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An improved protocol for quantification of root-lesion nematode infection in Barley

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Abstract

In green house conditions, an improved protocol for testing barley plants against root lesion nematode (RLN) had been standerdized. The plants were grown in 150 cm³ instead of 20 cm³ tubes and we increased the inoculum size from 400 to 1000 nematodes/plant in combination with a nutrient solution better adapted to the barley crop. Six barley accessions were tested with *Pratylenchus neglectus* and *Pratylenchus penetrans*. Tests were evaluated 7, 8, 9, and 10 weeks after inoculation. It was found; a shorter test period of 7 weeks is suitable for distinguishing resistant and susceptible genotypes. It was further demonstrated that 7 weeks after inoculations is sufficient to determine nematode numbers instead of 10 or 12 weeks after inoculation. Moreover, measuring the root fresh weight was necessary to calculate the relative number of nematodes/g root mass. In conclusion, results led to a more efficient, quicker and more accurate measurement of barley infections by *Pratylenchus* spp. which is important for selection during early generations of cultivar development.

Keywords: *Hordeum vulgare, Pratylenchus, P. penetrans, P. neglectus,* glasshouse test, nematode extraction, resistance breeding.

Root lesion nematodes (RLN) are migratory endoparasites, polyphagous in nature. They attack a wide spectrum of crops including barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), strawberry (*Fragaria* × *ananassa*), maize (*Zea mays* L.), potato (*Solanum tuberosum* L.), alfalfa (*Medicago sativa* L.), and carrots (*Daucus carota* L.) (Kimpinski & Willis 1981; Olthof, 1987; Smiley, 2009; Pudasaini *et al*., 2008). Three species *viz*., *P. neglectus*, *P. penetrans* and *P. thornei* are among the most important nematode pests worldwide. Often symptoms of root lesion nematode infections are indistinct and difficult to identify. They include poor vigor, stunted growth, poor tillering, and wilting as a result of reduced water uptake. In the field, symptoms can usually be seen in small patches throughout the field as nematodes tend to have a patchy distribution throughout the field.

Apart from wide crop rotations, growing resistant cultivars is the only practical and environmentally friendly way to control RLN in the field. In early generations, infection rates must be determined under standardized conditions in the glasshouse with a clearly defined nematode inoculum to distinguish between resistant and susceptible plant genotypes. Later, when enough seeds are available for replicated trials, selected genotypes were tested in the field (Kable & Mai 1968; Kimpinski & Willis, 1981; Umesh & Ferris, 1992; Olabiyi *et al*., 2009). Many field experiments have been performed with *P. neglectus*, *P. penetrans* and *P. thornei* to screen barley and wheat genotypes (Nicol *et al*., 1999; Smiley, 2009). When commercial wheat cultivars were tested for resistance to *P. thornei* in the field where significant variations in nematode population densities were found high

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population densities caused more extensive lesions and severe cortical degradation in roots of susceptible cultivars.

Besides, in susceptible cultivars there was a significant linear relationship between initial density (Pi) of *P. thornei* and yield (Nicol *et al*., 1999). The reproductive factor (Pf/Pi) was generally lower ($P < 0.05$) for the tolerant/moderately susceptible cultivar (Smiley, 2009). In conclusion, selection in the field was found to be difficult due to varying nematode population densities (Williams *et al.*, 2002). Moreover, damage caused by *Pratylenchus* infection is difficult to score as above ground plant parts mainly appear symptomless even if roots are heavily damaged. In addition, other root parasites as well as environmental factors (e.g. temperature, soil moisture, soil pH, and soil texture) may impact *Pratylenchus* multiplication rates resulting in high genotype × environment interactions. Different protocols have already been published to measure RLN infection in glasshouse grown plants. A panel of 565 barley accessions has been tested for resistance to *P. neglectus* and a number of accessions with low infection rates have been identified (Keil *et al.*, 2009). Continuous phenotypic variations were observed in infected doubled-haploid (DH) population segregating for resistance to *P. neglectus* and *P. penetrans*, indicating a quantitative inheritance of *Pratylenchus* resistance (Sharma *et al.*, 2011a; Galal *et al.*, 2014).

In wheat, a greenhouse bioassay was developed to identify resistance phenotypes of doubled-haploid populations (Williams *et al.* 2002). In barley, a glasshouse test to measure infections by *P. neglectus* and *P. penetrans* plants were grown in 20 cm^3 tubes filled with sterilized sand and inoculated with 400 nematodes 10 days after planting. After 9-12 weeks, this protocol gave reproducible results (Keil *et al.*, (2009).

