 to 74% annual declines of *H. avenae* populations in the presence of resistant hosts. These results indicate that with proper crop rotation most of the encysted eggs hatch within 1 year and the population densities decline sharply.

Diapause. Some nematodes are able to suspend their development (dormant state) during unfavorable conditions, a tactic that helps them to survive in the absence of a host plant. This suspension is removed and the nematodes are reactivated when favorable conditions return. Female cyst nematodes change into the cyst stage and can enter a dormancy phase in which the unhatched J2s remain dormant for many years. The dormant encysted J2s exhibit minimum metabolism leading to a marked reduction in metabolising vital energy reserves. Not all cyst nematode species exhibit dormancy, but some have a dormant state in their life cycle, a state that is subdivided into two types, diapause and quiescence. The difference between these two types of arrested development is that the strategy of dormancy in

quiescence is reversible when favorable conditions return, while in the diapause state the development does not continue until specific requirements have been satisfied, even if favorable conditions return. There are two types of diapause; obligatory, which is programmed into the life cycle, and facultative, which is triggered by environmental stimuli such as day length (Perry 2002).

Population dynamics of CCNs have been studied extensively. Most studies were on nematodes initially identified as *H. avenae*. There are fewer studies of other species, particularly as some of the studied populations of *H. avenae* have been redescribed recently as other species. These investigations indicate the presence of diapause in the life cycle of *H. avenae* and once the diapause ends, the emergence of J2s and further development of the life cycle is affected by environmental factors. A minimum period of 8 weeks at low temperature is needed for substantial hatching by *H. avenae* (Fushtey and Johnson 1966). Occurrence of diapause has been reported in Australian populations of '*H. avenae*' (Banyer and Fisher 1971a, b) and for two French *H. avenae* populations (Fr1 and Fr4) under in vitro and field conditions (Rivoal 1978, 1979, 1983). Obligate diapause of *H. avenae* in its first season of development has also been observed (Rivoal and Cook 1993). Obligate diapause is also present in the life cycle of the Chinese population of '*H. avenae*' in Shandong Province, China (Wu et al. 2014). Summer diapause similar to that found in populations in Australia and in southern France was also observed in the Shandong population, indicating that hatching processes do not continue until the soil temperature increases to 5 to 10°C during the spring.

Existence of diapause has also been reported in *H. latipons* populations. The population of *H. latipons* from the dry zone of Syria appeared to exhibit a facultative quiescence, which was related to metabolic processes rather than to developmental inhibition (Schölz and Sikora 2004). The breaking of quiescence was facilitated by cold temperatures, as with other species of CCNs.

In contrast, the Haymana population of *H. filipjevi* in Turkey does not appear to exhibit a diapause in that there was no apparent requirement of an incubation temperature of 5°C for hatching of J2s in that population (Şahin et al. 2010). The J2s emerged from cysts at 10 and 15°C.

Effects of environmental factors on emergence of J2s. Temperature plays a key role in the hatching process. Cotten (1962) was the first to suggest that a period of low temperature was essential for subsequent hatching of *H. avenae* cysts in England. Additional studies demonstrated that temperature requirements differ within species and among species (Rivoal and Cook 1993).

H. avenae. Fushtey and Johnson (1966) confirmed Cotten's finding that a prehatching incubation at low temperature (0 to 7°C) for a minimum period of 8 weeks was required to initiate the hatching of *H. avenae* J2s in Canada. The optimum temperature requirement for hatching after the cold incubation was at 10 to 15°C, and the maximum temperature for hatching was between 25 and 30°C (Fushtey and Johnson 1966). Subsequently it was demonstrated that a low temperature of about 10°C influences hatching of most populations of *H. avenae*, and activates the juveniles inside the eggshell to initiate transformation to a hatchable condition (Banyer and Fisher 1971b, 1980; T. D. Williams and J. Beane 1980).

The optimum temperature requirement for hatching juveniles may vary with population of the nematode. Infestations of Australian populations of '*H. avenae*' are present in cereal-growing regions of South Australia, Victoria, and Western Australia. Each region has a Mediterranean climate with hot, dry summers and cool, wet winters. In South Australia, hatching takes place in two phases. The first phase consists of larval development, which occurs optimally at 10°C. That phase is a prerequisite for the second phase in which juveniles emerge, a process for which the optimum temperature is 20°C (Banyer and Fisher 1971b). The hatching pattern of the Western Australian population differs slightly, with the optimum temperature for larval development also being 10°C, while the optimum for phase 2 is about 15°C (Stanton and Eyres 1994). The egg hatching rate for Chinese populations of '*H. avenae*' is higher at 15°C than at 5, 10, or 20°C (Fisher 1981; M. Wang and J. Yan 1993).

Two French populations of *H. avenae* also displayed a shift in the hatching process when they were moved from their origin to a region with a different climate. The southern population present in a Mediterranean-type climate (Mediterranean ecotype), hatched during winter. In contrast, the northern population present in a continental climate (oceanic- or temperate-type ecotypes) hatched in spring. These population traits were not altered by reciprocal transfers of populations or by transferring the populations to an intermediate location; however, the timing of the onset of hatching and the peak emergence of juveniles were each modified slightly, depending upon the population's origin and the location where it was incubated for hatching (Rivoal 1986). Two Spanish CCN populations (pathotypes Ha81 and Ha22) also hatched at different rates after 1 and 5 months of incubation at 20 and 10°C, respectively, indicating a difference in their temperature requirements (Zancada and Sanchez 1988).

These findings suggest that CCN populations in different regions adapt to local conditions and environments to synchronize the hatching process with favorable conditions for invasion of host plants and nematode reproduction. The lowest density of J2s in soil is generally found during growth of young seedlings and the peak density occurs at the tillering and stem elongation stages. The maximum population density of J2s is present in wheat roots during the booting stage when the soil temperature ranged from 12 to 14°C. Cysts were formed after the booting stage (X. H. Li et al. 2012b; Wu et al. 2014).

The relationship between developmental stages of the Chinese population of '*H. avenae*' in Shandong Province, phenology of winter wheat, and accumulation of daily heat units (degree days; DD) was studied by X. H. Li et al. (2012b) and Wu et al. (2014). The DD calculation involves subtraction of the daily low temperature from the daily high temperature, and dividing the difference by two. The calculation also assumes that there are no DDs accumulated below a certain temperature threshold for biological activities. That threshold differs for each type of organism or each stage in the life cycle of certain organisms. The number of DD required for development of different morphologic stages assumed a minimum temperature of 2°C for eggs to hatch (Fushtey and Johnson 1966) and a minimum temperature of 14°C for development of the J2 stage (X. H. Li et al. 2012b). The optimum temperature for penetration of roots was 16°C for a Chinese population of '*H. avenae*,' and the optimum range of temperatures for juveniles to go through molts as they develop into gravid females was 18 to 22°C (X. H. Li et al. 2012b). Therefore, under Shandong conditions, molting from the J2 to J4 stages required more than two continuous DD above the minimum 14°C (Wu et al. 2014). In a cold semicontinental climate of eastern Oregon, the J2s of *H. avenae* emerged from cysts very rapidly when weekly mean air temperatures rose to between 2 and 5°C, and the maximum J2 densities were observed in March and April (Fig. 8) when soil temperatures ranged from 10 to 15°C (Smiley et al. 2005). Similar observations for *H. avenae* in England were reported by Kerry and Jenkinson (1976).

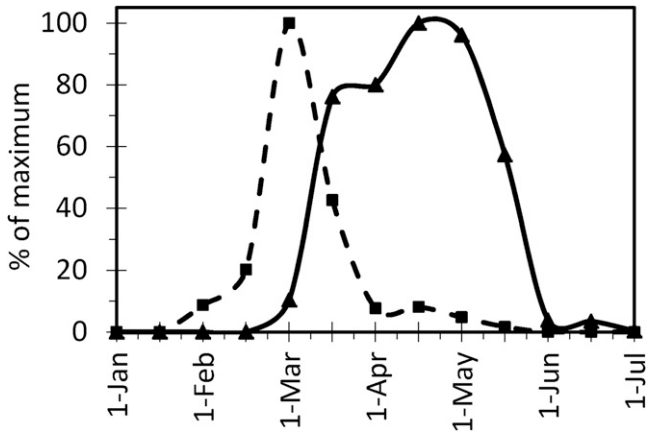

Fig. 8. Density of *Heterodera avenae* second-stage juveniles present in soil during the spring (January to June) in a mild oceanic climate of western Oregon (dotted line) and in a cold continental climate of eastern Oregon, U.S.A. (solid line) (Smiley 2016).

H. latipons. The influence of temperature on hatching of *H. latipons* was studied in the eastern Mediterranean. The optimum temperature for hatching in Syria was 10°C and the peak densities of hatched J2s in soil were in January and February in the dry and wet agro-ecological zones. Hatching decreased further and stopped in March or April in the wet and dry zone, respectively. It was concluded that hatching would not commence unless the soil temperature was below 15°C in the wet zone or below 10°C in the dry zone. In vitro tests showed that incubations at 2 and 5°C for 16 days initiated the hatching cycle (Schölz and Sikora 2004). Populations of *H. latipons*, like *H. avenae*, therefore appear to require a preincubation of low temperature to stimulate hatching. In Jordan, the optimal rate of invasion of roots by *H. latipons* was between 15 and 20°C (Al Abed et al. 2009).

Studies of the life cycle of *H. latipons* show that this species completed only one generation per growing season in Cyprus under field conditions on barley (Philis 1999) and in Syria on wheat and barley in both dry and wet agro-ecological zones of the Mediterranean-type climate (Schölz and Sikora 2004). This also occurred in Markazi Province of Iran with winter wheat under outdoor microplot conditions, where winters had minimum temperatures below –15°C (Hajihasani et al. 2011). The life cycle of *H. latipons* in Iran was 145 to 150 days on winter wheat under microplot conditions (Hajihasani et al. 2011). In Cyprus, it took 70 days on spring barley from the time when juveniles invaded the roots until eggs began to appear, and 98 days from invasion until development of embryonated eggs (Philis 1999).

Difference in life cycle lengths is a response to varying requirements for accumulation of DD to activate hatching and the additional molts to higher developmental stages. The time required for each developmental stage is directly related to the soil temperature and development of the host plant. The DD required for completion of a young female and a mature female containing developed eggs of *H. latipons* was 190 and 375 DD, respectively, using a base temperature of 8°C for that calculation (Hajihasani et al. 2011). The accumulated heat units above 7°C for development of a young female and a mature female containing embryos of *H. latipons* on barley were 215 and 386 DD in Cyprus (Philis 1999). It can be concluded that the hatching process of *H. latipons* is similar to that of *H. avenae* in that it hatches readily in tap water and host root diffusates do not enhance the rate of hatching (Al Abed et al. 2009; Schölz and Sikora 2004).

H. filipjevi. The life cycle of *H. filipjevi* has been studied in Iran, Russia, and Turkey (Hajihasani et al. 2010a; Şahin et al. 2010; Seifi et al. 2013; Tikhonova 1971). Like *H. avenae* and *H. latipons*, this species has only one generation on wheat per growing season and the mature cyst appears simultaneously with maturity of wheat grain. In Iran, the accumulated DD above 8°C, the minimal temperature requirement for development of white females and of embryonated eggs of *H. filipjevi*, was 209 and 358 DD, respectively (Hajihasani et al. 2010a). These values are higher than those needed for development of *H. latipons* (Philis 1999; Schölz and Sikora 2004), suggesting that *H. filipjevi* might need more energy for development and completion of its life cycle (Hajihasani et al. 2010a).

Hatching of *H. filipjevi* is significantly greater at lower temperatures (5, 10, and 15°C) than at higher temperatures of 20 and 25°C. As for *H. avenae*, the cysts of *H. filipjevi* require a preincubation at lower initial temperatures of 10 or 15°C for 58 days to obtain the highest initial hatching rates 1 week after exposure to the final temperature of 25°C (Şahin et al. 2010). Tikhonova (1971) found that *H. filipjevi* (reported as *H. avenae*) J2s in the continental climate of the Bashkir region of central Russia emerged from cysts at temperatures of 5°C and above. In Iran, *H. filipjevi* began hatching when soil temperatures warmed into the range of 2 to 6°C and the J2s were observed inside roots when the temperature reached 13°C (Seifi et al. 2013).

