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Investigation of resistance to *Pratylenchus penetrans* and *P. thornei* in international wheat lines and its durability when inoculated together with the cereal cyst nematode *Heterodera avenae*, using qPCR for nematode quantification

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Abstract The root lesion nematodes *Pratylenchus penetrans* and *P. thornei* cause high yield losses in rain-fed wheat fields in Morocco, as well as worldwide. Growing resistant varieties is one of the most effective methods for controlling nematodes. Therefore, a collection of 14 lines of spring wheat and 11 lines of winter wheat (*Triticum aestivum* and *T. durum*), provided by CIMMYT, were screened for resistance to *P. penetrans* and *P. thornei* in tubes ($15 \times 20 \times 120$ mm^3) under greenhouse conditions. The resistance level was evaluated based on the numbers of nematodes extracted from roots and soil 9 weeks after infestation. Three lines (L9, L12 and L13) were found moderately resistant (reproduction factor < 1) to *P. thornei* and one of these (L9) was also moderately resistant to *P. penetrans*. To investigate the durability of this resistance, we co-inoculated juveniles of *Heterodera avenae*, a cereal cyst nematode widely present in Moroccan wheat fields, and assessed the reproduction of both lesion nematodes on the three lines. Our results showed that the lines L9, L12, L13 remained moderately resistant in the presence of *H. avenae*. Moreover, the numbers of *Pratylenchus* spp. were generally lower when plants were co-inoculated with *H. avenae*. The number of *P. penetrans* or *P. thornei* were determined visually using a microscope and with a qPCR assay. The counts with qPCR were even lower than microscopic counts. These findings on resistance are promising but the field performance of these lines against root lesion nematode attacks should be evaluated.

Keywords Co-inoculation · Durability · qPCR · Screening

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Introduction

Root lesion nematodes (RLN) are considered among the most important groups of plant-parasitic nematodes attacking cereals on a worldwide basis (Smiley and Nicol 2009). They comprise a group of closely related *Pratylenchus* species that have caused economic losses for wheat crops in North Africa, Australia, the United States, and parts of Europe (Nicol et al. 2003; Thompson

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et al. 2008; Vanstone et al. 2008; Smiley and Nicol 2009). Eight species of RLN are known to be parasitic on small grain cereals. Of these, *P. thornei* and *P. neglectus* are considered the most economically important parasites of wheat, and are found in all wheat-growing regions of the world (Nicol and Rivoal 2008; Thompson et al. 2008). Economic losses to wheat production in Australia due to *P. thornei* have been estimated at $AU 69 million/year and $AU 190 million/year in the northern and southern/western grain growing regions, respectively (Thompson et al. 2008; Vanstone et al. 2008).

RLN feed, migrate and reproduce inside the host's root cortex, resulting in lesions and debilitated root systems that are inefficient in taking up nutrients and water from the soil. Evidence of the belowground pathogenic activity of the nematodes can be seen as aboveground symptoms of chlorosis and wilting of leaves, and reduced tillering (Moens and Perry 2009). In Morocco, *Pratylenchus* spp. are the most prevalent group of nematodes in different cereal growing regions followed by cyst nematodes of the *Heterodera avenae* group (Mokrini et al. 2009, 2012). The results of a survey of wheat producing regions in Morocco (Mokrini et al. 2012) demonstrated *P. penetrans* to be the most abundant and widespread species. It was recovered from 70% of the soil samples and its population densities varied between 32 and 123 nematodes 100 g^{-1} of soil and between 67 and 102 nematodes 10 g^{-1} of root of wheat. The related species *P. thornei* is also present in Morocco (19% of sampled localities) and can be found along with *P. penetrans* in a same field (Mokrini et al. 2012).

So far, many attempts have been made to control RLN in cereals around the world, including cultural practices, chemical control, and development of resistant varieties (Smiley and Nicol 2009; Dababat et al. 2011). The use of resistant accessions is considered the most economically feasible and environmentally sustainable method. Tolerant cultivars suffer little yield reduction even when their roots are invaded by nematodes, whereas resistant cultivars reduce the rate of nematode multiplication in the roots (Roberts 2002). Several wheat accessions resistant against *P. thornei* have been identified (Vanstone et al. 1998; Thompson et al. 1999; Toktay et al. 2012; Dababat et al. 2016). For example, the Iraqi landrace AUS4930 was found to be resistant to the root lesion nematode *P. thornei* as well as to the cyst nematode *H. australis* (Australian pathotype Ha13) (Nicol et al. 2009). The soil-borne pathogen program of CIMMYT (International Maize and Wheat Improvement Center)-ICARDA (International Center for Agricultural Research in the Dry Areas) annually screens about 1000 accessions from the International Winter Wheat Improvement Program (www.iwwip.org) for resistance to multiple diseases and pests (including *P. thornei* and *P. neglectus*) under growth room, greenhouse, and field conditions of various locations in Turkey (Dababat et al. 2015). However, effective nematode management in agricultural fields is constrained by the coexistence of a wide range of taxonomically diverse plant-parasitic nematodes (Stetina et al. 1997). This is particularly challenging when using resistant cultivars because most nematode resistance is targeted at one species, while other species that parasitize the resistant host are present in the field (Bradley and Duffy 1982). Competition between different soil-borne parasitic nematodes associated with economic damage has been reported for several crops (Yang et al. 1976; Lasserre et al. 1994; Moens and Perry 2009; Melakeberhan and Dey 2003). Gay and Bird (1973) found that the root-knot nematode *Meloidogyne incognita* was inhibited by the presence of *P. brachyurus* on cotton. Brinkman et al. (2005) reported that *P. penetrans* suppressed the abundance of *H. avenae* on dune grass *Ammophila arenaria*. Rivoal et al. (1995) recorded suppression of *P. neglectus* in the presence of *H. avenae* on oats. Lamberti et al. (2001) reported that *M. incognita* in olive suppressed reproduction of *P. vulnus*. Melakeberhan (1998) showed that infections by *M. incognita* and *P. penetrans* can affect resistance of soybean cultivars used to manage *H. glycines*. Interspecific competition has been suggested between *P. coffeae* and *M. exigua* on coffee in Costa Rica (Bertrand et al. 1998) and Guatemala (Hervé et al. 2005). Surveys of cereal fields in the major wheat and barley cultivating areas of Morocco showed that both *P. thornei* and *P. penetrans* are often present together with *H. avenae* (Znasni 2003; Mokrini et al. 2009, 2016). Similarly, Şahin et al. (2009) reported that *P. thornei* and *P. neglectus* can be present together with *H. filipjevi*. Thus, it is important to know when introducing or breeding for new cultivars if infection by both *Pratylenchus* species and *Heterodera* species influences the resistance level to RLN.

