



Risk assessment of *Aphanomyces euteiches* root rot disease: quantification of low inoculum densities in field soils using droplet digital PCR

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Abstract *Aphanomyces euteiches* Drechs is a pathogenic soil-borne oomycete that causes root rot of legumes, one of the most serious diseases affecting legume production worldwide. There is currently no registered pesticide and no available resistant pea cultivar. Avoidance of infested fields based on disease risk assessment prior to pea sowing remains the main method available to manage the disease, but time-consuming bioassays are required to assess disease severity on susceptible plants grown in field soil samples. Direct quantification of *A. euteiches* inoculum in the soil by targeting multicopy genomic sequences of the internal transcribed spacer 1 with a qPCR-based method has been proposed as a rapid alternative for disease prediction. However, the method lacks sensitivity to accurately quantify low inoculum levels from naturally infested fields. We developed a suitable methodology based on droplet digital PCR (ddPCR) to quantify low *A. euteiches* inoculum levels in naturally infested soils. The methodology was validated on 200 soil samples

taken from four naturally infested fields in the main pea cropping area in the north of France. The comparative analysis of inoculum density and disease severity of the 50 samples within each of the four fields revealed a non-homogeneous distribution of the *A. euteiches* population; this explains why the disease is visible in the form of foci. A significant relationship between pea root rot disease severity determined by bioassays and *A. euteiches* inoculum density was highlighted, and a linear mixed model is proposed to predict disease severity from inoculum density.

Keywords *Aphanomyces euteiches* · Droplet digital PCR · Pea root rot · Disease risk assessment · Oospore · Soil · Inoculum density

Introduction

Root rot of legumes caused by the pathogenic soil-borne oomycete *Aphanomyces euteiches* Drechs is one of the most serious diseases in legume production worldwide. This pathogen is present in many legume-growing areas including North America (Holub et al., 1991; Wu et al., 2018), Europe (Oyarzun & van Loon, 1989; Persson et al., 1997; Quillevere-Hamard et al., 2018), Oceania (Abbo & Irwin, 1990; Manning & Menzies, 1980) and Asia (Yokosawa et al., 1974).

A. euteiches infects different legume species such as pea (*Pisum sativum* L.; Jones & Drechsler, 1925), lentil (*Lens culinaris*; Vandemark & Porter, 2010), alfalfa

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(*Medicago sativa*; Delwiche et al., 1987), green bean (*Phaseolus vulgaris*; Pfender & Hagedorn, 1982), faba bean (*Vicia faba*; Lamari & Bernier, 1985), subterranean clover (Greenhalgh et al., 1988), red clover (*Trifolium pratense*; Tofte, 1990) and common vetch (*Vicia sativa*; Tsvetkova & Guseva, 1980). Its impact is particularly devastating on pea crops where it causes considerable yield losses (Gaulin et al., 2007).

The pathogen infects pea plants at any growth stage (Kraft & Pflieger, 2001). Initial symptoms appear in the form of yellowed to honey-brown spots on the roots. Lesions rapidly grow as the pathogen colonises the cortical tissues and then spreads through the taproot to the epicotyl that appears either softer or water-soaked. Other opportunistic organisms and root pathogens of the pea root rot complex such as *Pythium* spp., *Phytophthora* spp., *Fusarium* spp., *Didymella* spp., *Rhizoctonia solani* or *Thielaviopsis basicola* may invade the lesions. This increases disease severity and leads to root darkening (Chang et al., 2017; Gossen et al., 2016; Kraft & Pflieger, 2001; Willsey et al., 2018). The destruction of the plant root cortex and feeding rootlets generally results in plant chlorosis, withering and sometimes premature death before pods are formed (Kraft & Pflieger, 2001). In favourable conditions, oospores are formed in infested roots by sexual reproduction a few days after infection (Cunningham & Hagedorn, 1962). After plant death, oospores are dispersed heterogeneously via decomposing host tissues and are released in the soil. *A. euteiches* mycelium does not live as a saprophyte within dead plants, and oospores are the persistent form of the pathogen in the soil where they serve as primary inoculum (Kjoller & Rosendahl, 1998; Kraft & Pflieger, 2001; Schren, 1960). Their thick wall makes them resistant to drought and temperature fluctuations and allows them to survive more than 10 years in the soil (Papavizas & Ayers, 1974). Oospores germinate under the influence of root exudates from a nearby host plant, and form a zoosporangium. The zoosporangium releases more than 300 motile zoospores that reach the host roots by chemotactic attraction and get encysted on the rhizoplane, and a new infection cycle starts (Gaulin et al., 2007). Environmental conditions such as high soil moisture, poor drainage or soil compaction increase disease proliferation by favouring the movement of the motile zoospores toward plant roots in the moisture film surrounding soil particles (Wu et al., 2018).

Disease management is difficult because no effective option for *A. euteiches* control is presently available. Only a few pesticides are effective but they are either prohibited or undesirable in most pea-growing areas because of their toxicity and negative environmental impacts (Gaulin et al., 2007; Wu et al., 2018). Some biocontrol agents such as arbuscular mycorrhizal fungi, *Clonostachys rosea*, *Pseudomonas fluorescens*, and species belonging to the *Burkholderia cepacia* complex associated or not with seed treatment have shown interesting effects on pea emergence and root rot severity (Wu et al., 2018), but their large-scale use under field conditions remains hypothetical. Genetic selection of resistant pea varieties is a promising control option. Several quantitative trait loci (QTL) controlling partial resistance and valuable in pea breeding programs have been reported, but no resistant pea cultivar is available yet (Desgroux et al., 2016; Lavaud et al., 2015).

Avoidance of infested fields and crop rotation remain the only ways to manage the disease to date. Due to the *A. euteiches* life cycle, the frequency of susceptible legume crops is directly correlated to the inoculum increment in the soil and in turn to the increased incidence and severity of root rot disease on the next susceptible crop (Bodker et al., 1993; Chan & Close, 1987; Oyarzun et al., 1993). Rotations replacing susceptible plants by non-host species or resistant cultivars belonging to vetch or faba bean species contribute effectively to decrease the soil inoculum potential (IP) (Moussart et al., 2013). However, a limitation of this cropping management practice is the need for long-term rotations due to the survival of *A. euteiches* oospores in the soil for years coupled to the broad range of host species and susceptible cultivars that should be excluded from the rotation. This results in an increasing need for new cropping areas with a sufficiently safe low infestation level, a hard task for pea producers.

To be effective, avoidance of infested fields requires identifying suitable plots for pea cropping. Several methods based on disease severity indexing or IP measurements from field soil samples have been proposed (Chan & Close, 1987; Malvick et al., 1994; Pfender et al., 1981; Reiling et al., 1960; Sherwood & Hagedorn, 1958). In France, a specific test has been developed; this test uses bioassays to measure disease severity in optimal conditions for its expression on susceptible pea plants grown in field soil samples has been developed. Due to a linear relationship between the bioassay

results and disease severity in the field, this test is now recommended by most stakeholders of the sector (Moussart et al., 2009).

Bioassay-based methods are technically simple and predictive, but they are also burdensome, time consuming and not specific to a single pathogen. The direct quantification of *A. euteiches* inoculum in the soil by a molecular method could be a specific, accurate, reliable and faster alternative for disease prediction. Sauvage et al. (2007) proposed a real-time quantitative PCR (qPCR) assay to quantify *A. euteiches* oospores in the soil. However, this method originally developed for *in planta* quantification (Vandemark et al., 2000; Vandemark et al., 2002) was based on the detection of a DNA sequence specific to *A. euteiches* but present in a single copy in its genome, so that the limit of detection was 200 oospores *per* gram of soil. Gangneux et al. (2014) developed a more sensitive qPCR approach by targeting the multicopy ribosomal internal transcribed spacer 1 (ITS1) region of *A. euteiches*. This multicopy DNA target allowed for the detection of 10 oospores *per* gram of artificially inoculated soil (Gangneux et al., 2014). A clear relationship between disease severity on susceptible pea plants and oospore density was established in both approaches conducted in artificially inoculated soils, and was proposed to predict disease severity using oospore quantification. However, this relationship was not validated in field conditions.

Considering the heterogeneous distribution of *A. euteiches* in naturally-infested agricultural bulk soils and its ability to trigger severe infection from very low inoculum levels, the sensitivity threshold of the method is decisive for a routine use of molecular quantification to predict pea root rot disease. The sensitivity of a qPCR approach with a detection limit of 10 oospores *per* gram of soil is insufficient to obtain a reliable measure from low infested field soil samples (Heyman, 2008). Moreover, qPCR is subject to inhibition by soil substances such as humic acids, phenolic compounds, heavy metals or clay particles that are difficult to get rid of during DNA extraction and purification (Albers et al., 2013; Matheson et al., 2010).

Droplet digital PCR (ddPCR) is a highly sensitive and well adapted accurate tool for quantifying rare DNA targets (Hindson et al., 2011). This method is based on the partitioning of the PCR mixture into 20,000 individual droplets that randomly distribute the targets and the background DNA among them. An endpoint PCR amplification occurs in each positive droplet containing the

target. Poisson calculation of the ratio between the positive and negative droplets gives an accurate and absolute target quantification without requiring an external standard curve. Moreover, ddPCR is less subject to PCR inhibition by soil residues compared to qPCR (Racki et al., 2014; Wood et al., 2019). This way, the quantity of soil DNA matrix in the PCR mix can be increased, and this significantly improves the sensitivity of the method. Thus, quantification accuracy and resistance to PCR inhibitors make ddPCR a good candidate for the quantification of low microbial inocula in the soil (Gossen et al., 2019; Liu et al., 2020; Maldonado-Gonzalez et al., 2020; Wen et al., 2020).

