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Analysis of *Aphanomyces euteiches* in Swedish Commercial Pea Field Soils and in Legume Cover Crops Using Droplet Digital PCR

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ABSTRACT

Aphanomyces root rot (ARR), caused by *Aphanomyces euteiches*, is a major constraint in pea (*Pisum sativum*) production. This study aimed to improve molecular detection of *A. euteiches* in soil samples, evaluate the susceptibility of selected legume species, and assess their influence on soil inoculum levels. Twenty-four soil samples were analysed in bioassay 1, which was performed in two batches, using susceptible pea cv. Linnea to determine disease severity index (DSI). DNA analyses employed droplet digital PCR (ddPCR) and quantitative real-time PCR (qPCR). Susceptibility of 14 legume species was examined by artificial inoculation, and the influence of five species on ARR in a subsequent pea crop was tested in naturally infested field soil. The number of gene copies g^{-1} soil differed significantly among soil samples. Soil analysis by ddPCR allowed detection of *A. euteiches* at a level <10 gene copies g^{-1} soil, demonstrating high sensitivity. In bioassay 1, batch 2, DSI correlated significantly with *A. euteiches* gene copies g^{-1} soil quantified by ddPCR and qPCR. Visual assessment and ddPCR showed that lentil (*Lens culinaris*) and fodder vetch (*Vicia villosa*) were highly susceptible, whereas birdsfoot trefoil (*Lotus corniculatus*), sainfoin (*Onobrychis viciifolia*) and Persian clover (*Trifolium resupinatum*) were not susceptible to *A. euteiches* infection. Pea plants grown after fodder vetch had significantly higher DSI and number of *A. euteiches* gene copies. These findings underscore the importance of molecular soil analysis and knowledge of legume species susceptibility for effective ARR management.

1 | Introduction

Aphanomyces root rot (ARR) is a major limitation for pea (*Pisum sativum*) production worldwide (Wu et al. 2019). The responsible oomycete pathogen (*Aphanomyces euteiches*) was initially observed in pea fields in Norway in 1925 and subsequently documented in France in the early 1930s (Sundheim 1972). The disease has since been reported in canning and fodder pea cultivated in Sweden (Olofsson 1967) and later in several pea fields in southern Scandinavia. A new investigation of the distribution of *Aphanomyces* spp. in Sweden, Denmark and Lithuania, revealed *A. euteiches* as the dominant species limiting pea production in Sweden and Denmark (Wikström et al. 2025). The pathogen occurred in 31% of 42 soil samples collected from nine counties throughout Sweden, indicating a widespread occurrence of *A. euteiches* in Swedish pea fields (Wikström et al. 2025). Moreover, pathogenicity tests of Swedish isolates obtained from pea and other legumes revealed two pathotype groups: pea-specific and vetch-specific (Levenfors et al. 2003). Isolates originating from pea, blue lucerne (*Medicago sativa*), green bean (*Phaseolus vulgaris*) and yellow sweet-clover (*Melilotus officinalis*) were highly pathogenic to pea, whereas isolates originating from common vetch (*Vicia sativa*) were weakly pathogenic to pea and other legumes (Levenfors et al. 2003). Recent studies reported distinct

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genetic groups of *A. euteiches* throughout Europe, suggesting diverse regional dynamics (Kälin et al. 2022). Among these, Swedish isolates were found to belong to either a north-eastern European group or a central European genetic group (Kälin et al. 2022). Understanding the genetic structure and virulence in *A. euteiches* will aid in developing future management strategies.

There are currently no effective control measures against ARR available for growers. Chemical control may help to delay the infection but fails to completely suppress the disease (Wu et al. 2019). Pea genotypes with partial resistance to *A. euteiches* have been identified and proven to reduce ARR-mediated yield loss (Kälin et al. 2023). Still, pea cultivars with full resistance remain unavailable (Gossen et al. 2016). Cultural practices, such as crop rotation with non-hosts, are essential but the long-term survival of the oospores in the soil necessitates an extension in crop rotation, placing constraints on the total production capacity (Wu et al. 2018).

ARR can be managed by avoiding cultivation of pea and other host crops in infested fields (Moussart et al. 2009). This helps to prevent the buildup of soil inoculum, which is crucial for achieving satisfactory disease management (Oyarzun et al. 1993a). Disease prediction and management of ARR primarily focus on soil inoculum assessment, pathogen dynamics and environmental conditions conducive to disease outbreaks (Chatterton et al. 2023). In Sweden, ARR prediction is predominantly based on time-consuming soil analysis using a commercial greenhouse bioassay with a susceptible pea cultivar. Based on the root assessment, the bait plant is given a disease severity index (DSI) in a range from 0 to 100 (Olofsson 1967; Wikström et al. 2025). The DSI should not exceed a value of 15 in fields intended for canning pea cultivation (Mariann Wikström, Agro Plantarum AB). Multiple infections by other soil-borne pathogens may occur in Swedish pea fields, including *Phytophthora pisi*, *Phytophthora medicaginis* var. *pinodella* and *Fusarium solani* f. sp. *pisi* (Kälin et al. 2023). This renders visual disease assessment time-consuming as it must be followed by microscopic diagnosis to differentiate between, for example, *A. euteiches* and *P. pisi* (based on the shape of their oospores) (Heyman et al. 2013). Advancements in molecular diagnostic techniques have revolutionised the ability to precisely detect, quantify and monitor plant pathogens, thereby significantly contributing to the management of plant diseases (Wallenhammar et al. 2012). Quantitative real-time PCR (qPCR) is widely considered as the standard molecular diagnostic technique and different qPCR assays have been developed for soil-borne pathogens analyses, including *A. euteiches*. Sensitive qPCR assays targeting *A. euteiches* rDNA from the internal transcribed spacer (ITS) 1 region allowed detection of the pathogen below 10 oospores g^{-1} soil (Gangneux et al. 2014; Heyman 2008). Indeed, these studies also demonstrated a significant correlation between visual disease assessment (using DSI) and molecular detection (with qPCR) using field soil artificially inoculated with *A. euteiches* oospores and zoospores (Gangneux et al. 2014; Heyman 2008). Droplet digital PCR (ddPCR) is a novel PCR technique where the reaction volume is randomly divided in several thousand droplets prior to PCR (Piermatteo et al. 2022). Each droplet subsequently functions as a single amplification reaction, with the positive or negative droplets being identified post-PCR through the measurement of the fluorescent signal. The number of target gene copies is subsequently determined by Poisson statistics without the necessity for known standards (Piermatteo et al. 2022). This technique exhibits reduced sensitivity to inhibitors derived from biological and environmental samples, which is particularly important for analysing soil DNA (Hou et al. 2023; Huggett and Whale 2013; Sidstedt et al. 2015; Wallenhammar et al. 2012).

European cultivation of legume cover crops has increased in recent years driven by the EU CAP policy and the adoption of Conservation Agriculture with reduced tillage. Legume species that are used as cover crops or intercrops can be affected by ARR (Moussart et al. 2013). However, the extent to which certain legume species are susceptible to soil-borne diseases remain to be elucidated. *A. euteiches* produces resting oospores characterised by thick walls, facilitating their persistence in soil over several years (Billard et al. 2019). Consequently, susceptible legume species can become infected and contribute to the propagation of soil inoculum even after extended periods without the cultivation of host plants. The likelihood of an ARR infection escalates markedly when multiple susceptible crops are incorporated into the same crop rotation (Chan and Close 1987; Moussart et al. 2013). Investigations on the susceptibility of legume species for *A. euteiches* are thus crucial, alongside understanding their impact on soil inoculum and implications for ARR infection in subsequent pea crops.

The objectives of this study were to optimise DNA extraction from soil samples and thereby improve/lower the detection limit of *A. euteiches* in soil using ddPCR and qPCR. Further objectives were to investigate the susceptibility of legume species cultivated as cover crops and to assess the influence of selected legume species on ARR infection on a successive pea crop as well as on *A. euteiches* inoculum in soil. The hypotheses were that optimising soil DNA extraction would (i) lower the detection limit of *A. euteiches* inoculum in soil, (ii) improve the correlation between DSI and the number of *A. euteiches* gene copies in soil, and (iii) accurately identify legume species that impose risk for ARR infection in pea.

2 | Materials and Methods

2.1 | Assessment of *A. euteiches* DNA in Field Soil

2.1.1 | Soil Sampling

In total, 24 soil samples were collected from 15 pea fields in October and November 2022 (Table S1). Field selection was based on observations and history of ARR infection in pea crops previously grown in these fields (Table S1). Soil samples were collected from a 20 × 20 m area, in a W-pattern according to Wallenhammar et al. (2012) and marked with a GPS, using a soil auger (Trekanten, Galko Svets and Stålprodukter) with a diameter of 25 mm and a volume of 76 mL that covered the top 20 cm of soil. Each sample consisted of 40 subsamples pooled to one composite soil sample. Due to large field size, multiple samples were collected from fields A:S, B:S and D:K (Table S1). One additional pooled soil sample, composed of 10 subsamples from a depth of 0–20 cm, was collected from each field for chemical soil analysis (Eurofins Food & Agro Testing, Kristianstad, Sweden; Table S1). All soil samples were air dried at room temperature (approx. 22°C) for 6 weeks, crushed with a hammer, and sieved

(pore size of 3.4 mm). The method is hereafter referred to as soil homogenisation method C (Table 1). Each soil sample was subsequently divided in two parts: one used for DNA extraction and molecular analysis and one for the greenhouse bioassay 1.

2.1.2 | Soil Homogenisation

Ten randomly selected soil samples (soil sample number: 1–3, 7 and 10–15) treated with method C were used to study the effect of soil homogenisation on the concentration of extracted DNA and on the quantification of *A. euteiches* soil DNA determined by ddPCR. The soil samples were treated as follows: 100 g of sieved soil samples were freeze-dried at −97°C in a ScanVac coolSafe (Labogene) for 18 h. The samples were then transported to Intertek Scanbi Diagnostics AB, Lomma, Sweden, for homogenisation, according to two grinding methods (A and B) described in Table 1.

2.1.3 | Soil DNA Extraction and Concentration

DNA was extracted from all soil samples, three subsamples of 10 g dry soil each, using DNeasy PowerMax Soil Kit (Qiagen) according to the manufacturer's instructions, but with the following modification: in step 4, the samples were homogenised with a Mixer Mill 400 (Retsch) at 30 Hz for 60 s (repeated twice) and finally incubated at 65°C in a water bath for 10 min.

The eluted DNA was concentrated in two steps. In the first step, 0.2 mL 5 M NaCl (Invitrogen) and 3.5 mL 2-propanol (Thermo Scientific Chemicals) were added to DNA extract. Subsequently, the DNA mixture was pressed through NucleoBond Finalizer filters (Macherey-Nagel) and concentrated according to the manufacturer's instructions, but with one modification: after the washing step with ethanol the DNA was eluted in 250 μL TE buffer (Integrated DNA Technologies) without drying the filters to prevent DNA loss. In the second concentration step, 8 μL 5 M NaCl (Invitrogen) and 416 μL 99% ice-cold (−18°C) ethanol were added per sample. Thereafter, the samples were centrifuged at 14,000 *g* for 30 min and the supernatant was discarded. The pellet was washed with 70% ice-cold (−18°C) ethanol, centrifuged at 14,000*g* for 10 min, then dried in a vacuum desiccator (Nalgene) for 45 min and finally resuspended with 60 μL of 55°C Milli-Q water. The DNA concentration was measured after each step using NanoDrop (Thermofisher Scientific).

TABLE 1 | Treatment list of soil homogenisation.

Grinding method	Device	Device setting
A	Grindomix GM 200 (Retsch)	2 times reverse, 2000 rpm, 15 s; 2 times reverse, 4000 rpm, 15 s
B	1. Grindomix GM 200 (Retsch)	2 times reverse, 2000 rpm, 15 s; 2 times reverse, 4000 rpm, 15 s
	2. Geno/Grinder (SPEX SamplePrep)	1620 rpm for 2 min with steel balls
C	Air dried, crushed with a hammer and sieved soil	Sieve diameter 3.4 mm

Note: Soil samples were first treated with method C before being homogenised with either method A or B.