The correct identification and quantification of nematode species is a fundamental step in control strategies. Recently, quantitative PCR (qPCR) strategies have been developed for *P. penetrans* (Mokrini et al. 2013),

P. thornei (Mokrini et al. 2014), and *H. avenae* (Toumi et al. 2013), providing a rapid alternative to microscopic identification. Yan et al. (2008) reported differences in the estimates of *P. neglectus* and *P. thornei* obtained by microscopy versus qPCR. This discrepancy was attributed to the difficulty in identifying and quantifying both *Pratylenchus* species using traditional microscopy-based methods and also to the uneven distribution of nematodes in soil. However, Mokrini et al. (2014) concluded that there were no differences between the number of *P. thornei* counted using a microscope and numbers estimated by the qPCR assay from a suspension of cultured nematodes.

In view of these issues, the objectives of this study were to: (1) identify wheat lines with resistance to root lesion nematodes *P. thornei* and *P. penetrans* in pot experiments under greenhouse conditions; (2) investigate the effects of co-inoculating *H. avenae* on the reproduction of *P. penetrans* and *P. thornei* on resistant wheat lines under controlled conditions, hence on the stability of the resistance when more than one nematode species are present; and (3) compare the enumeration of vermiform stages of *P. thornei* and *P. penetrans* using traditional visual counting through microscopy and qPCR.

Material and methods

Screening for resistance against the root lesion nematodes *P. thornei* and *P. penetrans*

Plant material

Twenty-five wheat lines provided by CIMMYT were screened for resistance against a population of both *P. thornei* and *P. penetrans* (experiments 1 and 2, respectively). The collection comprised 14 spring wheat lines and 11 winter wheat lines (*Triticum aestivum* and *T. durum*) (Tables 1 and 2). Durum wheat cultivar Ourgh, susceptible to both *P. thornei* and *P. penetrans,* was used as a control. Two independent experiments were conducted to phenotype the collection against *P. thornei* (exp. 1) and *P. penetrans* (exp. 2) under greenhouse conditions. Surface-sterilized seeds of each line were placed on moistened filter paper in sterilized Petri dishes to enhance germination. Seeds were germinated at 23–25°C for 3–4 days. Winter wheat seeds had first been vernalized at 5°C for 3 weeks. One seedling of each line with 3 seminal roots was transplanted into a plastic folding tube ($15 \times 20 \times 120$ mm^3) filled with about 40 g of a mixture of sand, field soil, and organic matter (70:29:1 *v/v/v*). The field soil and sand had been sieved and sterilized at 100°C before use. For each line, 30 screening tubes (replicates) were divided equally into three pots (15 cm diameter), with the spaces around the tubes filled with sand to keep the tubes upright. Pots were arranged in a completely randomized design in a greenhouse at a temperature between 22 and 24°C. After nematode inoculation, plants were gently watered using an atomizer to provide adequate moisture to increase the efficiency of nematode penetration. Later, plants were watered whenever needed to keep the soil moist during the experiment. The same experimental protocol was used for both *P. thornei* and *P. penetrans*.

Nematode inoculum

Experiments were carried out using one population of each species, *P. penetrans* (PG18) and *P. thornei* (PZ2), collected from the Moroccan regions Gharb and Zaers, respectively (Mokrini et al. 2016). The nematode populations were maintained in vitro on carrot-disc cultures according to Moody et al. (1973). Nematodes were extracted by placing infested chopped carrot discs on Baermann funnels in a mist chamber (OEPP/EPPO 2013) for 3 days. Nematode suspensions, containing all vermiform stages, were prepared using tap water. One week after planting, each seedling was inoculated with a nematode suspension containing 400 individuals (all vermiform stages confounded) of either *P. thornei* or *P. penetrans*, which is an appropriate inoculum density for nematode screening in cereals (Keil et al. 2009; Toktay et al. 2012), corresponding with about 850 individuals per 100 cm^3 soil. Nematodes were applied with a pipette into three holes of 2 cm deep made at 0.5 cm distance from the stem base.

Assessment of resistance

Plants were harvested 9 weeks after inoculation and aboveground plant parts were removed. The sides of the plastic tubes were pressed to loosen the soil and ensure easy removal of the plants. Soil was then freed from the roots by shaking the plants gently and washing the roots from each plant. Soil and rinsing water were collected in a beaker. Nematodes were released from the roots by cutting the root system in 2-cm pieces and macerating them in water for 1 min at high speed in a

Table 1 Numbers of vermiform *Pratylenchus penetrans* per plant ($n = 30$) of different wheat lines, 9 weeks after inoculation with 400 vermiform stages of *P. penetrans* (exp. 1)