The objective of the present study was to propose a suitable method to quantify *A. euteiches* inoculum density (ID) in naturally infested field soils to improve risk assessment of *A. euteiches* root rot disease. This work was carried out in three steps: (a) we developed an accurate and sensitive duplex ddPCR approach to quantify multicopy ITS1 and single-copy *A. euteiches* sequences in soil DNA; (b) we optimised the DNA extraction method (soil quantity and homogenisation) to obtain a reproducible measure; and (c) we validated the suitability and the predictivity of the ddPCR method by characterising the relationship between the ID measured by ddPCR and disease severity in naturally infested soil samples.

Materials and methods

Field characteristics and soil sampling

The study focused on the legume production basin of the Hauts-de-France region (France). Four fields were chosen based on the established presence of *A. euteiches*-infested peas in the recent historical cropping record. Two highly infested fields, named Renescure and Verpillieres, are located in the north and in the south of the production basin, respectively, and were sampled in October 2017 (Table 1). Two low infested fields named Woestyne and Flers are located in the north and in the south of the production basin, respectively, and were sampled in November 2018. The historical cropping record of each plot was recorded with the farmers, and their respective GPS coordinates were scored with grower's agreement (Table 1). A 1-ha plot divided into 50 contiguous 14.14 m by 14.14 m quadrats was georeferenced in each of the four fields.

Four L of soil were collected systematically with a spade at the centre of each quadrat at -5 cm to -20 cm depth and transferred into plastic boxes. The spade was cleaned with absorbent paper and disinfested with 70% ethanol between two samplings. The soil samples were brought to the laboratory and air dried at room temperature (20 °C) for at least 8 days. The dried samples were sieved through a 4 mm mesh and mixed by hand to homogenise them. Subsamples made of 60 mL of each sieved soil intended for molecular analyses were lyophilised and stored at -20 °C. The remaining sieved samples intended for bioassays and physico-chemical analyses were temporarily stored in plastic boxes in a room at 18 °C.

Soil physico-chemical properties were determined on 10 soil samples out of the 50 samples from each field by the accredited INRAE Soil Analysis Laboratory (Arras, France) according to international (ISO) or French (NF) standardised methods. The samples were first ground to particle sizes <250 μm and prepared, and particle-size distribution, pH in water, total calcium carbonate (CaCO_3), organic carbon (C), organic matter (OM), nitrogen (N) and phosphorus (P_2O_5 - Olsen method) were analysed. The mean value of each physico-chemical parameter of the ten soil samples *per* field was calculated (Table 2).

Disease severity assessment

Disease severity was assessed for each soil sample in a greenhouse bioassay using an *A. euteiches*-susceptible pea cultivar (cv. Kayanne, KWS, France) and optimal conditions to favour infection according to Moussart et al. (2009). One-L pots containing 700 mL of sieved soil were used. Pea seeds were disinfested in a sodium hypochlorite solution (1.5 g L^{-1} of available Cl_2) for 20 min, rinsed three times with sterile deionised water, and hydrated in sterile deionised water for 12 h before sowing. Nine seeds were sown *per* pot and *per* soil for the soils collected in 2017 (Renescure and Verpillieres fields). For the two naturally low infested soils collected in November 2018 (Woestyne and Flers fields), the protocol was modified to improve the sensitivity of detection and quantification of a low level of root rot disease. Five pea seeds were sown in each pot *per* soil sample, in triplicate. All pots were placed in a greenhouse for 14 days and were watered daily to maintain a high soil moisture. Greenhouse conditions were $80 \pm 10\%$ humidity, with a thermoperiod of 25 °C day/ 23 °C

night and a photoperiod of 16 h light (with extra light intensity of 160 $\mu\text{E s}^{-1} \text{m}^{-2}$) / 8 h dark. Plants were removed from the pots after 14 days, and the roots were delicately washed under running tap water. A disease severity score was determined for each plant by visual assessment of root symptoms evaluated on a 0–5 scale (Moussart et al., 2009): 0 = no symptom; 1 = roots with a few discoloured to honey-coloured lesions on rootlets; 2 = discoloured to honey-brown zones covering at least half of the root system; 3 = honey-brown lesions covering at least half of the root system; hypocotyl or epicotyl discoloured and water-soaked in some cases; 4 = most of the root system honey-brown to dark brown and epicotyl softened or brown; 5 = dead plant. Aborted plants were considered as non-available (NA) values. A disease severity index (DSI) was defined as the mean value of the disease severity scores of all plants in one pot. For the Woestyne and Flers field soil samples, the mean value of the three pots was calculated for each soil sample.

Presence of *Aphanomyces euteiches* in symptomatic roots

The presence of *A. euteiches* in symptomatic roots recovered from the bioassay was determined with a duplex PCR test using the *A. euteiches*-specific primer pairs Ae_ITS1_39F / Ae_ITS1_167R and Ae_76bp_136_F / Ae_76bp_211_R designed by Gangneux et al. (2014) and Vandemark et al. (2000), respectively (Table 3). Symptomatic roots of five soil samples *per* soil were chosen so that a large DSI range from 0.58 to 4.25 was represented. Asymptomatic roots (DSI = 0) from two soil samples from the Woestyne and Flers fields were used as negative controls, and DNA from *A. euteiches* reference strain RB84 (Wicker et al., 2001) was used as a positive control. DNA was extracted from 50 mg of root tissue or from 50 mg of mycelium of strain RB84 using a FastDNA® SPIN kit (MP Biomedicals, Eschwege, Germany) according to the manufacturer's recommendations. DNA extracts were stored at -20 °C until PCR analyses. Duplex PCRs were performed in 25- μL reaction mixtures containing 150 μM of dNTPs, 0.3 μM of each primer, 3 U of *Taq* polymerase (MP Biomedicals), 1.5 mM MgCl_2 , 2 μL of DNA, and PCR buffer. The reactions were initiated with a 3 min incubation at 95 °C, followed by 35 cycles of 1 min at 95 °C,

Table 1 Geographic coordinates, areas and historical cropping records of the Renesecure, Verpillieres, Woestyne and Flers fields

Field name	Geographic coordinates	Field area (ha)	Sampling date	Tillage	Historical cropping record						
					2012	2013	2014	2015	2016	2017	2018
Renesecure	50°44' 34.4"N 2°21'45.2"E	18.1	oct-17	No till	Wheat (<i>Triticum aestivum</i>)	Bean (<i>Phaseolus vulgaris</i>)*	Wheat (<i>Triticum aestivum</i>)	Flax (<i>Linum usitatissimum</i>)	Wheat (<i>Triticum aestivum</i>)	Pea cv. Firenze (<i>Pisum sativum</i>)*	/
Verpilliere	49°39' 52.7"N 2°47'42.6"E	4.85	oct-17	Till	Spring barley (<i>Hordeum vulgare</i>)	Corn (<i>Zea mays</i>)	Wheat (<i>Triticum aestivum</i>)	Sugar beet (<i>Beta vulgaris</i>)	Wheat (<i>Triticum aestivum</i>)	Pea cv. Style (<i>Pisum sativum</i>)*	/
Woestyne	50°44' 55.7"N 2°23'04.1"E	29	nov-18	No till	Bean (<i>Phaseolus vulgaris</i>)*	Wheat (<i>Triticum aestivum</i>)	Sugar beet (<i>Beta vulgaris</i>)	Wheat (<i>Triticum aestivum</i>)	Flax (<i>Linum usitatissimum</i>)	Wheat (<i>Triticum aestivum</i>)	Pea cv. Messenger (<i>Pisum sativum</i>)*
Flers	50°03' 17.5"N 2°48'52.7"E	12	nov-18	Till	Corn (<i>Zea mays</i>)	Pea cv. Messenger (<i>Pisum sativum</i>)*	Wheat (<i>Triticum aestivum</i>)	Wheat (<i>Triticum aestivum</i>)	Sugar beet (<i>Beta vulgaris</i>)	Potato (<i>Solanum tuberosum</i>)	Wheat (<i>Triticum aestivum</i>)

*Legume species susceptible to *Aphanomyces eutiches*

Table 2 Field soil textures and main physico-chemical parameters

Field name	Texture				Physico-chemistry						
	Type	Clay (%)	Silt (%)	Sand (%)	pH	C (g. Kg ⁻¹)	N (g. kg ⁻¹)	C/N	OM (g. Kg ⁻¹)	P ₂ O ₅ (mg. Kg ⁻¹)	CaCO ₃ (g. Kg ⁻¹)
Renescure	Silty clay loam	30	62	8	5.7	25.7	2.9	8.8	44.4	75	< 1
Verpilliere	Silt loam	17	67	16	7	11.4	1.14	9.9	19.7	61	< 1
Woestyne	Silt loam	22	67	11	7.1	19.1	2.1	8.9	33.1	218	< 1.2
Flers	Silt loam	19	74	7	7.4	10.1	1.1	8.9	17.5	108	< 1.4

1 min at 60 °C, 1 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were checked by agarose gel electrophoresis.