2.1.4 | ddPCR

The ddPCR assays targeting the ITS-region 1 were performed in a Bio-Rad system with AutoDG Automated Droplet Generator at the Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences (Uppsala, Sweden). The master mix reaction (22 μL) contained 4× ddPCR multiplex supermix (5.5 μL) (Bio-Rad), 1.1 μL each of primer Ae 169F (5′-TCAGGGCTAGCCGAAGGTT-3′) and Ae 169R (5′-ACAAG CTTCATTTCTGATGCTAGTTTA-3′) (20 μM), 0.55 μL of FAM-labelled MGB probe Ae169 (5′-CGCAAGGAACCGATGTA-3′) (10 μM) (Heyman 2008), 3.75 μL Milli-Q water, and 10 μL DNA template (two technical replicates). A synthetic plasmid, pEX-A.Ae 169 (Eurofins Operon) with the *A. euteiches* target sequence was used as a positive control (8.9×10^3 copies μL^{-1}) and Milli-Q water as a negative control. PCRs were carried out in the 96-well plate cycler ProFlex (Thermo Scientific), with the following cycling conditions: 10 min at 95°C; 46 cycles of 30 s at 94°C and 1 min at 65°C; and finally 10 min at 98°C. Data acquisition and analysis performed by the QX200 Droplet Reader (Bio-Rad) and the threshold for positive or negative droplet reads was set manually in QX manager software v. 1.2. The number of gene copies g^{-1} soil was calculated according to the following equation:

$$\text{Gene copies} \times \left(\frac{\text{unit}}{\text{material}}\right)^{-1} = \frac{\frac{\text{Copies}}{\mu L} \times \text{total volume of PCR unit}}{\text{unit material}}$$

2.1.5 | Real-Time PCR

Aliquots of the concentrated soil DNA analysed by ddPCR were initially one-fold diluted with Milli-Q water, purified with Illustra MicroSpin S-300 HR columns (Cytiva), and further diluted (10^{-2}) prior to qPCR analysis. The TaqMan qPCR assay was performed in CFX Connect real-time system (Bio-Rad) according to Heyman (2008). For each reaction (25 μL) the master mix contained 2× TaqMan Universal PCR master mix (12.5 μL) (ThermoFisher Scientific), 0.75 μL of each primer Ae 169F and Ae 169R (20 μM), 0.5 μL of probe Ae169 (10 μM), 5.5 μL Milli-Q water, and 5 μL DNA template (three technical replicates per subsample, $n = 9$). Reaction conditions were 10 min at 95°C; followed by 45 cycles of 15 s at 95°C, and 1 min at 65°C. The number of gene copies g^{-1} soil was calculated from a standard curve constructed with the pEX-A.A 169 plasmid containing the target *A. euteiches* sequence at dilutions of 10^{-6}–10^{-9} (Heyman 2008).

2.2 | Qualitative Analyses of ARR in Field Soils

2.2.1 | Bioassay 1

Twenty-four soil samples were analysed for ARR infection in bioassay 1 and performed in two separate parts: batch 1 and batch 2 (because of the different availability of the soil

samples). Soil samples 1–15 were used for batch 1 and soil samples 16–24 for batch 2 (Table S1). Two soils were used as negative controls: an organogenic greenhouse soil 'U-Jord' designated as 'GH' (Hasselfors Garden) and a field soil (clay soil collected from a commercial field in Uppsala County, Sweden) designated as '0'. Infestation of pea plants with *A. euteiches* in soil '0' was considered unlikely due to the absence of disease indicators and cultivation history with non-hosts (Pfender and Hagedorn 1983). Seeds of the susceptible canning pea cv. Linnea (Nomad Foods Ltd., Findus Sverige AB) were surface sterilised with 70% ethanol for 1 min, 1% NaOCl for 5 min, and washed three times with sterile distilled water (Kälin et al. 2022). Five seeds were sown per plastic pot (9 × 9 × 11 cm), arranged in a randomised complete block design ($n = 5$) in a greenhouse at BioCenter, Swedish University of Agricultural Sciences (Uppsala). The growth conditions were a temperature of 20°C, 52% humidity, and a light circle with 16 h of light and 8 h of darkness. Pots were kept moist and watered with 50 mL tap water per pot. After 3 weeks, pea plants were uprooted, roots were washed under running tap water and symptoms were assessed in a 0–100 scale according to Kälin et al. (2022) (Figure S1). The shoots were excised per pot and dried for 16 h at 60°C in a drying chamber (Termaks). Roots were cut into small pieces and kept in a freezer (at −18°C) until molecular analysis. Batch 1 was repeated once ($n = 2$), while batch 2 was not repeated due to limited amount of collected soil.

2.2.2 | DNA Extraction From Roots

Pea roots were initially crushed in liquid nitrogen using a mortar and pestle. DNA was extracted from 100 mg of root material using FastDNA SPIN Kit (MP Biomedicals) according to the manufacturer's instructions (technical replicates $n = 2$). Tissue homogenisation in the extraction buffer was performed at 5500 rpm with two 35 s cycles using Precellys 24 tissue homogeniser (Bertin Technologies). DNA extracts were purified with Illustra MicroSpin columns (Cytiva).

2.2.3 | Conventional PCR and Gel Electrophoresis

DNA extracts from pea roots were analysed using conventional PCR performed with *A. euteiches* microsatellite marker Ae37 in SimpliAmp thermal cycler (Applied Biosystems) (Kälin et al. 2022; Mieuzet et al. 2016). The master mix reaction (25 μL) contained 2.5 μL Dream Taq buffer (10×) (Thermo Fisher Scientific), 2.5 μL dNTPs (10 mM), 1.5 μL each of forward and reverse primers (20 mM), 0.3 μL Dream Taq (2 U) (Thermo Fisher Scientific), 16.7 μL Milli-Q water and 1 μL DNA template. The reaction conditions were denaturation at 96°C for 5 min; followed by 30 cycles of 1 min at 95°C, 1 min at 58°C and 1.5 min at 72°C (Mieuzet et al. 2016). PCR products were separated by electrophoresis in 2% agarose gel and visualised in the GelDoc 2000 (Bio-Rad).

2.3 | Evaluation of Legume Species Susceptibility for *A. euteiches*

2.3.1 | Bioassay 2

Susceptibility of 14 legume species for *A. euteiches* was investigated in a greenhouse bioassay. The selected species were birdsfoot trefoil (*Lotus corniculatus*), common vetch (*Vicia sativa*), fodder vetch (*Vicia villosa*), lentil (*Lens culinaris*), blue lucerne (*Medicago sativa*), narrow-leaf lupin (*Lupinus angustifolius*), sainfoin (*Onobrychis viciifolia*), crimson clover (*Trifolium incarnatum*), Egyptian clover (*Trifolium alexandrinum*), Persian clover (*Trifolium resupinatum*), red clover (*Trifolium pratense*), squarrose clover (*Trifolium squarrosum*), Swiss red clover mattenklee (*Trifolium pratense*) and white clover (*Trifolium repens*) (Table S2). Pea cv. Linnea was included as a positive control. All seeds were surface sterilised in the same manner as bioassay 1, then placed on 0.8% water agar and left to germinate at 20°C for 2–4 days in darkness depending on the size of the seeds. A Swedish strain of *A. euteiches*, SE51, belonging to the broader central European genetic group, was cultured on corn meal agar (CMA, BD Biosciences) for 14 days at 20°C in darkness (Kälin et al. 2022). Plastic pots (9 × 9 × 11 cm) were filled with vermiculite (Sibelco) to about 3 cm under the pot rim. Five regularly interspaced agar plugs (10 mm diameter) were placed in the pot and covered with a thin (3 mm) layer of vermiculite. Subsequently, five seedlings of each legume species were sown, and the remaining space was filled with vermiculite ($n = 4$). The pots were placed in a greenhouse with similar growth conditions to bioassay 1. The plants were watered with a nutrient solution containing nitrogen (nitrate-$N = 80\ mg\ L^{-1}$; ammonium-$N = 41\ mg\ L^{-1}$) (Wallco), to compensate for the lack of biological nitrogen fixation. The plants were uprooted after 4 weeks, washed, and assessed for ARR in a 0–6 scale, to encompass discolouration in the epicotyl, where 0 = healthy plants; 1 = little discolouration; 2 = 25% discolouration of the root system; 3 = 50% discolouration of the root system; 4 = 75% discolouration of the root system; 5 = the entire root system is discoloured; 6 = the whole root system + the epicotyl is discoloured (Wikström et al. 2025). Disease severity index was calculated according to the following equation:

$$\mathrm{DSI} = \frac{\left((0 \times N_0) + (1 \times N_1) + (2 \times N_2) + (3 \times N_3) + (4 \times N_4) + (5 \times N_5) + (6 \times N_6)\right) \times 100}{n \times (\text{No. of classes} - 1)}$$

N = number of plants in each class per pot; n = total number of plants assessed per pot. The shoots were dried for 16 h at 60°C in a drying chamber (Termaks) and the dry weight was measured thereafter. The bioassay was repeated once ($n = 2$).

2.3.2 | ddPCR

DNA was extracted from the roots and purified as described previously. DNA from the infected roots of pea, lentil and birdsfoot trefoil was tested in two DNA concentrations, 10 and 20 ng μL^{-1}, to find the optimal DNA concentration prior to ddPCR analysis of all samples. The 10 ng μL^{-1} resulted in saturation of the ddPCR in certain subsamples of pea and lentil. Gene copies at the lowest DNA concentration in birdsfoot trefoil was still

detectable, 19 ng μL^{-1}, therefore DNA from all samples was diluted to 5 and analysed as mentioned previously. The number of *A. euteiches* gene copies was not normalised against plant DNA because the copy number of *cox* gene in the genome of certain tested legumes is unknown.

2.4 | Risk Assessment of ARR in Pea Following a Legume Cover Crop

In bioassay 3, new field soil samples were collected in November 2023 from the GPS-marked patches in field B:S where soil samples 8 and 9 were previously collected in 2022 (Table S1). The soil samples were homogenised by method C (Table 1). To confirm the amount of the inoculum in each soil sample, DNA was extracted from 10 g subsamples ($n = 3$) and analysed by ddPCR. The two soil samples were then mixed 1:1 to provide a composite soil with 19 gene copies g^{-1} soil, then filled in plastic pots (9 × 9 × 11 cm). Seeds of red clover, white clover, crimson clover, Egyptian clover and fodder vetch were surface sterilised, and 10 seeds were directly sown in each pot ($n = 4$). Seeds of pea cv. Linnea were sown as a positive control, and an additional control of unsown soil was included as a negative control. Following seed germination, the plants were thinned to five plants per pot and left to grow for 6 weeks. The plants were then uprooted, and the roots were cut into small pieces and mixed back into the soil per respective pot. Five seeds of pea, cv. Linnea, were sown in each pot, including the untreated control, and grown in the greenhouse for 3 weeks under the same previously mentioned conditions. Once this period ended, pea plants were uprooted, and the roots were washed and assessed for disease symptoms as for bioassay 2 (section 2.3.1). Lastly, the roots were ground, and 100 mg root tissue was used for DNA extraction as previously described ($n = 2$). Pea shoots were dried, and the dry weight was recorded. The remaining soil in each pot was air dried, homogenised by method C and 10 g was used for DNA extraction (two technical replicates). Soil and plant DNA were analysed by ddPCR with two technical replicates. The bioassay was repeated once ($n = 2$).