Code	Line	Wheat Type[a]	Root (Mean)	Root (Range)	Soil (Mean)	Soil (Range)	Total	Min-Max	Rf (Pf/Pi)	Reaction[b]
L1	6R (6D)	SW	261 g	203–290	379 k	350–460	640 ± 33.7	521–801	1.6	S
L2	FRAME	SW	198 i	165–231	522 j	385–632	720 ± 71.3	589–840	1.8	S
L3	SILVERSTAR	SW	523 b	480–571	997 c	796–1156	1520 ± 89.5	1287–1677	3.8	S
L4	VP5053	SW	453 c	390–497	907 d	652–986	1360 ± 68.6	1200–1528	3.4	S
L5	T-2003	SW	437 c	408–471	1243 b	1195–1289	1680 ± 35	1548–1796	4.2	S
L6	RAJ 1	SW	503 b	422–539	1497 a	1470–1534	2000 ± 32	1891–2126	5	S
L7	ID-2150	SW	209 i	171–242	791 ef	741–825	1000 ± 33.4	893–1130	2.5	S
L8	MILAN	SW	326 e	286–370	634 i	587–671	960 ± 30.2	885–1087	2.4	S
L9	AUS 4930.7/2 PASTOR	SW	98 k	71–124	262 l	220–320	360 ± 22.3	269–390	0.9	MR
L10	AUS GS50AT34/SUNCO	SW	201 i	167–240	439 j	382–511	640 ± 30.5	571–721	1.6	S
L11	VL411R	SW	232 h	183–270	608 i	421–657	840 ± 51.5	780–892	2.1	S
L12	CROC_1/AE.SQUARROSA (224)	SW	102 k	77–134	458 j	381–555	560 ± 48.5	519–661	1.4	S
L13	CROC_1/AE.SQUARROSA (224)	SW	283 f	241–317	357 k	311–432	640 ± 28	590–702	1.6	S
L14	VP1620	SW	204 i	170–240	636 i	590–681	840 ± 116	791–961	2.1	S
L15	F130 L1.12/ATTILA	WW	387 d	351–420	773 g	723–812	1160 ± 26.8	1002–1326	2.9	S
L16	SONMEZ	WW	178 j	127–242	822 e	780–898	1000 ± 46.5	927–1056	2.5	S
L17	CPI133859	WW	104 c	16–137	416 m	378–467	520 ± 35. 3	481–601	1.3	S
L18	CPI133872	WW	204 i	169–137	636 i	580–678	840 ± 38.5	791–902	2.1	S
L19	KATE A-1	WW	598 a	561–640	1202 b	1115–1245	1800 ± 37.7	1759–1902	4.5	S
L20	PRINS	WW	321 e	271–372	439 j	401–487	760 ± 29.4	744–802	1.9	S
L21	MIRZABEY2000	WW	309 e	266–365	691 h	641–735	1000 ± 33.8	956–1122	2.5	S
L22	AU/CO652337//2CA8–155/3/F474S1–1.1	WW	595 a	570–629	805 f	780–841	1400 ± 25.8	1321–1522	3.5	S
L23	F372	WW	384 d	322–451	856 e	801–923	1240 ± 31.2	1181–1382	3.1	S
L24	TAIKONG	WW	627 a	566–702	1501 a	1458–1589	2128 ± 36.7	1998–2320	5.3	S
L25	ZHONGYU	WW	523 b	408–611	923 d	845–1123	1446 ± 41.7	1234–1600	3.6	S
Ls	OURGH		395 d	376–482	895 d	812–976	1285 ± 29.9	1190–1367	3.2	S

[a] SW: Spring wheat, WW: Winter wheat

[b] R: Resistant, MR: Moderately resistant, S: Susceptible

Means with the same letter in the same column are not significantly different at $P < 0.05$, per Tukey's test. RF <1 = R; 0.5 < RF < 1 = MR; RF > 1 = S

Table 2 Numbers of vermiform *Pratylenchus thornei* per plant ($n = 30$) of different wheat lines, 9 weeks after nematode inoculation with 400 vermiform stages of *P. thornei* (exp. 2)

Code	Line	Wheat Type[a]	Root (Mean)	Root (Range)	Soil (Mean)	Soil (Range)	Total	Min-Max	Rf (Pf/Pi)	Reaction[b]
	6R (6D)	SW	140 k	91–183	460 g	406–503	600 ± 40	577–620	1.5	S
L2	FRAME	SW	300 g	266–361	1220 b	922–1303	1520 ± 78	1479–1602	3.8	S
L3	SILVERSTAR	SW	390 ef	289–432	530 f	477–581	920 ± 30	878–1001	2.3	S
L4	VP5053	SW	247 h	209–378	793 d	734–835	1040 ± 41	989–1191	2.6	S
L5	T-2003	SW	366 f	267–476	634 e	564–693	1000 ± 76	986–1020	2.5	S
L6	RAJ 1	SW	746 a	680–893	534 f	449–595	1280 ± 56	1202–1425	3.2	S
L7	ID-2150	SW	528 c	471–613	432 g	361–497	960 ± 54	891–1001	2.4	S
L8	MILAN	SW	610 b	566–673	1430 a	1377–1498	2040 ± 44	1901–2199	5.1	S
L9	AUS 4930.7/2 PASTOR	SW	84	56–140	156 k	83–223	240 ± 48	170–393	0.6	MR
L10	AUS GS50AT34/SUNCO	SW	147 jk	106–193	293 h	254–344	440 ± 37	370–563	1.1	MS
L11	VL411R	SW	319 g	270–372	641 e	578–699	960 ± 43	898–1042	2.4	S
L12	CROC_1/AE.SQUARROSA (224)	SW	173 j	134–237	107 l	70–143	280 ± 27	170–390	0.7	MR
L13	CROC_1/AE.SQUARROSA (224)	SW	156 jk	111–212	204 j	166–270	360 ± 36	277–524	0.9	MR
L14	VP1620	SW	211 i	170–256	789 d	731–882	1000 ± 37	941–1062	2.5	S
L15	F130 L1.12/ATTILA	WW	411 ed	371–455	429 g	355–479	840 ± 45	711–989	2.1	S
L16	SONMEZ	WW	433 d	386–478	327 h	291–377	760 ± 36	536–821	1.9	S
L17	CPI133859	WW	333 g	277–392	507 f	416–562	840 ± 41	790–910	2.1	S
L18	CPI133872	WW	211 i	165–264	789 d	722–845	1000 ± 35	885–1051	2.5	S
L19	KATE A-1	WW	283 g	222–345	237 i	173–290	520 ± 36	406–570	1.3	S
L20	PRINS	WW	174 j	143–241	306 h	243–352	480 ± 40	390–581	1.2	S
L21	MIRZABEY2000	WW	246 h	178–312	514 f	422–592	760 ± 48	663–820	1.9	S
L22	AU/CO652337//2CA8–155/3/F474S1–1.1	WW	423 d	361–487	897 c	853–945	1320 ± 39	1245–1563	3.3	S
L23	F372	WW	321 g	241–389	439 g	390–489	760 ± 52	604–811	1.9	S
L24	TAIKONG	WW	227 ih	162–376	293 h	209–379	520 ± 65	488–587	1.3	S
L25	ZHONGYU	WW	233 h	134–321	760 d	612–835	1093 ± 49	937–1123	2.5	S
Ls	OURGH		314 g	269–375	1490 a	1381–1557	1804 ± 78	1756–1980	4.7	S