The aim of the present study was to increase the efficiency and precision of standard procedures for testing resistance of barley towards root lesion nematodes. The main focus was on a shorter test period and improved accuracy. Therefore, three glasshouse experiments were conducted with standardized plant material and nematode inoculum (*P. neglectus* and *P. penetrans*). The modified protocol is expected to facilitate the selection for resistant barley genotypes among segregating populations.

Materials and Methods

Plant Material

Six barley accessions (AC Legenda, Barke, BYDV17, Clho 4285, Morex and Steptoe) from different geographical origin were used. They had been selected due to their contrasting response to *Pratylenchus* spp. infection (Keil *et al*., 2009).

Nematode inoculum

Two species of the genus *Pratylenchus* were used, i.e. *Pratylenchus penetrans* (INOC-388) which was obtained from HZPC Research B.V. (Netherlands) in 2008, and *Pratylenchus neglectus* kindly supplied by Prof. Dr. Richard Smiley (Oregon State University, Columbia Basin Agricultural Research Center, United States) in 2008.

Both populations were maintained and multiplied using the carrot disks method (Moody *et al.*, 1973) with the following modifications: i) Instead of inoculating the carrot disks with a sterilized nematode suspension, small pieces of previously infected carrot disk callus were used to inoculate the new carrot disks, and ii) after 12 weeks of inoculation, carrot disks were chopped and transferred to the misting chamber for 5 days to harvest the nematodes. The nematode suspension was adjusted to 1000 nematodes/ml. Ten days after germination; each plant was inoculated with 1000 nematodes using a Muto-syringe.

Glasshouse experiments

All experiments were conducted in a glasshouse with 23°C day and 18°C night temperatures, under long day conditions (16/8 h) with

supplementary lighting (Son-T Agro 400W, Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands). Seeds were germinated on wet filter paper at 26°C for one day in the dark. Seedlings were transplanted into 150 cm^3 plastic tubes (d = 4 cm, h = 15 cm) filled with heat-sterilized sand and watered with tap water until they were fully soaked. At the bottom of the tubes 20-µm sieves were fixed to prevent root outgrowth and nematode escape during the experiment. Seedlings were grown for 10 days in the glasshouse before nematode inoculation.

After inoculation, the sand was covered with black beads to avoid algal growth, and the tubes were arranged randomly with 8 x 8 cm spacing between tubes as described by Keil *et al*. (2009). Plants were irrigated once a week from below with a nutrient solution as described by Marshall & Ellis (1998). The pH in the solution was adjusted to 6 with 25% sulphuric acid. Nutrient solution was supplied from a 100-liter tank and the excessive water was pumped back into the tank for reuse. The nutrient solution was renewed every two weeks to avoid changes in nutrients concentrations.

Nematode extraction and counting

Between six and ten weeks after nematode inoculation, plants were uprooted and nematodes were extracted from soil and/or chopped roots. Roots were washed with tap water to remove adhering sand, and kept for 5 minutes on a paper towel to remove excessive water. Afterwards, the root fresh weight was determined. Nematodes were extracted only from chopped roots or from chopped roots and soil together using a Baermann funnel kept in a misting chamber for 5 days. Nematode suspensions were collected in plastic bottles and the volume was determined. The bottles were kept in the dark at 4°C until counting. To estimate the number of extracted nematodes, three subsamples (0.5 ml) were taken from each replicate (bottle). Nematodes were counted on a counting slide under a stereomicroscope at 40-fold magnification.

Experimental design and statistical analysis

Three different experiments were conducted in green house conditions.

Experiment 1: Nematode numbers of *P. neglectus* extracted from roots and soil together of each tube were evaluated 7, 8, 9, and 10 weeks post inoculation (WPI). This experiment was designed and analyzed as a completely randomized design (CRD) (Snedecor & Cochran 1967) with six cultivars and six replications. Nematode infection was determined as total nematodes extracted from roots and soil (NRS). Analysis of variance was performed with SAS PROC GLM (SAS version 9.1), where the factors test period and cultivar were treated as fixed.

Experiment 2: The experiment was conducted using *P. penetrans*. Nematode numbers were evaluated 6, 7, 8, 9, and 10 WPI from roots and soil separately. This experiment was also designed as a CRD with six barley accessions and six replications.

