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Rate of decline and survival of root-knot and lesion nematodes under cold storage conditions

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Summary – Storage of nematode-infected soil, roots and nematode suspensions is important in nematological research. The available storage methods are based on potato cyst nematodes, where cysts with viable eggs can be stored for long periods at 4°C. When dealing with other nematode species, understanding the effect of storage temperature is crucial. This study was designed to investigate the decline rate and survival of four root-knot and a lesion nematode of both temperate and tropical origin, when stored at 4°C in three substrates: water, soil and roots. The starting density (P_i) for each substrate was determined at $t = 0$ and survival of all nematode species was estimated at 10-day intervals for 100 days. During storage, population densities of all species declined in all substrates exponentially. A slower decline rate ($r_d = 0.988$-0.999) was observed for juveniles of *Meloidogyne fallax* in water, soil and roots compared to juveniles of *M. hapla* and *Pratylenchus penetrans*. *Meloidogyne incognita* was seriously affected by cold storage with the highest decline rate ($r_d = 0.919$-0.977) observed in all substrates. Only data on the root substrate were obtained for *M. javanica* with a decline rate of ($r_d = 0.977$) predicting zero survival at $t > 100$ days. Notable is the higher fraction of surviving *P. penetrans* ($P_i = 0.238$-0.545) in all substrates during the storage period, compared with all other species. Based on the results, it is recommended to process nematode samples in the three substrates as quickly as possible, as underestimation of the actual population densities is likely. Consequences of cold storage in handling and processing of samples from different substrates are discussed.

Keywords – exponential decline, *Meloidogyne fallax*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Meloidogyne javanica*, modelling, natural decline, population underestimation, *Pratylenchus penetrans*.

Root-knot nematodes (RKN) and root-lesion nematodes (RLN) are among those plant-parasitic nematodes that are known to cause quality and yield loss in many agricultural crops (Eisenback & Triantaphyllou, 1991; Bridge *et al.*, 2005). Both are geographically distributed worldwide, including in temperate and tropical regions. *Meloidogyne fallax*, *M. chitwoodi*, *M. hapla* and *Pratylenchus penetrans* are mostly found in temperate regions, whilst *M. javanica* and *M. incognita* are found in the tropics. There are several reports also indicating the prevalence of *P. penetrans* and *M. hapla* in the cooler highlands of the tropics. Širca *et al*. (2004) reported the occurrence of *M. incognita* in glasshouses in North Europe and in open fields in the southern parts of Europe. Also Bačić *et al*. (2016) reported the occurrence of *M. incognita* in Serbia (south east Europe) causing quality damage in potatoes.

Due to the economic damage caused to important crops, particularly potato, both *M. fallax* and *M. chitwoodi* have been quarantine nematodes in Europe since 1998 (Karrsen *et al*., 1995, 1996; Holterman *et al*., 2012). Root-knot nematodes are known to cause deformations to quality, mainly blisters on tuber crops *e.g.*, potato, which might cause total rejection by the market (Vovlas *et al*., 2005; Norshie *et al*., 2011; Wesemael *et al*., 2014; Teklu *et al*., 2022). They also cause malformation of other underground produce, *e.g.*, in carrots, which affects the shape and severely diminishes their quality (Heve *et al*.,

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2015). In maize, potato, onion, carrot, field beans, black salsify and many other field crops, *P. penetrans* is reported to cause significant yield losses depending on the initial population density (Patel *et al*., 2002; Pudasaini *et al*., 2007; MacGuidwin & Bender, 2016).

After sampling, storage of nematode-infested samples is a necessary step (Takemoto *et al*., 2010). Field and micro-plot research, pot experiments for resistance, host-status, population dynamics, *etc.*, as well as for detection or diagnostics, require large numbers of soil samples, which have to be processed (Neher & Campbell, 1996; Been & Schomaker, 1998). Frequently, infected soil and root samples have to be stored for a certain time before extraction, and nematode suspensions before counting (Barker *et al*., 1969). Hence, storage temperature is an important factor in maintaining the original population density for a certain period. This temperature might also depend on whether the nematode is of tropical or temperate origin. Deviation from their optimum storage temperature requirements may shorten the life span of RKN and RLN. Juveniles of *Meloidogyne* spp. and *P. penetrans* exposed to warmer storage temperatures are more active, use more energy and have a shortened life span (Pudasaini *et al*., 2008; Das *et al*., 2011).

By default, soil samples, roots and suspensions of nematodes in water are currently stored at 4°C (Hooper, 1986; Hunt & De Ley, 1996; Teklu *et al*., 2016; Elhady *et al*., 2018), and this applies also to RKN and RLN regardless of their origin (tropical or temperate). This temperature has been proved to be perfect for storing dried cysts of potato cyst nematodes (PCN) or their egg suspensions. However, for other nematode species this temperature might not be optimal, (Takemoto *et al*., 2010). As dead nematodes will not be recovered using the most prominent extraction methods, except centrifugation and flotation (McSorley & Walter, 1991), decline during storage will not be noticed and population densities will be underestimated. Consequently, erroneous conclusions might be reached through statutory soil sampling for detection in field, and in pot experiments, when carrying out plant-nematode interaction studies.

Therefore, this study investigated the survival of four *Meloidogyne* species (*M. fallax*, *M. hapla*, *M. incognita* and *M. javanica*) and *P. penetrans* when stored at 4°C for a period of 100 days. The first objective was to assess the decline rate and survival of these temperate and tropical nematode species when stored at 4°C in three substrates: water, soil and root. The second objective was to gain some insights regarding the consequences of sample processing for research (field or pot experiments) and management of (quarantine) nematodes.

Materials and methods

Experimental design

The experiments were undertaken at Wageningen University and Research, Plant Science Group. Three substrates were evaluated in the storage experiments: root, soil and water. Five nematode species were investigated, including temperate (*M. fallax*, *M. hapla* and *P. penetrans*) and tropical (*M. incognita* and *M. javanica*) nematodes. The nematodes were from stock cultures that had been identified both morphometrically and molecularly. Five replications per nematode species and per substrate were evaluated at intervals of 10 days for 100 days, except for *M. javanica* where only the root substrate could be evaluated. Therefore, for the three substrates, water (220), soil (220) and roots (275), a total of 715 samples were processed (nematodes were extracted from the roots and soil) and counted at each time interval.

Source of inoculum

*Meloidogyn*e species (*M. fallax*, *M. hapla*, *M. incognita* and *M. javanica*) were cultured on the susceptible tomato 'Moneymaker' (Teklu *et al*., 2016). *Pratylenchus penetrans* was multiplied on susceptible maize 'Husar'. For multiplication, 5 kg pots were filled with hydro-grains (25% of pot volume) and silver sand (75% of pot volume). The initial population density (P_i) used was 4 second-stage juveniles (J2) (g dry soil)$^{-1}$ for RKN and 4 mixed stages (g dry soil)$^{-1}$ for *P. penetrans*. The inoculated pots were maintained under glasshouse conditions to obtain sufficient inoculum, estimated at 9×10^5 J2 for RKN and 0.5 million *P. penetrans* (pot)$^{-1}$ (Teklu *et al*., 2016, 2022). After 12 weeks, both tomato and maize stems were removed. A 10 mm sieve was used to sieve the soil and collect the roots, which were then cut into small pieces of about 1 cm. Some of the chopped roots were placed in extraction sieves (20 cm diam., 150 μm gauze) and immediately put inside a Seinhorst spray mist-chamber for nematode extraction (Seinhorst, 1988). Nematode suspensions were collected and counted every day for 3 days and were immediately used to prepare both the water (Fig. 1A) and soil (Fig. 1B) samples. Details about the inoculum levels used for each substrate are presented in Table 2.