[a] SW: Spring wheat, WW: Winter wheat

[b] R: Resistant, MR: Moderately resistant, S: Susceptible

Means with the same letter in the same column are not significantly different at $P < 0.05$, per Tukey's test. RF <1 = R; 0.5 < RF < 1 = MR; RF > 1 = S

commercial blender (Waring). Nematodes were extracted from this mixture and also, separately, from the soil of each tube using an automated zonal centrifuge (Hendrickx 1995). Vermiform stages of *P. thornei* or *P. penetrans* in the obtained nematode suspensions were counted using a stereomicroscope. When there were less than about 1000 individuals, nematodes were counted in four batches. For more than 1000 individuals per sample, a dilution was made and 1 ml of the nematode suspension was counted in three replications. The number of extracted nematodes per plant was then calculated. For the evaluation of the susceptibility of the wheat lines against *P. penetrans* and *P. thornei*, the reproduction factor Rf = Pf/Pi was calculated for each plant, where Pf = total number of nematodes from both soil and roots in each tube at harvest and Pi = initial number of nematodes inoculated in the tube (i.e. 400). Wheat lines were classified as completely resistant (R), when no nematodes in plant roots and soil were observed; as moderately resistant if Rf was lower than 1 ($0.5 < Rf < 1$); and susceptible if the Rf was higher than 1 (Toktay et al. 2012). Wheat lines with Rf <1 for *P. thornei* and *P. penetrans* were re-phenotyped, using the same methodology, for data confirmation.

Dual inoculation of *H. avenae* and *P. penetrans* or *P. thornei*

Nematode inoculum

The *P. thornei* and *P. penetrans* populations used in the screening described above were also used in dual inoculation experiments with *H. avenae*. *Heterodera avenae* cysts were obtained from soil samples collected from a field in Marchoch, Zaers region, Morocco. They were extracted from the soil using a sieving and flotation method (Shepherd 1986), then surface sterilized with 0.5% NaOCl for 10 min and finally rinsed several times in distilled water. Cysts were stored for 2 months in a refrigerator at 4°C before they were transferred to room temperature to enhance hatching (Dababat et al. 2014). The second-stage juveniles (J2) were obtained after incubating the cysts in water; hatched J2 that were at most 2 days old were used as inoculum.

Plants and inoculation procedure

The three wheat lines (L9, L12, and L13) and one line (L9) found to be moderately resistant against *P. thornei* and *P. penetrans,* respectively, were tested once more under the same conditions as the first experiments, though each seedling was placed in a conical screening tube (100 mm long × 15 mm diameter) instead of a plastic folding tube. Two experiments (experiment 3 with *P. thornei*, experiment 4 with *P. penetrans*) were conducted in a growth chamber set at 16 h of artificial light, 21°C, and 70% relative humidity.

In experiment 3, the lines L9, L12, and L13 were inoculated with: (a) 400 *P. thornei* (all stages combined); (b) 400 J2 of *H. avenae*; or (c) 400 *P. thornei* (all stages) + 400 J2 of *H. avenae*. Similarly, in experiment 4, line L9 was inoculated with: (a) 400 *P. penetrans* (all stages combined); (b) 400 J2 of *H. avenae*; or (3) a mixture of 400 *P. penetrans* + 400 *H. avenae.* The susceptible durum wheat cv. Ourgh (control) was inoculated with 400 *P. thornei* (exp. 3) or 400 *P. penetrans* (exp. 4). Ten tubes (replicates) per inoculum treatment were arranged in a completely randomized design in tube racks placed above a shallow dish of water. The tubes were submerged about 2 cm into the water during the weekends, allowing plants to receive water as needed. On week days, plants were watered as needed. Experiments 3 and 4 were conducted simultaneously.

Evaluation of resistance

For both experiments 3 and 4, shoots were removed 9 weeks after inoculation and cysts were extracted from soil on 200-μm sieves using the flotation and sieving method (Shepherd 1986). The roots of each wheat line were washed separately and the rinsing water was added to the beaker with the soil. Roots were visually checked for presence of cysts to ensure that all cysts were extracted. Nematodes inside the roots were released by cutting the root system in 2-cm pieces and macerating them in water for 1 min at high speed in a commercial blender (Waring). Vermiform stages of *Pratylenchus* and *H. avenae* were extracted from the recovered soil and from the macerated roots (separate extractions) using an automated zonal centrifuge (Hendrickx 1995). The number of cysts (on roots and in soil), as well as the vermiform stages of *P. thornei*, *P. penetrans* or *H. avenae* in the obtained nematode suspensions (about 35 ml), were counted using a stereomicroscope. The number of extracted vermiform stages of *P. penetrans*, *P. thornei*, and *H. avenae* per plant was calculated. For each plant, a reproduction factor (Rf = Pf/Pi) was calculated to evaluate the susceptibility of the

wheat lines against *P. penetrans* and *P. thornei* (see below). In both experiments 3 and 4, the plant heights were measured and fresh roots were weighed.

qPCR methods for quantifying *P. penetrans* and *P. thornei*

The numbers of both *P. thornei* and *P. penetrans* obtained by counting were compared with those obtained with the qPCR methods for quantifying *P. penetrans* (Mokrini et al. 2013) and *P. thornei* (Mokrini et al. 2014). After counting the vermiform stages of *P. penetrans*, *P. thornei*, or *H. avenae* extracted from soil and roots by zonal centrifugation (Hendrickx 1995), the obtained nematode suspension was transferred into a 40-ml conical tube where nematodes were allowed to settle for 3 h. A 3 ml nematode suspension was then pipetted from the bottom of each tube and distributed equally in two microtubes of 1.5 ml. After centrifugation at 12,000 rpm for 10 min, the supernatant was removed. DNA was extracted from nematodes in each of the two microtubes as described by Holterman et al. (2006). The microtubes were incubated at 65°C for 1.5 h and 99°C for 5 min, consecutively. The reaction mixture and PCR-program were as described in Mokrini et al. (2013, 2014); the standard curves as obtained in Mokrini et al. (2013, 2014).