In microplot experiments with winter wheat, there were two peaks in egg hatching and presence of juveniles in soil, with the first following the emergence of seedlings and initial growth of the root system, and the second taking place after the melting of snow in winter, when winter wheat resumes active growth (Hajihasani et al. 2010a). Similar results were found in the cool-temperate climate of the Central Anatolian Plateau in Turkey, where there were two peak periods of J2 hatching, the first in October and the second in February (Şahin et al. 2010). The primary hatching of *H. filipjevi* under natural conditions in the Central Anatolian Plateau and in Markazi Province of Iran begins when the soil temperatures rise to between 5 and 15°C, with the most rapid emergence being at 10 and 15°C (Hajihasani et al. 2010a; Şahin et al. 2010). In Fars Province of Iran, under microplot conditions, hatching of *H. filipjevi* eggs began between 2 and 6°C, and J2s were found in roots when the soil temperature was 13°C (Seifi et al. 2013) with completion of its life cycle requiring 197 calendar days after winter wheat was planted.

Root invasion and induction of feeding sites. Motile J2s of *H. avenae* survive in the soil for 1 to 2 months under the influence of increasing temperatures during spring in England (Kerry and Jenkinson 1976) and are capable of invading roots for at least 3 weeks after they emerge from cysts in Australia (Davies and Fisher 1976). The J2s of *H. avenae* and *H. filipjevi* seek a host plant root and only invade at the root tip. Once in contact with the root tip, the J2 penetrates by using its strong stylet (Fig. 7E). After penetration, it migrates through cortical cells (Fig. 7F) and reaches the differentiating vascular cylinder and initiates the establishment of the feeding site, the syncytium. The protoplasts of the initial syncytial cell and the neighboring cells fuse together and the syncytium continues to expand by incorporating many adjacent cells, with wall dissolution occurring at pit fields (Bleve-Zacheo et al. 1995; Gheysen and Jones 2006; Jones 1981).

The histopathology of the syncytia induced by Australian populations of '*H. avenae*' in resistant and susceptible cultivars revealed that a syncytium was induced in both susceptible and resistant cultivars, but became evident 4 days after inoculation in susceptible plants and 14 days after inoculation in resistant plants (Grymaszewska and Golinowski 1991). In an earlier study of two susceptible (susceptible near-isogenic lines, Prins) and resistant (AUS 10894 × Prins) cultivars, it was found that males completed their life cycle in both susceptible and resistant cultivars but the female development was suppressed in the resistant cultivar at the J4 stage (K. J. Williams and J. M. Fisher 1993). The initiation and development of syncytia was similar in both cultivars until day 15, at which time in the resistant cultivar the syncytium started to degenerate, exhibiting coalescing large vacuoles and a reduction of cytoplasm, and complete degeneration by day 33 (K. J. Williams and J. M. Fisher 1993). Exit of J2s from roots of resistant hosts and repenetration into the root in search for a favorable host has also been reported (Koenning and Sipes 1998).

Whereas *H. avenae* and *H. filipjevi* invade only at the root apex, the site of invasion by *H. latipons* can be more distant from the root apex (Mor et al. 1992). Likewise, the tissue in which the syncytium is induced by *H. latipons* in wheat and barley roots differs from syncytia formed by *H. avenae* (Mor et al. 2008). *H. latipons* starts induction of a syncytium in a root cortical cell, and so the developing syncytium incorporates cells both of the cortex and the vascular cylinder. In contrast, *H. avenae* initially targets a vascular parenchyma cell and development of the syncytium then begins within the vascular cylinder. The development of *H. latipons* in resistant plants is similar to that of *H. avenae*, in that development is suppressed before the adult female becomes formed. In *Lolium rigidium*, which is resistant to *H. latipons*, the J2 penetrates to an endodermal cell or a cortical cell near the endodermis to initiate syncytial development, with changes including the density of cytoplasm and cell incorpation by 20 days after inoculation, after which the nematode stops developing at the J3 stage (Mor et al. 2008).

Disease symptoms. Symptoms of *H. avenae* and *H. filipjevi* invasion of cereal roots are usually a knotted appearance without necrosis or brown lesion. When the J2 enters the root tip, the growth of the tip stops and the root produces adventitious roots and root proliferation, which gives roots a bushy or knotted appearance (Fig. 1G to J). This is where syncytia are present and therefore also the location of most maturing white females. The young maturing females are initially white (Fig. 7I) and gradually become cream colored and then light to dark brown (Fig. 7J and K), at which time they die and become cysts. However the symptoms of root infection varies in different cereal hosts.

Heavy invasion of roots leads to above-ground symptoms that include stunting, reduced tillering, and smaller heads with shriveled grain kernels. CCNs may also cause a pale green discoloration of the leaves or even result in senescence of lower leaves. In severe infections of crops with a lax head type, when the heads contain small grains, the heads may remain upright compared with drooped heads of the healthy plants which contain heavier fully-ripened and developed seeds.

In contrast, because *H. latipons* invades roots at points more distant from the root apex, this species does not produce clearly visible root symptoms in the early seedling stage (Mor et al. 1992). Inhibition of foliar growth by *H. latipons* is also less severe than that caused by *H. avenae*, and it is possible that invasion of wheat by *H. latipons* may only become recognizable when cysts appear on the roots (Mor et al. 1992).

Detecting and Identifying Pathogens

Evidence for the presence of CCNs may not be noticed in most infested soils and in plant roots with low nematode numbers. Detecting and identifying nematodes in soil and plant tissues is therefore important to be able to determine their presence, to monitor their movement or introduction, to estimate population densities, and to make a correct identification, all of which are important in developing appropriate management schemes. While CCN species are often found as single infections in most fields, it is not uncommon to identify two species in the same field (Abidou et al. 2005; Holgado et al. 2009; Smiley and Yan 2015; Yan and Smiley 2010).

Sampling is the first step in detection. Soil samples are usually taken soon after harvest because the population densities are near their maximum levels and the analysis will provide timely information for the next season. However, sampling just before planting provides a better estimate of the initial nematode density and their possible impact on the crop that will be planted. Samples may also be taken at any time during the growing season because low, nondetectable population densities increase over time to levels at which damage to crops becomes visible. During that period, not only cysts with eggs and juveniles inside, but also other life stages can be found free in the soil and inside the roots. In this case, to have a proper idea of nematode densities, both a soil and a root sample should be taken (Barker and Campbell 1981).

Soil samples. Sampling can be accomplished using a variety of tools, ranging from a spade to special soil samplers or augers. In each case, soil samples are collected to a depth of 30 cm because most cysts or free-living nematodes are located in the upper 10 to 15 cm of soil (X. H. Li et al. 2014; Smiley 2016; Xiang et al. 2013). Different methods of sampling are recommended depending on the purpose of the investigation, the costs, and the available time. For a qualitative study (e.g., detection of CCN to determine presence or absence, but not quantity), the required accuracy can be relatively low and thus requires only a simple sampling plan. For this purpose, it is sufficient to collect a composite sample of 20 soil cores taken from the area surrounding patches of stunted plants, with or without some of the infected plants themselves. When the quantity of the population needs to be determined, as for precise advice regarding control measures or the selection of a crop or a cultivar, a higher degree of sampling intensity is required to achieve the intended level of accuracy. In this case, it is recommended that three samples per hectare are collected with up to 60 or 70 soil cores per sample, following a systematic sampling pattern. Random sampling is another option but it does not account for the patchy nature of nematode distribution and thus it is only representative if the sampling area is small. A systematic grid-like sampling pattern gives more reliable results. Accuracy increases by taking more samples and a larger volume of soil and/or more plant samples. However, the aim is to find the best balance characterized by a combination of highest possible accuracy and lowest possible cost and time (van Bezooijen 2006).

Soil samples are usually put in labeled, plastic sampling bags to retain the field level of moisture. This is particularly important when the samples contain life stages other than cysts. Temperature and direct sunlight are two critical factors affecting the survival of nematodes. Therefore, plastic bags should be stored in a cool, dark place (Elmiligy 1971).

After collecting samples, the nematodes should be separated from the soil ('mineral' fraction) or plant tissue ('organic' fraction) using a coarse sieve. The latter can be roots cut from plants or root pieces collected from the soil. In most cases, each fraction is analyzed separately. However, if a sample cannot be processed completely, a representative subsample should be taken. When this is done properly, analysis of one subsample per sample is sufficient (Carbonell and Angulo 1979).

Many extraction methods exist but all indirectly use one or a combination of the following principles: (i) the specific density of nematodes makes it possible to decant them from particles that settle faster while the nematodes remain floating in the water, whether or not the extraction is made with the aid of an undercurrent (elutriation), or with a liquid having a specific gravity higher than that of water and combined with a centrifugal force (flotation/centrifugation); (ii) the size and shape of the nematodes makes it possible to separate them from other particles by using a set of sieves with different mesh size (sieving); (iii) the motility of the nematodes makes it possible for them to migrate out of the matrix into a clear suspension through a filter positioned in a shallow water-filled tray or funnel (Baermann funnel), with or without the aid of a preceding maceration step (to make plant pieces smaller) and/or a fine mist sprayed onto the matrix (mistifier). The first two principles are usually applied for extracting infective juveniles and males from soil, while extraction methods for plant tissue are usually based on nematode mobility. The only exception on the latter is the blender flotation/centrifugation method which does not make use of nematode mobility as the nematodes are liberated from the plant tissue by blending first. This technique makes it possible to extract the swollen endo-parasitic stages of

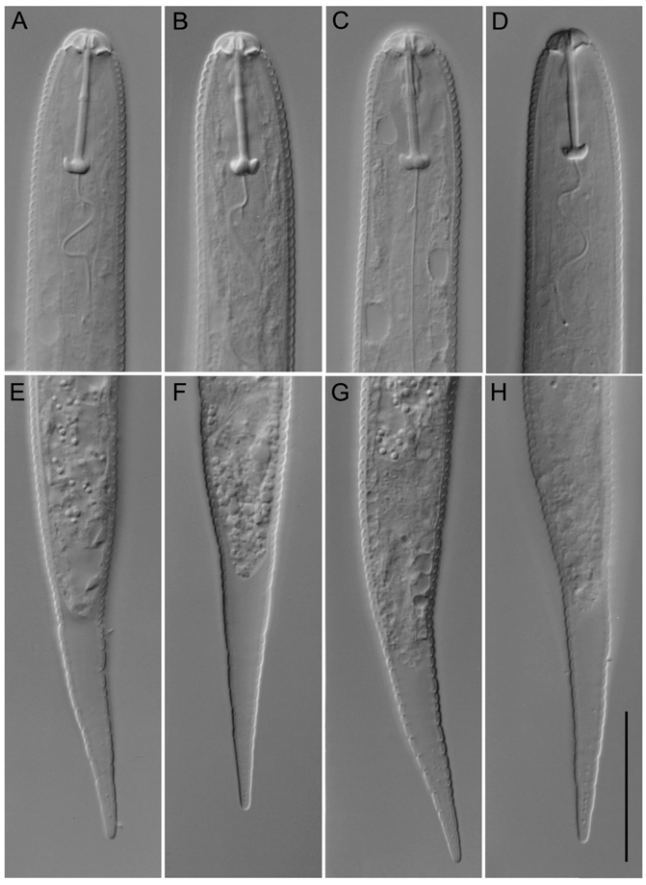

Fig. 9. Anterior and posterior ends of the second-stage juveniles of species belonging to the *Heterodera avenae* complex. **A, E,** *H. avenae*; **B, F,** *H. australis*; **C, G,** *H. filipjevi*: **D, H,** *H. sturhani*. Scale bar = 30 µm (after Subbotin et al. 2003).

CCN from plants. There are some excellent reviews that provide an overview of the different extraction methods, their principles, procedure, materials needed, advantages, and disadvantages (Hooper et al. 2005; Seinhorst 1988; Southey 1986; van Bezooijen 2006).