Data from roots and soil were statistically analyzed separately as different traits. Analysis of variance was performed with SAS PROC GLM where the factors test period and cultivars were treated as fixed. The coefficient of variation [$CV = (SD / \bar{x}) * 100$] and the coefficient of determination [$R^2 = 1 - (SS_{error} / SS_{total})$] were calculated to determine the variability within the data set of each trait.

Experiment 3: Experiment was carried out to compare nematode numbers extracted from roots and soil together with those extracted only from roots. This experiment was designed as a CRD with six barley cultivars in six replications. Barley genotypes were inoculated with *P. neglectus* as described above. Seven weeks after inoculation, nematodes were extracted from roots and soil together (NRS) or only from roots (NR). In order to relate nematode numbers and root growth, the relative number of nematodes per 1 g of root fresh weight (NgFW).

Analysis of variance was performed with SAS PROC GLM where the factors extraction method (NRS and NR), accessions and extraction method by accessions interaction were treated as fixed. A separate ANOVA was conducted for nematodes extracted only from roots with adjustment to root weight (NgFW). Pearson's correlation between root fresh weight and NRS, NR, and NgFR was calculated.

Results

Optimizing plant growth and nematode extraction

The aims of these experiments were to shorten the time period of the resistance test without affecting its efficiency, and to optimize nematode extraction and counting. Two experiments were carried out. In experiment 1, six barley accessions were inoculated with *P. neglectus*. Nematodes were jointly extracted from roots and soil 7, 8, 9, and 10 weeks post inoculation (WPI). The analysis of variance exhibited significant effects for time periods and barley accessions.

The interaction between accessions and test periods was not significant ($p = 0.414$), indicating that plants from different accessions were equally infected by *P. neglectus* irrespective of the test period (Table 1). The highest number of nematodes (3122, NRS) was found 7 weeks after inoculation. The difference in NRS after 7 and 8 WPI (3085) turned out not to be significant whereas the number of nematodes dropped to 2443 and 2570 nematodes in the 9^{th} and 10^{th} week after inoculation, respectively.

The optimum result to discriminate between resistant and susceptible genotypes was obtained 7 WPI (Fisher's Least Significant Difference, LSD, $\alpha = 0.05$). This time period is 3 weeks shorter as under previous test conditions (Table 1). Then we performed experiment 2 to measure the effects of different test parameters on the multiplication rates of *P. penetrans* using the same six barley accessions as in the previous experiment. Nematodes were extracted from roots and soil separately 6, 7, 8, 9, and 10 WPI. In the light of results from experiment 1 (highest infection rate before the 7^{th} WPI), the number of test periods was increased by one (6 WPI). The number of nematodes ranged from 2015 (6 WPI) to 6196 (10 WPI) in roots, and from 414 (6 WPI) to 2039 (7 WPI) in soil.

The differences between the root extractions 7 and 10 WPI (5917 and 6196) was not significant. However, the number of nematodes was significantly lower in the 8^{th} and 9^{th} WPI. In contrast, no significant differences were found in nematode numbers extracted from soil 7, 9, and 10 WPI (2039, 1947, and 1663; Table 2). In summary, the total number of *P. penetrans* nematodes in roots and soil had its minimum 6 WPI and peaked 7 WPI, followed by a decline in the 8^{th} and 9^{th} week. After 9 weeks the number of nematodes increased again (Fig.1).

Experiment 2 revealed highly significant differences for nematode numbers extracted from roots but not from soil The number of nematodes extracted from soil ranged from 1087 (BYDV17) to 1716 (AC Legenda) with an average of 1461 over the six accessions. In comparison, the number of nematodes in roots was much higher. It ranged from 2908 (BYDV17) to 6109 (AC Legenda) with an average of 4218 over all six accessions. The greatest differences between genotypes (LSD, $\alpha = 0.05$) were found 7 WPI (Table 4). The accessions × test period interaction was significant for both, NR ($p = 0.0387$) and NS ($p = 0.0003$) (Table 2).

Finally, the correlations between NR and NS was calculated, coefficients of variation and coefficients of determination within the data set of each trait (NR, NS) in order to test the precision and accuracy of the results. A significant ($P<0.05$) correlation (0.58) was detected between NR and NS which demonstrates mobility of the nematodes between both environments. The coefficient of variation calculated for NR (0.46) was lower than for NS

(0.63) whereas the coefficient of determination (R^2) for NR (0.59) was higher than for NS (0.48).