2.5 | Statistical Analysis

Statistical evaluation of the experimental results was determined using the programming language R (v. 4.4.1). The coefficient of variation (CV) was analysed to assess the variability of the amount of DNA extracted from differently ground soil samples. The CV was divided into three classes (1, 2 and 3) and applied to the variously treated soils of one soil sample. Class 1, which had the lowest CV, was attributed to the most consistent and reliable soil homogenisation method, while class 3, which had the highest CV, was considered the least reliable. The two batches of bioassay 1 were analysed separately. The correlation analyses of number of gene copies and DSI, and gene copies and shoot dry weight were determined through Spearman analysis. Significant effects of the soils on emergence, DSI and shoot dry weight were determined using ANOVA and post hoc Tukey's test ($p \leq 0.05$), presented with standard error. The effects of the legume species on the following pea crop, measured as DSI, shoot dry weight, emergence, and number of gene copies, were analysed using linear modelling with emergence of both legume species and pea as covariant (ANCOVA and post hoc Fisher's least significant difference, $p \leq 0.05$).

3 | Results

3.1 | Effect of Soil Homogenisation on Soil DNA and Detection of *A. euteiches*

No significant difference was shown in DNA concentration from soil samples 2, 3, 7 and 12 following soil grinding by method A and B compared to method C (Table 2). Methods A and B resulted in significantly higher DNA concentration from soil sample 1 and soil samples 10–11 compared to method C (Table 2). The positive impact of soil grinding on DNA concentration also led to lower variation between the subsamples of soil samples 1 (Figure S2), and 10–11 (Table 3). On the other

TABLE 2 | The effect of soil homogenisation, performed by soil grinding methods (A and B), compared to method C, on DNA concentration (ng μL^{-1}) of *Aphanomyces euteiches* in soil measured with NanoDrop ($n = 3$).

	Method A		Method B		Method C	
Soil ID	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
1	29.3	0.96 a	23.3	0.95 b	16.5	0.98 c
2	5.1	1.54 a	4.6	2.20 a	12.4	2.51 a
3	4.8	1.74 a	9.0	1.68 a	7.6	1.13 a
7	29.8	1.79 a	22.6	6.80 a	20.0	4.73 a
10	33.2	1.85 a	31.5	2.86 a	17.5	1.04
11	37.3	4.54 a	40.0	5.67 a	14.7	1.27 b
12	17.5	3.15 a	12.7	2.80 a	10.8	2.40 a
13	29.7	2.64 a	19.9	1.42 b	21.3	1.90 ab
14	28.5	0.88 a	21.8	2.18 a	21.8	2.91 a
15	19.3	0.64 ab	16.7	3.30 b	31.0	3.41 a

Note: Soil samples were first treated with method C before being homogenised with either method A or B. Different letters indicate significant differences between methods for each soil sample, according to Tukey's HSD test ($p \leq 0.05$).

TABLE 3 | The effect of soil homogenisation on DNA concentration ($ng^{-1}\mu L^{-1}$), performed by soil grinding methods (A and B) compared to method C, on coefficient of variation of DNA concentration ($ng^{-1}\mu L^{-1}$) measured with NanoDrop.

	Method A			Method B			Method C		
Soil ID	**DNA**	**Group**	**CV[a]**	**DNA**	**Group**	**CV**	**DNA**	**Group**	**CV**
1	29	2	0.05	23	1	0.06	17	3	0.08
2	5	2	0.43	5	3	0.68	12	1	0.29
3	5	3	0.51	9	2	0.26	8	1	0.21
7	30	1	0.09	23	3	0.43	20	2	0.33
10	33	1	0.08	32	3	0.13	18	2	0.08
11	37	2	0.17	40	3	0.20	15	1	0.12
12	17	1	0.25	13	2	0.31	11	3	0.31
13	30	3	0.13	20	1	0.10	21	2	0.13
14	28	1	0.04	22	2	0.14	22	3	0.19
15	19	1	0.05	17	3	0.28	31	2	0.16
Total		17	1.80		23	2.59		20	1.90

Note: Soil samples were first treated with method C before being homogenised with either method A or B.
[a]The coefficient of variation (CV) of the DNA concentrations was determined. The homogenisation method with the lowest variance within the soil sample was labelled and grouped as 1, and the highest as 3. The total values per treatment method are indicated.

TABLE 4 | The effect of soil homogenisation, performed by soil grinding methods (A and B) compared to method C, on quantification of *Aphanomyces euteiches* DNA in soil measured by droplet digital PCR as gene copies g^{-1} soil ($n = 3$).

	Method A		Method B		Method C	
Soil ID	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
1	211	5.73 a	272	115.01 a	42	40.84 a
2	49	23.70 a	15	3.87 b	9	1.32 b
3	254	22.65 b	172	20.94 a	106	2.11 a
7	38	20.89 a	167	21.17 a	2054	1854.85 a
10	31	6.36 a	26	5.16 b	17	8.53 b
11	7	2.51 b	1	0.95 a	0	0.07 a
12	1	0.08 a	0	0.06 a	0	0.11 a
13	12	0.93 a	14	0.66 b	86	76.56 b
14	47	46.92 a	0	0.45 a	162	44.52 a
15	2	1.97 a	0	0.48 a	0	0.07 a

Note: Soil samples were first treated with method C before being homogenised with either method A or B. Different letters indicate significant differences between methods for each soil sample according to Tukey's HSD test ($p \leq 0.05$).

hand, there were no significant differences regarding the number of gene copies after soil grinding including samples 1 (Figure S2), 2, 10–11 and 15, although the number of gene copies g^{-1} soil were higher for method A than for method C (Table 4). Grinding method A resulted in a significantly higher number of gene copies in samples 3 and 12, compared to methods C and B (Table 4). In soil sample 14, method B generated just 0.7 gene copies 100 ng^{-1} DNA, which was significantly lower than for method C (Table 4).

3.2 | Qualitative Assessment of ARR in Field Soils

3.2.1 | Disease Severity Index and Pea Shoot Dry Weight

Visual ARR assessment in pea plants tested in batch 1 of bioassay 1 (soil samples 1–15) showed that pea plants sown in soil samples 1, 3 and 6 showed a DSI > 70 (Figure 1A). These indices were significantly higher than DSI for the untreated control plants ($p \leq 0.05$) (Figure 1A). Pea plants grown in soil samples

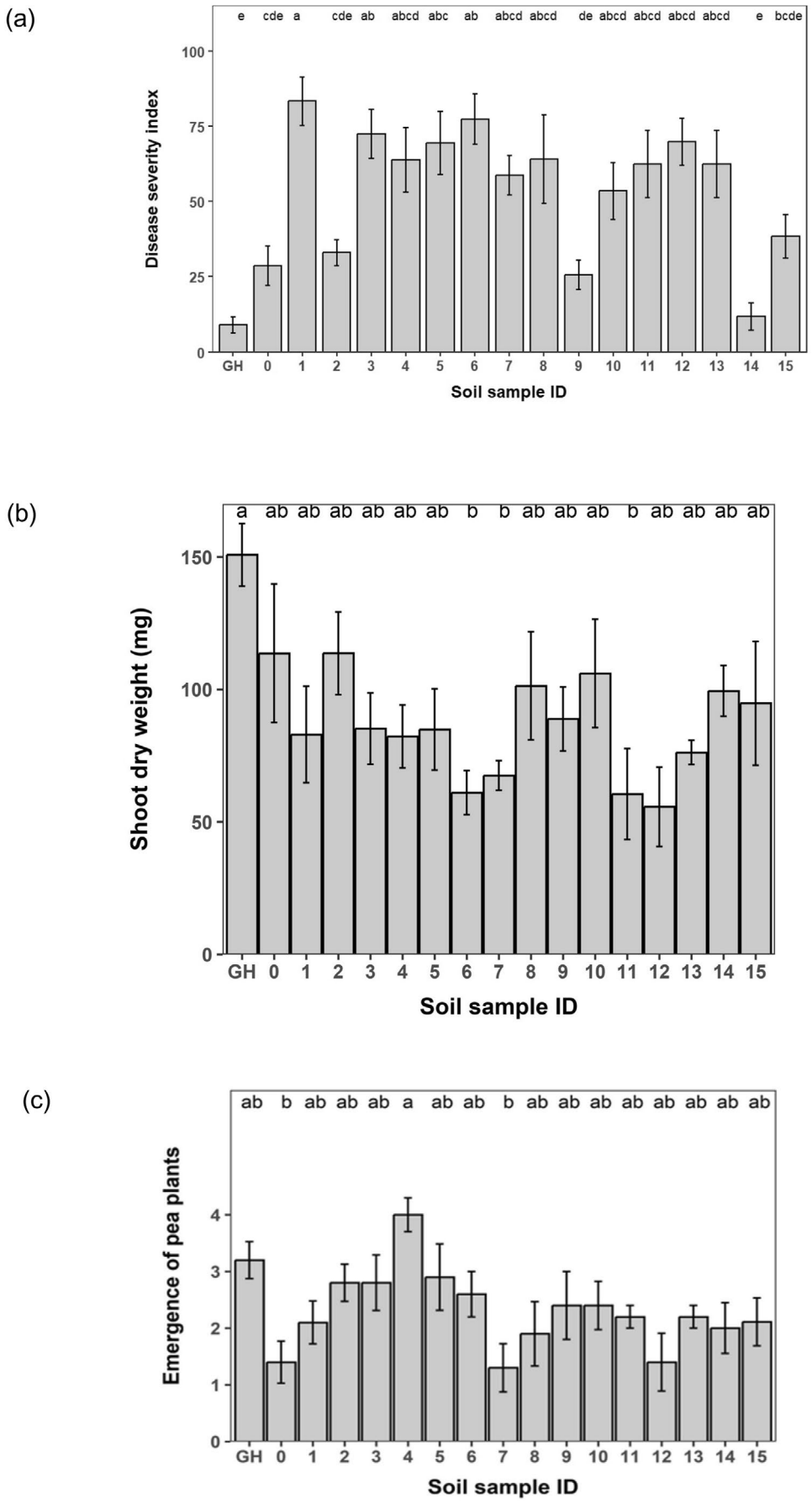


FIGURE 1 | Legend on next page.

FIGURE 1 | Assessment of Aphanomyces root rot in susceptible pea cv. Linnea sown in natural field soil samples 1–15 performed in batch 1 of bioassay 1. (a) Disease severity index, (b) shoot dry weight (mg) and (c) emergence measured after 21 days. The whiskers on the bar represent the standard error of the mean (SE), and different letters indicate significant differences as determined with ANOVA and post hoc Tukey HSD test ($p \leq 0.05$). Mean values of two greenhouse experiments.

4–5, 7–8 and 10–13 exhibited a DSI > 50, significantly higher than DSI of pea plants grown in untreated control soil 'GH', but not for control soil '0' ($p \leq 0.05$) (Figure 1A). The lowest DSI was obtained in soil 14 and was not significantly different from soils 'GH' and '0', as was the case for soil samples 2 and 9 (Figure 1A). In contrast to DSI, soils exerted less influence on shoot dry weight (Figure 1B). The highest shoot dry weight was obtained for plants grown in soil 'GH' (120 mg plant^{-1}), which is significantly higher than plants grown in soil 6 ($p \leq 0.05$) (Figure 1B). The shoot dry weights ranged from 60 to 70 mg per plant for the other plants (Figure 1B). Plant emergence was particularly high in soil 4 and untreated control soil "GH" (> 3 plants per pot out of 5 seeds per pot) ($p \leq 0.05$) (Figure 1C).

Likewise, DSI of plants grown in the untreated control soils in batch 2 of bioassay 1 were significantly lower compared to soil samples 16–24, except samples 16, 19 and 23 ($p \leq 0.05$) (Figure 2A). DSI of plants grown in soil samples 20 and 21 were significantly higher compared to the DSI of the plants grown in the other soil samples (Figure 2A). Additionally, the shoot dry weight was particularly low in plants grown in soil samples 20 and 21, with an average dry weight of 10 mg and 60 mg per plant, respectively (Figure 2B). The shoot dry weight of plants grown in control soil 'GH' (140 mg per plant) was significantly higher compared to plants grown in soil samples 17–18 and 20–21 (Figure 2B). Seedling emergence was significantly lower in soil samples 18, 21–22 and 24 compared to soil 'GH' (Figure 2C).