For CCNs, specialized extraction methods exist for cysts because their size, shape, and weight differ significantly from life stages of other nematodes. These extraction methods can be subdivided into methods using dry soil and those using dry or wet soil. Cyst extraction methods using dried soil are based on the fact that cysts usually contain some air and therefore float on water. However, it should be noted that the viability of the eggs and juveniles inside the cyst will decrease drastically if the soil is dried for several days at temperatures above 35°C. Another disadvantage is the fact that young, egg-filled cysts do not float as well as older cysts with fewer eggs, and the young full cysts can therefore be lost. As a consequence, more empty or half-empty cysts are isolated, which results in an underestimation of the population. Therefore, when it is intended to use CCN cysts for further multiplication or inoculation, or when densities should be determined precisely, a 'wet' extraction method should be selected. Such methods apply an undercurrent or a solution with a higher density than the cysts to keep them afloat in the suspension or on top of the solution respectively, while allowing soil particles to settle (van Bezooijen 2006).

Plant samples. Plant samples, gently rinsed in water to remove soil and other particles, can also be examined directly after sampling. Young, rounded white females or encysted brown females full of eggs and juveniles break through the surface of roots and thus can be detected by eye or using a magnifying glass. The presence of other life stages in plant tissues can be determined by microscopy after staining with cotton blue or acid fuchsin, which stain the nematodes while plant tissue remains relatively clear (Southey 1986). For further processing, cysts and young females or other life stages can be isolated by dissection.

Morphology-based identification methods. After extraction, a nematode suspension or a mix of cysts and organic matter (the 'float') are retained from samples. The nematode suspension can be stored at 4°C, but should be processed within a few days as fungal and bacterial growth can reduce the quality and quantity of the nematodes (van Bezooijen 2006). A subsample is taken and all CCN, still alive, are counted using a dissecting microscope (10 to 50×). This can be repeated several times to increase accuracy. The total number of nematodes in the sample is calculated by multiplying the mean number of nematodes in the subsamples with the number of subsamples that can be taken to complete the sample. The nematode suspension can also be preserved using a fixative for later evaluation (Hooper 1986). Identification of J2s (Fig. 9) usually requires a combination of the following morphological characteristics: body length, stylet length, tail length, length of hyaline part of the tail, stylet knob shape, and shape of tail terminus (Sturhan 1982; Subbotin et al. 2010b).

Cysts present in the 'float' are also counted. The organic matter is spread on a counting plate under a dissecting microscope. To investigate the cyst contents, they need to be separated from the 'float,' usually using forceps, a small paint brush, or a more robust hair (e.g., an eyebrow hair or a pig hair in a holder). The cysts are then crushed manually with a scalpel, or a 'cyst squasher,' by gently rubbing them between two glass slides. Pieces of the cyst shell should be removed before counting eggs and juveniles (van Bezooijen 2006). Identification can be done as described for J2s. Alternatively, a vulval

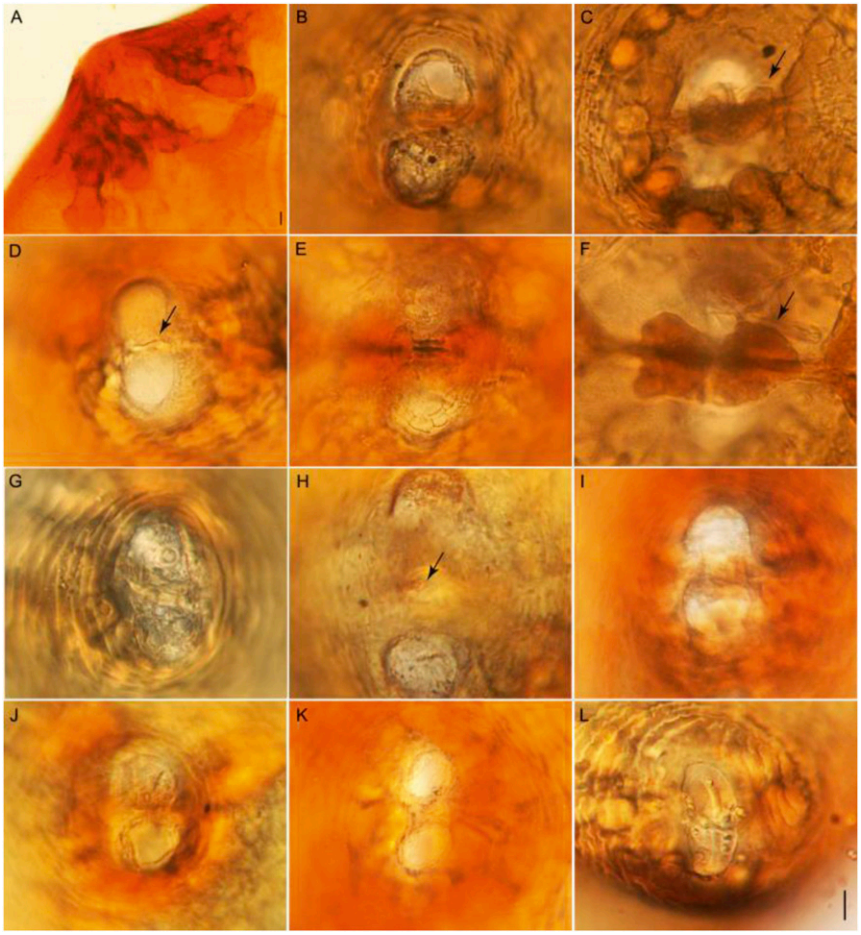

Fig. 10. Vulval plates of species from the Avenae group. **A,** Lateral view of vulval cone with bullae; **B,** *Heterodera filipjevi*, anterior view; **C,** *H. filipjevi*, underbridge level view (underbridge is indicated by arrow); **D,** *H. avenae*, anterior view (vulval slit is indicated by arrow); **E,** *H. hordecalis*, anterior view; **F,** *H. hordecalis* (underbridge with pronounced thickenings is indicated by arrow); **G,** *H. aucklandica*, anterior view; **H,** *H. latipons*, anterior view (vulval slit is indicated by arrow); **I,** *H. mani*, anterior view; **J,** *H. sturhani*, anterior view; **K,** *H. pratensis*, anterior view; **L,** *H. ustinovi*, anterior view. Scale bars = 10 μm.

cone, consisting of the terminal region of the cyst, can be prepared and examined (Cooper 1955; Subbotin et al. 2010a). Four morphometrical characters of the vulval cone (Fig. 10) are diagnostically valid: fenestra length, mean semifenestral width, vulval bridge width, and vulval slit length. Also useful are the presence of an underbridge and size and shape of bullae (Handoo 2002). However, morphological analysis requires a trained scientist because these nematode species do not show a broad anatomical variation. Sometimes only small but consistent differences are important for distinguishing certain species. In addition, environmental factors and the fixation process can affect nematode dimensions. Because of these issues, nematologists are increasingly turning to molecular methods for identification.

Nematode vitality. It is easy to determine whether nematodes in a suspension are alive or dead. Living plant parasitic nematodes actively move and exhibit gentle, sinusoidal, dorsoventral waves along their entire body (Wallace 1968). It is more difficult to determine whether eggs and juveniles inside cysts are alive. Various methods have been used but none are entirely satisfactory. Stains like phloxine B (Fenner 1962) or Meldola blue (Ogiga and Estey 1975) can differentiate between living and dead nematodes because the dye only enters a dead nematode if internal membranes are damaged. Many samples can be examined with relative ease and speed by staining; however, this may not indicate true viability as, for example, nematodes treated with nematicides may appear viable, but eventually die.

Another method for assessing viability is measurement of ATP, which is present only in living tissue (H. J. Atkinson and A. J. Ballantyne 1977). However, this method requires specialized equipment and nematodes that are not contaminated by microorganisms, since ATP from the microbes will influence the results. Two more recent methods include measuring the trehalose content of the cysts (Perry et al. 1983; Yen et al. 1996), or using reverse transcription quantitative PCR (RT-qPCR) (Christoforou et al. 2014). Trehalose, which acts as a protectant, is converted into glucose just before hatching, leaving behind an empty cyst without trehalose. Alternatively, due to a change in the permeability of the membrane of dead eggs and juveniles, the trehalose leaks out leaving behind a full 'dead' cyst without trehalose. The RT-qPCR is designed to detect mRNA of a certain gene indicating gene expression and thus activity of the nematode. In dead organisms, mRNA degenerates quickly, resulting in a negative signal. Unfortunately, neither of these methods can currently substitute for the visual inspection because the variance of the RT-qPCR is too high and the density range of the trehalose assay is too small (Beniers et al. 2014).

DNA-based identification methods. Molecular techniques are becoming the most important tools to detect and identify nematodes, including CCN. General protein and specific enzyme banding patterns obtained after iso-electric focusing (IEF), an electrophoretic method capable of separating amphoteric molecules like proteins, has been applied to distinguish *Heterodera* species. An extensive evaluation of five different isozymes within species of the CCN complex confirmed previous morphological characterizations that separated *H. avenae*, *H. filipjevi*, *H. latipons*, and *H. mani* (Andrés et al. 2001b). For many years, IEF was considered to be a simple and efficient method for nematode diagnostics (Gäbler et al. 2000; Rumpenhorst 1985; Sturhan and Rumpenhorst 1996; Subbotin et al. 1996). Nevertheless, it was evident that results could be influenced by sample preparation, storage, and the developmental stage of the nematode. This approach has now been replaced by more sensitive DNA-based methods (Subbotin et al. 2010a; Waeyenberge and Viaene 2015). Initially, PCR-RFLP profiling and DNA-barcoding based on the rDNA cistron were applied. These techniques only required DNA extracted from few to one isolated juvenile or cyst. PCR-ITS-RFLP diagnostics profiles have been developed for most *Avenae* group species (Bekal et al. 1997; Gäbler et al. 2000; Madani et al. 2004; Rivoal et al. 2003; Subbotin et al. 1999, 2002, 2003; Tanha Maafi et al. 2003; and others). However, other molecular techniques were needed to speed up acquisition of results and to reduce costs. This has led to the development of species-specific primers for end-point PCR and DNA-probes for quantitative PCR. These assays are currently available for several CCN species such as *H. avenae*, *H. latipons*, and *H. filipjevi* (H. Peng et al. 2013; Qi et al. 2012; Toumi et al. 2013a, 2013b, 2015; Yan and Smiley 2010; Yan et al. 2013). The assays can detect and, in case of qPCR, quantify (Fig. 11) one or two different CCN species simultaneously in a nematode suspension. This means that these methods can eliminate the need to isolate and, in case of qPCR, count these species. Further developments are already available but are not yet routinely applied to CCNs. Direct DNA extraction from soil and loop-mediated isothermal amplification (LAMP) techniques are particularly interesting. The latter method will enable on-site diagnosis as the amplification runs at a single temperature, avoiding the need for specialized equipment.

Direct extraction of DNA from soil facilitates the processing of soil samples without the need to extract, isolate, or count individual nematodes or cysts. A high-throughput method to quantify DNA simultaneously for CCN and the most important other soilborne pathogens of small grains and other field crops was developed in Australia. The PreDicta B commercial assay (http://pir.sa.gov.au/research/services/molecular_diagnostics/predicta_b) produces quantitative data that closely approximates the densities of living pathogens in soil (McKay et al. 2008; Ophel-Keller et al. 2008). However, the PreDicta B assay does not differentiate species within the CCN complex (A.C. McKay, SARDI, Adelaide, Australia, personal communication, 2009). The PreDicta B

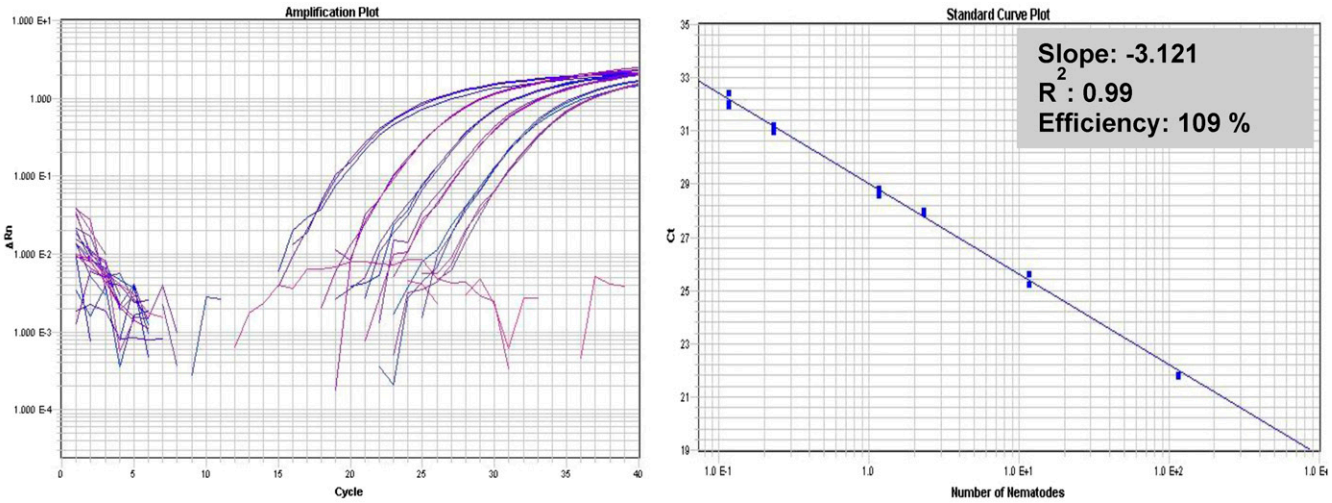

Fig. 11. Panel on the left: qPCR amplification plot from DNA extracted out of different number of *Heterodera filipjevi* nematodes to obtain the number of PCR cycles needed (Ct-values) to generate a signal above a certain threshold. Panel on the right: Ct-values versus the number of *H. filipjevi* nematodes resulting in a standard curve for quantification of samples with unknown number of *H. filipjevi* juveniles (F. Toumi, personal communications).

service has been used for research in several countries, including the U.S. A. (Smiley et al. 2016), and prompted the development of species-specific DNA-based assays to detect and distinguish between *H. avenae* and *H. filipjevi* in the U.S.A. (Figs. 12 and 13; Yan and Smiley 2010; Yan et al. 2013). Those tests are now available as a commmercial service to farmers and scientists at several nematode testing laboratories in the U.S.A. (Smiley 2016).