Fig. 1. A: Water substrate in 100 ml bottles; B: Biological bags with 100 g inoculated soil.

Preparation of the water substrate samples

Using a glass pipette, a 2 ml well-homogenised nematode suspension was added to tap water (50 ml) in 100 ml glass bottles. The nematodes were kept to a Poisson distribution using a perforated plunger to reduce counting error. Bottles were then closed tightly using parafilm, containing one hole in the middle for aeration. They were stored in a dark cold room at 4°C (Teklu *et al*., 2018). At $t = 0$, nematodes were immediately counted after inoculation using a compound light microscope, as a starting point. With five replications, 11 time points and four nematode species, a total of 220 samples were used for the water substrate.

Preparation of soil substrate samples

The soil substrate for the storage experiment was prepared by mixing silver sand, hydro-grains and clay at a ratio of 6:1.5:1 (Teklu *et al*., 2014). The soil mixture also contained NPK (14:13:14) and Steiner's nutrient solution (Steiner, 1968). In total, 24 kg soil substrate was required for the experiment. Small transparent biological plastic bags (25 × 15 cm) with micro-pores for aeration, were filled with 100 g of the soil mixture and inoculated with 3-4 ml of a well-homogenised nematode suspension using a glass pipette. Bags were immediately closed with sealing tape and stored in a dark cold room at 4°C (Elhady *et al*., 2018). The moisture content of the soil, adjusted to 12% at the start of the experiment, could be maintained in the sealed plastic bags until the end of the experiment.

A higher P_i was used for the soil substrate, compared to the water substrate, to compensate for the extraction losses during the processing of the soil samples, thereby avoiding lower nematode numbers and higher counting errors. Again, at $t = 0$, nematodes were immediately extracted and counted as a starting point. In total, 220 samples were used for this storage experiment.

Preparation of infected root substrate

Infected roots of tomato 'Moneymaker' and maize 'Husar', freshly harvested from the multiplication pots, were chopped to about 1 cm. After mixing, batches of 4-8 g of roots were collected (same weight per species, see Table 2) and placed in small transparent biological plastic bags (25 × 15 cm) with micro-pores for aeration, closed using sealing tape and stored in the cold room at 4°C. At $t = 0$, nematodes were immediately extracted from the roots for each nematode species using the Seinhorst spray mist chamber (Seinhorst, 1988; Teklu *et al*., 2016) as a starting point. There were five replications, 11 time points and five nematode species, resulting in a total of 275 samples for the root substrate experiment.

Nematode extraction from soil and roots

Nematodes were extracted from the complete soil sample (100 g) using the Seinhorst elutriator (Seinhorst, 1988). The details of the procedure are described by Teklu *et al*. (2014). All roots (4-8 g) were used to extract the nematodes to minimise variation. The roots were fresh when harvested, with visible galls for those infected with RKN and lesions for those infected with RLN. Chopped roots were placed in 9 cm diam., 150 μm extraction sieves after each storage time. The sieves were then put on top

Table 1. Overview of variables and parameters used.

Variable	Parameter	Description	Dimension
P_{i}		Initial population density used	Number of nematodes
$P_{\mathrm{i,max}}$		Maximum population density at $t = 0$	Number of nematodes
S		Fraction if survived nematodes at time (t)	Fraction
t		Storage time	Days
	r_{d}	Rate of decline (slope of the line)	$0 \leqslant r_{\mathrm{d}} < 1$
	$P_{\mathrm{i},s}$	Fraction of survived nematodes when $t \to \infty$	$0 \leqslant P_{\mathrm{i},s} < 1$

of 12 cm extraction dishes and were kept in a spray-mist chamber (Seinhorst, 1988) for 4 weeks. The temperature in the mist-chamber was kept at 20°C and was well aerated. Hatched J2 were collected at 7-day intervals for 4 consecutive weeks. The mist-chamber was programmed to spray for 15 min at intervals of 30 min, continuously. No growth of fungi or rotting of the roots was observed during extraction in 28 days.

Nematode counting

To obtain uniform volumes of suspension when estimating the nematode population density, the volume was reduced to a minimum of 150 ml for those collected from soil samples and 100-200 ml for those collected from roots. Volumes were gently reduced by pressure suction after the nematodes had settled to the bottom of the flasks for 24 h (at room temperature of 18-20°C). The aim was to count at least 200 nematodes per ml to keep the counting error low and the coefficient of variation around 7%, based on a Poisson distribution of nematodes in the suspension. To fulfil this requirement, the solution was randomised using a perforated plunger before a subsample of 1 ml was taken using a pipette to estimate nematode population density. A 1 ml counting dish was used to count the nematodes under a compound light microscope. When densities of nematodes of >500 nematodes ml^{-1} were encountered, the mother suspension was diluted to adjust the number of nematodes to approx. 200 nematodes ml^{-1}. On the other hand, if densities <100 nematodes ml^{-1} were encountered, the mother suspension was left to settle for 24 h followed by another reduction in volume to concentrate nematodes and count around 200 nematodes ml^{-1}. The goal of 200 nematodes ml^{-1} was not always feasible, especially when the population density was declining during storage. Adults and juveniles were counted separately for samples of *P. penetrans* to compare their survival over storage time separately. Juveniles of *P. penetrans* in this paper refers to second-stage to fourth-stage (J2-J4) life stages. Survival of nematodes is herein defined in reference to an active vermiform nematode after storage in each substrate.

Data analysis and model fitting

Scripts for data analysis and modelling were written in R Studio version 1.3.959 and run using the R console version 4.0.2 (Venables & Smith, 2013). As initial population densities at the start of the experiment differed for all species, normal comparison would be impossible. Hence, the relative number of the surviving nematodes was calculated by dividing the number of nematodes at any time ($t > 0$) by the number of nematodes at the start, time ($t = 0$). The data of the relative densities of surviving juveniles were log-transformed to stabilise variance, averaged over the five replications, and back-transformed. The exponential model (Equation 1) was fitted to the data. Nonlinear regression analysis was conducted to estimate the parameters $P_{\mathrm{i,max}}$, $P_{\mathrm{i},s}$ and r_{d} directly from the data. When necessary, the least significance difference (LSD) was estimated between parameter estimates to compare both fraction of surviving nematodes and the rate of decline with the water substate as a reference at 5% level of uncertainty. An overview of the variables, parameters and their dimension is provided in Table 1.

$$S = P_{\mathrm{i,max}} * (P_{\mathrm{i},s} + (1 - P_{\mathrm{i},s}) * r_{\mathrm{d}}^{t}) \qquad (1)$$

Results

The model fit

During storage, population densities of all species declined in all substrates. The exponential model fitted well to the relative survival of all nematode species in the three substrates with $0.71 \leqslant R^2 \leqslant 0.99$. One exception, where no regression could be established, was the relationship between the relative number of surviving

Table 2. List of nematode species evaluated, volume of water suspension, weight of the soil, weight of infected root used and initial population (P_i) for each substrate at inoculation.