3.2.2 | Detection of *A. euteiches* DNA in Pea Roots

PCR and gel electrophoresis confirmed infection by *A. euteiches* in the roots of pea plants grown in soil samples 1–8, and 10–11, but not the roots of plants grown in soil samples 9 and 12–15 in batch 1 of bioassay 1 (Figure 3A). In batch 2 with soil samples 16–24, *A. euteiches* DNA was detected in the roots of pea plants grown in soil samples 16–18 and 20–22 (Figure 3B). It should be noted that an amplification product with a DNA band size of 500 bp was detected in most of the samples, which does not indicate infection by *A. euteiches*. In this context, the presumed absence of *A. euteiches* in control soils 'GH' and '0' was confirmed in both parts of the bioassay (Figure 3A,B).

3.3 | Quantification of *A. euteiches* Gene Copies in Soil

The highest average number of gene copies quantified by ddPCR was obtained in soil sample 14 (1816 g^{-1} soil) followed by soil sample 21 (1522 g^{-1} soil) and soil sample 7 (1389 g^{-1} soil) (Figure 4A). The respective values in the other samples ranged from 0 to 799 gene copies g^{-1} soil with untreated control soil 'GH' and '0' showing the lowest average number of gene copies (< 0.5 copies μL^{-1} reaction) (Figure 4A). If the threshold line in ddPCR software QX manager was set manually to 0.5 copies μL^{-1} reaction, both control soils, along with soil samples 9, 11–12 and 23–24, could be considered negative or under the detection limit for quantification of *A. euteiches* DNA using ddPCR.

qPCR failed to produce any amplification when using the same DNA samples that were analysed by ddPCR. Therefore, it was necessary to further purify and dilute the DNA before any detection could be achieved. The qPCR analysis revealed a large variation in the number of *A. euteiches* gene copies across the various soil samples. *A. euteiches* DNA was undetectable in soil samples 9, 11–12 and 23–24, as well as the untreated control soil 'GH' (Figure 4B). The occurrence of *A. euteiches* in control soil '0' and in soil samples 15 and 17–19 was under the detection limit with DNA amplification from only one of nine technical replicates, hence also considered negative as shown in Figure 4B. The highest average number of gene copies g^{-1} soil was determined in samples 21 (1044 gene copies g^{-1} soil) and 20 (917 gene copies g^{-1} soil), significantly higher than the respective values in both soil controls. The corresponding values for the other soil samples ranged from 187 to 825 gene copies g^{-1} soil (Figure 4B).

A tendency of positive correlation between DSI and the number of gene copies g^{-1} soil quantified by ddPCR was observed in batch 1 of bioassay 1. On the other hand, the correlation with shoot dry weight was negative, but the Spearman correlation did not reach statistical significance (Figure 5A,B). Similar pattern of correlations was obtained for DSI and shoot dry weight with number of gene copies g^{-1} soil quantified by qPCR (Figure 5C,D). Conversely, in batch 2, a significant positive correlation was obtained between DSI and gene copies g^{-1} soil quantified by ddPCR ($R^2 = 0.65$; $p = 0.0044$) (Figure 6A). A significantly negative correlation was obtained between the gene copies g^{-1} soil (ddPCR) and shoot dry weight ($R^2 = 0.5$; $p = 0.019$) (Figure 6B). The quantified gene copies using qPCR correlated significantly with DSI in batch 2 ($R^2 = 0.6$; $p = 0.0051$) (Figure 6C), but not in batch 1 (Figure 5C). No significant correlation was found between gene copies g^{-1} (qPCR) and pea shoot dry weights across all 24 samples, as evidenced by the R^2 values < 0.5 (Figures 5D and 6D).

3.4 | Evaluation of Legume Species Susceptibility for *A. euteiches*

Visual assessment of ARR in the susceptible pea cv. Linnea, and the 14 investigated legume species demonstrated strong discolouration on the roots of inoculated plants of pea, lentil and fodder vetch in bioassay 2 (Table 5, Figure S3). Conversely, Egyptian clover, white clover, sainfoin, as well as birdsfoot trefoil and red clover exhibited weaker symptoms (Figure S4). The roots of untreated control plants and those of inoculated plants of Persian cover and squarrose clover were strongly discoloured.

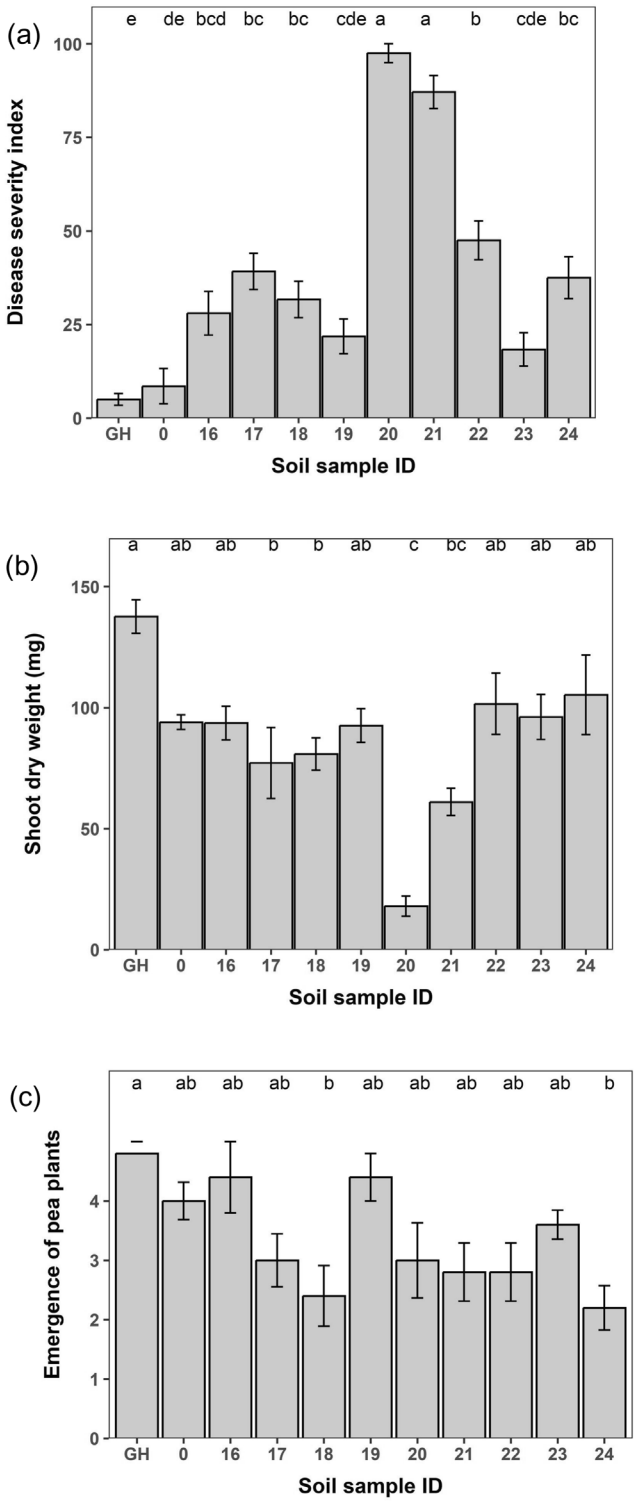


FIGURE 2 | Assessment of Aphanomyces root rot in plants of susceptible pea cv. Linnea sown in natural field soil samples 16–24 performed in batch 2 of bioassay 1. (a) Disease severity index, (b) shoot dry weight (mg) and (c) emergence measured after 21 days. The whiskers on the bar represent the standard error of the mean (SE), and different letters indicate significant differences as determined with ANOVA and post hoc Tukey HSD test ($p \leq 0.05$). Mean values of one greenhouse experiment for batch 2.

The mean value of DSI for all control plants was ≥ 20 (Table 5). The highest DSIs of inoculated legume plants were observed in pea, fodder vetch, lentil and squarrose clover, which were significantly different from the other legume species in the pairwise comparisons ($p < 0.0001$) (Table 5).

The highest numbers of *A. euteiches* gene copies mg^{-1} in root tissue of the tested legume species were obtained for pea and lentil ($p < 0.0001$) (Table 5). The corresponding values for blue lucerne, crimson clover and narrow-leaf lupin were < 20 gene copies mg^{-1} root (Table 5). Despite high DSI, a low number of gene copies were quantified in squarrose clover. The mean values for Egyptian clover, Persian clover, red clover, white clover, mattenklee, birdsfoot trefoil, common vetch and sainfoin were ≤ 1 gene copies mg^{-1} roots (Table 5). Furthermore, the DNA of *A. euteiches* was undetectable in the roots of all untreated control plants (Table 5).

3.5 | Risk Assessment of Cultivating Pea After a Legume Cover Crop

Pea plant roots exhibited pronounced discolouration when cultivated in field soil after succession with pea plants or fodder vetch plants in bioassay 3. This resulted in significantly higher DSI compared to that of pea plants grown in the untreated soil control or after crimson clover, Egyptian clover, red clover and white clover ($p \leq 0.05$) (Figure 7A). Correspondingly, the shoot dry weight of pea grown after pea (60 mg per plant) was the lowest among the treatments yet was only significantly different from shoot dry weight of pea plants grown after crimson clover (111 mg per plant) and Egyptian clover (122 mg per plant) ($p \leq 0.05$) (Figure 7B).

The lowest emergence, out of five seeds per pot, was observed in pea grown in succession with Egyptian clover, only significantly different from the emergence of pea grown in the negative control ($p \leq 0.05$) (Figure 7C).

The highest average number of gene copies g^{-1} soil was observed for the soil where pea was grown in succession with pea, significantly different from the corresponding values for the negative control, crimson clover, Egyptian clover and white clover ($p \leq 0.05$) (Figure 7D). Fodder vetch and red clover typically increased soil inoculum, but the corresponding values of average gene copies g^{-1} soil were not significantly different from the other treatments (Figure 7D). Quantification of *A. euteiches* DNA in the pea roots measured as gene copies mg^{-1} of root was highest in pea plants grown after pea (34,000 gene copies mg^{-1} of root). This result was significantly different from the corresponding values of pea grown in the negative control and for pea grown after crimson clover and white clover ($p \leq 0.05$) (Figure 7E).

4 | Discussion

ARR is a significant challenge in pea production areas, with control strategies restricted due to the lack of effective fungicides and the absence of complete genetic resistance. The primary management strategy is field avoidance, supported by soil testing to identify low-risk sites prior to seeding (Olofsson 1967). Soil inoculum is often assessed visually in a bioassay, where disease symptoms on bait plants are scored