Impacts on Grain Yield and Quality

Estimates of crop damage caused by CCNs were published in recent workshops of the International Cereal Nematodes Initiative (Dababat et al. 2015b, 2017; Riley et al. 2009). Other reports were summarized in Dababat et al. (2014), McDonald and Nicol (2005), Nicol and Rivoal (2008), Rivoal and Cook (1993), Smiley and Nicol (2009), Vanstone et al. (2008), and others.

Magnitude of yield reduction. CCNs have reduced yields in individual research trials or fields by as much as 20% in Pakistan, 50% in Australia, 50% in Turkey, and 90% in Saudi Arabia (Dababat et al. 2015b; Riley et al. 2009). More than half the fields are reported to be infested by CCNs in selected cereal-producing regions of Turkey (Abidou et al. 2005; Elekçioğlu et al. 2009), Iran (Tanha Maafi et al. 2009), the U.S.A. (Smiley et al. 1994), and Europe (Rivoal and Cook 1993). All wheat fields are infested by CCNs in several provinces of China (D. L. Peng et al. 2009). A bibliography of 123 CCN publications relating to all aspects of CCN biology and management in China, from 1991 to 2014, was published by Riley and Qi (2015).

Reports of crop losses at the magnitudes shown above do not accurately depict the magnitude of economic losses at the regional or national level because documentation was based mostly on research plots located in infested areas of fields. Since the nematode density varies greatly across most fields, published estimates nearly always fail to represent field-wide yield reductions, which are rarely documented. A further complication is that some reports initially attributed to yield reduction by '*H. avenae*' are now known or assumed to have been attributable to species recently reclassified as *H. australis*, *H. filipjevi*, *H. latipons*, or *H. sturhani*. Nevertheless, several reports of regional or national crop losses caused by CCNs are available. In Australia, annual yield losses due to Australian populations of '*H. avenae*' were estimated at 300 kt (Murray and Brennan 2009). Losses in Australia were at one time much higher but have been reduced greatly by deploying resistant varieties because only one biotype is present (Lewis et al. 2009; Riley and McKay 2009). Yield losses in three provinces of China, caused by '*H. avenae*,' were estimated at 1.2 Mt, assuming that 22% of the production area was infested and that the overall yield reduction was 10% in those areas (D. L. Peng et al. 2009). National production of cereals in Norway was estimated to be reduced by 1 to 5% by multiple CCN species (Holgado et al. 2003). Losses from *H. avenae* and *H. filipjevi* in four northwestern states of the U.S.A. are estimated at 22 kt, assuming that 0.04% of the wheat and barley fields are infested and the average field-wide yield reduction in infested fields is 10% (Smiley 2009; Smiley, unreported data). Economic losses in other infested regions of the western U.S.A. and globally are poorly documented (Dababat et al. 2015b, 2017; Riley et al. 2009).

Yield reduction relative to population density. A definite relationship between the number of nematodes and either the magnitude of yield suppression or an economic threshold is difficult to generalize because yield responses are strongly influenced by interactions between climate, crop variety, management practice, nematode distribution and density within the field, and chemical, biological, and physical properties of soil. For instance, the importance of a given density of nematodes at the time of planting will become greater if affected plants are later subjected to drought, inadequate nutrition, impediment to root penetration into soil, or adverse temperature. The potential for damage may also differ among varieties if, for example, those varieties have different abilities to replace damaged roots. Nevertheless, associations have been made throughout the history of CCN research to demonstrate a generally linear relationship between initial population density and potential for reduced grain yield and reductions of other growth and yield components.

As an example of the relationship between CCN density and grain yield, *H. filipjevi* in Iran reduced the yield of rainfed winter wheat at all densities ranging from 2.5 to 20 eggs plus J2s/g of soil, with the lowest and highest densities causing 11 and 48% reductions in yield, respectively (Hajihasani et al. 2010b). In the northwestern U.S.A., rainfed wheat yields are generally reduced when the number of *H. avenae* eggs plus J2s from extracted cysts, plus J2s present within the soil matrix, exceeds 3/g of soil (Smiley, unpublished). Densities of five *H. avenae* or *H. filipjevi* J2s/g of soil are capable of causing economic damage to irrigated wheat in India (Singh et al. 2009). Andersen (1961) reported that the numbers of *H. avenae* cysts produced on barley were 12, 24, 26, and 33 cysts/plant at initial densities of 1, 2.5, 5, and 10 eggs plus J2s/g of soil.

Influence of edaphic factors. Effects of temperature, precipitation, soil texture, soil microbiota, and other edaphic factors on the life cycle and crop damage caused by CCNs have been reported in papers shown in Dababat et al. (2015b, 2017), Riley et al. (2009), and Riley and Qi (2015). Other reports were summarized in Andersson (1982), Dababat et al. (2014), McDonald and Nicol (2005), Nicol and Rivoal (2008), Rivoal and Cook (1993), Smiley and Nicol (2009), and others. Complex interactions among edaphic factors are thought to be largely responsible for observations that, in many areas, multiyear periods of high CCN densities and crop damage appear to alternate with multiyear periods of low CCN densities and little crop damage. Andersson (1982) stated that this phenomenon appears to represent a dynamic balance between the pathogen and its parasites and predators.

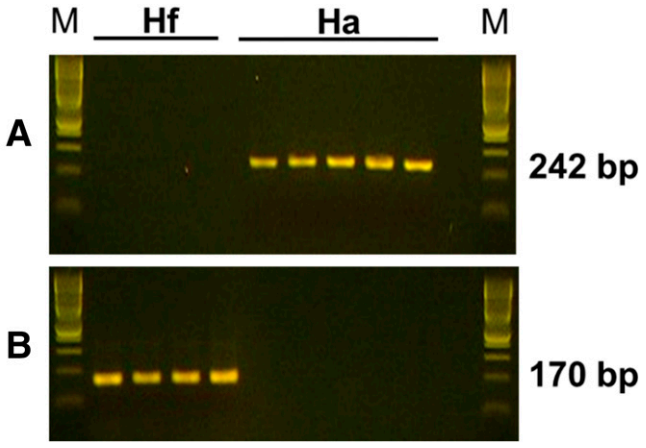

Fig. 12. Distinguishing *Heterodera avenae* (Ha) and *H. filipjevi* (Hf) using species-specific PCR assays of DNA extracted from soil; **A,** detection of *H. avenae* at the 242 bp band position; **B,** detection of *H. filipjevi* at the 170 bp band position (Yan et al. 2013).

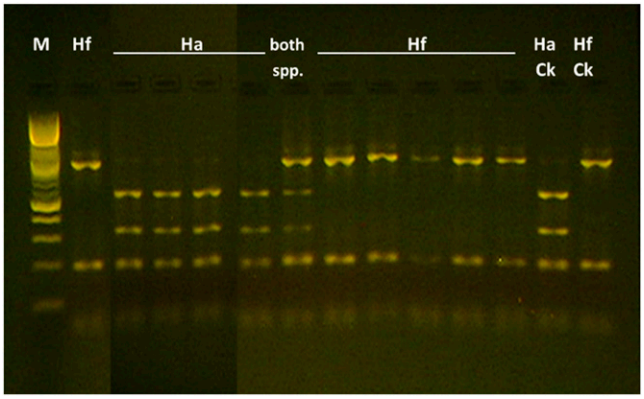

Fig. 13. Distinguishing *Heterodera avenae* (Ha), *H. filipjevi* (Hf), and mixtures of these species in wheat field soils from Washington State, U.S.A. (Smiley and Yan 2015), using an PCR-RFLP assay developed by Yan and Smiley (2010).

Temperature. It is important to recognize that temperature and, to a lesser extent, moisture drive the hatching process and that it can therefore vary greatly in chronological time during different seasons (Andersen 1961). Hatching patterns for CCN populations in representative countries were reviewed in the previous section on effects of environmental factors on emergence of J2s. In short, peak hatching of these nematodes occurs in synchrony with planting and growth of cereals in each region of the world (Rivoal and Cook 1993), including different climate types within comparatively small geographic regions (Fig. 8).

Precipitation. Moisture is almost always present when small grains are planted, and the moisture films in soil are essential for mobility of these nematodes. Excessively thick films of moisture can impede nematode mobility and the ability of J2s to invade root tips.

Grain yield losses due to CCN species are often more economically important in rainfed wheat production systems than in irrigated systems (Nicol and Rivoal 2008). For instance, damage to wheat by *H. filipjevi* was greater when wheat was irrigated only four times rather than the seven times considered to be agronomically optimal in Isfahan Province of Iran (Fard et al. 2015).

Soil physical factors. CCNs may be present in soils with textures ranging from sand to clay. Production of cysts and eggs/cyst are generally greatest in sandy soils and least in clay loams (Al Abed et al. 2009; Andersson 1982; X. H. Li et al. 2012a). Likewise, the number of new cysts formed can become much greater after soil from a no-till field is cultivated than when it remains undisturbed (Al Abed et al. 2009). This may take place because soil structure is important for hatching in fine-textured soils. The greatest number of cysts are formed when most soil pores are in the range of 30 to 100 µm, which coincides with the porosity that is most favorable for movement of J2s (Andersson 1982).

Soil chemical factors. CCNs are not limited within the range of chemical characteristics required to produce their hosts. However, more cysts are often produced on roots of plants in soils with more favorable nutrition, which is thought to be an indirect effect of such plants producing larger root systems, providing more sites for invasion by J2s (Andersson 1982). While adequate supplies of nitrogen may promote production of more cysts, it is also true that higher levels of nutrition often are used to mask some of the deleterious effects of root destruction. Plants are better able to generate additional roots if they have adequate nutrition and water. Grain yield and CCN population dynamics can therefore be complex and not always directly related.

Soil microbiological factors. Many members of the soil microflora and soil microfauna have been implicated as parasites or predators of CCN eggs or juveniles (Kerry 1987; Kerry and Crump 1998). All stages of the CCN life cycle have been evaluated for possible exploitation of interactions between the pathogen and one or multiple parasites. Eggs within cysts appear to be particularly susceptible to parasitism by fungi and bacteria (Ismail et al. 2001). Studies of interactions between CCNs (*H. avenae*, *H. filipjevi*, and *H. latipons*) and parasitic fungi and bacteria were recently reviewed by Dababat et al. (2014). High levels of nematode mortality have been reported in numerous studies. Of particular interest was a field study in Tunisia, which showed that the presence of both a fungal parasite (*Pochonia chlamydosporia*; = *Verticillium chlamydosporium*) and a bacterium (*Rhizobium radiobacter*) led to the greatest parasitism of *H. avenae* eggs, and that suppressive soils characterized by high rates of egg mortality were correlated with the highest densities of the fungal parasite (Mensi et al. 2011). Many more reports of research on interactions between CCNs and bacteria, mychorrizae, and other soil fungi in China are reported in Riley and Qi (2015). Effects of abiotic edaphic factors on the efficiency of interactions between fungal parasites and CCNs were reviewed by Andersson (1982).