Isolation and molecular identification of oomycetes, and characterisation of their pathogenicity

Oomycetes were isolated from one pea root bunch showing typical *A. euteiches* honey-brown symptoms

per soil sample. Symptomatic roots were disinfected in 70% ethanol for 10 s, rinsed in sterile deionised water, and dried on sterile filter paper. Three 10-mm root fragments were excised and plated onto semi-selective corn meal agar medium (CMA, 10 g.L⁻¹, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) added with agar (10 g.L⁻¹, Conda, Madrid, Spain), metalaxyl (30 mg.L⁻¹), benomyl (5 mg.L⁻¹) and rifampicin (25 mg.L⁻¹) (CMA-MBR, Malvick et al., 1994), and

Table 3 Primers and probes used in this study

Target (use)	Primer/probe	Nucleotide sequence	Reference
Ae ITS1 specific of <i>A. euteiches</i> (ddPCR - 132 bp multicopy sequence <i>per</i> haploid genome)	Forward primer Ae ITS1_39F	5'- TGAGGCTTGCTCTTTTCA - 3'	Gangneux et al., 2014
	Reverse primer Ae ITS1_167-R	5'- GAAAGTTGTATAGAATTGAC AAGCT-3'	Gangneux et al., 2014
	Probe Ae ITS1_39T	5' - /56 FAM/GCAAGGAAC/ZEN/-CGATGTAATTTTAAATCCCT /3IABkFQ/- 3'	This study
Ae_76bp sequence specific of <i>A. euteiches</i> (ddPCR - 76 bp single copy sequence <i>per</i> haploid genome)	Forward primer Ae_76bp_136_F	5'- GACTGCAATGTCGTCCAAGACTT - 3'	Vandemark et al., 2000, 2002
	Reverse primer Ae_76bp_211_R	5' - ACAAAGCTGAGATGAAGAGA TCGA - 3'	Vandemark et al., 2000, 2002
	Probe Ae_76bp_161-T	5'-/5Hex/CAACCACC G/ZEN/AGCGAGCCGC/3IABkFQ/-3'	Sauvage et al., 2007
Internal transcribed spacer 1 (oomycete identification)	Forward primer ITS-6	5'- GAAGGTGAAGTCGTAACAAGG-3'	Cooke et al., 2000
	Reverse primer ITS-4	5'-TCCTCCG CTTATTGATATGC - 3'	White et al., 1990
Cytochrome c oxidase subunit I (COI) (oomycete identification)	Forward primer OomCoxI--Levup	5'-TCAWCWMGATGGCTTTTTTCA AC-3'	Robideau et al., 2011
	Reverse primer Fm85mod	5'-RRHWACKTGACTDATRATACC AAA-3'	Robideau et al., 2011

incubated at 25 °C for 48 h. One colony *per* morphotype showing coenocytic hyphae was subcultured onto CMA medium. All the cultures were maintained at 4 °C on CMA and in tubes containing 10 sterilised hemp seeds and 10 mL of deionised water for long-term storage in the Microorganisms of Interest for Agriculture and Environment (MIAE) collection (INRAE Dijon, France). Isolates from each field were chosen so as to be representative of morphotype diversity for further identification and pathogenicity assessment. Each isolate was cultured at 25 °C on potato dextrose agar (PDA, 39 g L⁻¹, Sigma-Aldrich) plates for 15 days, and the mycelium was scraped directly from the agar plates. Fifty mg of mycelium were used for DNA extraction using a FastDNA® SPIN kit according to the manufacturer's recommendations. DNA extracts were stored at -20 °C until PCR analyses. The ITS region was amplified by PCR using primers ITS-6 and ITS-4 (Cooke et al., 2000; White et al., 1990; Table 3). The mitochondrial encoded gene cytochrome c oxidase subunit I (COI, *COXI*) was used to further identify *Pythium* isolates at the species level using the OomCoxI-Levup and Fm85mod primers (Robideau et al., 2011, Table 3). PCRs were performed in 25-µL reaction mixtures with the same composition as above. PCRs were initiated with a 3-min incubation at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 50 °C for ITS amplification or 1 min at 55 °C for COI amplification, 1 min at 72 °C, and a final extension of 10 min at 72 °C. PCR products were visualised on 2% agarose gels, and sent to Eurofins Genomics Germany GmbH (Ebersberg, Germany) for Sanger sequencing in forward and reverse directions using the PCR primers as sequencing primers. For each PCR product, sequences from both strands were assembled using SEQMAN 6.0 (DNASTAR Lasergene, GATC Biotech). Sequence identities were determined using BLAST analysis from the National Center for Biotechnology Information (NCBI). Taxonomic identification was based on the sequence similarity of the amplified ITS or COXI region with sequences available in the database and associated with a publication. The sequences were deposited in GenBank (GenBank accession numbers MZ027692 to MZ027703 and MZ054442 to MZ054448).

The pathogenicity of the identified isolates was assessed *in vitro*. The *A. euteiches* RB84 reference strain was used as a positive control. Pea seeds (cv. Firenze, Vilmorin, France) were disinfected in a sodium hypochlorite solution (1.5 g L⁻¹ available Cl₂) for 20 min,

rinsed three times in sterile deionised water, and plated onto PDA until germination. Germinated seeds without apparent contamination symptoms were transferred into sterile glass tubes (20 cm deep, 3 cm diameter) containing 30 mL of Hoagland's No.2 Basalt salt mixture (1.6 g L⁻¹) (Sigma-Aldrich Chemie GmbH) added with 8 g L⁻¹ agar (Conda) and inoculated at $t = 7$ days post germination with a total of 10⁵ zoospores using a water-zoospore suspension. Eight tubes *per* isolate were prepared. Symptoms were assessed 14 days later using the previously described scale.

Optimisation of DNA extraction from field soils

Homogenisation of field soil samples and different soil quantities for DNA extraction were assessed to obtain reproducible ddPCR measurements from naturally infested soil samples. Two hundred and fifty g of two naturally infested soil from Verpillieres (sample V141) and Renescore (sample R63) were sieved (4 mm mesh), or sieved and mechanically milled with a blender (Waring Laboratory Blender, Sigma) using 4 runs of 5 s at maximum speed separated by 10 s breaks. Soil DNA extractions were performed using an adapted procedure of the GnS-GII protocol described by Plassart et al. (2012) on 1 g, 2 g and 5 g of sieved soil samples, and 1 g, 2 g and 5 g of sieved and milled soil samples, with six replicates in each case. A lysing matrix containing 4 g of 0.1-mm diameter silica beads (Lysing matrix B bulk, MP-Biomedicals, New York, USA), 5 g of 1.4-mm diameter ceramic beads (Lysing matrix D bulk, MP-Biomedicals), four 4-mm diameter glass beads and 8 mL of lysis buffer containing 100 mM Tris-HCl (pH 8), 100 mM EDTA (pH 8), 100 mM NaCl, 2% SDS and ultra-pure water were added to each 1-g soil sample. The quantities of lysing matrix and lysis buffer were increased proportionally to the soil quantity for the 2-g and 5-g soil samples. Total DNA was extracted by mechanical lysis using FastPrep-24™ Classic Instrument (MP-Biomedicals). DNA was purified with a NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. DNA samples were eluted in 60 µL of SE elution buffer of the NucleoSpin® Soil kit and stored at -20 °C until analyses. Purified DNA samples were quantified by fluorometry using a QuantiFluor staining kit (Promega, Madison, Wisconsin, USA) and a TECAN system according to the manufacturer's instructions. DNA samples were analysed using the optimised ddPCR

conditions described below, in the presence or in the absence of *EcoRI*. The optimised procedure was used to extract DNA from the 200 soil samples, in triplicate.

Development and optimisation of a droplet digital PCR assay to quantify *Aphanomyces euteiches* in field soil samples

A duplex TaqMan approach was used to quantify two *A. euteiches*-specific DNA fragments. The first target was a 132-bp sequence of *A. euteiches* ITS1 (Ae_ITS1) present in multicopy in the genome of *A. euteiches* and appropriate to quantify low inoculum levels in the soil (Gangneux et al., 2014). PCR primers Ae_ITS1_39F and Ae_ITS1_167R proposed by Gangneux et al. (2014) were used to amplify this target (Table 3). A TaqMan fluorogenic probe (Ae_ITS1_39T) was designed to be specific to this sequence. The specificity and identity of the probe with the targeted sequence were verified using BLASTN program (NCBI). Blast results with 100% of similarity with the probe corresponded exclusively to *A. euteiches* ITS sequences. Sequences of organisms other than *A. euteiches* that matched the probe had only 96% of similarity or less with the probe. The second target was a 76-bp fragment (Ae_76bp) present in a single copy in the haploid genome of *A. euteiches* (Vandemark et al., 2000, 2002). It was used to approximate the ITS1 copy number in *A. euteiches* genomic DNA. PCR primers Ae_76bp_136_F and Ae_76bp_211_R (Vandemark et al., 2000, 2002) and probe Ae_76bp_161T (Sauvage et al., 2007) were used to target and quantify this fragment (Table 3).