Statistical analysis

Data of the screening tests (experiments 1 and 2) were analysed with a one-way analysis of variance (ANOVA) using SPSS software for Windows (SPSS Inc., Illinois, USA). Differences in nematode reproduction between wheat accessions were checked using Tukey's test for comparison of means, with a significant *F*-value at $P < 0.05$. The reproduction of *P. thornei* on three resistant wheat lines (exp. 3), counted using the microscope and by the qPCR method was subjected to a two-way-factorial ANOVA to determine the effects of the nematode treatment and wheat lines. The Student Newman-Keuls test ($P < 0.05$) was used for mean separation. Differences between the two nematode treatments in experiment 4 were assessed using a one-way ANOVA analysis and the means were separated using Student Newman-Keuls test. The influence of the nematode treatments on the vegetative growth was analysed with a two-way ANOVA using SPSS software for Windows and a one-way ANOVA, for experiments 3 and 4, respectively. Correlation between the two estimates of nematode numbers, i.e. qPCR and traditional visual counting through microscopy, was determined.

Results

Screening wheat lines for resistance to *P. penetrans* and *P. thornei* (experiments 1 and 2)

Both *P. penetrans* and *P. thornei* were able to survive and even increase on most lines. Nine weeks after inoculation, the number of vermiform stages of *P. penetrans* and *P. thornei* in the 25 wheat lines ranged from 360 to 2128 and 240–2040 per plant, respectively (Tables 1 and 2). On the control line (Ourgh), the average final numbers of *P. penetrans* and *P. thornei* per plant were 1285 and 1804, respectively. The lowest average number of nematodes per plant was found in L9 (360 *P. penetrans* in soil and root), whereas the highest average number of nematodes per plant was found in line L24 (2128 *P. penetrans* in soil and root) (Table 1). The corresponding Rf varied from 0.9 (L9) to 5.3 (L24). The total number of nematodes (roots and soil) on L9 was significantly lower than on other lines; L9 was the only line with an Rf less than 1. No lines showed immunity to *P. penetrans*.

The Rf of *P. thornei* on the 25 lines varied from 0.6 (L9) to 5.1 (L8) (Table 2). Three lines (L9, L12, and L13) had an Rf < 1, with L9 exhibiting the lowest number of *P. thornei* both in roots (84) and soil (156). The lines L12 and L13 had similar numbers of *P. thornei* in their roots, but more nematodes were found in the soil of L13, resulting in Rf values of 0.7 and 0.9 for L12 and L13, respectively. The highest number of nematodes in soil and roots was found in line L8 (2040 *P. thornei* per plant) (Table 2).

Because of the relatively low reproduction rate of *P. thornei* (Rf <1) on L9, L12, and L13, these lines were tested again, along with the control line (Ourgh). For *P. penetrans*, only L9 was retested because the Rf of both L12 and L13 was higher than 1. Nine weeks after inoculation, the mean number of *P. penetrans* in L9 roots and soil was 321/plant. The final numbers of *P. thornei* on lines L9, L12, and L13 were 288, 250, and 310, respectively. The corresponding Rf varied from 0.6 to 0.8. These results confirmed the moderate resistance of a number of lines to *P. penetrans* (L9) and *P. thornei* (L9, L12, and L13).

Interaction between *P. thornei* and *H. avenae* and their influence on vegetative growth of wheat (experiment 3)

Significant differences in *P. thornei* reproduction were found both between nematode treatments and between wheat lines. When *P. thornei* was inoculated alone, there were significant differences ($P = 0.04$) between the number of *P. thornei* extracted from the three resistant wheat lines: 143 (L9), 160 (L12), and 303 (L13) (Table 3). The corresponding Rf values were 0.3, 0.4, and 0.8 for L9, L12, and L13, respectively. When mixed with *H. avenae*, significant differences ($P < 0.0001$) were still observed in the total number of *P. thornei* among the lines tested. When *P. thornei* and *H. avenae* cohabited the root, the number of *P. thornei* extracted from roots decreased in lines L9 and L12, but not in L13, compared to the single inoculation of *P. thornei*. The total number of *P. thornei* per plant was reduced to 45, 92, and 280 for lines L9, L12, and L13, respectively (Table 3).

Plants were smaller in both height and weight in mixed inoculations than when inoculated with just *P. thornei* (Table 4). Under single inoculations, there was no significant difference in root weight between L9, L12, and L13. In general, root weight in mixed inoculations was slightly less than root weight in single inoculations of either *P. thornei* or *H. avenae.*

Interaction between *P. penetrans* and *H. avenae* and their influence on vegetative growth of wheat (experiment 4)

The numbers of *P. penetrans* extracted from soil and roots of the moderately resistant L9 were reduced when *H. avenae* and *P. penetrans* were simultaneously inoculated, compared to when *P. penetrans* was inoculated alone (Table 3). A total of 315 *P. penetrans* nematodes per plant were found in the single inoculation, compared with 167 nematodes when inoculated with equal numbers of *P. penetrans* and *H. avenae.* Consequently, the *P. penetrans* Rf reduced from 0.8 under single inoculation to 0.4 in the presence of *H. avenae*.

Plant height was significantly reduced from 34.2 cm under single inoculations to 29.3 cm under mixed inoculations. Root weight, however, was not affected. Under single inoculations, *H. avenae* caused a significant reduction in plant height of L9 compared to *P. penetrans* (Table 4).

Relationship between numbers of nematodes detected by qPCR and microscope

Resistance (R: resistant, MR: moderately resistant, or S: susceptible) of L9, L12, and L13 was evaluated based on the numbers of nematodes extracted from roots and soil. These numbers were also determined using qPCR assays (Table 3). There was a positive correlation between the numbers of *Pratylenchus* spp. detected with the qPCR assay and the numbers counted under the microscope in single inoculations, as well as in nematode suspensions where *H. avenae* was present (Fig. 1). In single inoculations with *P. thornei* (Fig. 1a) there was a strong positive correlation ($R^2 = 0.96$, $P < 0.001$, $n = 10$) between the results of the qPCR and those obtained by visual observation; counts based on qPCR underestimated the numbers of *P. thornei*. In mixed inoculations, there was also a positive relationship ($R^2 = 0.88$; $P < 0.001$, $n = 10$) between the data generated by the two methods (Fig. 1b); *P. thornei* estimates were again higher using the microscopic method than qPCR. When quantifying *P. penetrans*, the estimates based on qPCR and those obtained by counting were not very well correlated ($R^2 = 0.64$; $P < 0.05$, $n = 10$), but this relationship was better under mixed inoculations ($R^2 = 0.80$; $P < 0.05$, $n = 10$) (Fig. 1d). Estimates of *P. penetrans* in mixed inoculations were greater for the microscopic method than qPCR, as observed for *P. thornei*.