Improving the nematode census protocol

Experiment 3 was carried out as a verification experiment to test the hypothesis that NR is a reliable indicator for the multiplication of *Pratylenchus* in barley plants. Moreover, the experiment was aimed to identify the most reliable method for estimating multiplication rates with a maximum of efficiency and precision.

The six barley accessions were inoculated with *P. neglectus*, and nematodes were extracted either from roots + soil together (NRS) or only from roots (NR) 7 WPI.

Variances for extraction methods (NRS and NR) and genotype (barley accessions) were highly significant ($P<0.05$) whereas their interaction was not significant ($p = 0.3321$).

The average number of nematodes in all 6 barley accessions was 2493 for NRS and 1940 for NR, which corresponds to 361 NgFW (Figure 2). The average root fresh weight (FW) differed markedly between the barley accessions ranging from 3.98 g (Morex) to 7.20 g (Clho 4285). Pearson's correlation coefficients between FW and NRS, NR, and NgFW were not significant ($r = 0.05$, - 0.10 and - 0.49).

These results revealed a clear impact of the test system on nematode multiplication rates. Based on NRS, Steptoe (FW = 4.98) showed moderately low infection by *P. neglectus* but multiplication rates were highest based on NR and NgFW. In contrast, accession Clho 4285, which produced the highest FW (7.20 g), was classified susceptible based on NR and NgFW but resistant based on NRS (Fig. 2).

Table 1. Average number of *Pratylenchus neglectus* (NRS) extracted from roots and sand from 6 barley accessions over four-time periods (7, 8, 9, and 10 WPI) (experiment 1)

Test period	AC Legend	Barke	BYDV17	Clho4285	Morex	Steptoe	Mean
7 weeks	3613ab*	1853 c	1885 c	4623 a	3810 ab	2950 bc	3122 A*
8 weeks	3091 *abc*	1979 *c*	2267 *bc*	3382 *ab*	4015 *a*	3772 *a*	3085 A
9 weeks	2544 abc	1535 c	1784 b	2938 ab	3240 a	2618 abc	2443 B
10 weeks	2249 *ab*	2199 *ab*	1846 *b*	2464 *ab*	3300 *a*	3360 *a*	2570 B
Mean[1]	2874 *B**	1892 *C*	1945 *C*	3352 *AB*	3591 *A*	3175 *AB*	2805

* Means with the same letter within each test period or within the right column and bottom row do not differ significantly (Fisher's Least Significant Difference at $\alpha = 0.05$).

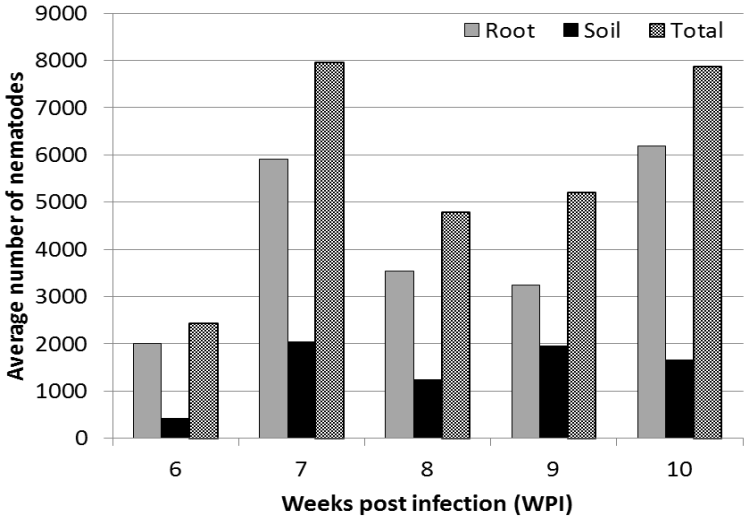

Fig. 1. Nematode (*P. penetrans*) detection efficiency depending on test period (weeks after infection, WPI) and source where the nematodes originated from root or sand (experiment 2). The average number of nematodes (as an average of 6 barley accessions) extracted from roots and soil separately and their sum is given for each treatment.