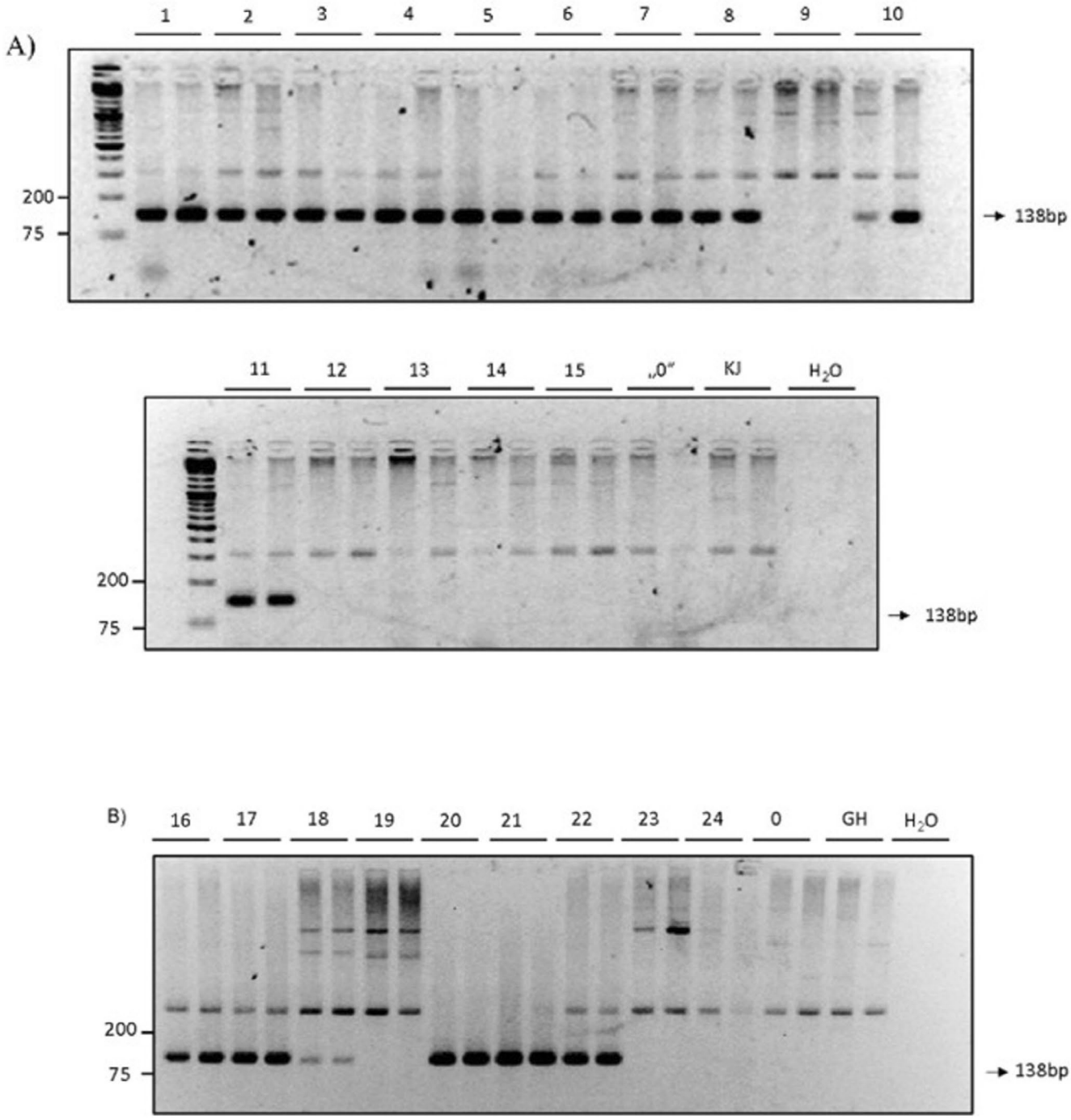


FIGURE 3 | Qualitative assessment of *Aphanomyces euteiches* infection in pea roots (cv. Linnea) analysed by PCR and the amplicons visualised using gel electrophoresis. The DNA band, which indicates a positive infection, has a molecular weight of 138 bp. (A) Detection of *A. euteiches* in roots of plants grown in soil samples 1–15. (B) Detection of *A. euteiches* in roots of plants grown in soil samples 16–24.

after incubation. In contrast, molecular methods assess inoculum by detecting pathogen DNA directly in soil, offering greater sensitivity and specificity. DNA extraction is the first step in molecular detection of soil-borne pathogens, which typically relies on extraction from small soil subsamples of approximately 0.25–1 g (Wallenhammar et al. 2012). *A. euteiches* can cause infection in pea and other susceptible legumes at very low inoculum levels. To address the challenge of low DNA-yield and suboptimal detection limit of soil samples, we proposed that increasing the soil sample size and applying mechanical soil grinding would enhance *A. euteiches* detection. It is widely acknowledged that soil grinding prior to DNA extraction reduces soil heterogeneity, facilitates lysis of microbial cell walls, and enhances DNA release (Frostegård et al. 1999). The influence of soil grinding on the detection of *A. euteiches* remained largely inconclusive, even though the effect was more evident in specific soil samples, for example sample 1 collected near the edge of field A:S, therefore rejecting hypothesis (i). One potential explanation for the inconsistent effect of soil grinding across most of the

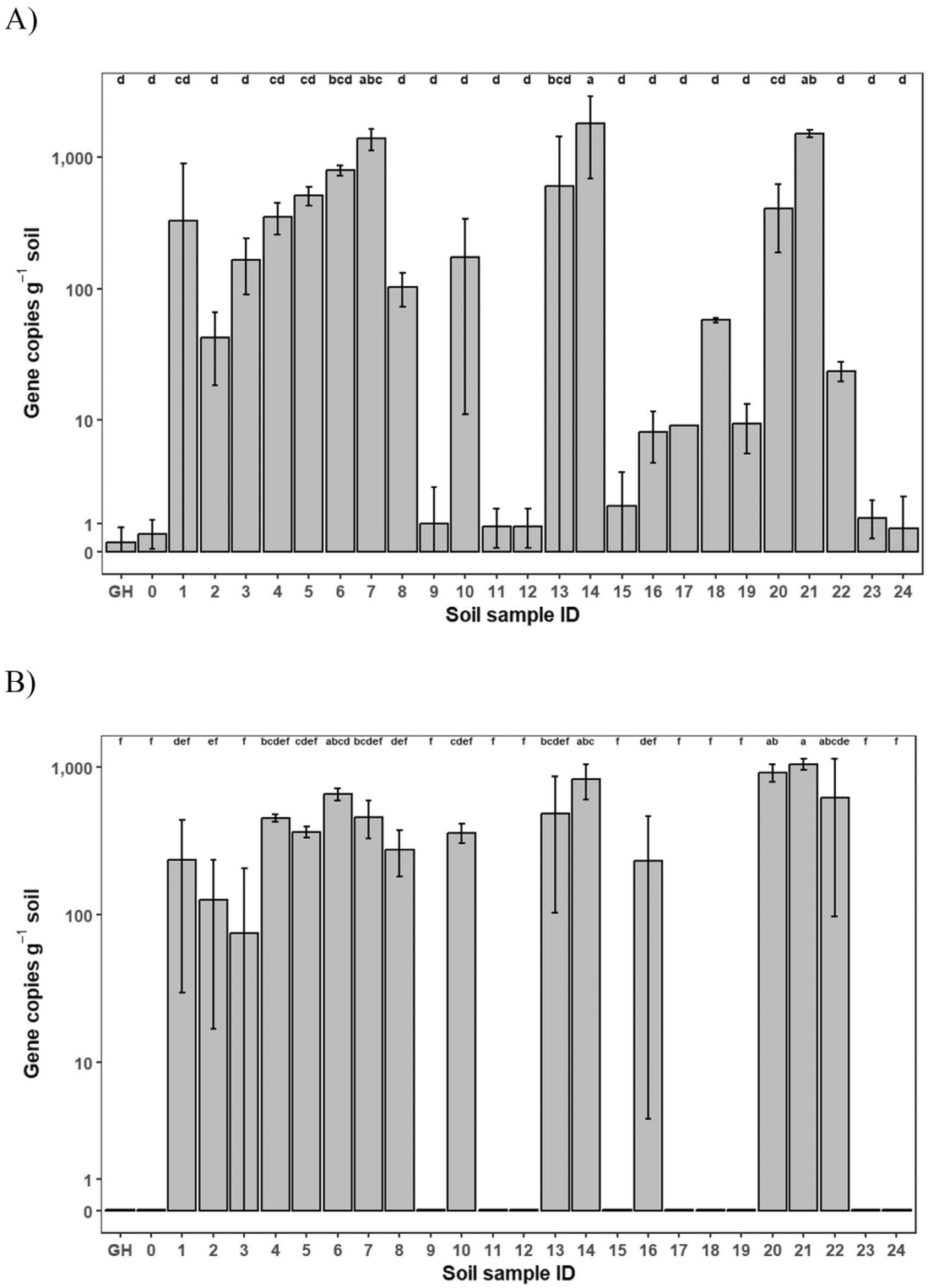


FIGURE 4 | Occurrence of *Aphanomyces euteiches* in 24 field soil samples, control greenhouse soil 'GH' and uninfected field soil '0' quantified as gene copies g^{-1} soil. (a) Number of gene copies quantified by droplet digital PCR, (b) number of gene copies quantified by qPCR. Log scale in *y*-axis. ANOVA and post hoc Tukey HSD test ($p \leq 0.05$).

24 soil samples is the use of larger soil quantities for DNA extraction, combined with mechanical bead-beating and chemical lysis during the DNA extraction process, which may have compensated for the effects of soil grinding (Plassart et al. 2012).

The adopted methodologies for optimisation of soil DNA extraction were anticipated to lower the detection limit of the qPCR assay. The inherent sensitivity of qPCR to soil contaminants hindered identification of pea fields with low inoculum (Sidstedt et al. 2015). In contrast, ddPCR confirmed the presence

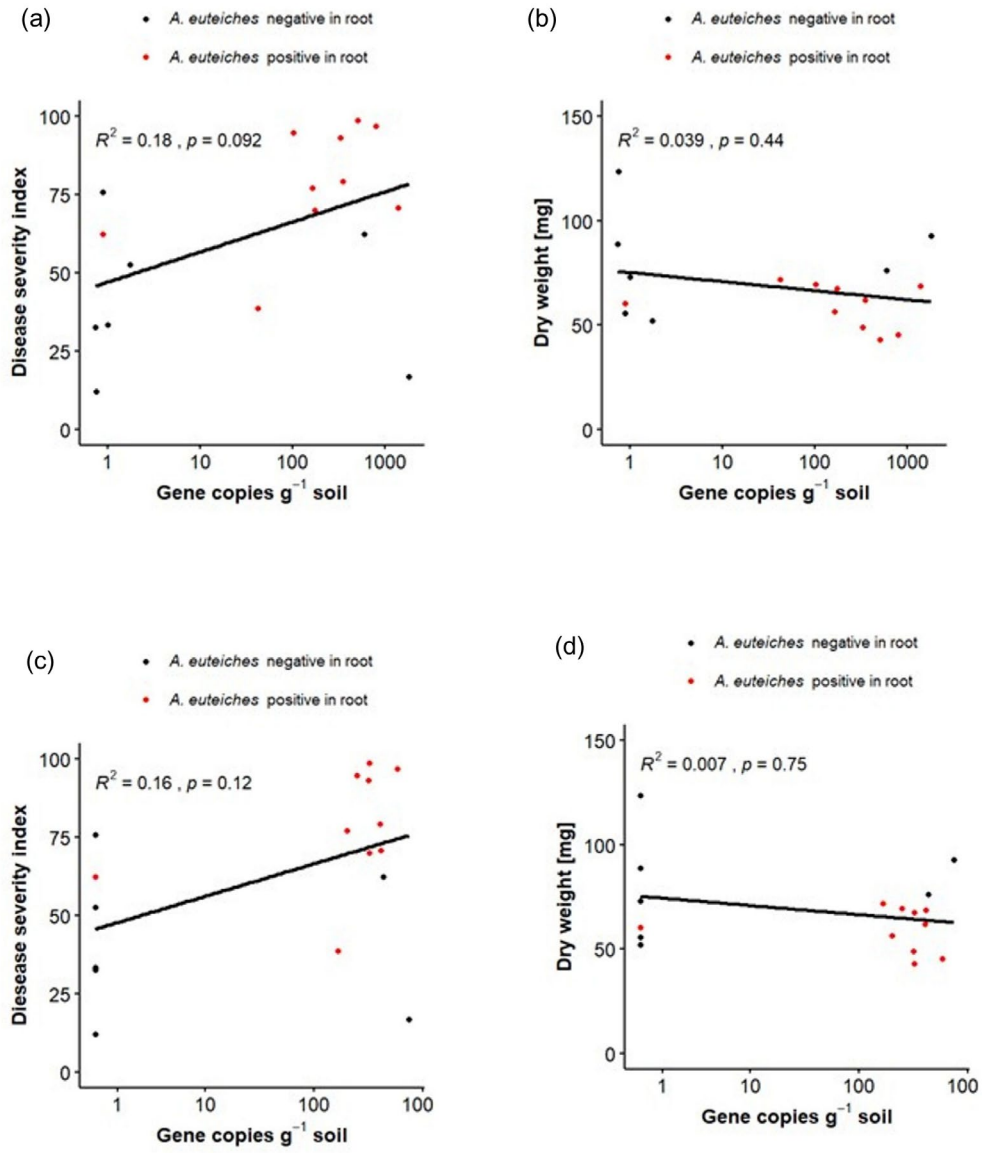


FIGURE 5 | Correlation between number of gene copies g^{-1} soil for *Aphanomyces euteiches* determined by droplet digital PCR for soil samples 1–15 (batch 1 of bioassay 1, $n = 2$) and (a) disease severity index (DSI), or (b) shoot dry weight (mg). Correlation between *A. euteiches* gene copies g^{-1} soil determined by real-time quantitative PCR for soil samples 1–15 and (c) DSI, or (d) shoot dry weight (mg). Datapoints from samples that were positive for *A. euteiches* infection in pea roots are coloured red and the black datapoints represent root samples negative for *A. euteiches* according to PCR and gel electrophoresis analysis depicted in Figure 3A. Correlations were determined using Spearman. The coefficient of determination (R) and the significance are given (p-value). Log scale on x-axis.

of *A. euteiches* DNA in the examined field soil samples detecting levels < 10 target gene sequence g^{-1} soil (96 bp fragment). However, employing multiplex ddPCR to analyse multiple soilborne pathogens within a single sample may result in a reduction of sensitivity compared to singleplex qPCR (Chatterton et al. 2023).