Influence of cereal growth habit. In general, in regions having cold winters, invasion of roots by a given density of CCN will have a greater impact on the yield of a spring-planted cereal than of an autumn-planted cereal. This occurs because winter wheat already has a well-established root system at the time when most J2s emerge from cysts during the spring. Juveniles are already present in soil when spring grains are planted, and continue to emerge from cysts when roots of spring grains are at their most vulnerable stage of development. This relationship is exemplified by comparisons of damage relationships for spring wheat and winter wheat in eastern Oregon (Fig. 14). However, it is also true that higher rates of J2 invasion are often measured in the seminal roots than in the nodal roots of spring cereals (Kerry and Hague 1974) because the seminal roots are developing at the time of highest J2 density in soil, whereas the nodal roots of spring cereals often develop mostly after the J2 density in soil has diminished. While nodal and seminal root branches are each developing on winter cereals when densities of J2 in soil are highest, leading to high rates of invasion of each component of the root system, the main axis of seminal roots and of many coronal roots of winter cereals have grown well below the zone of highest J2 concentration (the 20 cm nearest the surface) by the time the J2 density is highest during the spring.

Interactions of CCNs with other plant-parasitic nematodes and plant-pathogenic fungi. The ability of plant-parasitic nematodes to interact with other plant pathogens has long been recognized (Pitcher 1978; Powell 1971). Invasion of roots by a nematode can increase, decrease, or have no affect on the other pathogen. Interactions of the two pathogens is usually indirect, being affected by changes they each cause on the root substrate upon which they both depend. This differs from relationships discussed previously, regarding direct

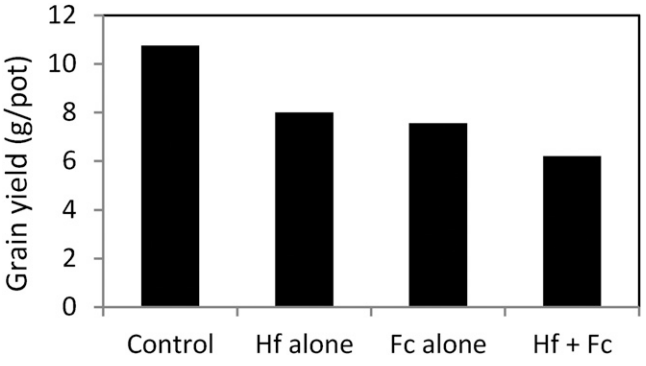

Fig. 15. Influence of *Heterodera filipjevi*, *Fusarium culmorum*, or presence of both pathogens on yield of winter wheat in outdoor pot trials incubated outdoors in Iran; means of two years of data, LSD 0.05 = 0.3 (Hajihasani et al. 2013).

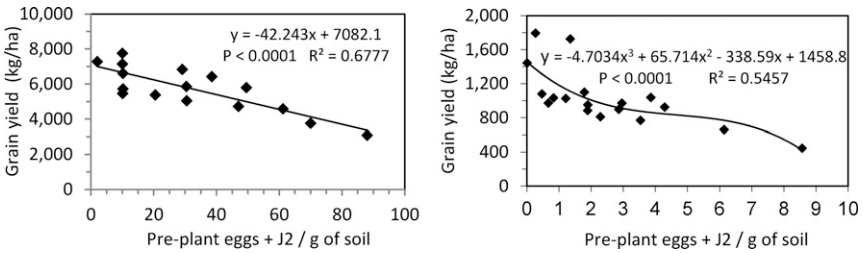

Fig. 14. Relationship between yield of irrigated winter wheat (left) or rainfed spring wheat (right) and the preplant density of *Heterodera avenae* (from extracted cysts) in two fields in eastern Oregon, U.S.A. (modified from Smiley 2016).

influences on the parasitic nematode by other components of the soil microbiota and microfauna.

While interactions among pathogens are well known, very little research has been focused upon interactions involving CCNs. Rivoal et al. (1995) reported that *Pratylenchus neglectus* numbers in soil became elevated after a *H. avenae*-resistant oat variety was released, suggesting that *H. avenae* suppressed multiplication of *P. neglectus*. However, Nombela and Romero (1999, 2001) found no relationship between *H. avenae* and *P. thornei* on wheat.

Cook (1970) reported that severe instances of take-all, caused by *Gaeumannomyces graminis* var. *tritici*, were always associated with low densities of *H. avenae* in England (Cook 1970). That association in the field was then confirmed in pot experiments. Smiley et al. (1994) used regression analyses to determine that the greatest amount of yield reduction for winter wheat in field trials was associated with combined damage from *H. avenae* and *G. graminis* var. *tritici*, although the nematode was individually responsible for more damage than the fungal pathogen. They also reported that the greatest negative effect on numbers of roots was associated with a combination of *H. avenae* and *Pythium* spp. When effects of the nematode were reduced through application of a nematicide, there was a corresponding increase in root damage by *Rhizoctonia solani* and *Pythium* spp., resulting in no yield improvement. In Australia, Meagher and Chambers (1971) demonstrated that a reduction of wheat tillering, plant height and weight, and number and length of roots were greater when plants were inoculated with *R. solani* AG-8 and an Australian population of '*H. avenae*' than when inoculated with either pathogen alone. The dual influence of these pathogens was synergistic for plant height and for number and length of roots. Subsequent research under field conditions confirmed these relationships (Meagher et al. 1978).

Potential mechanisms of synergism between nematode and fungal pathogens were discussed by Back et al. (2002). Schölz et al. (1998) demonstrated in pot cultures that yields of barley were decreased more by a combination of *H. latipons* and *Cochliobolus sativus* than by either pathogen alone. The presence of both pathogens reduced the reproductive rate (final density divided by initial density) of the nematode by 50%, as compared with the presence of the nematode alone. The fungal pathogen caused a greater severity of infections to the crown, subcrown internode, and seminal and nodal roots when the fungus was in the presence of the nematode, as compared with infection by the fungus alone. Hajihasani et al. (2013) showed that dual inoculations of *H. filipjevi* and *Fusarium culmorum* caused a greater reduction in rainfed winter wheat growth and yield parameters than inoculation with either pathogen alone. Reductions were measured for plant height, shoot weight, root weight, and grain weight (Fig. 15) of wheat grown in terra cotta pots in an outdoor nursery in Iran. The effects of these pathogens on plants were additive rather than synergistic. In the same study, coinoculation of *H. filipjevi* with *C. sativus* did not cause additive deleterious effects on growth of wheat. Wheat is known to be more tolerant than barley to *C. sativus*, which may explain the differences between results of Schölz et al. (1998) and Hajihasani et al. (2013). But both *F. culmorum* and *C. sativus*, when coinoculated with *H. filipjevi*, reduced the reproductive capacity of the nematode (Hajihasani et al. 2013).

Disease Management

Damage from CCNs can be most efficiently managed using an integration of multiple strategies, as reviewed by Andersson (1982), Brown (1987), Dababat et al. (2014, 2015a, b, 2017), McDonald and Nicol (2005), Nicol and Rivoal (2008), Riley and Qi (2015), Rivoal and Cook (1993), Sikora (1988), Smiley and Nicol (2009), Vanstone et al. (2008), Whitehead (1998), and others.

First, it is important to protect noninfested fields from becoming infested, because it is seldom possible to eradicate CCNs. After fields are infested, the management objective is to reduce the pathogen density to a level lower than the threshold that causes economic damage. Since that threshold varies widely depending on climate, soil and crop variables, and nematode species and virulence characteristics, integrations of several simultaneous management strategies provides the most stable and reliable protection. Practices that may potentially be integrated involve components of the following strategies.

Sanitation. Phytosanitary quarantines are effective at the country and state or provincial levels. Sanitary procedures can be helpful at the local level, but prevention of localized spread is very difficult or impractical. These nematodes become disseminated by all processes that move soil (Dawabah and Al-Hazmi 2007; Smiley et al. 1994); as on equipment, animals, plant products, water, or wind. Many years are typically required before the nematode becomes apparent or detectable in infested fields, allowing much time for additional spread before the problem is realized.

Crop rotation and host frequency. To achieve effective control of CCNs, it is necessary to reduce the population below the economic threshold for damage. This requires definitive studies on population dynamics and yield losses on representative local cultivars under natural field conditions. Cultural practices based on rotational combinations of nonhosts (noncereals), resistant cultivars, and clean fallow can effectively suppress densities of CCNs in soil. Rotation of cereal crop hosts with two years of nonhost broadleaf crop species or fallow have long been recommended to reduce the density of CCNs on highly infested fields (Andersson 1982; Fisher and Hancock 1991; Rivoal and Sarr 1987; Smiley et al. 1994; Tikhonova et al. 1975). However, the 3-year rotation for this purpose alone is often not profitable in regions where cereals are the main or the only adapted and profitable crop.

Resistance and tolerance. The use of host-plant resistance plus tolerance is one of the most effective methods of controlling CCNs (Trudgill 1991). Multiple plantings of resistant wheat or barley crops have successfully reduced CCN densities to very low levels in many countries (Andersen and Andersen 1970; Lewis et al. 2009; Rathjen et al. 1998; Riley and McKay 2009; Rivoal et al. 1991). Resistance is characterized by cultivars that suppress or prevent reproduction of the nematode (Cook and Evans 1987). Sources of resistance to CCN populations worldwide have been collated and reviewed and, where possible, have had their genetic location and gene designation reported (McDonald and Nicol 2005; Nicol 2002; Nicol et al. 2003; Nicol and Rivoal 2008; Rivoal et al. 2001; Smiley and Nicol 2009). All resistances reported against CCNs in commercial cultivars have been based on introgressions of single dominant genes.

Resistance. Resistance to *H. avenae* in barley was first discovered in 1920 in Sweden and was characterized in 1961 (Andersen 1961). Resistance of barley to the CCN has been a specific crop improvement objective in many countries for more than five decades (Andersen 1961; Andersen and Andersen 1973; Cook 1974; Cotten 1967; Holgado et al. 2009; Nielsen 1982; O'Brien and Fisher 1974; Smiley et al. 2011b; Valocká et al. 1994; T. D. Williams 1970; Yavuzaslanoğlu et al. 2016).

The best currently characterized sources of resistance to '*H. avenae*' in barley are the genes mapped to the *Ha2* locus on chromosome 2H (Kretschmer et al. 1997) and to the *Ha4* locus mapped on chromosome 5H (Barr et al. 1998). According to the gene nomenclature system of Moseman (1972), these gene loci are designated as *Rha2* and *Rha4*, respectively.

Six *Cre* genes for *H. avenae* resistance in wheat (*Cre2* to *Cre7*) and the *Rkn2* gene for resistance to both *H. avenae* and *Meloidogyne naasi* (Jahier et al. 1998) were derived from *Aegilops* species. Other resistance genes were derived from *Triticum aestivum* (*Cre1* and *Cre8*) and *Secale cereale* (*CreR*). Several other sources of resistance (*CreX* and *CreY*) are also reported, but their genetic control and gene designation are still unknown. Most of these resistance genes have been introgressed into hexaploid wheat. The *Cre1* gene is highly effective against populations of *H. avenae* from Europe, North Africa, and North America and moderately effective or ineffective to populations of CCNs in Australia and Asia (Mokabli et al. 2002; Rivoal et al. 2001). Populations of *H. filipjevi* in India and *H. latipons* in Syria differ in virulence to the *Cre1* gene, compared with *H. avenae* (Mokabli et al. 2002). In Turkey, the *Cre1* gene appears partially affective against *H. filipjevi*, but *Cre3* is not. The *Cre3* gene is effective against Australian populations of '*H. avenae*' (Vanstone et al. 2008) but not European populations of *H. avenae* (de Majnik et al. 2003; Safari et al. 2005) or *H. filipjevi* in Turkey. The *Cre2* and *Cre4* resistance

genes from *Aegilops* and an unidentified resistance gene from the wheat line AUS4930 offer promise against an array of *Heterodera* species and pathotypes (Nicol et al. 2001). An international root disease resistance nursery containing seven of the known *Cre* genes is coordinated by CIMMYT to establish the value of these genes in different regions of the world. CCN resistance QTLs for entries in the nursery were also mapped to chromosomes 1A, 1D, 4D, 5A, 5B, 5D, 6A, 6B, 7A, and 7D (Dababat et al. 2016; Mulki et al. 2013). Eleven DArT markers were also reported (Dababat et al. 2016). These resources identified new resistance loci and tools that may soon become useful in wheat breeding programs.