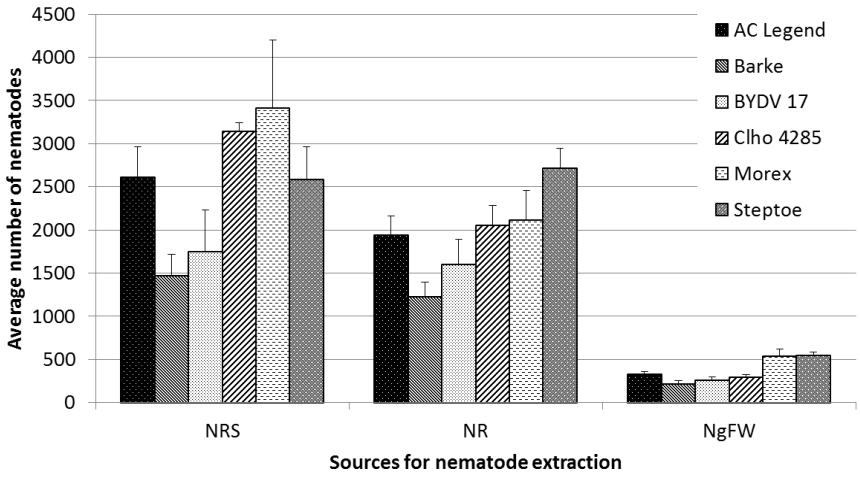

Fig. 2. Nematode (*P. neglectus*) infection 7 WPI (experiment 3). Each column is the mean of nematodes of an individual barley accession (as an average of 6 replicates). Nematodes were extracted from roots and soil together (NRS) or only from roots (NR), and the relative number of nematodes (NgFW) was estimated.

Table 2. Average number of *Pratylenchus penetrans* (NR, NS) extracted from 6 barley accessions over five-time periods (6, 7, 8, 9, and 10 WPI) (experiment 2).

Treatments	Period	AC Legend	Barke	BYDV17	Clho4285	Morex	Steptoe	Mean
Nematode extraction from roots (NR)	6 weeks	2734 a*	1489 a	1605 a	2585 a	2168 a	1509 a	2015 C*
	7 weeks	7215 *ab*	5725 *bc*	3757 *cd*	8727 *a*	3441 *d*	6637 *a*	5917 A
	8 weeks	6833 a	1596 c	2489 bc	4113 b	4253 b	2965 bc	3708 B
	9 weeks	4658 *a*	2292 *b*	2614 *a*	4612 *a*	2505 ab	2830 *a*	3252 B
	10 weeks	9108 a	6256 bc	4073 c	6335 b	5688 bc	5719 bc	6196 A
	Mean	6109 *A**	3471 *B*	2908 *B*	5274 *A*	3611 *B*	3932 *B*	4218
Nematode extraction from soil (NS)	6 weeks	404 a	332 a	508 a	469 a	388 a	382 a	414 C
	7 weeks	1526 *b*	3344 *a*	1150 *b*	1476 *b*	1722 *b*	3019 *a*	2039 A
	8 weeks	3003 a	708 b	685 b	860 b	1077 b	1117 b	1242 B
	9 weeks	1691 *ab*	1671 *ab*	1512 *b*	2590 *a*	1935 *ab*	2284 *ab*	1947 A
	10 weeks	1955 a	1426 a	1584 a	1601 a	1825 a	1590 a	1663 AB
	Mean	1716	1496	1087	1399	1390	1678	1461

* Means with the same letter within each test period or within the right column and bottom row do not differ significantly (Fisher's Least Significant Difference at $\alpha = 0.05$).

Discussion

Resistance is the ability of a host plant to reduce the multiplication rate of a parasite and to hamper its development after infection. In plant nematology, the number of nematodes per plant is an appropriate estimate of plant resistance (Farsi *et al*., 1995). In the present study, we aimed to improve the efficiency, robustness and precision of the morphological quantification-based glasshouse protocol by Keil *et al*., (2009) for resistance evaluation of barley to two *Pratylenchus* species. This experiments lead to some essential modifications of the previous protocol, i.e., the use of 150 cm^3 instead of 20 cm^3 tubes in combination with a nutrient solution better adapted to the barley crop and an increase in nematode inoculums density from 400 to 1000 nematodes per plant. The major achievements are the reduction of the time period from 9-12 to 7 weeks. Furthermore, it has been found that extracting nematodes from roots alone (NR) gives a better measure of nematode multiplication rates in barley plants compared to extracting nematodes from both roots and soil. The life cycle of RLN consists of several developmental stages. As the nematode develops in the egg, it molts to change from a first stage juvenile (J_1) to a second stage juvenile (J_2) which hatches from the egg. The nematode grows and molts three more times to develop into J_3, J_4 and adults. All life stages outside the egg are infective to roots (Castillo and Vovlas, 2007). The life cycle of *Pratylenchus* spp. responds remarkably to environmental factors.