Discussion

This study aimed to evaluate the resistance of wheat lines (*T. aestivum* and *T. durum*) to *P. penetrans* and *P. thornei*, the most important *Pratylenchus* species in wheat-growing areas of Morocco (Mokrini et al. 2016). This was done by estimating the density of nematodes in both soil and roots, as *P. penetrans* and *P. thornei* are migratory endoparasites. The evaluation took place 9 weeks after inoculation, the standard screening time used at CIMMYT when evaluating the susceptibility of wheat lines against *Pratylenchus* spp. Evaluating two harvesting times for screening tests with *P. thornei*, Toktay et al. (2012) found that harvesting after 9 weeks was enough to indicate resistance as less nematodes were found in resistant germplasm than in susceptible germplasm, and this with a lower standard error than harvesting after 13 weeks. Also Keil et al. (2009)

Table 3 Interaction of *Pratylenchus thornei* or *P. penetrans* with *Heterodera avenae* in three lines of wheat resistant to *P. thornei* (exp. 3) and one line resistant to *P. penetrans* (exp. 4)

Nematode	Wheat lines	Root-lesion nematodes (all vermiform stages)					*H. avenae*	
		Root[c] (per plant)	Soil[c] (tube)	Total[c] Counted	Rf (Pf/Pi)	Total qPCR[d]	Cysts (soil)	Vermiform stages (root and soil)
Pth[a]	L9	41 b	99 b	143 c ± 40	0.3	107 bc ± 40	–	–
	L12	25 a	135 c	160 d ± 49	0.4	140 c ± 48	–	–
	L13	24 a	279 d	303 f ± 52	0.8	278 d ± 49	–	–
	Control[b]	666	1151	1817 ± 64	4.6	1779 ± 75	–	–
Pth + Ha	L9	19 a	26 a	45 a ± 11	0.1	26 a ± 7	10 ± 1.7	410 ± 39
	L12	17 a	75 b	92 b ± 19	0.2	72 b ± 15	13 ± 2.7	453 ± 40.4
	L13	95 c	185 c	280 e ± 86	0.7	249 d ± 68	7 ± 2.1	558 ± 47
Ha	L9	–	–	–	–	–	6 ± 1.6	438 ± 51,2
	L12	–	–	–	–	–	6 ± 1.9	504 ± 27.7
	L13	–	–	–	–	–	7 ± 2.2	391 ± 47
	Control	–	–	–	–	–	12 ± 2.8	821 ± 56
Ppen	L9	86 b	229 b	315 b ± 49	0.8	236 b ± 76	–	–
	Control	567	1373	1940 ± 31	4.9	1214 ± 57	–	–
Ppen + Ha	L9	65 a	102 a	167 a ± 42	0.4	126 a ± 41	6 ± 1.4	504 ± 12
Ha	L9	–	–	–	–	–	9 ± 2.3	754 ± 32

[a] Pth: *P. thornei*, Ppen: *P. penetrans*, Ha: *H. avenae*

[b] Control refers to the susceptible wheat cv. Ourgh

[c] Means in column per nematode treatment followed by different letters are significantly different ($n = 10$), according to Student Newman-Keuls test. Means of totals are followed by standard error

[d] Soil and root. The calculated number of *P. thornei* or *P. penetrans* is the number of individuals for the whole sample (as described in Mokrini et al. 2014)

reported that the best time to estimate *P. thornei* multiplication on wheat is between 8 and 12 weeks. Although a reproduction factor of 5 on the susceptible reference Ourgh can be considered low, we consider it sufficient to detect differences with lines where reproduction is lacking (Rf <1), thus showing resistance. The reproduction obtained on the susceptible lines demonstrated that nematodes were able to multiply in the relative short time frame of 9 weeks.

We could show that the wheat lines varied from moderately resistant to very good hosts for both *P. penetrans* and *P. thornei.* Three lines, viz. L9 (AUS 4930.7/2 PASTOR), L12 (CROC_1/AE.SQUARROSA (224); 20,215), and L13 (CROC_1/AE.SQUARROSA (224); 20,216) were moderately resistant to *P. thornei*. L9 (AUS 4930.7/2 PASTOR) was also moderately resistant to *P. penetrans*. Nematode resistance in wheat has been reported many times, either from pot experiments (glasshouse and growth chamber) or from field experiments (Zwart et al. 2005; Thompson et al. 2015; Dababat et al. 2016). Resistance to *P. thornei* was reported from Turkey (Toktay et al. 2012), Australia (Thompson et al. 2009; Thompson and Seymour 2011) and India (Kranti and Kanwar 2012). Kranti and Kanwar (2012) tested 20 wheat lines against *P. thornei* and reported several lines resistant to an Indian population of *P. thornei*, including CROC_1/AE. SQUARROSA (224)//OPATA. Our study confirmed the resistance of this line: L12 (CROC_1/AE.SQUARROSA(224); 20,215) and L13 (CROC_1/AE.SQUARROSA (224); 20,216), with a Moroccan population of *P. thornei*. Line L9 (AUS 4930.7/2 PASTOR) was also found resistant to both *P. thornei* and *P. penetrans* by Thompson et al. (2010).

Concomitant infestations of wheat fields with different species of plant-parasitic nematodes are common. In Morocco, combinations of *P. penetrans* or *P. thornei* and *H. avenae* occur with high frequency in wheat fields (Znasni 2003; Mokrini et al. 2012). To unravel the interaction between these genera with different feeding

Table 4 Effects of the interaction of *Pratylenchus thornei* (exp. 3) or *P. penetrans* (exp. 4) with *Heterodera avenae* on plant growth

Nematode	Wheat lines	Plant growth	
		Height (cm)[c]	Root weight (g)
Pth [a]	L9	32.2 a	1.19 ab
	L12	41.1 b	1.3 b
	L13	44.7 b	1.3 b
	Control[b]	24.5	0.9
Pth + Ha	L9	30.3 a	1.15 a
	L12	33.3 a	1.27 ab
	L13	33.2 a	1.24 ab
Ha	L9	30.0	1.4
	L12	33.7	1.4
	L13	33.0	1.8
	Control	22.3	0.8
Ppen	L9	34.2 b	1.25 b
	Control	22.2	1.15
Ppen + Ha	L9	29.3 a	1.28 b
Ha	L9	30.0 a	1.1 a