Wheat, barley, or oat cultivars resistant to *Heterodera* populations in one region may be fully susceptible to populations in other regions. This was shown for Australian wheat cultivars evaluated in Israel (Bonfil et al. 2004) and for the cultivar Raj MR1 in India, which is effective in Rajasthan but not in Punjab. Oat cultivars exhibiting resistance and tolerance to '*H. avenae*' in Australia were susceptible and intolerant to *H. avenae* in Britain (Cook and York 1988). The use of resistance requires a sound knowledge of the virulence spectrum for the targeted species and pathotypes present in each region. Also, although not frequently reported, repeated plantings of wheat, barley, and oat cultivars with a single gene for resistance to *H. avenae* have led to selection of new virulent pathotypes over prolonged time periods, overcoming host plant resistance (Cook and Noel 2002; Lasserre et al. 1996).

Molecular markers have been developed to identify genes for resistance to *H. avenae* complex in barley and wheat (Barloy et al. 2007; Barr et al. 1998; Eagles et al. 2001; Eastwood et al. 1994; Kretschmer et al. 1997; Martin et al. 2004; Ogbonnaya et al. 2001a, 2001b; Paull et al. 1998; K. J. Williams et al. 1994, 2006). Some of these markers have been used in marker-assisted selection and for pyramiding genes for resistance.

Marker-assisted breeding to improve genetic resistance is being applied, but effective resistance genes for CCNs are not yet available for all crops and are not effective against all pathotypes. Large scale tube, pot, or field test screening to identify lines of wheat, barley, oats, and triticale resistant to Australian populations of '*H. avenae*' have been undertaken in Australia for more than 30 years (Lewis et al. 2009). Initially, the pot test was the method of choice, with resistance determined by white cysts counted on the surface of root balls enabling up to 600 pots to be evaluated each day. Up to 130,000 plants per annum have been screened in this way, resulting in the release of many cultivars resistant or moderately resistant to the Australian populations (Lewis et al. 2009). However, this approach is labor intensive and time consuming, taking a full growing season to complete. With the development and validation of codominant molecular markers for resistance to the Australian populations of '*H. avenae*,' these can be applied to leaf samples from small seedlings, and the tests can be automated to determine presence of resistance genes in 1 to 2 days, with substantial savings in costs and time. As a result, marker-assisted selection for resistance to CCNs in wheat is now used routinely in Australia to identify resistant germplasm in breeding programs.

The combination of pot tests and marker-assisted breeding has been used very successfully to reduce infestation levels and losses caused by the CCN in Australia (Ogbonnaya et al. 2009). The strategy followed for marker-assisted selection involves two phases: prebreeding, to identify and characterize resistance sources and the development of linked markers, followed by their incorporation by backcrossing into advanced breeding lines used in breeding programs, including pyramiding of resistance genes from different sources (e.g., *Cre1* on chromosome 2BL, *Cre3* on chromosome 2DL, and *Cre8* on chromosome 6B), using specific linked PCR markers to follow each gene (Ogbonnaya et al. 2009). Deployment of resistant cultivars, starting in about 1975, was also responsible for a strong decrease in damage caused by *H. avenae* in Sweden (Andersson 1982).

Tolerance. Resistance must be combined with tolerance to attain optimal yield performance and stability while simultaneously reducing the risk to subsequent plantings of intolerant cultivars or crops (Andersen 1961; Brown 1987; Cook and Evans 1987; Fisher 1982). Tolerant cultivars are characterized as having an ability to withstand or recover from nematode invasion and to yield well in comparison with noninvaded plants (Cook and Evans 1987; Fisher 1982). Tolerance is usually estimated in the field by comparing the yield of a specific plant cultivar in a naturally infested soil that is either left untreated or is treated with a nematicide such as aldicarb to reduce the impact of the existing nematode population (Brown 1987; Meagher et al. 1978; Smiley 2009; Smiley and Marshall 2016).

Roots of both resistant and susceptible cultivars are initially invaded by J2s, which may result in an intolerant reaction prior to the expression of resistance in a resistant cultivar (Andersson 1982; O'Brien and Fisher 1977; Ogbonnaya et al. 2001a; Oka et al. 1997). The tolerance trait has been attributed to characteristics of root growth and physiological response to invasion (Stanton and Fisher 1988; Volkmar 1990). The intolerant reaction is characterized by a closely grouped proliferation of adventitious roots at locations where female nematodes have established a feeding syncytium, resulting in a bushy or knotted appearance to the root. Invaded roots often fail to continue growing deeply into the soil. Some resistant cultivars are unable to produce competitive grain yields when compared with susceptible cultivars that are grown without nematode pressure (Wilson et al. 1983). Growers are often reluctant to plant resistant cultivars that produce lower yield than will susceptible cultivars in noninfested soils (Rivoal and Cook 1993).

Barley is generally more tolerant of *H. avenae* than wheat or oats. Andersen (1961) reported comparative damage thresholds 5, 1, and 0.2 eggs plus J2s per gram of soil for these crops, respectively. Because barley is more tolerant than wheat, it was predicted that there would be a greater potential in barley than in other cereal species to identify cultivars that are both resistant and tolerant of these nematodes (Andersson 1982; O'Brien and Fisher 1977). This expectation was confirmed in selections of barley and wheat cultivars in the U.S.A. (Marshall and Smiley 2016; Smiley and Marshall 2016). The mechanism whereby barley is generally more tolerant than wheat was postulated to be the production of crown roots earlier in barley seedlings than in wheat seedlings, which enables the crown roots to compensate more rapidly and more completely for early damage on seminal roots (T. D. Williams and G. A. Salt 1970).

It is also possible to manage damage by rotating resistant cereals with susceptible crop species. However, local knowledge of resistance reactions is essential for effective use of this practice. For instance, rye (*Secale cereale* L.) and certain cultivars of triticale (*Triticosecale rimpaui* Wittm.) are resistant. Oat is resistant to Australian populations of '*H. avenae*' and to CCNs in several Mediterranean countries, but it is susceptible to *H. avenae* in northern Europe (McDonald and Nicol 2005). Moreover, resistant cultivars from one region may be exposed to mixtures of species in other regions, as exemplified in Israel by oat cultivars that are resistant to *H. avenae* and susceptible to *H. latipons* (Mor et al. 1992).

Resistance plus tolerance. Additional production efficiency and profitability will be attained by improving the combined level of nematode resistance plus tolerance in small grain cultivars produced on highly infested fields. However, most wheat breeding programs in states or regions harboring CCNs are not yet breeding for resistance or selecting for tolerace to CCNs. Reasons include a lack of understanding of the importance of the issue, limited financial, technical, or institutional support for this demanding disciplinary research, lack of field test sites with adequate uniformity of infestation, or lack of field sites that do not also subject plants to significant impacts by other parasitic nematode species or soilborne fungal pathogens. Development of additional and improved molecular markers will allow more rapid integration of resistance into tolerant cultivars. Genetic transformations may also aid in the development of nematode-resistant germplasm. Molecular techniques will also facilitate greater precision in studies of resistance mechanisms (Andrés et al. 2001a; Montes et al. 2003, 2004; Seah et al. 2000).

Nematicides. Application of a chemical nematicide has repeatedly been shown to be capable of providing effective control of CCNs in wheat (Brown 1987; Dababat et al. 2014; Fard et al. 2015; King et al. 1982; Riley and Qi 2015; Singh et al. 2009; Smiley et al. 1994, 2012; Wu et al. 2007). Aldicarb is a preplant applied nematicide that has been very effective and used most frequently. However, after a period of repeated use to control *H. avenae* in irrigated wheat in Saudi Arabia, aldicarb became ineffective as it became subjected to an enhanced rate of microbial deactivation (Dawabah et al. 2015). In the region where aldicarb had become compromised, multiple foliar

applications of oxymyl were successful in reducing the density of *H. avenae* and increasing the yield of wheat. However, the yield response to oxyml was not superior to that from growing wheat after two crops of alfalfa (Dawabah et al. 2015). Oxamyl was therefore recommended for periodic use only as a component of a well-defined integrated CCN management system that included crop rotation, management of soil fertility and field sanitation, and rotating the chemistry of nematicides. Economic constraints and environmental and safety concerns associated with early generation nematicides have eliminated them as a viable alternative for use by most farmers. In rainfed agricultural systems, effective and economical chemical or biological nematicides are not currently available to manage CCNs.

Efforts to develop nematicides that may provide effective control are continuing. Abamectin (mixture of avermectin B_{1a} and avermectin B_{1b}) was evaluated as a seed treatment to manage CCNs but benefits were negligible to marginal in wheat fields infested with *H. avenae* in Israel (Oka et al. 2009) and the U.S.A. (Smiley et al. 2012). However, grain yields were significantly increased when a higher rate of abamectin was applied in furrows below the seed in China (Zhang et al. 2017). A formulation of *Bacillus firmus* spores applied as a biological seed treatment in the U.S.A. also had negligible effect on grain yield and postharvest density of *H. avenae* (Smiley et al. 2012). Foliar applications of spirotetramat, a broad-spectrum insecticide/nematicide with both phloem and xylem mobility (Safferling 2008), reduced the postharvest density of *H. avenae* by up to 78% but did not reduce the amount of knotting on roots or improve the yield of spring wheat (Smiley et al. 2011a). Cui et al. (2017) found that treating winter wheat seed with a mixture of either methylene (bis)thiocyanate plus thiamethoxam, or of fipronil plus chlorpyrifos, increased wheat yield and reduced the number of cysts in soils that were infested by Chinese populations of either '*H. avenae*' or *H. filipjevi*. Different formulations of these and other modern nematicides warrant further evaluations in fields infested with CCNs.

Natural biological suppression. Dababat et al. (2014) and Tian et al. (2007) reviewed reports in which CCN populations were reduced to nondamaging densities by fungal and bacterial parasites of eggs and J2s. These organisms reduced the number of cysts formed, the capacity of eggs to molt, and the vitality of J2s. At present, however, it has not been demonstrated that this phenomenon can be effectively manipulated as a practical management strategy in most regions of the world. There have apparently been no successful commercial applications of biological suppression of CCNs.

Avoidance. It is typically not possible to adjust planting times of crops so that fewer J2s are in soil when the crop is planted. However, a management option that can be effective in cool, temperate regions, where the hatch occurs mostly during the spring, is to plant winter cereals during the autumn to provide deeply penetrating roots before the peak rate of hatching. While less efficient than crop rotation or genetic resistance, this strategy can be a useful component of an integrated pest management approach to CCN control.

Masking. The greatest crop loss from CCNs is measured when nutrients or water become limiting for maximum plant growth potential at any point during the growing season. Crop damage is therefore minimized by supplying optimal plant nutrition and, where possible, supplemental water during intervals of drought (Fard et al. 2015; Singh et al. 2009).

Trap crops. *Heterodera* populations can also be reduced by planting a susceptible host as a trap crop prior to the major hatching period, thereby encouraging invasion of roots in plants that are then killed before newly developing white females can produce viable eggs (Stone 1961).

Future Directions With Molecular Approches

Adoption of molecular methods by commercial nematode diagnostic labs. Just as marker-assisted selection can speed up identification of resistance genes in germplasm, commercial diagnostic labs have moved from conventional soil sampling tests for nematode identification and population estimates to molecular testing methods. Similarly, these can be more cost-effective when many samples have to be processed. Molecular diagnostics can be used to identify different species present in a single sample faster than using conventional morphometric identification of nematodes, and this can also help in planning and deploying appropriate control strategies for effective nematode control.