The ddPCR assay was optimised by testing different amounts of soil, whether milled or not (see above), different amounts of DNA, and in the presence or absence of restriction enzymes in the ddPCR mixture. Total DNA was extracted from 2 g of milled samples from Verpillieres soil (sample V45) and Renescure soil (sample R125) infested by *A. euteiches*, using the procedure described above. Totals of 50 ng, 100 ng and 200 ng of DNA from these two soil samples were tested for ddPCR, with five replicates *per* sample. The *EcoRI* and *SalI* restriction enzymes were tested on three replicates of the same DNA samples and quantities in order to cut *A. euteiches* DNA between the tandem repeats of operons of ribosomal RNA-encoding genes and allow for a random distribution of the Ae_ITS1 target among the droplets. The absence of *EcoRI* and *SalI* restriction

sites inside the Ae_ITS1 and Ae_76bp sequences was previously checked.

ddPCR were performed in 22- μ L mixtures containing 11 μ L of ddPCR supermix for probes (no dUTP) (Bio-Rad Laboratories, Hercules, USA), 900 nM of each primer, 250 nM of each probe, 1 U of restriction enzyme *EcoRI* or *SalI* or no restriction enzyme, and variable quantities of template DNA. Twenty μ L of the PCR mixture were pipetted into the sample chambers of a Droplet Generator DG8 Cartridge (Bio-Rad, cat no. 1864008), added with 60 μ L of droplet generation oil (Bio-Rad, Cat No. 1863005) and placed in the QX100 Droplet Generator (Bio-Rad) for droplet generation. Forty μ L of created droplets were transferred into a 96-well ddPCR plate (Bio-Rad, cat no. 12001925), sealed with a heat seal foil for PCR plates (Bio-Rad, cat no. 1814040), and transferred to a T100 thermal cycler (Bio-Rad) for amplification. For each ddPCR plate run, at least two no-template controls (containing DNA-free water instead of soil DNA) and two positive controls (containing 5 ng of *A. euteiches* genomic DNA) were used. Thermal cycling conditions consisted of initial denaturation and DNA polymerase activation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s for denaturation and 60 °C for 1 min for hybridisation and elongation (ramp 2 °C s⁻¹), and a final step at 98 °C for 10 min for droplet stabilisation. ddPCR were performed using the Bio-Rad QX100 ddPCR system at the DTAMB platform of Claude Bernard Lyon 1 University (Développement de Techniques et Analyse Moléculaire de la Biodiversité, Villeurbanne, France). The optimised ddPCR assay was performed on DNA extracts from the 200 soil samples in triplicate.

ddPCR analysis

QuantaSoft Analysis Pro software version 1.0.596 (Bio-Rad) was used to analyse ddPCR results. The threshold for the classification of droplets as positive or negative was set manually above the cluster of negative partitions for all DNA samples at amplitudes around 900 for the Ae_ITS1 target (Channel 1, fluorescein [FAM]) and around 1450 for the Ae_76bp target (Channel 2, hexachlorofluorescein [HEX]). Results were obtained as number of targeted copies *per* μ L of reaction mixture and were first converted into number of copies *per* ng of soil DNA (Table 4). To standardise and compare *A. euteiches* abundance in the fields, results were then converted into number of diploid genomes *per* gram of

dry field soil based on the quantity of extracted DNA and on the mean number of Ae_ITS1 copies *per* haploid genome in the *A. euteiches* population of each field (see below).

Assessment of ITS1 copy numbers *per* haploid genome of *Aphanomyces euteiches*

The number of ITS1 copy numbers *per* haploid genome was assessed for one or two *A. euteiches* isolates collected from each field. *A. euteiches* genomic DNA was extracted and quantified using the previously described protocols. Duplex ddPCRs were performed using the primer sets and methodology described above on a dilution series from 2.5 ng to 0.625 ng of genomic DNA, in triplicate. A dilution level inside the recommended dynamic range from 1 to 120,000 copies of each target *per* 20- μ L reaction mixture (Digital Droplet PCR Application Guide, Bio-Rad) was selected for each isolate. The ratio of the mean copy number of Ae_ITS1 to the mean copy number of Ae_76bp was calculated for each isolate. Results were expressed as mean \pm standard deviation following three independent measurements.

The mean number of Ae_ITS1 sequences *per* *A. euteiches* haploid genome was estimated for each field directly from the ddPCR results, following the analysis of the DNA extracted from the 200 field samples. For each field and for each replicate, the ratio of the sum of Ae_ITS1 copies to the sum of Ae_76bp copies detected in all samples ($n = 50$) was calculated according to the formula:

$$N_{ITS1} = \frac{\sum_{i=1}^n x_i}{\sum_{i=1}^n y_i}$$

where N_{ITS1} is the mean number of Ae_ITS1 sequences *per* haploid genome in the *A. euteiches* field population, n is the number of soil samples analysed by duplex ddPCR, x is the number of Ae_ITS1 copies detected in each sample, and y is the number of Ae_176bp copies detected in each sample. Results were expressed as mean \pm standard deviation of the three replicates.

Statistical analyses

All statistical analyses were conducted and visualised using R statistical software version 4.0.1 for Windows (R Core Team 2020, Online Resource 1). The readxl

package V1.3.1 and the tidyverse package v1.3.0 were used to import and to analyse the dataset (Wickham & Bryan, 2019; Wickham et al., 2019). The packages ggplot2 v3.3.2, cowplot v1.1.0 and lemon v0.4.5 were used for data visualisation and graphic constructions (Edwards, 2020; Wickham, 2016; Wilke, 2020). Variability among conditions was compared graphically using coefficients of variation (CVs) and tested using the Brown-Forsythe test with the leveneTest function of the package lawstat v3.4 (Gastwirth et al., 2020) to optimise DNA extraction and ddPCR.

To compare our results with previous studies and with pea-producing stakeholder practices, the bounded pseudo-quantitative DSI variable defined by Moussart et al. (2009) was treated as a quantitative variable. Spearman Rho coefficient was calculated, and non-parametric locally weighted scatterplot smoothing regression (LOESS) was used to illustrate the monotonic relationship between DSI and *A. euteiches* soil-borne ID in each field. Due to their heavy influence on LOESS regression results, 2 points above 125 diploid genomes.g⁻¹ of dry field soil, corresponding to soil samples R1 and W5, were considered as outliers and removed from this analysis.

The lmer function in R package lme4 (Bates et al., 2015) was used to apply linear mixed models to test the effect of *A. euteiches* ID on the DSI response variable measured on the field soils. Models were fitted using the restricted maximum likelihood (REML) method and compared based on Akaike information criterion (AIC) and Bayesian information criterion (BIC) using the maximum likelihood (ML) estimation method. The selected model was under the form:

$$\text{logit} (\text{DSI}/5) = (b_{\text{intercept}} + b_{\text{field}}) + b_{\text{ID}} \cdot \text{log}_{10} (\text{ID} + 1) + \epsilon$$

where DSI and ID represent the mean DSI values and ID values within technical replicates, respectively, $b_{\text{intercept}}$, b_{ID} and b_{field} represent the coefficients of the model (the intercept, the slope and the field random-effects coefficients, respectively), and ϵ represents the model residuals with $\epsilon \sim N(0, \sigma)$.

DSI and ID were logit and log-10 transformed, respectively, for linearisation. DSI values of 0 were adjusted to 0.025 using the logit function in the car package (Fox & Weisberg, 2019). Outlier data were removed from the dataset based on Cook's distance. The model residuals were tested for assumptions of independency,

homogeneity of variance and normality using Shapiro test and graphical analyses. The `r.squaredLR` function of the Mumin package (Barton, 2020) was used to calculate the pseudo- R^2 of the model (Nagelkerke adjusted). The model parameters and quality criteria were formatted using the `stargazer` package v5.2.1 (Hlavac, 2018). The `predict` function of the `lme4` package was used for DSI predictions (with the `re.form` argument set to NULL for predictions conditional on field random-effects, and set to NA for predictions made at the field population level (i.e. unconditional)) (Bates et al., 2015). The `predict` and `bootMer` functions of the `lme4` package were used to define the confidence interval of the model (Bates et al., 2015).

Results

Disease severity measured on field soil samples

Typical *A. euteiches* symptoms were observed on pea seedlings grown on soil samples from each field. However, symptoms sometimes turned darker, indicating that other pathogens potentially colonised the root systems. A high proportion of seedlings grown on the Woestyne (27.1%) and Flers (20.7%) soil samples underwent damping-off or aborted before emergence. These damping-off symptoms were likely caused by fly larvae that were observed in the fields at the time soils were collected. The proportion of aborted plants was only 3.1% in the Verpillieres soil samples and 2.1% in the Renescure soil samples. Two Woestyne samples and one Flers sample did not provide enough plants to assess the DSI.

As expected, the infestation levels of the Renescure and Verpillieres samples collected in 2017 were higher than those of Woestyne and Flers sampled in 2018. The DSIs measured on the Renescure soil samples ranged from 0.1 to 4.3 (median value 3.0) (Table 5). The DSIs measured on the Verpillieres soil samples ranged from 1.1 to 4.3 (median value 3.2). No disease was observed on the pea seedlings grown in 5 Woestyne soil samples and 14 Flers soil samples. The DSIs measured on the Woestyne soil samples ranged from 0 to 3.3 (median value 1.1). The DSIs measured on the Flers soil samples ranged from 0 to 4.0 (median value 1.3).

Pea root rot pathogens

The involvement of *A. euteiches* in the observed symptoms was confirmed by amplification of the `Ae_ITS1` and `Ae_76bp` targets. Duplex PCR performed on the DNA extracted from *A. euteiches* strain RB84 and from symptomatic pea roots yielded PCR products of the expected sizes (132 bp and 76 bp for `Ae_ITS1` and `Ae_76bp`, respectively). No amplification occurred when the DNA extracted from asymptomatic pea roots was used (not shown).

A. euteiches was isolated from symptomatic pea roots from the DSI bioassays of the four fields (Table 5). The *in vitro* pathogenicity tests confirmed that all *A. euteiches* isolates were pathogenic on pea. Based on pathogenicity scores, there was no significant difference in aggressiveness among all *A. euteiches* isolates (Table 5). Five other oomycetes isolated from symptomatic pea roots were identified as *Pythium terrestris* using the additional COI marker, and were isolated from the four fields (Table 5). The pathogenicity of all five isolates on pea was confirmed by the *in vitro* pathogenicity tests. They caused slight honey-brown coloured rot symptoms on pea roots that looked similar to the symptoms caused by *A. euteiches* strains at the early stage of infection (not shown). However, the *in vitro* pathogenicity test confirmed that *A. euteiches* had the highest pathogenicity score among the isolated taxa (Table 5). Other isolates belonging to phytopathogenic species such as *Pythium intermedium* (syn. *Globisporangium intermedium*) and *P. conidiophorum* were also isolated from symptomatic pea roots but with a low frequency, so their pathogenicity on pea was not assessed.