[a] Pth: *P. thornei,* Ppen: *P. penetrans,* Ha: *H. avenae*

[b]: Control refers to the susceptible wheat cv. Ourgh

[c]: Means in column per nematode treatment followed by different letters are significantly different ($n = 10$) according to Student Newman-Keuls

patterns, they were simultaneously inoculated on wheat lines with resistance to either or both RLN species. On L9 and L12, numbers of *P. thornei* and *P. penetrans* were greater when only these root lesion nematode species were inoculated, compared to when they were inoculated in combination with *H. avenae*. The reduction of *P. thornei* in mixed inoculations was significant in the roots of L9 and L12 and in the soil of all three lines (L9, L12, and L13). These results mean also that the wheat lines L12, L13 and L9 kept their resistance when exposed to a mixed inoculation with *H. avenae* and one of the *Pratylenchus* species. This type of result was observed for other crop species inoculated with other nematode species combinations. For instance, Niblack et al. (1986) showed that the soybean resistance to either *M. incognita* or *H. glycines* was unaffected in concomitant infections with these nematodes. However, nematode interaction could be affected by the timing of inoculation. We inoculated with J2 of *H. avenae*, because juveniles from inoculated cysts would have hatched gradually over time and could influence the competition with the *Pratylenchus* juveniles. Inoculating with cysts might be more reflecting the real field situation, but then nematode parameters fluctuate more between replications and lines, making it difficult to compare treatments.

Competition in varying degrees, between two or more nematodes species has been demonstrated in several other studies. Estores and Chen (1972) reported that *P. penetrans* and *M. incognita* depressed each other's population in tomato. Similarly, O'Bannon et al. (1976) found that a mixed inoculation of *Radopholus similis* and *P. coffeae* on citrus resulted in lower populations of each species than in separate inoculations. However, Melakeberhan and Dey (2003) reported that the rate of *P. penetrans* infection increased with increasing *H. glycines* proportions, up to the 50% level, and that the infection rate of *H. glycines* decreased with increasing proportion *P. penetrans* on soybean cultivars.

On *H. avenae* susceptible wheat cultivars in the field, Lasserre et al. (1994) reported that *P. neglectus* was inhibited throughout the long growing period of the host. These observations are similar to ours where co-infection with *H. avenae* of wheat generally reduced the numbers of lesion nematodes. In contrast, on *H. avenae* resistant cultivars, they observed that loss of the inhibitory effect associated with cyst nematode development can lead to greater numbers of *Pratylenchus.* The authors concluded from a split-root experiment, that the effects of *H. avenae* on *P. neglectus* were indirectly mediated as the two species were confined to separate halves of the root system, thus preventing direct competition. Similarly, it was suggested that the role of translocatable compounds resulting from the establishment of feeding sites was implicated in interactions between *Pratylenchus* and the sedentary endoparasite *M. incognita* on tomato and soybean (Estores and Chen 1972). It is possible that the establishment of a syncytium by *H. avenae* in our experiments had a negative influence on root penetration or feeding by *P. penetrans* and *P. neglectus*. Khan (1993) already suggested that the complex nematode-host relationship established by sedentary species may alter host physiology, making it either more or less suitable for migratory endoparasites.

Several studies have shown the inverse, viz. that *Pratylenchus* spp. can inhibit *Heterodera* spp. and *Meloidogyne* spp. (Brinkman et al. 2005; Eisenback 1993; Lasserre et al. 1994; Umesh et al. 1994). We did not elaborate on the multiplications of *H. avenae* with and without *Pratylenchus* spp., as our aim is to evaluate the durability of resistance to *Pratylenchus*