PreDicta B, discussed previously, is one such commercial DNA-based soil testing service developed to detect and quantify Australian populations of '*H. avenae*' for growers in cropping regions in southern Australia by the Molecular Diagnostic Centre (MDC) of the South Australia Research and Development Institute (SARDI). The MDC offers commercial diagnostic services to the grain and potato industries through the PreDictaB and PreDictaPt tests. These services report on detection and quantification of a range of plant-parasitic nematodes and plant-pathogenic fungi relevant to the respective industries. The commercial assays are done by qPCR and most are species-specific. For nematodes, eight commercial assays are provided for growers. These include assays for '*H. avenae*,' root lesion nematodes *P. neglectus*, *P. thornei*, *P. quasitereoides*, and *P. penetrans*, and root knot nematodes *M. fallax*, *M. hapla*, and the *M. javanica* complex (*M. javanica*, *M. incognita*, and *M. arenaria*). Additional assays for other plant-parasitic and free-living nematode species are under development and are currently in use for research only, but with the potential for commercial development. The results are provided on a risk-level basis (high, medium, low levels of infestation), with advice on what control methods need to be undertaken depending on the levels found (McKay et al. 2008; Kathy Ophel-Keller, DMC-SARDI, personal communication, 2016).

At least two commercial nematode diagnotic laboratories in the western U.S.A. now also provide molecular services to distinguish between the two species (*H. avenae* and *H. filipjevi*) known to be present in wheat and barley fields of the northwestern states (Smiley 2016; Smiley, personal communication). As in Australia, these labs also identify locally important species of *Pratylenchus* and of soilborne fungal pathogens of wheat and other crops.

Effectors. The term "effector" can be defined as a protein, peptide, or other small molecule that can modify host cell structure or function (Hogenhout et al. 2009). Effectors or the genes encoding them are required for successful nematode parasitism and are the foci of major studies in which the aim is to understand their mechanism of action and whether they could be targets for nematode control. Most nematode effector genes are expressed and translated in the dorsal or subventral gland cells of the nematode and are delivered through the stylet into host plant cells. Effectors can also be secreted from the amphids or possibly via the cuticle. Effectors of cyst nematodes generally fall into the categories of cell wall modifying enzymes, those that help the nematode to evade or avoid host defenses, and those that are required for the induction or maintenance of syncytia (or similar feeding cells for other sedentary endoparasites) (Jones 1981; Jones and Dropkin 1975; Jones and Goto 2011). Although cyst nematode J2s migrate intracellularly using their stylet to cut through cell walls, cell wall modifying enzymes may also be employed at this stage, and they may also be involved in softening or modifying cell walls during the induction of syncytia (Yang et al. 2017).

Plants can recognize and initiate defense mechanisms against invading pathogens at various levels. Signals that plant cells can respond to include damage-associated molecular patterns (DAMPS), such as cell wall oligosaccharides generated by cell wall modifying enzymes, or specific nematode effectors (pathogen associated molecular patterns, or PAMPS). The nematode must counteract host defense responses, and in some cases there is good evidence that effectors secreted by nematodes can counteract plant defense mechanisms by suppressing or even mimicking plant proteins or peptides to bypass or negate the recognition step (Gheysen and Mitchum 2011).

Identification of nematode effectors has usually involved either making an EST library from gland cells (Gao et al. 2003; Huang et al. 2003), or next generation sequencing and bioinformatic analyses that identify proteins or peptides with features such as a signal peptide for secretion, but no transmembrane domain (Fosu-Nyarko et al. 2016; Kumar et al. 2014). This provides a list of potential effectors, and further evidence that they may be secreted can be obtained by in situ hybridization showing that expression is limited

to the gland cells. In a comparison of transcripts sequenced from secretory glands, unique sequences were identified in potato cyst nematode (*G. rostochiensis*) and soybean cyst nematode (*H. glycines*), which indicate a different repertoire of effector proteins when compared with migratory nematodes (Gao et al. 2003; Maier et al. 2012).

In a recent detailed genomic study on the cyst nematode *G. rostochiensis* pathotype R01, Eves-van den Akker et al. (2016) found that horizontal gene transfer appeared to have contributed 3.5% of the predicted genes of this species, of which about 8.5% were thought to function as effectors. More than a third of the effector genes were clustered in 'effector islands' in the genome. Upstream of the genes encoding the majority of effector 'families,' they identified a dorsal gland promoter element motif termed a 'DOG box,' and this sequence could be used to predict novel effectors. We can predict that future in-depth genomic studies of CCNs will generate similar data on effector repertoires and functions, but also highlight specific differences between the cyst nematode genera that will relate factors such as host range and specific lifestyles.

Since there has been less research undertaken on effectors of CCNs, to a certain extent, it is necessary to extrapolate from findings for other cyst nematodes such as that for *G. rostochiensis* (Eves-van den Akker et al. 2016). Many effectors are likely to be common to other cyst nematodes, whereas others may be unique to CCN species. For example, genes encoding cell wall modifying enzymes include β-1,4-endoglucanases, pectate lyases, expansins, and cellulose-binding protein have been identified in a range of cyst nematodes (Cotton et al. 2014; Fosu-Nyarko et al. 2016; G. Wang et al. 2014).

Thus far, effectors identified and characterized in cyst nematodes, which modify host physiology and defense mechanisms, include CLAVATA3/ESR (CLE)-like proteins, which affect meristem differentiation, and HgGLAND18, which suppresses plant innate immunity

Table 2. Some studies in which gene silencing (RNAi) has been applied to cyst nematodes. Results are from in vitro soaking experiments except where marked with asterisk (*) which are from HIGS.

Gene description	Gene name or symbol	Species	Phenotype/effect	Reference
C-type lectin	*hgctl*	*H. glycines*	Reduced transcript level. 41% less infection.	Urwin et al. (2002)
Major sperm protein	*msp*	*H. glycines*	Reduced transcript level	
Cysteine proteinase	*Gpcp-I*	*G. pallida*	A shift in sexual fate (21% less females)	
	Hgcp-I	*H. glycines*	A shift in sexual fate (25% less females)	
Multiple functions	Aminopeptidase	*H. glycines*	61% reduced infection on soybean roots	Lilley et al. (2005)
Cell wall degrading enzyme	B-1,4, endoglucanase	*G. rostochiensis*	Reduced infection	Q. Chen et al. (2005)
Amphid secreted protein	*ams-1*		Reduced root invasion	
Major sperm protein	MSP	*H. glycines*	68% reduction in cyst formation	Steeves et al. (2006)
Ribosomal protein	*Hs-rps-23*	*H. glycines*	Lethal	Alkharouf et al. (2007)
Pharyngeal gland cell proteins	*hg-eng-1*, *hg-syv46*	*H. glycines*	Reduced plant infection establishment	Bakhetia et al. (2007)
	hg-gp, *hg-cm*, *hg-pel*		Increased male to female ratio	
FMRFamide-like neuropeptide	*Gp-flp-1*, *Gp-flp-6*, *Gp-flp-12*, *Gp-flp-14 and Gp-flp-18*	*G. pallida*	Defective locomotion, motor dysfunction and increased neuronal RNAi	Kimber et al. (2007)
Esophageal gland protein	*Hspel2*	*H. schachtii*	50% less infection and decreased transcript level	Vanholme et al. (2007)
Esophageal gland protein	*Hg-pel-1*	*H. glycines*	203-fold decrease in transcript level	Sukno et al. (2007)
	Hg-4E02		51-fold decrease in transcript level	
Dorsal pharyngeal gland proteins	*Dg13*	*H. glycines*	High male to female ratio	Bakhetia et al. (2008)
	Dg14		High male to female ratio	
	Dg21		High nematode establishment and male to female ratio	
Ribosomal protein 3a*	*rps-3a*	*H. glycines*	87% reduced cyst formation	Klink et al. (2009)
Ribosomal protein 4*	*rps-4*		81% reduced cyst formation	
Spliceosomal SR protein*	*spk-1*		88% reduced cyst formation	
Synaptobrevin*	*snb-1*		93% reduced cyst formation	
Ubiquitin-like protein*	*4G06*	*H. schachtii*	23 to 64% reduction in developed females	Sindhu et al. (2009)
Cellulose binding protein*	*3B05*		12 to 47% reduced infection	
SKP1-like protein*	*8H07*		>50% reduced infection	
Zinc finger protein*	*10A06*		42% reduced infection	
Nematode secreted peptide*	Hssyv46	*H. schachtii*	36% reduced cyst formation	Patel et al. (2008)
Nematode secreted peptide*	Hs5d08		20% reduced cyst formation	
Nematode secreted peptide*	Hs4e02		20% reduced cyst formation	
Nematode secreted peptide*	Hs4F01		55% reduced cyst formation	
Parasitism effector*	(*30C02*)	*H. schachtii*	92% reduced cyst formation	Hamamouch et al. (2012)
FMRFamide-like neuropeptide	*Gp-flp-12*	*G. pallida*	Decreased transcript level and inhibition of migratory ability	Dalzell et al. (2010)
FMRFamide-like neuropeptide	*Gp-flp-32 and Gp-flp-32R*	*G. pallida*	55% and 75% reduction in transcript respectively	L. E. Atkinson et al. (2013)
Hypervariable extracellular effector	*Gp-hyp*	*G. pallida*	50 to 60% reduction in nematode infestation	Eves-van den Akker et al. (2014)
Expansin-like protein	*HaEXPB2*	*H. avenae*	74% reduction in transcript level	Liu et al. (2016)
			53% reduction in nematodes in roots	
FMRFamide-like neuropeptide	*FLP-4*	*H. avenae*	Up to 40% reduced survival of nematodes	M. Zheng et al. (2015)
	Calpain homolog			

(Noon et al. 2016; J. Wang et al. 2011; X. Wang et al. 2005). These mimic plant CLE-like peptides and interact with the plasma membrane to form and maintain feeding cells (Y. Guo et al. 2011). Effectors like Hs 19C07 and chorismate mutase modify host hormonal balance (Doyle and Lambert 2003; Lee et al. 2011). Recently, C-terminally encoded peptides (CEPs) secreted by *Rotylenchulus reniformis* have been demonstrated to upregulate nitrate transporters and limit the size of syncytial feeding sites as an adaptation to obligate parasitism (Eves-van den Akker et al. 2016). The SPRYSEC (SPRY-domain containing) effectors initially identified in potato cyst nematode are proposed to be involved in suppression of plant innate immunity by interacting with plant protein complexes (Cotton et al. 2014; Diaz-Granados et al. 2016). Although bioinformatics analyses indicate the presence of a set of effectors in CCNs similar to those in other phytonematodes, limited functional analysis has been undertaken for CCN species, except for effectors such as β-1,4-endoglucanases, cathepsin S-like cysteine proteinase, expansin, and annexin (C. Chen et al. 2015; Liu et al. 2016; Long et al. 2012, 2013; Thakur et al. 2014).

HATdb: transcriptomic repository for CCN. Transcriptome data for J2s and adult stage *H. avenae* is now publically available (HATdb; http://insilico.iari.res.in) and this is a useful genetic resource available for this species complex that will help contribute to future molecular studies and functional analysis of genes and the mechanisms involved in host interactions (Kumar et al. 2014). Transcriptomic analysis of the available CCN data identified 39 candidate secreted effectors, of which 12 were unique to CCNs (Liu et al. 2016). RNA-seq analysis of a Chinese population of '*H. avenae*' during plant infection identified 122 unigenes with signal peptides, and included effectors similar to *flp* and *clp*. Up to 85% mortality of treated nematodes occurred when *flp* and *clp* were targeted by RNAi with 24 h soaking in siRNAs homologous to target genes (M. Zheng et al. 2015). Advances in understanding molecular processes in CCNs will accelerate as the technologies of next generation sequencing advance. RNA-seq and deep sequencing of small RNAs can provide data on populations of siRNAs generated in transgenic plants, and whether there is a correlation with effectiveness of target gene down-regulation with specific siRNA sequences and amounts. Such data will help in designing the most effective sequences to silence target genes.