Sample no	Nematode species	Substrate	Amount per replication	Unit	P_i
1	*Meloidogyne fallax*	Water	50	ml	9181
2	*M. hapla*	Water	50	ml	7335
3	*Pratylenchus penetrans*	Water	50	ml	4610
4	*M. incognita*	Water	50	ml	4142
5	*M. fallax*	Soil	100	g	13 771
6	*M. hapla*	Soil	100	g	14 669
7	*P. penetrans*	Soil	100	g	9220
8	*M. incognita*	Soil	100	g	8284
9	*M. fallax*	Root	5	g	76 102
10	*M. hapla*	Root	8	g	161 723
11	*P. penetrans*	Root	7	g	2487
12	*M. incognita*	Root	6	g	190 323
13	*M. javanica*	Root	4	g	10 940

The ratio of juveniles to adults of *P. penetrans* inoculated were 1.96, 0.59 and 2.03 in water, soil and root substrates, respectively, at $t = 0$.

juveniles of *P. penetrans* in the soil and storage time. Most of the model fit provided a fraction of surviving nematodes, $P_{i,s} > 0$, indicating that numbers do not decline to zero, but reach a certain asymptote at time, $t > 100$ days. Only in three instances for RKN (*M. fallax* (in water substrate), *M. hapla* and *M. javanica* (both in root substrate)) did the model predict a fraction of surviving nematodes $P_{i,s} = 0$. Lower fractions of surviving nematodes $0 \leqslant P_{i,s} \leqslant 0.437$ were observed for all RKN compared to $0.030 \leqslant P_{i,s} \leqslant 0.820$ for RLN in all substrates. Within the RKN species, the rate of decline was faster ($r_d \leqslant 0.930$) for the tropical RKN compared to the temperate RKN ($r_d \leqslant 0.999$) in all the substrates. Comparing the difference between the initial inoculum (P_i) in Table 2 at $t = 0$ and the parameter ($P_{i,max}$) in Table 3, estimated according to the model (Equation 1), resulted in a relative difference of <13% in ten instances out of 13. Only, in three instances out of 13 was the relative difference >13%.

Survival of temperate nematodes

Meloidogyne fallax

The results show different rates of decline of *M. fallax* per substrate (Fig. 2). The decline rate in water was significantly slower ($r_d = 0.999$) compared with that of soil ($r_d = 0.988$) and roots ($r_d = 0.992$). The fraction of surviving juveniles in roots ($P_{i,s} = 0.097$) and soil (0.037) was not significantly different compared with that of water ($P_{i,s} = 0$); see Table 3.

Meloidogyne hapla

Unlike *M. fallax*, the rate of decline for *M. hapla* was significantly faster (Fig. 3) in water with a decline rate of ($r_d = 0.948$) compared with that of soil ($r_d = 0.969$) and roots ($r_d = 0.988$). The fraction of surviving nematodes in soil and roots differed with $P_{i,s} = 0.437$ and $P_{i,s} = 0$, respectively (Table 3), but was not significantly different from that of the surviving nematodes in the water substrate ($P_{i,s} = 0.135$).

Total Pratylenchus penetrans *(adults + juveniles)*

In Figure 4, decline rates of all stages of *P. penetrans* were comparable to those of *M. hapla*, with the fastest rate of decline observed in water ($r_d = 0.955$), followed by soil ($r_d = 0.966$) and roots ($r_d = 0.975$). The fraction of surviving nematodes in water ($P_{i,s} = 0.545$) was significantly higher than those in the soil ($P_{i,s} = 0.438$) and roots ($P_{i,s} = 0.238$) substrates (Table 3).

Pratylenchus penetrans *adults*

In Figure 5, the relationship between the fraction of surviving *P. penetrans* adults and storage time is presented showing a decline rate r_d of 0.980, 0.977 and 0.982 in water, soil and roots, respectively. No significant differences in the decline rate were observed between the soil or root substrates when compared to the water substrate (Table 3). A significantly lower fraction of adults of *P. penetrans* survived in the roots ($P_{i,s} = 0.030$) compared with that of water and soil substrate ($P_{i,s} = 0.262$ and 0.117, respectively), when $t > 100$ days.

Table 3. Parameter estimation of the exponential model fitted to the data of relative number of nematodes survived in time for three substrates: water, soil and roots.

Nematode species	Substrate	$P_{i,max}$	$P_{i,s}$	r_d	SE			LSD		df	R^2
					$P_{i,0}$	$P_{i,s}$	r_d	$P_{i,s}$	r_d		
Meloidogyne fallax	Water	9437	0.000	0.999	210.87	0.53	0.0001	–	–	8	0.90
M. fallax	Soil	14 221	0.037	0.988	940.06	0.28	0.0057	0.385	0.0038*	8	0.89
M. fallax	Roots	76 102	0.097	0.992	5882.17	0.48	0.0061	0.450	0.0041*	8	0.76
M. hapla	Water	6472	0.135	0.948	759.09	0.06	0.0153	–	–	8	0.89
M. hapla	Soil	11 720	0.437	0.969	1391.93	0.12	0.0224	0.086	0.0172*	8	0.71
M. hapla	Roots	161 724	0.000	0.988	8324.50	0.22	0.0041	0.151	0.0104*	8	0.95
Pratylenchus penetrans total	Water	4123	0.545	0.955	273.87	0.05	0.0162	–	–	8	0.85
P. penetrans total	Soil	3444	0.438	0.966	339.76	0.09	0.0191	0.065*	0.0157	8	0.78
P. penetrans total	Roots	2562	0.238	0.975	182.63	0.08	0.0074	0.060*	0.0115*	8	0.94
P. penetrans adults	Water	1538	0.262	0.980	168.56	0.22	0.0141	–	–	8	0.79
P. penetrans adults	Soil	2158	0.117	0.977	160.61	0.12	0.0077	0.160	0.0102	8	0.93
P. penetrans adults	Roots	803	0.030	0.982	68.99	0.20	0.0081	0.187*	0.0103	8	0.90
P. penetrans juveniles	Water	2666	0.612	0.916	155.79	0.05	0.0296	–	–	8	0.82
P. penetrans juveniles	Soil	1260	0.820	NA	NA	NA	NA	NA	NA	8	NA
P. penetrans juveniles	Roots	1758	0.297	0.972	100.40	0.07	0.0077	0.054*	0.0202*	8	0.94
M. incognita	Water	4711	0.042	0.919	67.84	0.01	0.0029	–	–	8	0.99
M. incognita	Soil	5693	0.097	0.927	248.22	0.02	0.0089	0.014*	0.0061*	8	0.98
M. incognita	Roots	190 322	0.214	0.930	16 892.22	0.04	0.0227	0.027*	0.0153	8	0.928
M. javanica	Roots	10 939	0.000	0.977	1130.06	0.16	0.0086	–	–	8	0.91

$P_{i,max}$ is the number of nematodes at $t = 0$, $P_{i,s}$ the fraction of survived nematodes, r_d the rate of decline; SE, standard error; df, degrees of freedom; R^2, the coefficient of determination and LSD, the Least Significant Difference of a parameter compared with the water substrate is provided. The ratio of juveniles to adults of *P. penetrans* are: 1.43, 0.58 and 2.19 for water, soil and root substrates, respectively, based on the model estimates.

Pratylenchus penetrans *juveniles*

Juveniles of *P. penetrans* declined significantly faster in water ($r_d = 0.916$) compared with that of the root substrate ($r_d = 0.972$). The rate of decline on the soil was not estimated as no relationship could be established between the relative number of surviving *P. penetrans* juveniles and storage time for the soil substrate (Fig. 6B). A significantly higher fraction of juveniles of *P. penetrans* survived in the soil ($P_{i,s} = 0.820$), compared to water ($P_{i,s} = 0.612$) and the lowest survival was in the roots ($P_{i,s} = 0.297$).