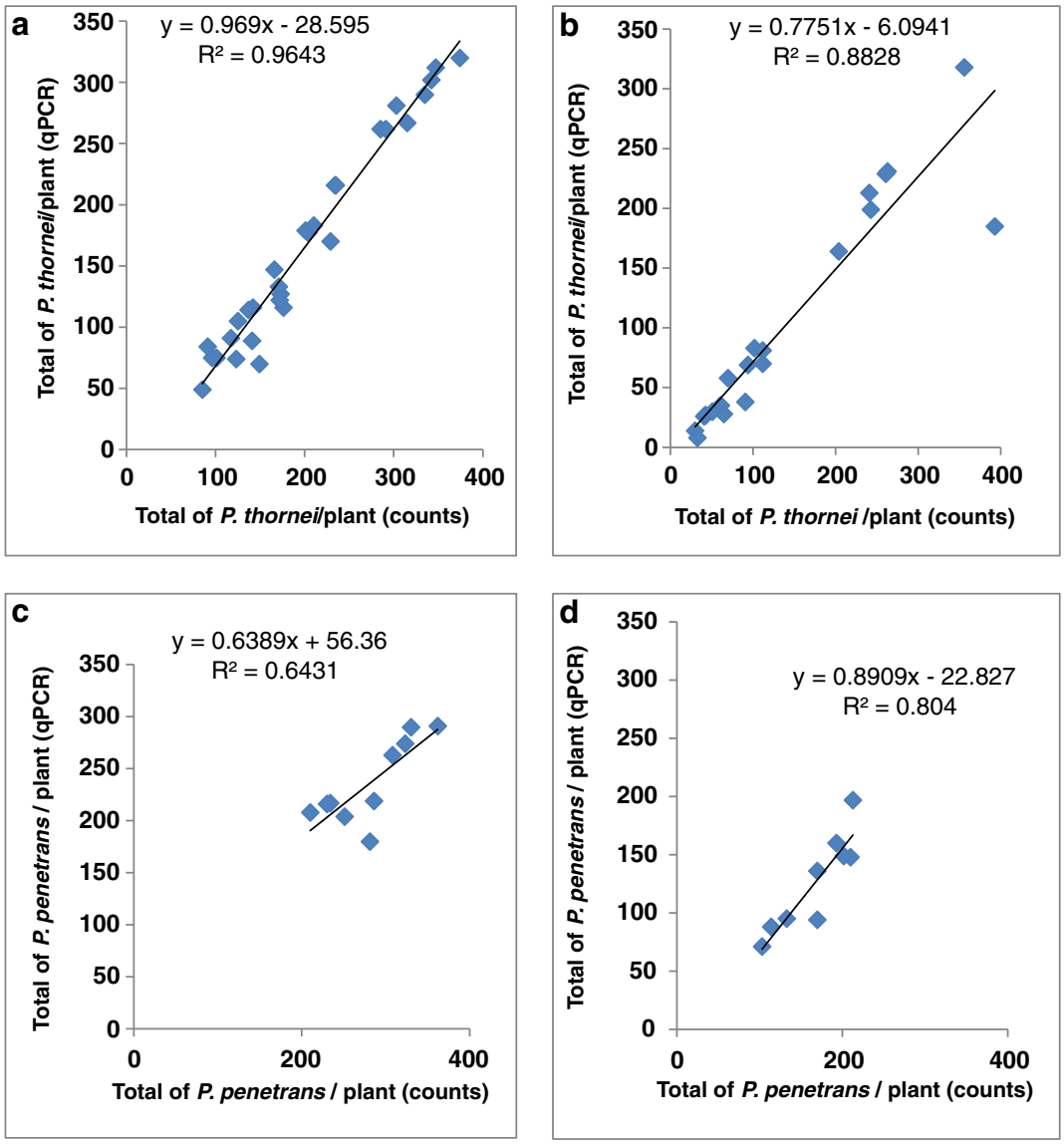

Fig. 1 Relationship between nematode population sizes assessed by microscopy (counts) and qPCR. **a** Total number of *Pratylenchus thornei* per plant in three moderately resistant lines of wheat after single inoculation; **b** Total number of *P. thornei* in three moderately resistant lines of wheat after mixed inoculation with *Heterodera avenae*; **c** Total number of *P. penetrans* per plant in a single line after single inoculation; **d** Total number of *P. penetrans* per plant in a single line after mixed inoculation with *H. avenae*

spp., not *H. avenae*. Inter-species effects are generally related to the nature of the parasitism and competition is assumed to be greatest between species with similar feeding habit (Eisenback 1985). The author reported that there is an interspecific relationship between sedentary and migratory endoparasites; the movement of migratory endoparasitic nematodes generally disrupts root tissues and often disturbs feeding by sedentary endoparasitic nematodes.

In our tests, mixed inoculations influenced plant growth more than did single species inoculations; plants were smaller when grown with mixed inoculations than when inoculated with a single nematode species. The reduced plant growth may result from increased competition between two species to penetrate roots (Powell 1979). Migratory endoparasites are less advanced parasites than sedentary endoparasites, which establish a very complex relationship with the host and alter plant

physiology. However, the sedentary endoparasite penetrates the plant at the root tip (Wyss and Zunke 1986), whereas the migratory endoparasites enter the root mainly in the region of root hair development and, to a lesser extent, in the root elongation zone (Zunke 1990). Several studies in agricultural systems show that *Pratylenchus* spp. inhibit both *Heterodera* spp. and *Meloidogyne* spp. (Eisenback 1993; Lasserre et al. 1994; Umesh et al. 1994); the influence of both endoparasites on plant growth might be reduced. Brinkman et al. (2005) studied the effects of intra and interspecific competition on the abundance of endoparasitic nematodes and assessed the consequences for biomass production of the dune grass *A. arenaria*. They showed that *P. penetrans* and *H. arenaria* reduced plant biomass and that biomass was not differently affected by adding one or two nematodes species.

Next to the classical counting of nematodes using a microscope, numbers of both *P. thornei* and *P. penetrans* in single and mixed inoculations with *H. avenae* were also estimated from soil and root samples using qPCR. There was a strong positive correlation between the numbers detected by qPCR and those obtained through traditional visual microscopy methods, though the qPCR generally gave lower nematode counts than microscopic observations. Several researchers have reported under or overestimation of nematode numbers using qPCR. Min et al. (2012) reported a higher density of *P. penetrans* based on DNA extracted directly from soil and used in qPCR, compared with visual counts of nematodes obtained from the soil using the Baermann method. Ophel-Keller et al. (2008) also reported that a qPCR assay tended to overestimate population densities of *P. thornei* compared with microscopic counting. Yan et al. (2012) reported a significant positive correlation between the numbers of *P. thornei* based on qPCR and visual counts of nematodes extracted with the Whitehead tray. The authors showed that the counts determined by the qPCR were larger than the numbers derived from the visual counts, but that this overestimate was not significant. Berry et al. (2008), however, found that qPCR tended to underestimate the numbers of nematodes (*M. javanica*, *P. zeae*, and *Xiphinema elongatum*). Our earlier data obtained with real-time PCR (Mokrini et al. 2013, 2014) indicate that life stages of *P. penetrans* and *P. thornei* in individual samples do not affect qPCR detection and quantification and are therefore unlikely to explain the under-estimation of nematodes calculated using qPCR, compared to the counts with the microscope. However, in the case of *P. penetrans*, Sato et al. (2007) found that the cycle threshold values of larger body sizes (male and female) were significantly lower than those from a small juvenile. Thus the population density of *P. penetrans* may change, depending on the composition of the life stages (male, female, and juveniles) present in a sample. In our study, the lower numbers of nematodes detected with qPCR can probably be attributed to the dilution effect when preparing the samples for qPCR. For microscopy, nematodes were enumerated from the whole suspension, whereas for qPCR the nematode suspension was first transferred into a 40-ml tube where nematodes were allowed to settle for 3 h before a 3 ml nematode suspension was pipetted from the bottom of the tube for DNA extraction. It is possible that some nematodes had not settled into the lower 3 ml, or that nematodes were lost during pipetting. In any case, qPCR can offer an alternative assay to the time-consuming traditional method of morphological counting.

Of the 25 lines of wheat that were screened, three lines (L9, L12 and L13) were found moderately resistant, even in the presence of *H. avenae*. These are promising results for enhanced wheat breeding. However, Nombela and Romero (1999) reported that the introgression wheat line H93–8 found resistant to *P. thornei* in the growth chamber turned out to be susceptible in a 5-months field experiment. It is therefore crucial that the field performance of these lines against RLN should be evaluated before they are released to the farmers.

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Compliance with ethical standards This study does not involve human participants nor vertebrate animals, so no informed consent is needed.

Conflict of interest The authors declare that they have no conflict of interest.

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