Gene silencing. Plant-parasitic nematodes can take up macromolecules from the plant cell cytoplasm when feeding, but initially it was thought that J2s would not ingest external solution when outside a host plant. It is now evident that J2s can take up external solutions containing dsRNA when they are 'soaked' or incubated in a solution containing dsRNA (Urwin et al. 2002), with some studies showing that the addition of neurostimulants like octopamine to the soaking solution can enhance the uptake of solution and thus dsRNA by plant nematodes. This finding enabled study of gene silencing through in vitro soaking or feeding of dsRNA. As a result, many effectors and other genes involved in biochemical and developmental processes have been targeted in functional studies to determine how their down-regulation can affect nematode parasitism and viability. In general, in vitro studies involve soaking of motile nematodes in dsRNA solutions for up to 24 h, followed by determining phenotypic effects (e.g., reduced motility or rigidity, aberrant behavior, reduced attraction to roots, reduction in migration in roots), measuring changes in the expression of the targeted gene, and quantifying the reduction in reproduction when treated nematodes are used to infect host plants (Fosu-Nyarko and Jones 2016; Tan et al. 2013). Such studies target genes involved in parasitism, development, locomotion, and important biological pathways. Table 2 summarizes some gene silencing studies that have been undertaken for cyst nematodes, with the main focus initially on the soybean cyst nematode, but now with major studies on the potato cyst nematode *G. rostochiensis*. This table includes data from experiments of dsRNA soaking of nematodes and those of host-induced gene silencing (HIGS) where dsRNA is delivered to target genes in the nematode through transgenic plants. To date, there have been relatively few studies on gene silencing for CCNs, partly because these species are relatively difficult to handle and culture, and because genomic or transcriptomic data were rather limited.

Host-induced gene silencing (HIGS). Conventional breeding for pest resistance either requires large scale screening of genotypes to identify suitable resistance genes and multiple generations of plants to incorporate the resistance, or more recently, the application of marker-assisted selection to combine validated resistance genes. Transgenic plants provide an attractive alternative strategy to develop new forms of resistance, by transfer of functional resistance genes directly, or by developing 'synthetic' resistance genes that interfere with vital processes in the nematode-plant interaction.

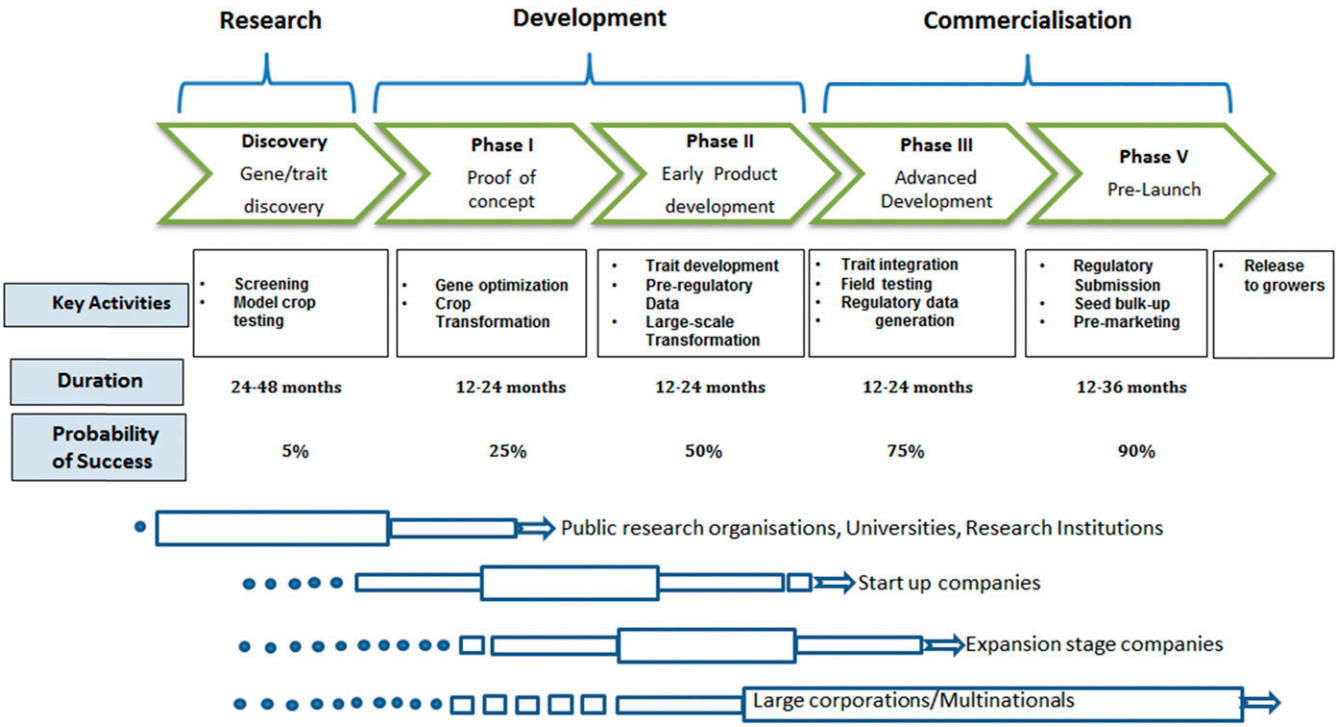

Fig. 16. Pathway to commercialization of a biotechnology trait conferring resistance to plant parasitic nematodes (modified from Fosu-Nyarko and Jones 2015).

Studies in which transgenic plants have been generated with various forms of synthetic resistance indicate that varying but significant levels of reduction in nematode cyst development can be achieved (e.g., Table 2). Many such studies have focused on root knot nematodes because of their broad host range and relative amenability to culture, but data are now emerging on applying this approach to cyst and root lesion nematodes (Samac and Smigocki 2003; Sindhu et al. 2009; Tan et al. 2013; Walawage et al. 2013), and to a range of other species such as *Rotylenchulus reniformis*, *Radopholus similis*, and *Bursaphelenchus xylophilus* (H. J. Atkinson et al. 2004; Tripathi et al. 2015; Urwin et al. 2000).

In most of these studies, the aim has been to achieve in planta knockdown of vital genes in nematodes to reduce their ability to parasitize the plant, by interfering with attraction to host roots, migration, development, or reproduction (reviewed by Dutta et al. 2015). For example, some data indicates that more than a 90% reduction can be achieved in cyst development for *H. schachtii* on *Arabidopsis* (Fosu-Nyarko and Jones, unpublished), and a reduction in cyst formation of up to 94% was found after silencing the synaptobrevin (*snb-1*) gene in soybean cyst nematode through transgenic roots (Klink et al. 2009). An important consideration in the choice of target gene sequences to use is to undertake bioinformatic analyses to choose sequences with no off-target effects. In addition, this approach can be used to silence genes in plants that respond to nematode-secreted effectors. For example, the CLE-like nematode effectors are recognized by plant CLE receptors that are required for syncytium development in plants (Replogle et al. 2011, 2012). Knockdown of these receptors in soybean roots resulted in reduced soybean cyst nematode infestation (X. Guo et al. 2015). However, reducing expression of plant genes involved in the nematode-plant interaction is likely to be detrimental to plant crops, since the plant genes will have a functional role in the plant, and interfering with this could reduce field performance.

Transgenic resistance from expression of cystatins and peptides that interfere with root invasion. RNAi is not the only transgenic approach to confer resistance to nematode pests. Other well-documented approaches include expression of an antifeedant cysteine proteinase inhibitor in plants to interfere with nematode digestion, and an antiroot invasion nonlethal synthetic peptide (H. J. Atkinson et al. 2004; Tripathi et al. 2015). Cysteine proteinases are major digestive enzymes of many nematodes and small protein inhibitors (cystatins) from plants have mediated nematode resistance when expressed in several crops (e.g., tomato, rice, potato, banana, plantain). Cystatin is a normal part of the human diet, is not allergenic, and is rapidly degraded by gastric juices, indicating there is no biosafety issue: the peptide is too small to be allergenic and is degraded in the human small intestine. Similarly, there is no evidence for environmental safety concerns (Tripathi et al. 2015).

Combining different resistance strategies. It is normally the case that HIGS for nematode resistance using RNAi does not confer 100% resistance to nematodes. There are a number of possible explanations for this. The first is the specific target gene chosen; is its expression vital, is it unique, or does it belong to a multigene family? Other factors that affect the extent of gene silencing include where and when the target gene is expressed, the specific dsRNA sequence chosen, the stability of target mRNA or encoded protein, the presence of a 'recovery' phenomenon, and experimental variables such as the vigor of the nematodes treated, and differences in the RNAi machinery (e.g., for systemic spread of the silencing signal) between genera or species (Jones and Fosu-Nyarko 2014). One way to improve transgenic resistance to nematodes is to combine two different modes of resistance, such as a cystatin and anti-invasion peptide as described above (Tripathi et al. 2015), and a combination of RNAi and one of the latter approaches may both give more effective and more durable transgenic resistance. Durability of resistance to pests and diseases is an important consideration, and where RNAi-based traits have already been deployed commercially, the expression of the trait seems to be consistent in following generations (Waltz 2015a, b).

Commercialization of transgenic nematode resistance traits. The general approach to commercializing a transgenic nematode resistance trait has been discussed by Fosu-Nyarko and Jones (2015). An overview is presented in Figure 16. Basic discovery research is undertaken, and if that is promising the technology moves from the discovery phase to proof-of-concept, early and advanced stages of product development, to a prelaunch phase, and finally to commercial release to growers. The activities to be undertaken in each phase are indicated in Figure 16, with time scales and probability of success. The discovery or trait moves along the pipeline often via a 'start-up' or 'expansion stage' company, until the trait is either licensed to a large corporation or multinational company or the company is bought by such companies for advanced development, prelaunch, and commercial release to growers.

The added value of a nematode resistance trait will be a major determinant on whether it will be deployed commercially. However, for CCN resistance, this decision will also be influenced by international factors, such as the acceptance of transgenic wheat or barley locally or in importing countries: thus both national regulations and acceptance of genetically modified crops will also influence commercial decisions to develop transgenic CCN resistant wheat or barley.

Nontransgenic delivery of gene silencing triggers for nematode control. Although at present about 10% of the world's crops are transgenic, the costs and issues that must be overcome to deploy any form of transgenic crop resistance to nematodes, and to CCN in particular, are not insubstantial. Hence there is current research aimed at delivering dsRNA in spray form ('ectopic delivery') rather than by transgenic plants. This strategy requires low cost production of dsRNA sequences, methods to stabilize them for field delivery, uptake of dsRNA by leaves, its systemic basipetal movement through plants to roots, and uptake by nematode on feeding (Fosu-Nyarko and Jones 2015; Naz et al. 2016).

If the technical aspects of ectopic delivery of dsRNA can be overcome in a cost-effective manner, this could bypass the issues of RNAi-based transgenic nematode control. In addition to ectopic delivery of RNAi, gene silencing technology can be used to determine targets for new nematicides; this direction, called genome-enabled novel chemical nematicides (Fosu-Nyarko and Jones 2015), is being followed by Danchin et al. (2013).

Genome editing. Genome editing is an exciting and powerful new alternative to RNAi for gene silencing. It is essentially targeted mutagenesis, in which mutations can be induced in target genes in a nontransgenic manner termed nonhomologous end joining (NHEJ). Alternatively, by insertion of oligonucleotide sequences with ends homologous to each side of the cut site, specific additions to a sequence can be made, termed homologous end joining (HEJ). The technology has passed through a number of iterations, with the use of a 'guide' RNA sequence directing a dsDNAse enzyme (CRISPR-Cas9) to cut a target sequence at a specific site now most widely used (Lozano-Juste and Cutler 2014). In NHEJ, the cell repair enzymes frequently make a mistake in joining the ends, resulting in a targeted mutation or total inactivation of the gene. When a cassette consisting of a selectable marker gene with CRISPR and Cas 9 is used to select edited cells for regeneration to plants, the site of gene editing will be elsewhere in the genome from the editing cassette. Hence, for cereals, it is possible to generate edited genotypes that no longer contain an editing cassette. This can be achieved by making a cross with the original (or another) genotype, and identifying genotypes with the edited gene but without the introduced cassette.

A plant with a targeted mutation but lacking any introduced DNA may well be regulated as non-GM. However, whereas in HIGS the silencing trigger is delivered from the plant to the pest, it is not possible to deliver a (nontransgenic) genome editing signal in this way. To apply this technology to nematode control reverses some of the current strategies, in that it will be necessary to find a nonvital host plant gene whose expression is needed for nematode parasitism, and edit it to be nonfunctional. The recent availability of genome sequences for hosts of CCNs could well lead to the development of nontransgenic cereals genome edited for resistance (Mayer et al. 2014; International Barley Genome Sequencing Consortium 2012).

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