Survival of tropical nematodes

Meloidogyne incognita

In Figure 7, a significantly faster rate of decline for *M. incognita* was also observed in water ($r_d = 0.919$), followed by soil ($r_d = 0.927$) and roots ($r_d = 0.930$) compared to all other nematode species (Table 3). A significantly higher fraction of nematodes survived in the root substrates ($P_{i,s} = 0.214$) compared with water ($P_{i,s} = 0.042$) and soil substrates ($P_{i,s} = 0.097$). Compared to all species of nematodes, the $P_{i,s} = 0.214$ was the highest for *M. incognita*, followed by that of *M. fallax*.

Meloidogyne javanica

Due to shortages of inoculum the experiment with the water and soil substrates had to be omitted. In the root substrate a decline rate of $r_d = 0.977$ was observed for *M. javanica* (Fig. 8). The model predicted a fraction of surviving nematodes of $P_{i,s} = 0$, like in that of root substrate for *M. hapla* (Table 3).

Discussion

The model used and its prediction

The model did not predict a complete decline of all nematode species to zero but instead predicted a remaining fraction – higher for RLN compared to RKN — in time. This is counter-intuitive, as energy is consumed

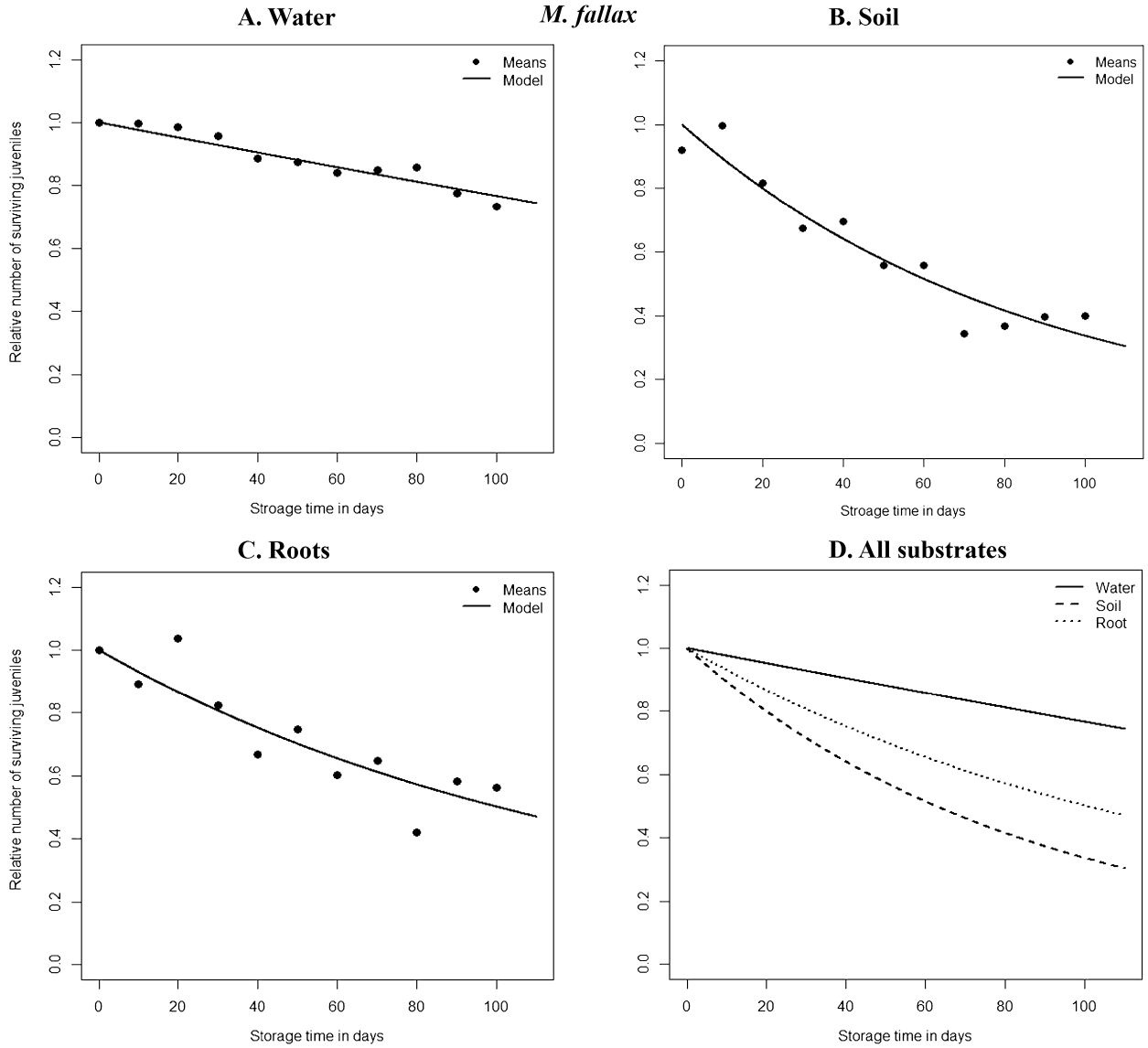

Fig. 2. The relationship between the relative number of surviving juveniles of *Meloidogyne fallax* and storage time: in water (A); in soil (B); in roots (C); and all substrates compared (D).

all the time and, when depleted, should result in the death of the nematodes. One reason might be that other processes, occurring after the storage time monitored (at $t > 100$ days), may follow a different model of decline. On the other hand, this phenomenon of nematodes declining to an asymptote not equal to zero has been reported before. Das *et al*. (2011) reported that *M. fallax*, *M. chitwoodi*, *M. hapla* and *M. minor* stored at 4°C for 12 weeks did not decline to zero percentage survival, except for *M. hapla* in water substrate. Seinhorst (1956), when studying the natural decline of the stem nematode *Ditylenchus dipsaci* in fields without hosts, from autumn to spring, observed their numbers to stabilise around 50 nematodes (500 g dry soil)$^{-1}$ on heavy clay soil. Following the natural decline of *P. penetrans* in field experiments with potato, maize, carrot and black salsify, Pudasaini *et al*. (2007) demonstrated that densities also levelled off at a non-zero asymptote. The relative difference in P_i estimated at $t = 0$ and the one estimated from the model (Equation 1), $P_{f,max}$, was >13% only in