Duplex ddPCR on *Aphanomyces euteiches* genomic DNA

The ddPCR assay was performed on genomic DNA of *A. euteiches* isolates from the DSI bioassays and on the reference strain RB84. The two targets were suitably quantified at the selected dilution (i.e. inside the recommended ddPCR dynamic range from 1 to 120,000 copies of each target *per* 20- μ L reaction mixture) from all genomic DNAs (not shown). The cluster of positive droplets exhibited a greater fluorescence amplitude and was clearly distinguishable from the cluster of negative droplets for both the `Ae_ITS1` (FAM positive, Channel 1) and `Ae_76pb` (HEX positive, Channel 2) targets

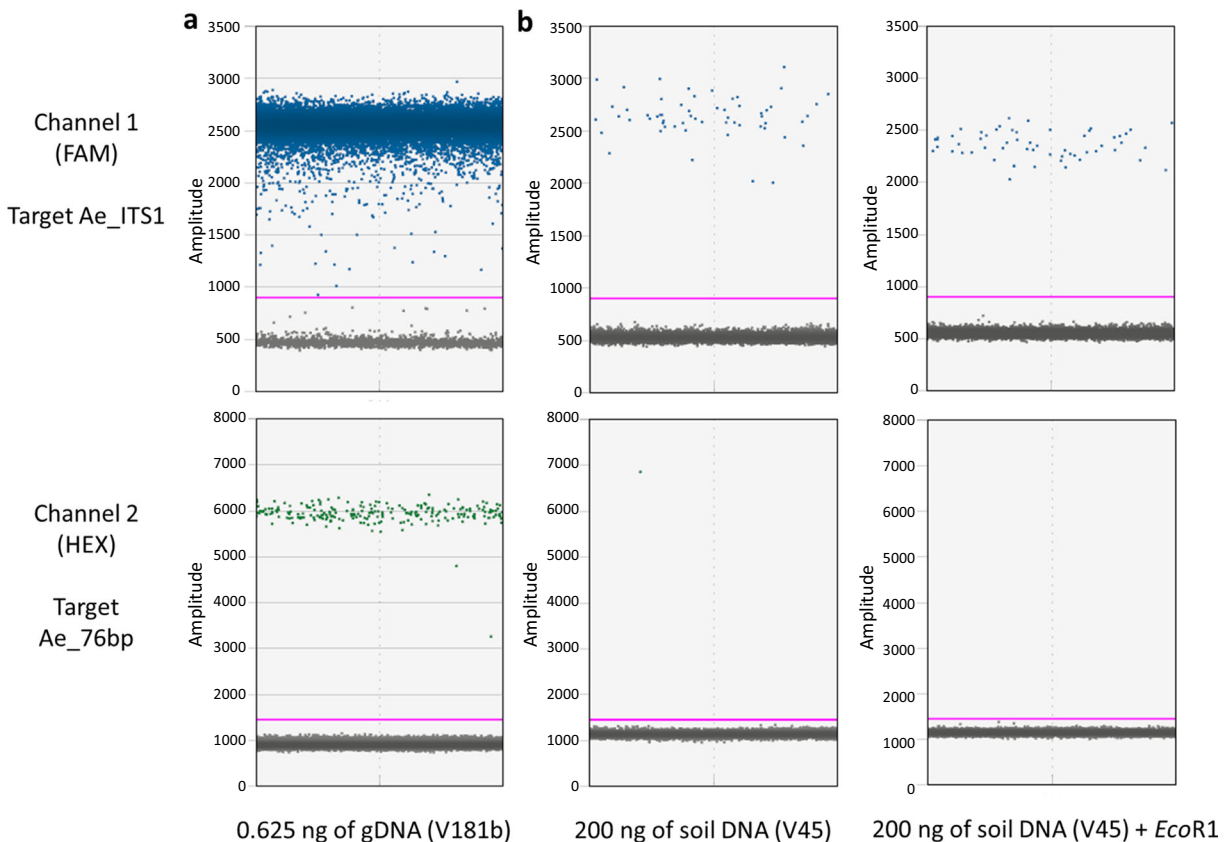


Fig. 1 Examples of distribution diagrams of droplets obtained after duplex digital Taqman PCR assay performed on (a) *Aphanomyces euteiches* strain V181b genomic DNA or (b) dry soil DNA from sample V45 in the absence or in the presence of the *EcoR1* restriction enzyme. Blue and green dots above the

threshold (pink line) represent positive droplets for the PCR amplification of the Ae_ITS1 target (Channel 1, [FAM]) and of the Ae_76bp target (Channel 2, [HEX]), respectively. Black dots represent negative droplets

(Fig. 1a). However, some rain droplets were detected at an amplitude in-between the clusters of positive and negative droplets; they were mainly considered positive owing to the positioning of the threshold (Fig. 1a).

The ITS1 copy number *per* haploid genome was calculated from the ddPCR results for all *A. euteiches* isolates from the DSI bioassays. This number varied from 8.5 ± 0.3 copies *per* haploid genome for isolate R185b from the Renescure field to 239 ± 45 copies *per* haploid genome for isolate W65a from the Woestyne field (Table 5).

Optimisation of field soil DNA extraction and of the ddPCR assay

The ddPCR assay was optimised with repeated measures performed on 50 ng, 100 ng and 200 ng of DNA from the infested soil samples of Verpillieres (V45) and

Renescure (R125) in the presence or in the absence of the restriction enzymes *EcoR1* or *SalI* in the ddPCR mixture (Fig. 2a, Online Resource 2). As for ddPCR performed on *A. euteiches* genomic DNA, the fluorescence amplitude of the positive droplets was clearly distinguishable from the cluster of negative droplets for both the Ae_ITS1 (Channel 1) and Ae_76bp (Channel 2) targets (Fig. 1b). However, the targets were at low concentrations, and Ae_76bp was only detected at very low levels ($< 2 \times 10^{-2}$ copies *per* ng of DNA) from the infested soil of Verpillieres using 200 ng of DNA in the absence of a restriction enzyme and 50 ng of DNA in the presence of the restriction enzyme *SalI* (Fig. 2a). The detection limit of the ddPCR assay was thus too high to accurately and reproducibly quantify this single copy *per* genome target with one measurement performed on the soil DNA quantities we tested. However, the multicopy Ae_ITS1 target was detected in all conditions

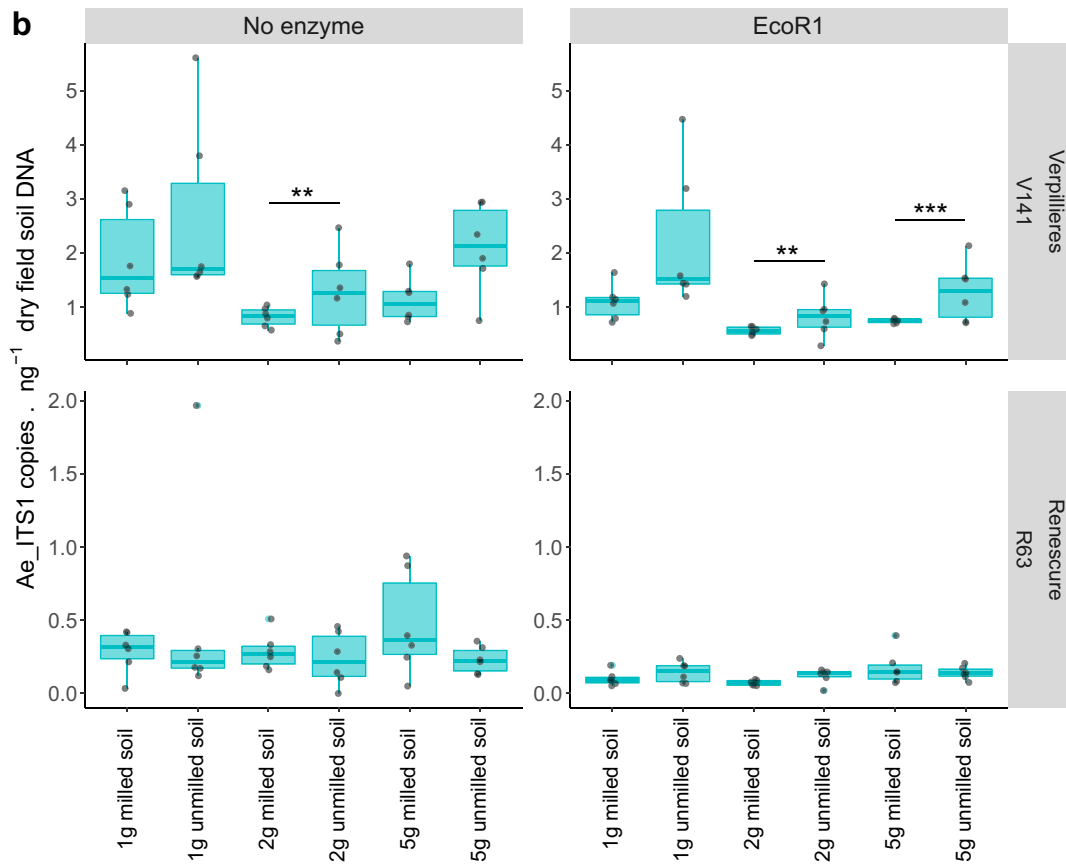
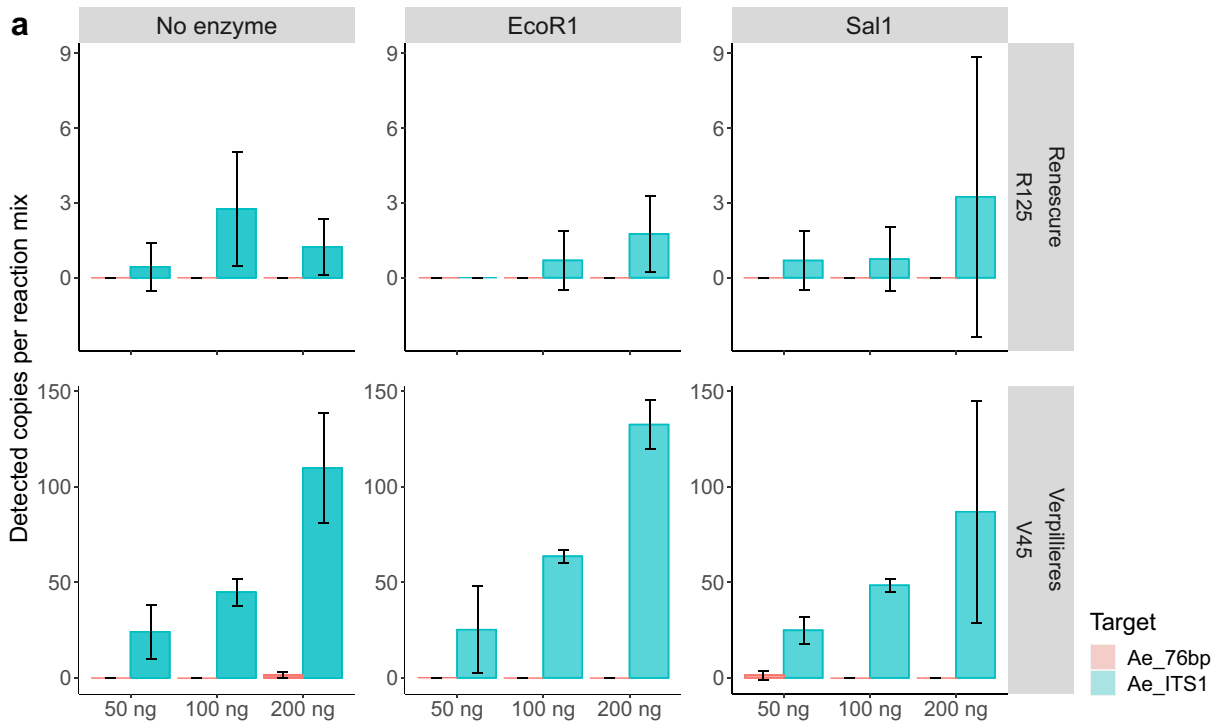