In our study, both qPCR and ddPCR were performed as singleplex assays and compared for the detection and quantification of *A. euteiches*. The two techniques may not be directly comparable, as dilution of DNA prior to qPCR may have reduced detection sensitivity, rendering soil samples with low inoculum level undetectable. For instance, while the presence of *A. euteiches* DNA in soil samples 17–19 was below the detection limit in the qPCR assay, it was successfully quantified using ddPCR. The number of gene copies obtained by qPCR and ddPCR assays are presented without conversion into oospores g^{-1} soil as the ITS copy number can vary among isolates (Gangneux et al. 2014). Considering that the actual number of oospores within the 24 soil samples is unknown, it is difficult to assess whether quantification by qPCR and ddPCR overestimated or underestimated the respective number of oospores in each soil sample. The conversion of gene copies into number of oospores could be possible if known numbers of oospores were added to the soil as was performed by Chatterton et al. (2023).

To refine the conversion of ITS copy number into oospores counts, Heyman (2008) quantified the ITS copy numbers in field soil artificially inoculated with oospores. In another experimental set, oospores and mycelium obtained from liquid

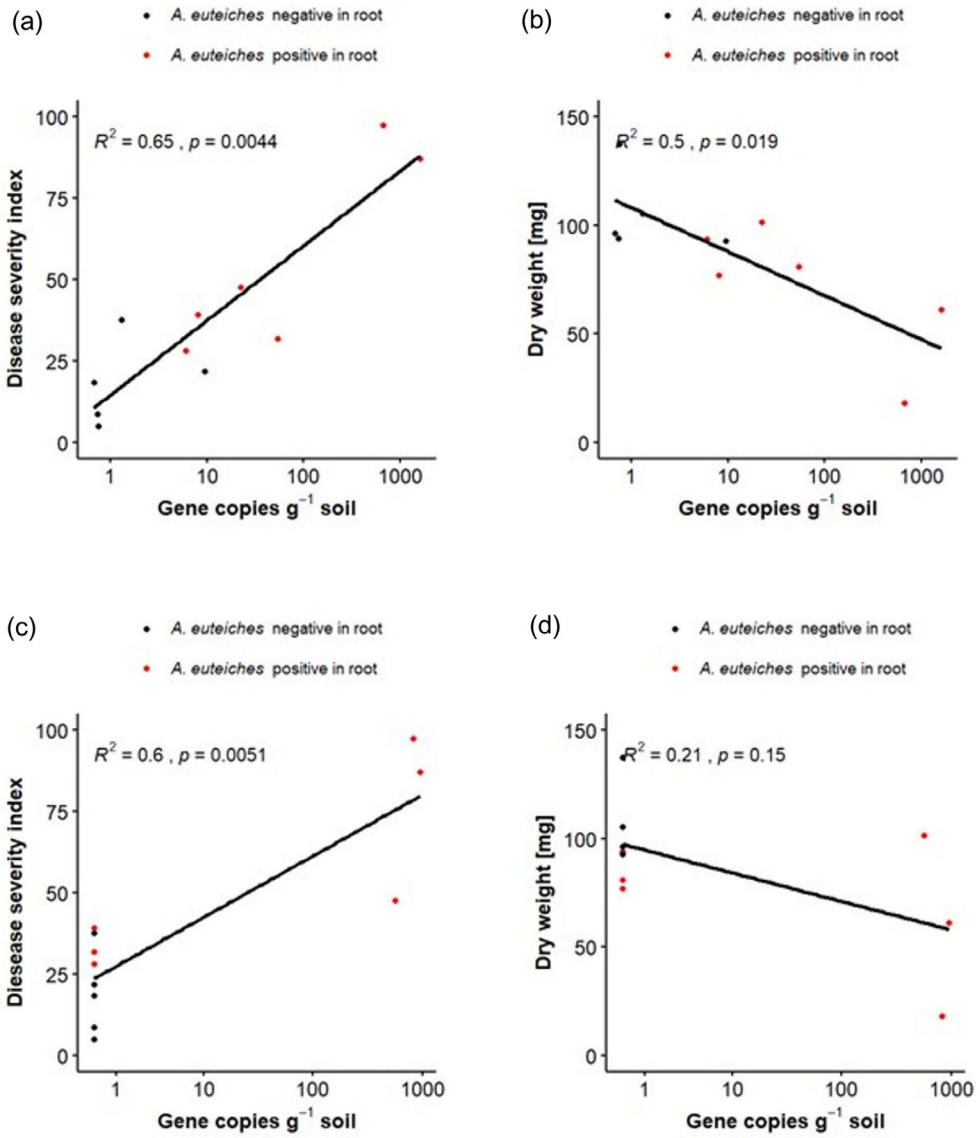


FIGURE 6 | Correlation between number of gene copies g^{-1} soil for *Aphanomyces euteiches* determined by droplet digital PCR for soil samples 16–24 (batch 2 of bioassay 1, $n = 1$) and (a) disease severity index (DSI), or (b) shoot dry weight (mg). Correlation between *A. euteiches* gene copies g^{-1} soil determined by real-time quantitative PCR soil samples 16–24 and (c) DSI, or (d) shoot dry weight (mg). Datapoints from samples positive for *A. euteiches* infection in pea roots are coloured red and the black datapoints represent root samples negative for *A. euteiches* according to PCR and gel electrophoresis analysis depicted in Figure 3B. Correlations were determined using Spearman. The coefficient of determination (R) and the significance are given (p-value). Log scale on x-axis.

cultures were incorporated into 350 mg soil in DNA extraction tubes (Heyman 2008). In the first case, the estimated number of gene copies per oospore was 126 and in the second case the estimated copy number of gene copies per oospore was 585. Using an average of these two values, the corresponding target gene copies per oospore is determined to be 356, which is higher than the average ITS copy number of 190 copies (Chatterton et al. 2023). In soil samples with <10 copies g^{-1} soil, as was observed in samples 16–17 and 19, the corresponding number of oospores should therefore be <1 oospore g^{-1} soil. Similarly, a French study reported successful detection and quantification of *A. euteiches* in naturally infested soils at a level below 10 oospores g^{-1} soil using ddPCR targeting the rDNA ITS sequence (Gibert et al. 2021). In fact, the investigated soil samples were collected from four fields located in the same geographical region of Hauts-de-France. Pea was cultivated immediately prior to soil sampling in three of the four fields. In contrast, in the present study, soil samples were obtained from 15 different fields across five counties of Sweden, each characterised by different ARR history (Table S1). Despite the capacity to detect *A. euteiches* at low levels in soil by ddPCR, high variation among subsamples was observed, except where occurrence levels were high (soil samples 20 and 21; >1522 g^{-1} soil).

The inoculum density of *A. euteiches* in soil can be estimated through ocular direct counting of oospores in naturally infested soil, facilitating ARR prediction through regression analysis and correlation between the number of oospores as well as DSI (Chan and Close 1987). In other cases, a correlation was established in

TABLE 5 | 'Heat map' of legume cover crops susceptibility to *Aphanomyces euteiches* strain SE51 evaluated visually as disease severity index and as gene copies mg^{-1} roots measured by droplet digital PCR.

	Disease severity index		Gene copies (mg^{-1} roots)[a]	
Legume	***A. euteiches*+**[b]	***A. euteiches*−**[c]	***A. euteiches*+**	***A. euteiches*−**
Pea	92 a	30 bc	80 a	0
Lentil	78 ab	21 c	74 a	0
Fodder vetch	67 b	31 bc	57 ab	0
Squarrose clover	67 b	52 a	4 c	0
Red clover	44 c	27 bc	0 c	0
Persian clover	43 c	27 bc	1 c	0
Crimson clover	43 c	36 abc	9 bc	0
Matttenklee	41 c	45 ab	0 c	0
Egyptian clover	38 c	27 bc	0 c	0
Common vetch	37 c	37 abc	0 c	0
Narrow-leaf lupin	36 c	24 c	14 bc	0
Blue lucerne	27 c	29 bc	7 bc	0
White clover	25 c	28 bc	1 c	0
Sainfoin	24 c	26 bc	1 c	0
Birdsfoot trefoil	22 c	20 c	0 c	0

Note: Data are mean values from two greenhouse experiments. Different letters indicate significant differences within treatments according to Tukey's HSD test ($p \leq 0.05$). The most intense colouration indicates highest DSI or gene copy values.
[a]*A. euteiches* gene copies quantified in mg^{-1} roots but not normalised by plant DNA.
[b]Plants inoculated with *A. euteiches*.
[c]Control plants non-inoculated with *A. euteiches*.

artificially infested soils (Gangneux et al. 2014; Heyman 2008). However, the influence of soil type on this relationship remains a subject of research, even in cases where oospores were directly added to the soil (Chatterton et al. 2023). Indeed, as reported by Gibert et al. (2021), a significant correlation was identified between $\log_{10}$-transformed gene copies of *A. euteiches* in soil and logit-transformed DSI, suggesting the utilisation of a mixed linear model for ARR prediction. Various physicochemical and biological characteristics of the soil were set as random effects, while DSI and the number of gene copies were fixed effects. The correlations reported in this study were generated without data transformation, which could otherwise have both aided in normalising skewed data and linearised the relationships between variables (Zwonitzer et al. 2024). The correlations demonstrated in Figures 5 and 6 explain the discrepancy in the detection of *A. euteiches* DNA in pea roots by conventional PCR (Figure 3) in comparison to the detection and quantification in soil by ddPCR and qPCR. The disagreement in the results was more pronounced in soil samples with low inoculum levels, such as soil samples 12 and 23–24, where the number of gene copies ranged from 0 to $2\,g^{-1}$ soil as in ddPCR. The occurrence of *A. euteiches* in soil relative to ARR infection in pea roots could have been accurately comparable if the root DNA had been analysed by qPCR or ddPCR, rather than the conventional PCR.

Moreover, the characteristics of the 24 collected soil samples were not accounted for by Spearman's correlation as a statistical model. The correlation between the number of gene copies in soil and DSI may be related to factors including the history of ARR in the sampled fields, the occurrence of other soil-borne pathogens and oospore viability as opposed to specific soil type (Pacheco-Moreno et al. 2025). For instance, in bioassay 1 (batch 1), DSI of bait plants for all soil samples (except for soil sample 14) remained above the threshold level that is generally used for ARR prediction in Sweden (DSI > 15). This specific soil sample exhibited an average target gene copy above 1800 copies g^{-1} soil, and the field observation indicated ARR infection when pea was cultivated in 2013 (Table S1). The low DSI for soil sample 14 (DSI = 9), despite the high abundance of pathogen DNA, may indicate that oospores have lost virulence and/or viability (Grau 1991). Another disagreement between DSI and the number of gene copies was shown for soil sample 12 collected from field D:K where the DSI value was > 60, even though the corresponding average gene copies value was extremely low. The observed symptoms might indicate multiple infection by several soil-borne pathogens (Pacheco-Moreno et al. 2025), potentially leading to an overestimation of the DSI due to the presence of blackening symptoms in the roots and hypocotyl. Such symptoms are not typically caused by *A. euteiches* (Oyarzun et al. 1993b). Furthermore, synergistic interactions between *A. euteiches* and other pathogens, such as *Fusarium* spp., have been demonstrated to result in increased disease severity (Willsey et al. 2018).