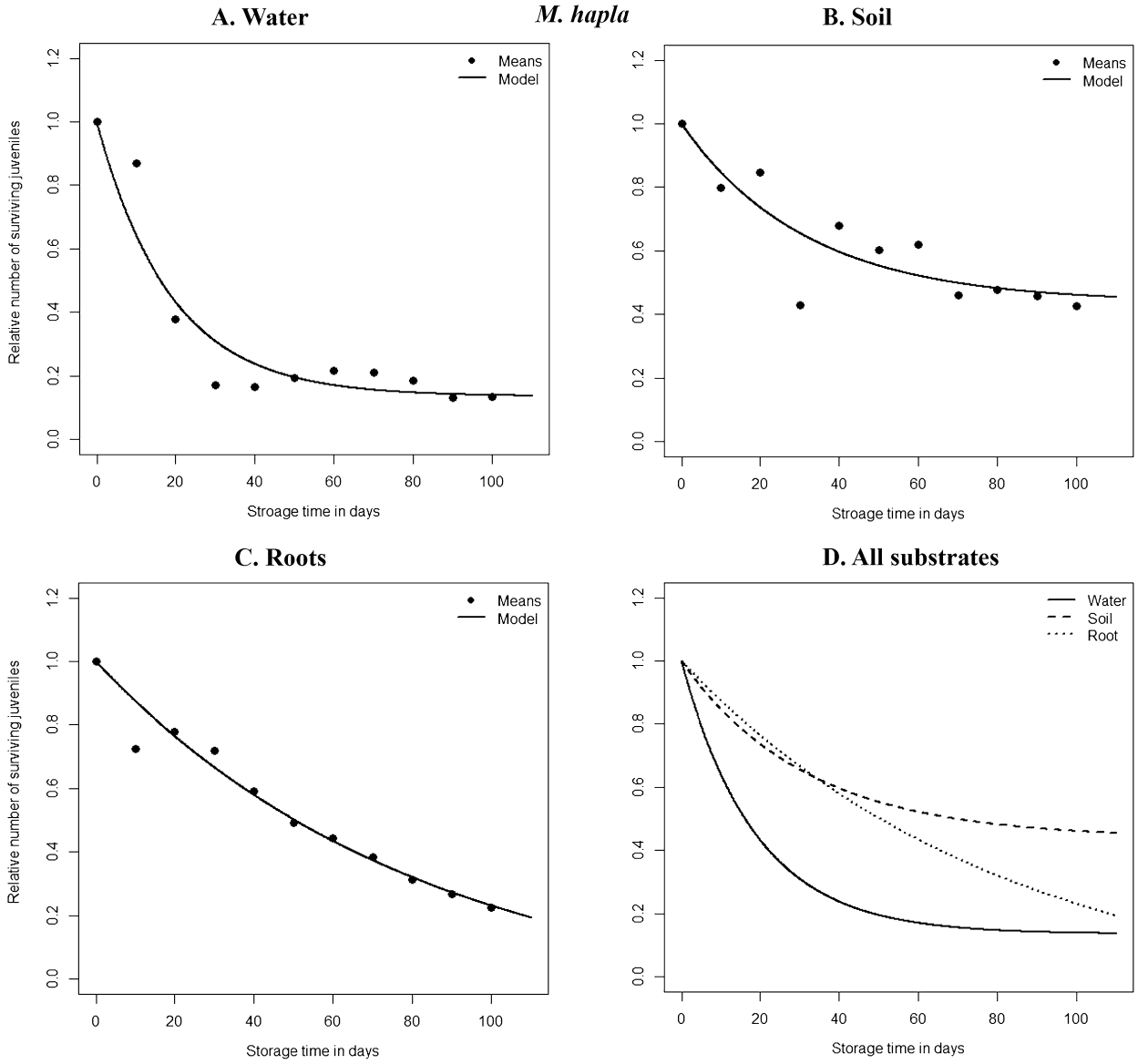

Fig. 3. The relationship between the relative number of juveniles of *Meloidogyne hapla* and storage time: in water (A); in soil (B); in roots (C); and all substrates compared (D).

three instances in the soil substrate, which is probably due to the variation encountered during extraction from soil involving several steps when using elutriators.

TEMPERATE NEMATODES

Survival of Meloidogyne fallax *and* M. hapla

The decline rate of *M. fallax* was the slowest of all species tested in all substrates recorded in this experiment ($0.988 \leqslant r_d \leqslant 0.999$), which might indicate that this nematode is the best adapted to temperate conditions. In theory, *M. fallax* and *M. hapla* are temperate nematodes and are expected to have the ability to tolerate low temperatures during storage. However, a faster rate of decline of *M. hapla* ($r_d = 0.948$-0.988) was observed in all substrates but with a higher fraction of surviving juveniles in both water and soil. A similar observation was noted by Das *et al.* (2011), where the survival rates

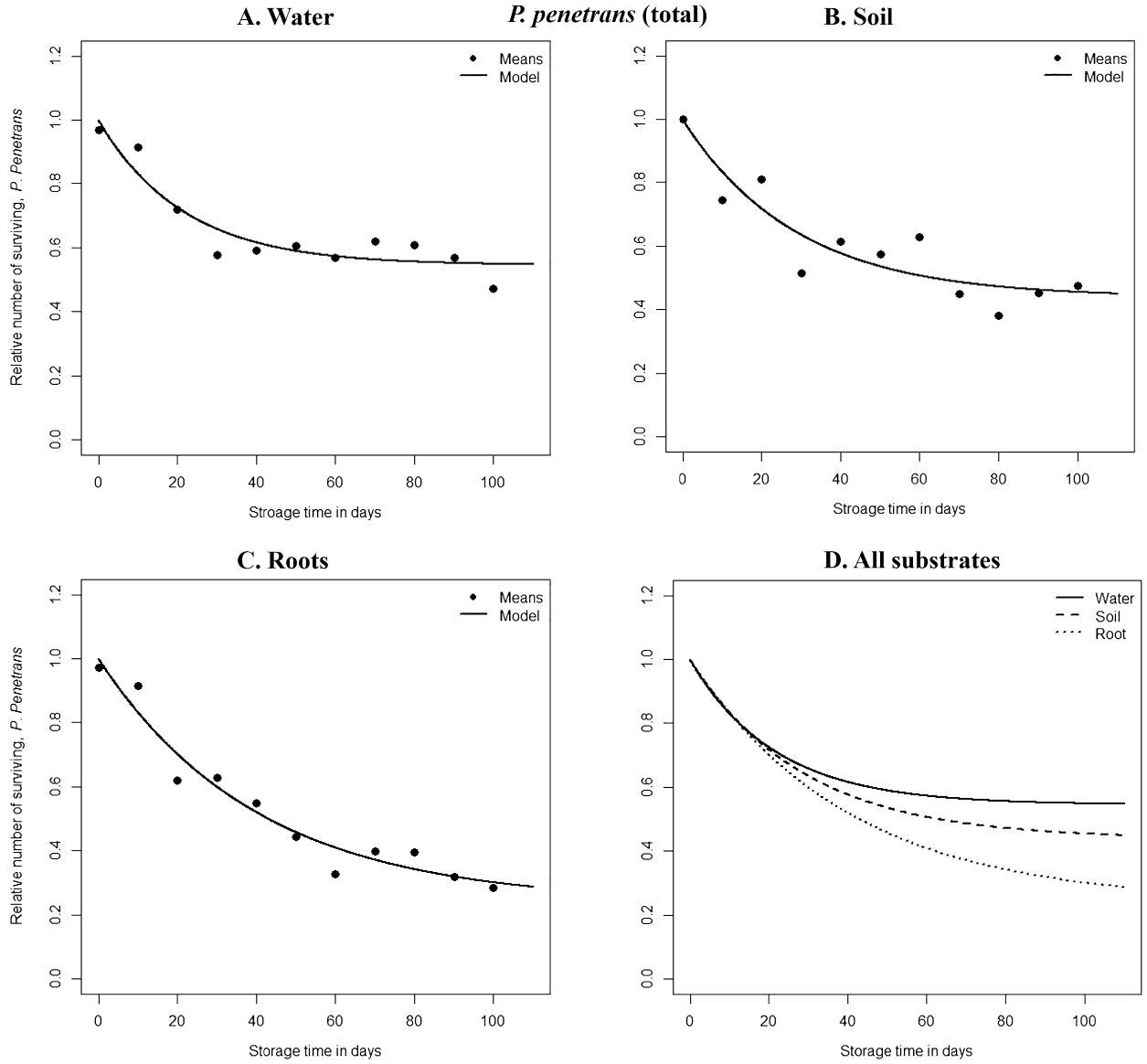

Fig. 4. The relationship between the relative number of surviving total (adults + juveniles), *Pratylenchus penetrans* and storage time: in water (A); in soil (B); in roots (C); and all substrates compared (D).

of *M. fallax* and *M. hapla* juveniles were monitored in distilled water at 4, 10 and 20°C over a period of 12 weeks. Das *et al*. (2011) noticed that at 4°C the rate of lipid depletion in the body of *M. fallax* juveniles was less because of reduced mobility compared with that of *M. hapla*. This survival strategy of restricting their energy utilisation was also described by Perry (2002) and Khan *et al*. (2014).