Fig. 2 Technical optimisation of the quantification of *Aphanomyces euteiches* inoculum density in naturally infested soil samples. **a** - Evaluation of the quantity of dry field soil DNA needed for a reproducible quantification with the multicopy Ae ITS1 and single copy Ae 76bp targets ($n = 5$ for the no-enzyme condition; $n = 3$ for the *EcoR1* and *Sal1* conditions). **b** - Impact on measured reproducibility of i) dry soil quantities and milling before DNA extraction, and ii) the use of the *EcoR1* restriction enzyme during Ae ITS1 quantification by droplet digital PCR. Measurements were performed in the presence or in the absence of *EcoR1* on six DNA extract replicates from a sieved soil or a sieved and milled soil from Renescure and Verpillieres fields. Brown-Forsythe test was used to test the pairwise equality of variance of the milled and unmilled conditions (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

in the two naturally infested soils samples using 100 ng and 200 ng of DNA (Fig. 2a). Less than one copy of this target *per* ng of dry soil DNA was detected in all measurements. The use of the restriction enzyme *EcoR1* improved measurement repeatability and fitted with the proportions of matrix DNA quantities compared to the *Sal1* or no-enzyme conditions.

The field soil quantity and homogenisation required for DNA extraction and the use of the restriction enzyme *EcoR1* during ddPCR influenced the variability of quantifications in the two Renescure (R63) and Verpillieres (V141) soils (Fig. 2b, Online Resource 3). Quantifications after DNA extraction from only 1 g of soil showed the greatest variability for the Verpillieres soil. The measurements were more reproducible when DNA from 2 g and 5 g of soil matrix was used. Soil homogenisation also greatly reduced the variability of the results for each Verpillieres soil quantity. This was highly significant when 2 g (Brown-Forsythe test, $p = 0.041$) and 5 g of dry soil (Brown-Forsythe test, $p = 0.003$) were used. Lastly, the improved reproducibility of quantification by adding the *EcoR1* enzyme in the ddPCR mixture was confirmed for the two soil samples whatever the condition compared to ddPCR in the absence of a restriction enzyme (Fig. 2b). This reduced variability was significant for 2 g of unmilled soil from Renescure (Brown-Forsythe test; $p = 0.009$) and 5 g of milled soil from Verpillieres (Brown-Forsythe test; $p = 0.006$). Finally, variability among replicates was low when DNA extracted from 2 g and 5 g of milled dry soil was amplified in the presence of *EcoR1*. For these conditions, CV values were 13.1% and 5.4% for 2 and 5 g of the Verpillieres soil, respectively, and 25.9% and 67.6% for 2 and 5 g of the Renescure soil, respectively.

Thus, DNA extracted from 2 g of milled dry soil and amplified in the presence of the *EcoR1* enzyme was effective for a reproducible quantification of *A. euteiches* inoculum in naturally infested field soil and was the best compromise in terms of cost and efficiency. This optimised procedure was applied in triplicate to extract total DNA from the 200 soil samples and quantify *A. euteiches* inoculum by ddPCR.

Sensitivity and accuracy of the ddPCR assay on naturally infested field soils

The soil DNA extractions revealed a higher molecular biomass level in the field soils rich in OM of the north of the pea production basin (Woestyne and Renescure) than in those with a lower level of OM of the south of the pea production basin (Verpillieres and Flers) (Table 4). The mean molecular biomass of the 50 samples from each field ranged from 38.5 ± 2.5 μg of DNA *per* gram of dry soil for Verpillieres to 73.3 ± 8.3 μg of DNA *per* gram of dry soil for Renescure.

The multicopy Ae ITS1 target was detected in most of the 200 samples (Online Resource 4). The target was accurately quantified from 0 to $882 \pm 27 \times 10^{-3}$ copies *per* ng of dry field soil DNA from the four soils (Table 4). This low rate below one copy *per* ng of dry soil DNA confirmed the low inoculum levels of naturally infested field soils and the sensitivity of ddPCR. However, ddPCR was not sensitive enough to quantify the single copy marker of *A. euteiches* in field soil samples with these low inoculum levels. The Ae 76bp target was detected in only 52 of the 200 soil samples, at very low densities from 2.2×10^{-3} to 1.4×10^{-2} copies *per* ng of dry soil DNA (Table 4). However, the sum of the detected copies of this target in the 50 soil DNA samples analysed *per* field corresponded to a 50-fold increase of the quantity of matrix DNA compared to a single soil DNA sample, so it was possible to obtain a representative value of the inoculum level at the field scale. Based on this mean value calculated for each target, the mean copy number of Ae ITS1 sequences *per* haploid genome in the *A. euteiches* population of each field was calculated. This number varied from 81 ± 15 in Flers to 184 ± 43 in Renescure (Table 4). These mean copy numbers were used to convert IDs expressed as Ae ITS1 copies *per* ng of dry field soil DNA to IDs expressed as *A. euteiches* diploid genomes *per* gram of dry field soil (Table 4).

Table 4 Statistical parameters of the distribution of the disease severity index (DSI), molecular biomass, Ae_76bp and Ae ITS1 copy numbers measured by digital droplet PCR at the field scale, and *Aphanomyces euteiches* diploid genomes. Results are expressed as mean values of technical replicates \pm standard deviation

Parameter	Statistics	Resequence (n=50)	Verpilliere (n=50)	Woestyne (n=50)	Flers (n=50)
DSI	Median	3.0	3.2	1.1	1.3
	Range (min. - max.)	(0.1–4.3)	(1.1–4.3)	(0–3.3 \pm 0.8)	(0–4.0 \pm 0.4)
	Mean	2.7 \pm 0.9	3.2 \pm 0.7	1.3 \pm 1.0	1.5 \pm 1.4
Molecular biomass ($\mu\text{g DNA} \cdot \text{g}^{-1}$ dry soil)	Median	74.7	38.4	57.9	41.2
	Range (min. - max.)	(49.2 \pm 3.4–86.6 \pm 5.3)	(33.4 \pm 2.0–43.5 \pm 1.9)	(47.12 \pm 5.7–69.8 \pm 1.0)	(32.8 \pm 4.4–49.3 \pm 4.0)
	Mean	73.3 \pm 8.3	38.5 \pm 2.5	58.9 \pm 4.5	41.0 \pm 4.0
Ae ITS1 (10^{-3} copies. ng^{-1} soil DNA)	Median	164.3	128.5	125.0	46.6
	Range (min. - max.)	(34.9 \pm 19.9–689.2 \pm 208.5)	(24.6 \pm 42.6–357.6 \pm 185.0)	(0–882.3 \pm 27.2)	(0–188.1 \pm 109.4)
	Mean	185.9 \pm 142.3	145.0 \pm 82.9	186.9 \pm 215.3	61.5 \pm 60.3
Ae_76bp (10^{-3} copies. ng^{-1} soil DNA)	Median	0	0	0	0
	Range (min. - max.)	(0–6.1 \pm 5.4)	(0–14.1 \pm 12.3)	(0–7.1 \pm 12.3)	(0–7.5 \pm 12.9)
	Mean	1.1 \pm 1.8	1.6 \pm 2.9	1.1 \pm 2.1	8.2 \pm 1.9
Mean number of ITS1 copies per <i>A. euteiches</i> haploid genome in field populations	Median	184 \pm 43	105 \pm 44	181 \pm 16	81 \pm 15
	Range (min. - max.)	(4.9 \pm 11.2–141.1 \pm 208.5)	(4.8 \pm 8.2–59.2 \pm 11.8)	(0–145.9 \pm 3.9)	(0–51.8 \pm 2.5)
	Mean	36.7 \pm 27.3	16.8 \pm 14.5	32.7 \pm 37.3	15.6 \pm 15.5