The correlations also exhibited divergent patterns between batch 1 and 2, partially supporting hypothesis (ii). This discrepancy

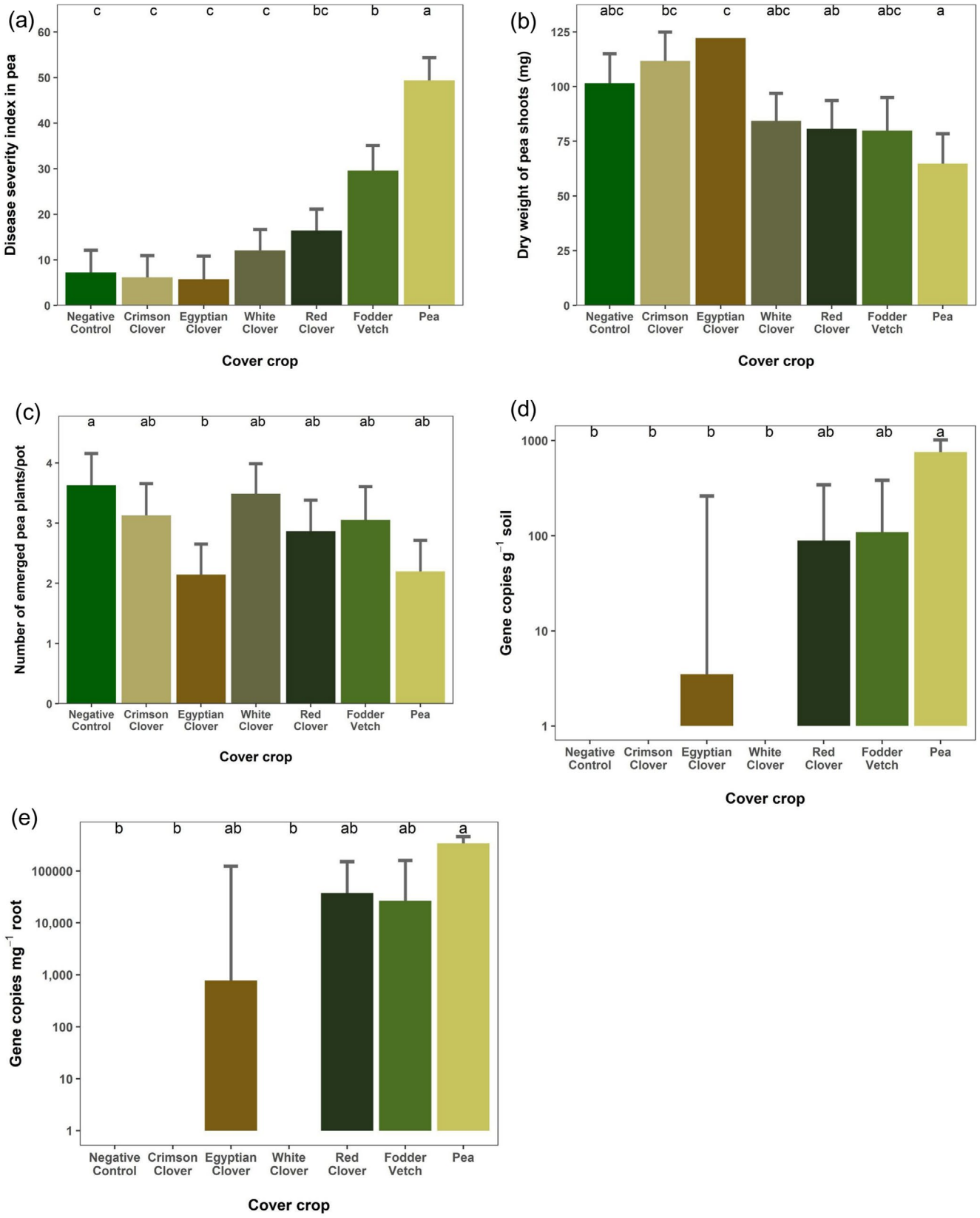


FIGURE 7 | Risk assessment of Aphanomyces root rot (ARR) infection performed in a bioassay for cultivating pea in succession with selected legume species assessed as (a) disease severity index, (b) shoot dry weight, (c) number of plants that emerged from five seeds, (d) amount of soil inoculum measured as gene copies g^{-1} soil using droplet digital PCR after each legume species, and (e) ARR infection level measured as *Aphanomyces euteiches* gene copies mg^{-1} root using droplet digital PCR. Statistical analysis using ANCOVA and post hoc Fisher's least significant difference. Different letters indicate $p \leq 0.05$. Mean values of two greenhouse experiments.

can potentially be attributed to the differences in ARR infection history among the sampled fields. In certain fields where samples were collected for batch 1, ARR infection was observed in 2009, 2013 and 2017, whereas for batch 2, ARR infection was noticed or confirmed either in 2020 or during the year of sampling in 2022 (Table S1).

The disagreement in the results between molecular methods and the bioassay suggest that molecular detection alone may not adequately reflect the infection potential in soil, thereby reducing predictive value for ARR risk in certain fields. Further, the present results facilitate relative comparisons among soil treatments, but the absence of conversion to oospore equivalents requires careful consideration when applying these findings to practical field recommendations regarding inoculum levels. Nevertheless, farmers growing susceptible crops may still benefit from ddPCR's ability to identify fields with low levels of gene copies, particularly those below 10 gene copies g^{-1} soil. Our results underscore the complexity of the disease dynamics and that ARR severity is influenced by multiple factors beyond the presence of *A. euteiches* DNA in soil.

Susceptibility for *A. euteiches* has been demonstrated for several legume species used as cover crop, among which common vetch, lentil (Vandemark and Porter 2010) and blue lucerne (Giles et al. 2022). The present study confirms that lentil and fodder vetch were susceptible to *A. euteiches*, whereas common vetch remained uninfected. This outcome contradicts earlier findings by Levenfors et al. (2003). Red clover was not susceptible in our assay despite previous reports of infection by *A. euteiches* (Hughes and Grau 2007). Such differences are presumably indicative of pathogen pathotype composition and the origin of the *A. euteiches* strains used in different experiments (Giles et al. 2022; Kälin et al. 2022). Differences in pathogenicity among the strains may also result in different interactions with the tested legume species as reported by Hughes and Grau (2007). The *A. euteiches* strain SE51, a low-virulent strain with relatively small oospores, has been used in pea breeding programmes in Sweden alongside virulent strains (Kälin et al. 2024). Limited access to more diverse strains restricted our analysis, but a broader strain panel would better capture the pathogen's full virulence range. The plant genotype can also play role in defining susceptibility for *A. euteiches*. Despite birdsfoot trefoil being among the most insensitive species analysed here, both susceptible and resistant cultivars may occur (Giles et al. 2022).

Another factor that may have influenced our results is infection by other pathogens. Although the seeds of the tested legumes were surface sterilised and bioassay 2 was conducted in a soilless system, untreated controls of crimson clover, mattenklee and squarrose clover displayed pronounced root discolouration. This observation suggests a seed-borne infection caused by other pathogen(s), for example, *Fusarium* spp., *Phoma medicaginis*, *Alternaria alternata* or *Botrytis* spp. (Lager and Johnsson 2002). Root discolouration observed in control plants was not linked to *A. euteiches*, as ddPCR analysis did not detect its DNA in roots, confirming that *A. euteiches* is not a seed-borne pathogen (Papavizas and Ayers 1974).

In general, a diversified crop rotation with non-hosts can be adopted to avoid build-up of soil inoculum of *A. euteiches* and other soil-borne pathogens (Giles et al. 2022; Wallenhammar et al. 2024). On the other hand, integrating host species in pea crop rotations may impose risk for ARR infection, thereby increasing soil inoculum (Moussart et al. 2013). In naturally infested soil, DSI increased for pea grown following fodder vetch, aligning with the susceptibility observed in bioassay 2. While red clover showed no susceptibility to strain SE51, an increase of pea DSI was observed when pea was cultivated after red clover in naturally infested soil, indicating that more virulent strains may exist within field pathogen populations. Conversely, DSI and soil inoculum levels were reduced after short rotations with crimson clover and Egyptian clover—even though oospores can survive for several years in soil. Oospores reduction was also observed in the untreated control containing uncultivated soil during the initial cultivation cycle. Although the bioassay was conducted over only two consecutive cycles, oospore formation can occur in pea roots within 7–10 days following inoculation (Larsen et al. 2000), indicating the possibility of oospore presence in the roots of legume species and pea plants used in the current bioassay after a 6-week incubation period. The rapid reduction of oospores may also be attributed to the disruption of soil structure under artificial greenhouse conditions (Moussart et al. 2013). Given the current findings and the wide host range of *A. euteiches*, it is crucial to gain more knowledge on the susceptibility of legume species to avoid risk of ARR and propagation of soil inoculum. Moreover, the ability of ddPCR to accurately identify legume species that impose risk for ARR infection in pea crops supports hypothesis (iii).

Our research highlights the significance of molecular analysis of soil DNA of *A. euteiches* for ARR disease prevention. Quantifying the number of gene copies of *A. euteiches* in soil samples by the presented assays will provide valuable information for pea growers. The combination of soil analysis and disease history, as well as bioassays, would likely give more accurate prediction of ARR. The method developed for DNA extraction and ddPCR analysis must be verified for a wider range of soil types prior to adoption for commercial use. Susceptibility of the legume species studied was accurately assessed using ddPCR. However, it is preferable to evaluate their response across multiple virulent strains and include several cultivars. While legume cover crops can maintain soil health and crop productivity, susceptible species such as fodder vetch can impose risk for infection of ARR in legume-based agricultural systems.

Author Contributions

Eivor Sofia Dubusc: investigation, methodology, validation, writing – original draft. **Anders Jonsson:** conceptualization, funding acquisition. **Zahra Saad Omer:** conceptualization, methodology, investigation, funding acquisition, writing – original draft, writing – review and editing, project administration, supervision. **Eva Edin:** conceptualization, funding acquisition, writing – original draft, writing – review and editing, investigation. **Ann-Charlotte Wallenhammar:** conceptualization, funding acquisition, writing – original draft, writing – review and editing, investigation. **Magnus Karlsson:** writing – original draft, writing – review and editing, supervision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data supporting this research are included in this article or attached as a Supporting Information.

References

Billard, E., A. Quillévéré-Hamard, C. Lavaud, M. L. Pilet-Nayel, and C. Le May. 2019. "Testing of Life History Traits of a Soilborne Pathogen In Vitro: Do Characteristics of Oospores Change According the Strains of *Aphanomyces euteiches* and the Host Plant Infected by the Pathogen?" *Journal of Phytopathology* 167: 313–320. https://doi.org/10.1111/jph.12799.

Chan, M. K. Y., and R. C. Close. 1987. "Root Rot of Peas 2. Some Pasture Legumes and Weeds as Alternative Hosts for *Aphanomyces euteiches*." *New Zealand Journal of Agricultural Research* 30: 219–223. https://doi.org/10.1080/00288233.1987.10430500.

Chatterton, S., T. D. Schwinghamer, A. Pagé, R. B. Davidson, and M. W. Harding. 2023. "Inoculum Dose–Disease Response Relationships for the Pea Root Rot Pathogen, *Aphanomyces euteiches*, Are Dependent on Soil Type and Other Pathogens." *Frontiers in Plant Science* 14: 1115420. https://doi.org/10.3389/fpls.2023.1115420.

Frostegård, Å., S. Courtois, V. Ramisse, et al. 1999. "Quantification of Bias Related to the Extraction of DNA Directly From Soils." *Applied and Environmental Microbiology* 65: 5409–5420.