Survival of Pratylenchus penetrans

Rates of decline of adults + juveniles of *P. penetrans* (r_d = 0.955-0.975) were similar to those of *M. hapla* (r_d = 0.948-0.988) in all substrates. However, the fraction of surviving adults + juveniles for *P. penetrans* ($P_{i,s}$ = 0.238-0.545) was higher compared with all other nematode species tested in all substrates. Densities of *P. penetrans* decreased (r_d = 0.976) in both roots and soil by

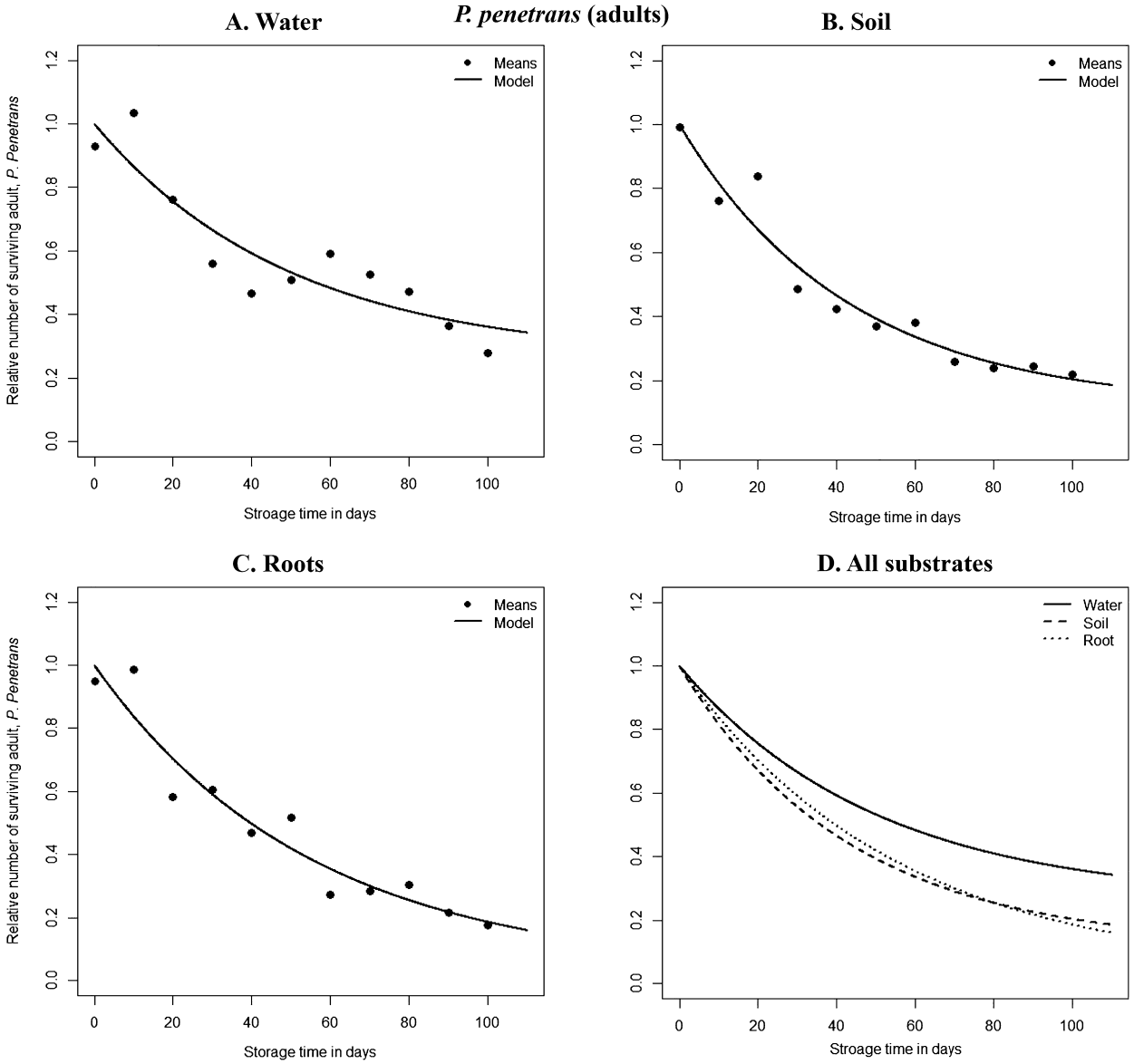

Fig. 5. The relationship between the relative number of surviving adults of *Pratylenchus penetrans* and storage time: in water (A); in soil (B); in roots (C); and all substrates compared (D).

40% in the first 3 weeks and were stable afterwards. In theory, the lesion nematode, *P. penetrans*, like *M. hapla and M. fallax* a temperate nematode but also adapted to the cooler highland of the tropics, is a migratory endoparasitic nematode, a fact that enhances survival in the field as well as in the storage with root substrates (Zunke, 1990; Prasad *et al*., 1999; Elhady *et al*., 2018).

When adults and juveniles of *P. penetrans* were analysed separately, the decline rate of adults (r_d = 0.977-0.982) was slower compared to that of juveniles (r_d = 0.916-0.972) in all the substrates, which is explained by the higher activity and hence energy depletion of the juveniles compared to the adults. When investigating the fraction of surviving juveniles of *P. penetrans*, we noticed a faster decline rate and an early stabilisation at 60% of the original population in the water substrate, which differed from that of the adults. No regression could be found between storage time and surviving juveniles of *P. pene-*