Table 5 Pathogenic oomycetes isolated from disease severity bioassays, estimated ITS1 copy numbers *per* haploid genome of *Aphanomyces euteiches*, and pathogenicity scores. Results are expressed as means of the replicates \pm standard deviation

Field of origin	Species	MIAE accession number ^a (original code)	ITS1 copy number <i>per</i> haploid genome of <i>Aphanomyces euteiches</i> ^b	Pathogenicity score
Renesecure	<i>Aphanomyces euteiches</i>	MIAE07676 (R83a)	42.3 \pm 3.5	5.0 \pm 0.0
	<i>A. euteiches</i>	MIAE07677 (R185b)	8.5 \pm 0.3	5.0 \pm 0.0
	<i>Pythium terrestris</i>	MIAE07678 (R143c)	ND	0.9 \pm 0.7
Verpillieres	<i>A. euteiches</i>	MIAE07679 (V105c)	204.9 \pm 40.8	5.0 \pm 0.0
	<i>A. euteiches</i>	MIAE07680 (V181b)	167 \pm 4.4	5.0 \pm 0.0
	<i>P. terrestris</i>	MIAE07681 (V143a)	ND	1.3 \pm 1.0
Woestyne	<i>A. euteiches</i>	MIAE07682 (W65a)	239.1 \pm 45.1	5.0 \pm 0.0
	<i>A. euteiches</i>	MIAE07683 (W163a)	149.7 \pm 46.7	5.0 \pm 0.0
	<i>P. terrestris</i>	MIAE07684 (W29a)	ND	0.9 \pm 1.2
Flers	<i>A. euteiches</i>	MIAE07685 (F85a)	10.6 \pm 0.7	5.0 \pm 0.0
	<i>P. terrestris</i>	MIAE07686 (F47a)	ND	1.6 \pm 0.8
	<i>P. terrestris</i>	MIAE07687 (F49a)	ND	1.7 \pm 1.1
Reference strain	<i>A. euteiches</i>	MIAE01625 (RB84)	21.5 \pm 3.1	5.0 \pm 0.0

^aMIAE: Microorganisms of Interest for Agriculture and Environment, INRAE Dijon, France

^bND: not determined

The converted IDs data ranged from 0 to 146 \pm 4 *A. euteiches* diploid genomes *per* gram of dry field soil. The CVs of the measured IDs ranged from 2% to 173% in all datasets, with a median value of 44%. The CVs were higher in the low infested fields of Woestyne and Flers, with median values of 71.8% and 55.3% compared to 39.4% and 35.5% for Renesecure and Verpillieres, respectively (Fig. 3). This difference was partly due to the presence of several CVs greater than 150% in these two fields, which corresponds to inoculum densities less than 5 *A. euteiches* diploid genomes *per* gram of dry field soil.

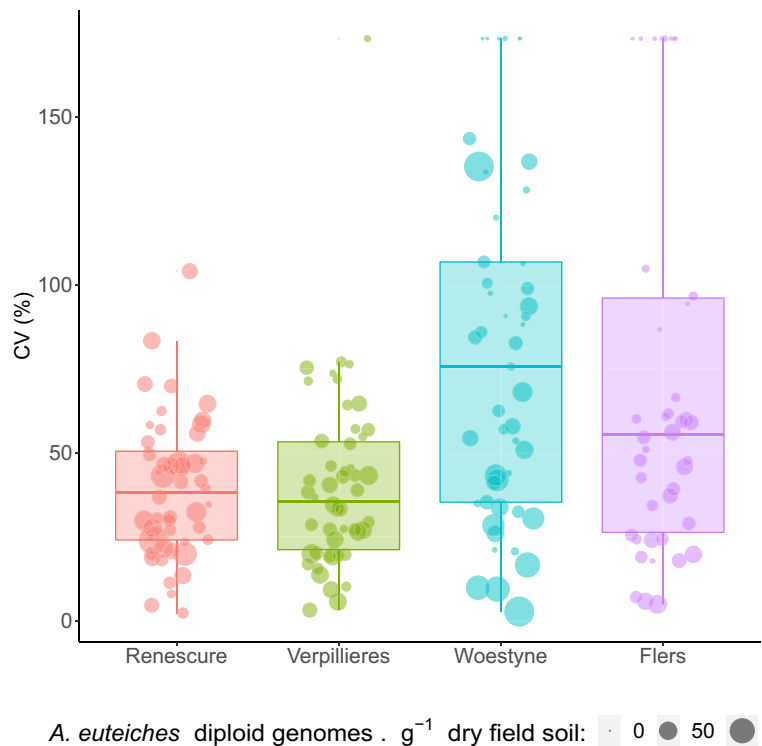
Relationship between the inoculum density and the disease severity index in naturally infested fields

Spearman rank correlation coefficients showed a significant positive correlation between DSIs and *A. euteiches* soil-borne IDs in Woestyne ($\rho = 0.85$; $p < 0.001$), Flers ($\rho = 0.76$; $p < 0.001$), Verpillieres ($\rho = 0.45$; $p < 0.001$) and Renesecure ($\rho = 0.38$; $p < 0.01$). Locally weighted regression (LOESS) was used to illustrate the relationship between IDs and DSIs in each of the four fields (Fig. 4).

The linear mixed regression analysis showed a significant linear relationship between Log_{10} -transformed *A. euteiches* IDs and logit-transformed DSIs, with a pseudo- R^2 equal to 0.77 (Fig. 5). The model fixed effects corresponding to the effects of *A. euteiches* IDs in the soil samples on the DSIs measured on susceptible pea plants were significant ($p < 0.001$). Similarly, the random effects due to specificities of each field were significant ($p < 0.001$). The field random effects, i.e. the random effects linked to the biotic and abiotic properties of each field, explained approximately 42% of the total variance of the model. A summary of the estimated conditional and unconditional parameters and of the quality criteria of the model is presented in Table 6. A 95% confidence interval of unconditional predicted values (i.e. taking the ID effect uncertainty and field random effect variance into account) is represented in Fig. 5b. Considering the model unconditional equation, the DSI threshold of 1, considered as the value not to be exceeded in France for an acceptable risk for pea cropping, corresponded to an ID of 5 ranging between 2 and 13 diploid genomes *per* gram of dry field soil.

A graphical mapping of the spatial distribution of the measured DSIs and of the model conditional and

Fig. 3 Boxplot representation of the distribution of the coefficients of variation (CVs) of *Aphanomyces euteiches* inoculum density measurements based on technical replicates



unconditional predicted DSI values reflects the diversity of situations (Fig. 6). The four field soils showed different levels of expression of the disease, but they also showed a heterogeneous distribution of foci as measured by the DSIs (Fig. 6a). The heterogeneity of this distribution of disease foci, estimated by measuring *A. euteiches* ID by ddPCR, is represented by both the conditional (Fig. 6b) and unconditional model predictions (Fig. 6c). However, the conditional model reflects the risk of pea root rot more strongly than the unconditional model does.

Discussion

This study validates a whole methodology based on ddPCR to accurately and reproducibly quantify low and heterogeneously distributed *A. euteiches* inoculum present in naturally infested field soils. A linear mixed model was built to predict disease severity from *A. euteiches* inoculum densities, which highlighted the importance of field random effects on disease expression.

Droplet digital PCR assay design and optimisation

The objective of this study was to develop a rapid and sensitive molecular quantification tool as an alternative to DSI bioassays commonly used for pea root rot disease risk assessment in naturally infested pea fields (Moussart et al., 2009). From this perspective, an important work was achieved by Sauvage et al. (2007) and Gangneux et al. (2014) using qPCR quantification tools. In both cases, the designed primers and their targeted DNA markers proved to be *A. euteiches* specific and made it possible to successfully and accurately quantify oospores from artificially inoculated soils (Gangneux et al., 2014; Sauvage et al., 2007). An encouraging limit of detection of 10 oospores *per* gram of dry soil was obtained by Gangneux et al. (2014) by targeting the multicopy ITS1 marker of *A. euteiches*. This greatly improved sensitivity compared to a test based on single-copy sequences. Therefore, we focused our efforts on the development of a ddPCR methodology based on these two previously used targets to quantify *A. euteiches* inoculum in naturally infested field soils.