Gangneux, C., M.-A. Cannesan, M. Bressan, et al. 2014. "A Sensitive Assay for Rapid Detection and Quantification of *Aphanomyces euteiches* in Soil." *Phytopathology* 104: 1138–1147.

Gibert, S., V. Edel-Hermann, R. Moussa Mcolo, et al. 2021. "Risk Assessment of *Aphanomyces euteiches* Root Rot Disease: Quantification of Low Inoculum Densities in Field Soils Using Droplet Digital PCR." *European Journal of Plant Pathology* 161, no. 3: 503–528. https://doi.org/10.1007/s10658-021-02325-5.

Giles, J. M., C. L. Tordsen, T. R. Rebstock, B. Bucciarelli, D. A. Samac, and A. E. Sathoff. 2022. "Management Strategies and Distribution of Aphanomyces Root Rot of Alfalfa (*Medicago sativa*), a Continuing Threat to Forage Production in the United States." *Plant Pathology* 71: 1231–1240. https://doi.org/10.1111/ppa.13563.

Gossen, B. D., R. L. Conner, K.-F. Chang, et al. 2016. "Identifying and Managing Root Rot of Pulses on the Northern Great Plains." *Plant Disease* 100: 1965–1978. https://doi.org/10.1094/PDIS-02-16-0184-FE.

Grau, C. R. 1991. "Variability in Virulence of *Aphanomyces euteiches*." *Plant Disease* 75: 1153. https://doi.org/10.1094/PD-75-1153.

Heyman, F. 2008. "Root Rot of Pea Caused by *Aphanomyces euteiches*: Calcium-Dependent Soil Suppressiveness, Molecular Detection and Population Structure." [Thesis] Swedish University of Agricultural Sciences.

Heyman, F., J. E. Blair, L. Persson, and M. Wikström. 2013. "Root Rot of Pea and Faba Bean in Southern Sweden Caused by *Phytophthora pisi* sp. nov." *Plant Disease* 97: 461–471. https://doi.org/10.1094/PDIS-09-12-0823-RE.

Hou, Y., S. Chen, Y. Zheng, X. Zheng, and J.-M. Lin. 2023. "Droplet-Based Digital PCR (ddPCR) and Its Applications." *Trends in Analytical Chemistry* 158: 116897.

Huggett, J. F., and A. Whale. 2013. "Digital PCR as a Novel Technology and Its Potential Implications for Molecular Diagnostics." *Clinical Chemistry* 59: 1691–1693. https://doi.org/10.1373/clinchem.2013.214742.

Hughes, T. J., and C. R. Grau. 2007. "Aphanomyces Root Rot or Common Root Rot of Legumes." *Plant Health Instructor*. https://doi.org/10.1094/PHI-I-2007-0418-01.

Kälin, C., A. Berlin, A. Kolodinska Brantestam, et al. 2022. "Genetic Diversity of the Pea Root Pathogen *Aphanomyces euteiches* in Europe." *Plant Pathology* 71: 1570–1578. https://doi.org/10.1111/ppa.13598.

Kälin, C., A. Kolodinska Brantestam, A.-K. Arvidsson, M. Dubey, M. Elfstrand, and M. Karlsson. 2023. "Evaluation of Pea Genotype PI180693 Partial Resistance Towards Aphanomyces Root Rot in Commercial Pea Breeding." *Frontiers in Plant Science* 14: 1114408. https://doi.org/10.3389/fpls.2023.1114408.

Kälin, C., E. Piombo, S. Bourras, et al. 2024. "Transcriptomic Analysis Identifies Candidate Genes for *Aphanomyces* Root Rot Disease Resistance in Pea." *BMC Plant Biology* 24, no. 1: 144. https://doi.org/10.1186/s12870-024-04817-y.

Lager, J., and L. Johnsson. 2002. "Seed-Borne Fungi Affect Field Emergence in Red Clover/Samenbürtige Pilze Beeinträchtigen den Feldaufgang von Rotklee." *Journal of Plant Diseases and Protection* 109: 350–356.

Larsen, J., K. Mansfeld-Giese, and L. Bødker. 2000. "Quantification of *Aphanomyces euteiches* in Pea Roots Using Specific Fatty Acids." *Mycological Research* 104, no. 7: 858–864. https://doi.org/10.1017/s0953756299002075.

Levenfors, J. P., M. Wikström, L. Persson, and B. Gerhardson. 2003. "Pathogenicity of *Aphanomyces* spp. From Different Leguminous Crops in Sweden." *European Journal of Plant Pathology* 109: 535–543.

Mieuzet, L., A. Quillévéré, M. L. Pilet, and C. Le May. 2016. "Development and Characterization of Microsatellite Markers for the Oomyceta *Aphanomyces euteiches*." *Fungal Genetics and Biology* 91: 1–5.

Moussart, A., M. N. Even, A. Lesné, and B. Tivoli. 2013. "Successive Legumes Tested in a Greenhouse Crop Rotation Experiment Modify the Inoculum Potential of Soils Naturally Infested by *Aphanomyces euteiches*." *Plant Pathology* 62: 545–551. https://doi.org/10.1111/j.1365-3059.2012.02679.x.

Moussart, A., E. Wicker, B. Le Delliou, et al. 2009. "Spatial Distribution of *Aphanomyces euteiches* Inoculum in a Naturally Infested Pea Field." *European Journal of Plant Pathology* 123: 153–158. https://doi.org/10.1007/s10658-008-9350-x.

Olofsson, J. 1967. "Root Rot of Canning and Freezing Peas in Sweden." *Acta Agriculturae Scandinavica* 17: 101–107. https://doi.org/10.1080/00015126709433144.

Oyarzun, P., M. Gerlagh, and A. E. Hoogland. 1993a. "Relation Between Cropping Frequency of Peas and Other Legumes and Foot and Root Rot in Peas." *Netherlands Journal of Plant Pathology* 99: 35–44. https://doi.org/10.1007/BF01974783.

Oyarzun, P., M. Gerlagh, and A. E. Hoogland. 1993b. "Pathogenic Fungi Involved in Root Rot of Peas in The Netherlands and Their Physiological Specialization." *Netherlands Journal of Plant Pathology* 99: 23–33. https://doi.org/10.1007/BF01974782.

Pacheco-Moreno, A., S. Bruty, H. Tidd, E. Morka, and S. Arora. 2025. "Loop Mediated Isothermal Amplification (LAMP) as a Rapid and Portable Diagnostic Tool for the Detection of Pea Root Rot Pathogens." *Scientific Reports* 15: 34904. https://doi.org/10.1038/s41598-025-18738-9.

Papavizas, G. C., and W. A. Ayers. 1974. "*Aphanomyces* Species and Their Root Diseases in Pea and Sugarbeet: A Review." Agricultural Research Service.

Pfender, W. F., and D. J. Hagedorn. 1983. "Disease Progress and Yield Loss in Aphanomyces Root Rot of Peas." *Phytopathology* 73: 1109–1113.

Piermatteo, L., R. Scutari, R. Chirichiello, et al. 2022. "Droplet Digital PCR Assay as an Innovative and Promising Highly Sensitive Assay to Unveil Residual and Cryptic HBV Replication in Peripheral Compartment." *Methods* 201: 74–81.

Plassart, P., S. Terrat, B. Thomson, et al. 2012. "Evaluation of the ISO Standard 11063 DNA Extraction Procedure for Assessing Soil Microbial Abundance and Community Structure." *PLoS One* 7: e44279. https://doi.org/10.1371/journal.pone.0044279.

Sidstedt, M., L. Jansson, E. Nilsson, et al. 2015. "Humic Substances Cause Fluorescence Inhibition in Real-Time Polymerase Chain Reaction." *Analytical Biochemistry* 487: 30–37.

Sundheim, L. 1972. "Physiologic Specialization in *Aphanomyces euteiches*." *Physiological Plant Pathology* 2: 301–306. https://doi.org/10.1016/0048-4059(72)90013-6.

Vandemark, G. J., and L. D. Porter. 2010. "First Report of Lentil Root Rot Caused by *Aphanomyces euteiches* in Idaho." *Plant Disease* 94: 480.

Wallenhammar, A.-C., C. Almquist, M. Söderström, and A. Jonsson. 2012. "In-Field Distribution of *Plasmodiophora brassicae* Measured Using Quantitative Real-Time PCR." *Plant Pathology* 61: 16–28. https://doi.org/10.1111/j.1365-3059.2011.02477.x.

Wallenhammar, A.-C., E. Edin, and A. Jonsson. 2024. "Susceptibility of Oilseed Radish (*Raphanus sativus* subsp. *oleiferus*) Cultivars and Various Brassica Crops to *Plasmodiophora brassicae*." *Pathogens* 13: 739.

Wikström, J., S. Chaudhary, L. Persson, M. Wikström, J. Fatehi, and M. Karlsson. 2025. "Distribution of Plant Pathogenic *Aphanomyces* Species in Sweden, Denmark and Lithuania and its Relationship With Soil Factors." *Journal of Plant Pathology* 108, no. 1: 211–221. https://doi.org/10.1007/s42161-025-01835-z.

Willsey, T. L., S. Chatterton, M. Heynen, and A. Erickson. 2018. "Detection of Interactions Between the Pea Root Rot Pathogens *Aphanomyces euteiches* and *Fusarium* spp. Using a Multiplex qPCR Assay." *Plant Pathology* 67: 1912–1923. https://doi.org/10.1111/ppa.12895.

Wu, L., K.-F. Chang, R. L. Conner, et al. 2018. "*Aphanomyces euteiches*: A Threat to Canadian Field Pea Production." *Engineering* 4: 542–551. https://doi.org/10.1016/j.eng.2018.07.006.

Wu, L., K.-F. Chang, S.-F. Hwang, et al. 2019. "Evaluation of Host Resistance and Fungicide Application as Tools for the Management of Root Rot of Field Pea Caused by *Aphanomyces euteiches*." *Crop Journal* 7: 38–48. https://doi.org/10.1016/j.cj.2018.07.005.

Zwonitzer, K. D., L. G. Tressel, Z. Wu, et al. 2024. "Genome Copy Number Predicts Extreme Evolutionary Rate Variation in Plant Mitochondrial DNA." *Proceedings of the National Academy of Sciences of the United States of America* 121: e2317240121. https://doi.org/10.1073/pnas.2317240121.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Disease severity index (DSI) of Aphanomyces root rot was assessed by the proportion of browning of the root system, 0: healthy, 25: slight root discolouration, 50: mild to moderate discolouration along with discolouration of the stem, 75: moderate browning of root and signs of wilt in shoot, 100: severe root browning leading to root degradation and wilted shoot. **Figure S2:** Amplitude 1 diagram of droplet digital PCR analysis illustrating the impact of soil homogenisation on detection of *Aphanomyces euteiches* DNA in soil sample 1 using three homogenisation methods: A = soil ground with method A, B = soil ground with method B and C = soil crushed with a hummer and sieved. Soil samples were first treated with method C before being homogenised with either method A or B. **Figure S3:** Susceptibility of peas (*Pisum sativum*), lentil (*Lens culinaris*) and fodder vetch (*Vicia villosa*) to *Aphanomyces euteiches* strain SE51. (a) Peas: non-inoculated plants, (b) peas: inoculated plants, (c) lentil: non-inoculated plants, (d) lentil: inoculated plants, (e) fodder vetch: non-inoculated plants, (f) fodder vetch: inoculated plants. **Figure S4:** Susceptibility of red clover (*Trifolium pratense*) and birdsfoot trefoil (*Lotus corniculatus*) to *Aphanomyces euteiches* strain SE51. (a) Red clover: non-inoculated plants, (b) red clover: inoculated plants, (c) birdsfoot trefoil: non-inoculated plants, (d) birdsfoot trefoil: inoculated plants. **Table S1:** Location, history of Aphanomyces root rot (ARR) and chemical properties of soil samples collected in Sweden in 2022. **Table S2:** Legume species studied in bioassay 2 for susceptibility against *Aphanomyces euteiches*.