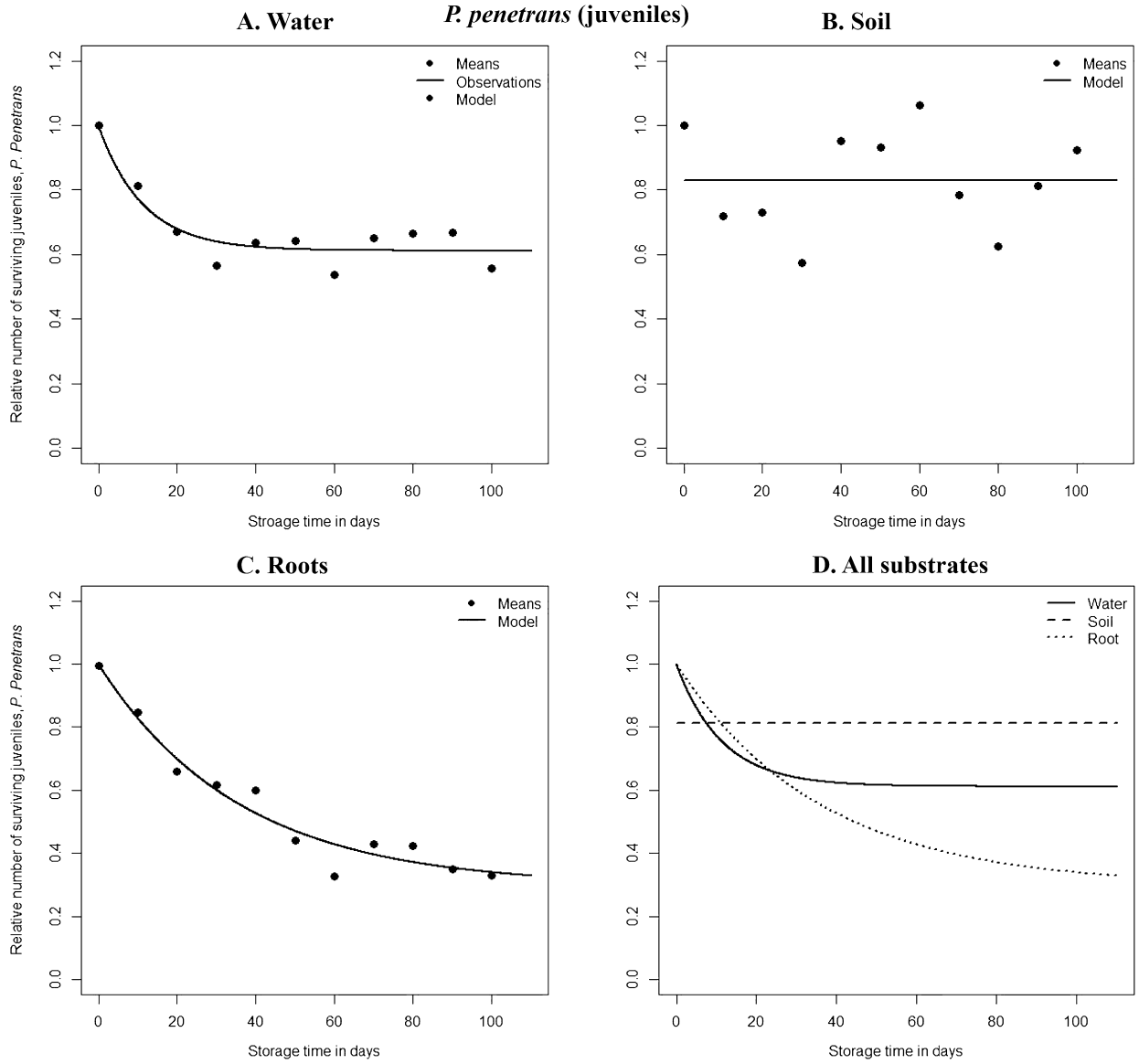

Fig. 6. The relationship between the relative number of surviving juveniles of *Pratylenchus penetrans* and storage time: in water (A); in soil (B); in roots (C); and all substrates compared (D).

trans in the soil substrate. The current result provides an average survival of 82% of the inoculated juveniles of *P. penetrans* over the first 100 days. The variation encountered was large and this finding is counter-intuitive to our expectation that densities should decline. One reason for the variation might be the low number of juveniles used in the inoculum at $t = 0$ (1260; Tables 2, 3), combined with the fact that early-stage juveniles might be lost during extraction, resulting in very low numbers to be counted. This was also indicated in the lower juvenile to adult ratio of 0.59 at $t = 0$ in the soil substrate compared with 1.96 and 2.03 in the water and soil substrates, respectively. Despite the variation encountered, the results may indicate that juveniles of *P. penetrans* are the most tolerant life-stage to survive cold storage temperature.

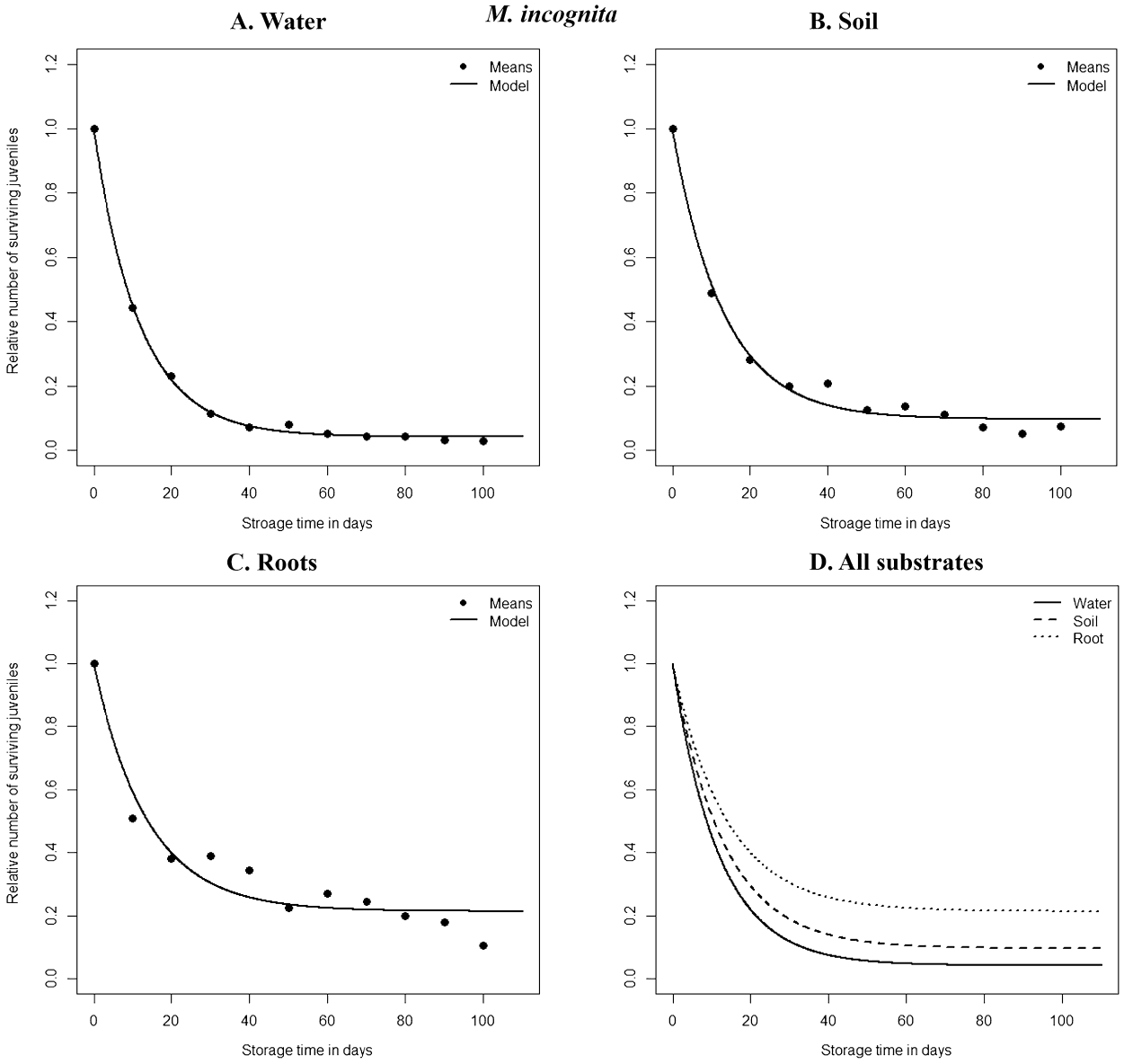

Fig. 7. The relationship between the relative number of surviving juveniles of *Meloidogyne incognita* and storage time: in water (A); in soil (B); in roots (C); and all substrates compared (D).

TROPICAL NEMATODES

Rate of decline of Meloidogyne incognita *and* M. javanica

A faster rate of decline was reported by Tsai (2008) and Ohba *et al*. (1982) after storing juveniles of *M. incognita* for 20-21 days at 5°C in water and observing a 99.3% mortality, compared to 10, 15 and 20°C with 41.3%, 1.5% and 1.7 % mortality, respectively. This agrees with the faster rate of decline of *M. incognita* juveniles observed in this experiment, when compared with all the tested nematode species in all substrates. A population decline of *M. incognita* and *M. javanica* in the absence of host in a severe winter season in Canada was reported by Sayre (1964) and Johnson & Potter (1980).