In an *in-vitro* experiment with ladino clover (*Trifolium repens* L.) roots, raising the temperature from 17°C to 27°C shortened the life cycle of *P. penetrans* from 46 days to 22 days (Mizukubo & Adachi, 1997). Evaluation of barley resistance to *Pratylenchus* spp. by morphological quantification of nematodes is a

time-consuming process especially when screening thousands of plants during a breeding program. In this study, the *P. penetrans* population size in roots and soil peaked 7 and 10 WPI followed by a decline 8 - 9 WPI. The *P. neglectus* population size peaked 7 - 8 WPI followed by a reduction 9 - 10 WPI. These dynamics in nematode population size over the test periods could be due to the proportion of adult and advanced juvenile nematode stages (J_3 and J_4) which affects the number of offspring. The higher the proportion of adult and advanced larvae stages, the higher the number of offspring will be and *vice versa*.

The decline in population size 8 - 9 WPI could be ascribed to higher mortality of adult nematodes which developed 7 WPI or earlier. In support of this hypothesis, in an *in-vitro* experiment with *P. neglectus* on barley roots the first generation J_2 larvae developed faster as second generation J_2 larvae and those that emerge directly from (eggs within) the root. This time lag was explained by the shock of handling and finding a suitable feeding site the nematode has to overcome (Umesh & Ferris, 1992).

Resistance to root lesion nematodes is determined as a quantitative value by extracting nematodes either from both roots and soil (Kimpinski & Willis 1981; Griffin & Gray 1990; Keil *et al.* 2009; Sharma *et al.* 2011) or only from roots (Kable & Mai, 1968; Williams *et al.*, 2002). The reproduction rate of nematodes is determined as the ratio between the final nematode population and the initial nematode population (Pf/Pi ratio) (Smiley *et al.*, 2005; Sharma *et al.*, 2011).

In the present study, both roots and soil were used as sources for nematode extractions. However, the following lines of evidence suggest that quantification of nematodes within roots alone is sufficient as a precise indicator for screening barley resistance to *Pratylenchus* spp.; i) the non-significant differences observed between accessions regarding the number of nematodes extracted from soil, and ii) the low number of nematodes extracted from soil compared to that extracted from roots (approximately 1/3) which is in consistence with field results reported by LaMondia (2002). This finding was consistent among accessions. Since *Pratylenchus* spp. are migratory endoparasites, the number of nematodes extracted from both roots and soil is not a precise indicator for resistance and leads to false positive results. Thus, it has been proposed that the resistance to root lesion nematodes can be best determined as the number of nematodes in roots. Low root infection, which is due to a reduction in reproduction rate of nematodes, is indicative for reduced susceptibility of a barley genotype.

It has been demonstrated before that infection of plants with *Pratylenchus* species does not significantly affect root mass (Santo *et al.* 1980; Griffin & Gray 1990; Umesh & Ferris, 1992). In field experiments at three locations, treating wheat varieties with the nematicide aldicarb against *P. thornei* did not affect the root fresh weights compared to the untreated control (Smiley *et al.*, 2005). In investigation the impact of root vigor (indicated by FW) on nematode number was determined. The hypothesis has been established that a larger root system makes the plant better tolerant to nematode infection.

This, however could mask the effect of resistance genes. A negative correlation (- 0.49) was shown between FW and NgFW. This could indicate that genotypes with bigger roots can better fight the invading pests. This could also mean that selecting for higher root mass would go along with better RLN tolerance. Therefore, when selecting for resistant genotypes it is suggested to calculate the relative number of nematode (NgFW) in order to identify the tolerant genotypes.

In conclusion, it is suggested to measure RLN infections at 7 WPI in a 150 cm^3 pot system and to count nematodes only from the roots. For scientific reasons, additional measuring of FW is necessary to calculate the relative number of nematodes per gram root fresh weight. However,

for practical reasons, during routine selection the breeder can rely on measuring the nematode numbers from roots only and take them as a proxy for selecting resistant genotypes from segregating populations.

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