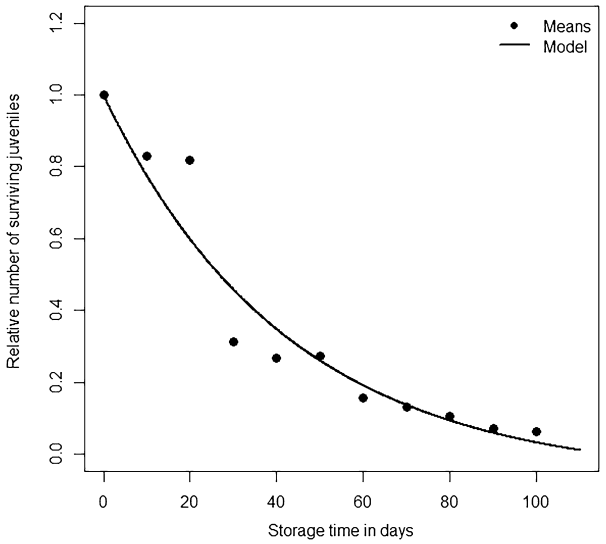

Fig. 8. The relationship between the relative number of surviving juveniles of *Meloidogyne javanica* and storage time in the root substrate.

Some practical implications

The fraction of surviving nematodes was lower in the root substrates of all nematode species except for *M. fallax* and *M. incognita* compared with that in water and soil. One aspect of both RKN and RLN not taken into consideration is the presence of free eggs and eggs inside egg masses in the root system. Though eggs are the life-stage most resilient to cold temperatures (Perry, 2002), prolonged storage at 4-5°C was reported to prevent hatching of *M. incognita* and *M. javanica* (Ohba *et al*., 1982; Stephan, 1982). A difference in tolerance to low temperature conditions even within the developmental stages of eggs as embryonic and differentiated J1/J2 was observed for *M. incognita* and *M. hapla* by Vrain *et al*. (1978). Embryonic eggs might not have well-developed and functioning haemostasis mechanisms developed early and are less tolerant to cold temperatures (Vrain *et al*., 1978)

No literature seems to be available on how eggs of *M. fallax* and *M. chitwoodi* are affected by storage. There are reports that the hatching of *M. hapla* differs depending on their geographic origin. Juveniles of *M. hapla* originating from England and Canada hardly hatched at storage temperatures <10°C (Stephan, 1982) while juveniles from North Carolina hatch at all temperatures between 5 and 30°C. The inhibition to hatch after cold storage might be a reason explaining the lower fraction of surviving nematodes obtained in the roots of most of the nematode species evaluated in the current research, although further confirmation is needed. Extraction of the nematodes from the roots using incubation in a mist-chamber lasting 28 days at 20°C, as in the current experiment, should provide enough time after cold storage for all live juveniles to hatch.

The rate of decline of all nematode species, except *M. fallax* and adults of *P. penetrans*, was faster in water, followed by soil and lowest in roots. According to Bélair (1985), the decline during cold storage is buffered by soil and even more in the presence of roots. Survival in soil, especially in root debris, provides a reservoir of nematodes (eggs and juveniles) in spring of the following year to invade new hosts. Previous work conducted by Been *et al*. (2007) on the over-wintering of *M. chitwoodi* when developing the sampling strategy, and Pudasaini *et al*. (2007) when studying the influence of host-status and temperature on the vertical distribution of *P. penetrans*, both highlighted the significant importance of organic matter for their survival. Similar results of better survival during a mild winter of *M. incognita*, *M. javanica* and *M. hapla* in the roots of perennial hosts, such as peach and asparagus, were also reported by Sayre (1964) and Johnson & Potter (1979). Therefore, detection probabilities by soil sampling will always be improved: *i*) when organic debris is also included in the extraction of the researched nematodes, particularly for quarantine nematodes; and *ii*) when soil samples are processed as soon as possible after sample collection, considering the results obtained in this experiment.

Little information is available on the vitality of stored nematodes. It was confirmed that *M. chitwoodi* (sibling of *M. fallax*) can survive storage in infected seed tubers, stored at 4°C to prevent sprouting, for 240 days. Although 91% mortality was observed, the vitality of the surviving juveniles was not significantly different from that of freshly hatched juveniles (Teklu *et al*., 2018). We assume that the infectivity of surviving juveniles and eggs of *M. fallax*, *M. hapla* and *P. penetrans* after cold storage in roots will not differ. There is also evidence of juveniles of *M. incognita* remaining infective after storage at 4°C (Vrain *et al*., 1978). A decline in the infectivity of surviving juveniles was reported for *M. incognita* and *P. penetrans* when stored at higher temperature >20°C, where their energy was depleted because of increased metabolism (Pudasaini *et al*, 2008; Tsai, 2008).

When maintaining cultures for future use as a source of inoculum, storage of infected roots is preferable to soil or water suspensions for both RKN and RLN. Avoiding

disturbance of the roots in the pots after harvesting the shoot of the host and keeping the soil moisture at 12-15% improves survival. However, decline is always to be expected dependent on the species and time of storage (Johnson & Potter, 1979; Been *et al*., 2007; Kroese *et al*., 2016).

Conclusions

The primary objective of this study was to investigate the rate of decline and survival of RKN and RLN under the commonly used cold storage temperature of 4°C and, if possible, provide recommendations on sample handling and processing. From the results, we can clearly conclude that storage at 4°C is not always beneficial for maintaining the correct population density, particularly for longer periods. In fact, numbers decline rapidly, which will seriously hamper both the detection of quarantine nematodes by providing false negative detection results (0 nematodes) and the correct estimation of population densities required for decision support systems on management of nematodes.

With the current knowledge we recommend processing samples (extraction and counting) as soon as possible after the samples are taken, to shorten the storage period, thereby avoiding significant population decline and erroneous density estimations in field and pot experiments. This could be realised easily with relatively few samples to be processed. Handling large numbers of samples is not always easy and may take time depending on the capacity of the extraction techniques available. When processing many samples, we can try to avoid bias in field and pot experiments by reducing the effect of storage time between the first and the last samples to be processed. Consider processing a series of treatments, *e.g.*, different nematode densities/plots, per replication, rather than to process all samples per replication at random. The effect of storage time is then mainly effective per replication, but the relation of the averaged nematode infestation /plots and the treatments remains intact. Also, if possible, samples should be processed with low nematode densities first and those with higher densities later. This will help to prevent finding zeros in samples after storage in pot and field experiments.

In both water and soil substrate only hatched juveniles were used, while in the infected roots, mixed-stages, including eggs shielded by egg masses of RKN and free eggs of RLN, could be present. As the number of J2 retrieved from roots in the field in spring is quite large, higher storage temperatures might help to maintain the population by lowering mortality of eggs. Additional work needs to investigate a suitable storage temperature that might improve the survival of temperate and tropical nematode species. Long storage at 4°C should be avoided, if possible, particularly for the tropical RKN. A recommended storage temperature might be 10-15°C for tropical RKN (Tsai, 2008; Takemoto *et al*., 2010), though these temperatures are not species-specific, and survival might depend on storage time, since after a certain period energy depletion occurs. Further research is required to substantiate this observation and to pinpoint the optimum storage temperature of these species by using a wider range of temperatures and even longer storage periods to further research survival in time.

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