However, numerous biological and technical barriers need to be overcome for reproducible and accurate

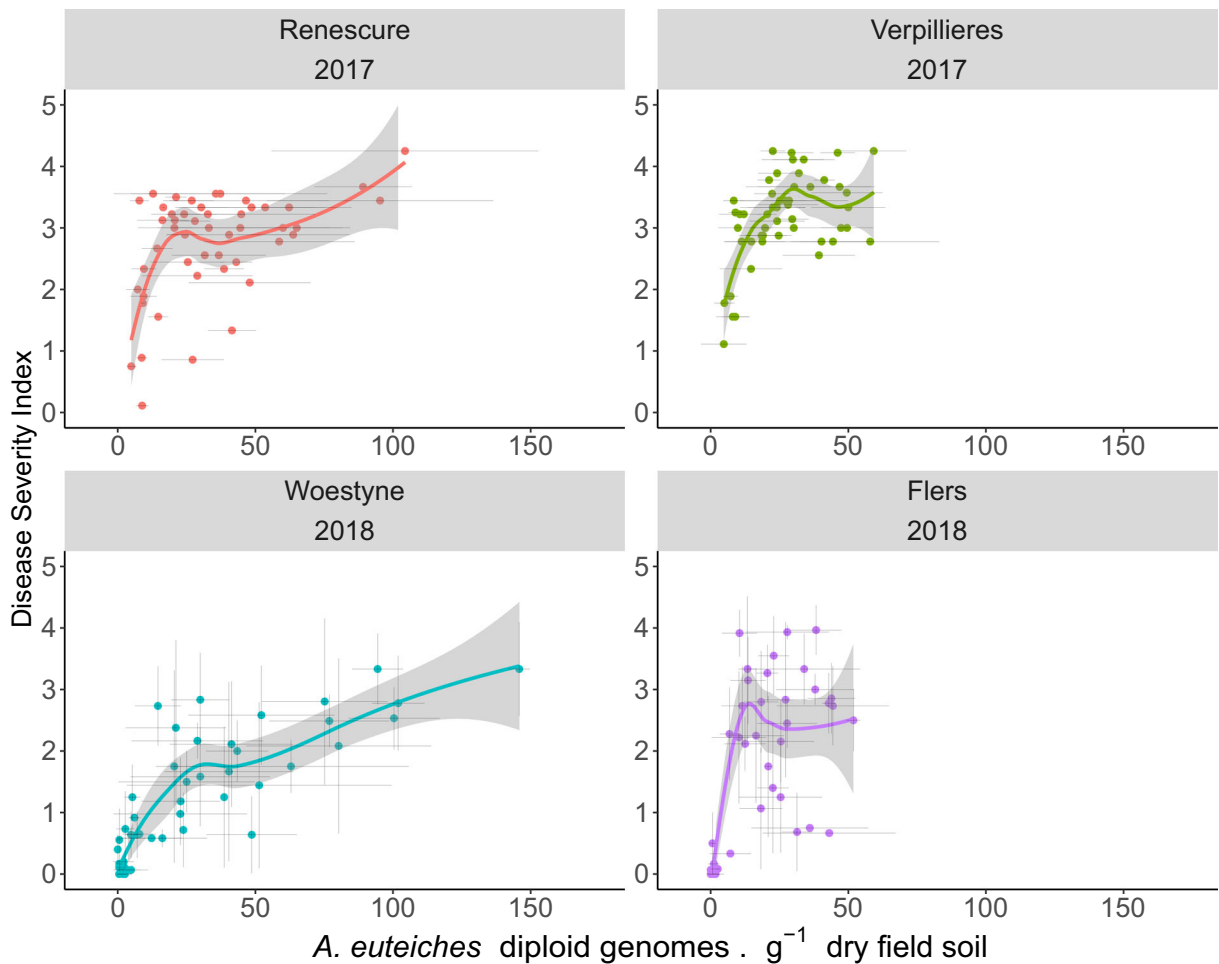


Fig. 4 Locally weighted scatterplot smoothing regression (LOESS) showing the relationship between disease severity indexes (DSIs) and *Aphanomyces euteiches* inoculum densities (IDs) in each of the four fields. Soil *A. euteiches* IDs were

measured by droplet digital PCR targeting *Ae_ITS1* sequences. Shaded ribbons represent 95% pointwise confidence intervals. Vertical and horizontal error bars represent standard errors of the mean values of the technical replicates

quantification from naturally infested soils. The first one is that *ITS1* copy numbers vary among *A. euteiches* isolates, which questions the choice of this target for a quantification method. A previous study reported a distribution range from 49 to 145 copies *per* haploid genome, with a mean number of 95 ± 22 copies among 40 *A. euteiches* isolates from different countries (Gangneux et al., 2014). According to the authors, this observation leads to an inherent error margin of 52% for each quantification, and calls for a direct assessment of *ITS1* copy number variation for each quantification. To this end, the two primer/probe sets were used in a duplex TaqMan ddPCR assay targeting the multicopy *Ae_ITS1* and the single copy *Ae_79bp* DNA fragments.

The concomitant measurement of the two targets from the same samples and the compartmentalisation of DNA copies in ddPCR preserved their respective proportions and their amplifications without creating any competition between the targets (Denis et al., 2018). Due to the tandem repeated structure of the operon of ribosomal RNA-encoding genes within eukaryotes, a restriction enzyme was also required to cut *Ae_ITS1* so as to release each copy from its neighbours and to allow for their random distribution among droplets (Droplet Digital PCR Applications Guide, Bio-Rad). Restriction enzymes are also adapted to the use of large amounts of DNA in PCR by digesting DNA and thereby promoting accessibility by *Taq* polymerase (Denis et al., 2018).

Table 6 Summary of the estimated unconditional parameters with standards errors, estimated conditional parameters with standard deviations and quality criterion summary of the linear mixed model

Parameter	Dependent variable
	Mean DSI
Inoculum Density ($\text{Log}_{10}(\textit{A. euteiches}$ diploid genome. g^{-1} dry field soil +1))	2.07* (0.11)
Constant	- 3.06* (0.35)
Field random effects	
Renescure	0.19 (0.10)
Verpillieres	0.75 (0.10)
Woestyne	- 0.70 (0.11)
Flers	- 0.24 (0.10)
Observations	195
Log Likelihood	-226.12
Akaike Inf. Crit.	460.25
Bayesian Inf. Crit.	473.34
Pseudo- R^2 (Nagelkerke adjusted)	0.77

* $p < 0.001$

The use of the restriction enzyme *EcoR1* in this study greatly improved the reproducibility of ddPCR quantification.

The two targets of our study were suitably quantified from genomic DNA of all tested *A. euteiches* isolates. However, with considerably different copy numbers for each target, an adequate dilution of genomic DNA had to be determined for each *A. euteiches* isolate to remain within the recommended ddPCR dynamic range (1 to 120,000 copies *per* 20 μL reaction - Digital Droplet PCR Application Guide, Bio-Rad). ITS1 copy numbers varied from 9 to 239 copies *per* haploid genome among *A. euteiches* isolates from the different fields. This 26-fold factor range was much greater than previously reported (Gangneux et al., 2014). This supports the need to take this parameter into account for disease prediction. Furthermore, ITS1 copy numbers also varied among *A. euteiches* isolates from a given field. This result confirms the absence of a relationship between geographical origin and ITS copy numbers, and supports the relevance of an estimation of mean values of *A. euteiches* populations at the field scale rather than of a single *A. euteiches* isolate. However, because this method remains limited by the scarcity of the single copy target, a large soil sampling size is necessary in each field to obtain an accurate estimation.

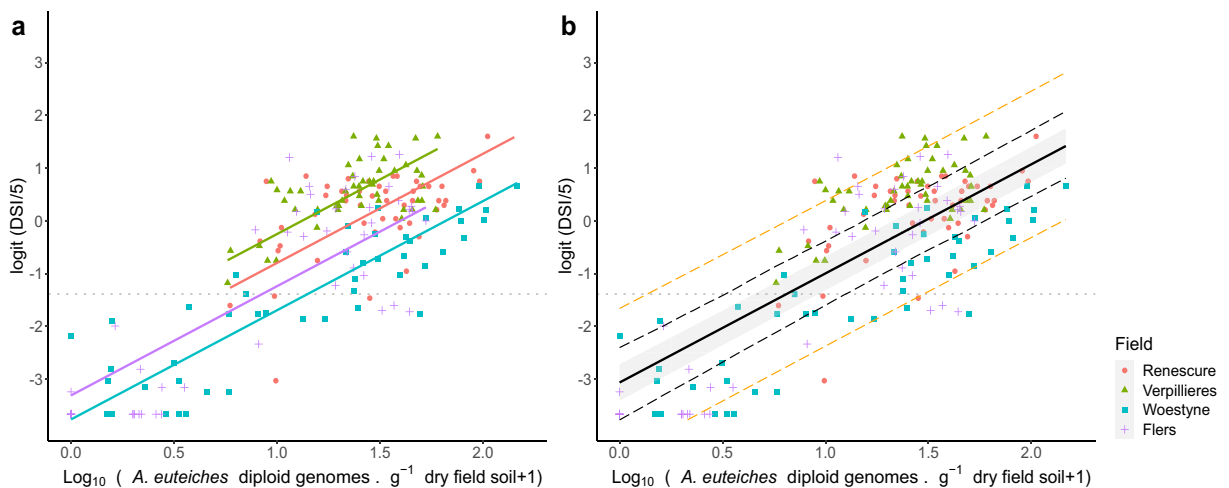


Fig. 5 Linear mixed model representation of the relationship between mean disease severity indexes (DSIs) and *Aphanomyces euteiches* soil-borne inoculum densities (IDs). **a** - Lines are predicted values of mean DSIs conditional on the mean random effect of each field. **b** - Grey shaded ribbons and black dashed lines represent the standard error and the 95% confidence intervals of the predicted mean DSIs based on inoculum densities fixed-effects uncertainty, respectively. Orange dashed lines represent the 95%

confidence intervals of the predicted mean DSI values based on inoculum densities fixed-effects uncertainty and field random-effects variance. In each graph, *A. euteiches* IDs are represented by the Log_{10} of the mean number of *A. euteiches* diploid genomes *per* gram of dry field soil measured in triplicate from each soil sample. Field effects were considered as random. Dotted lines represent the recommended risk threshold for pea cropping in France. Outlier values were excluded from the analyses

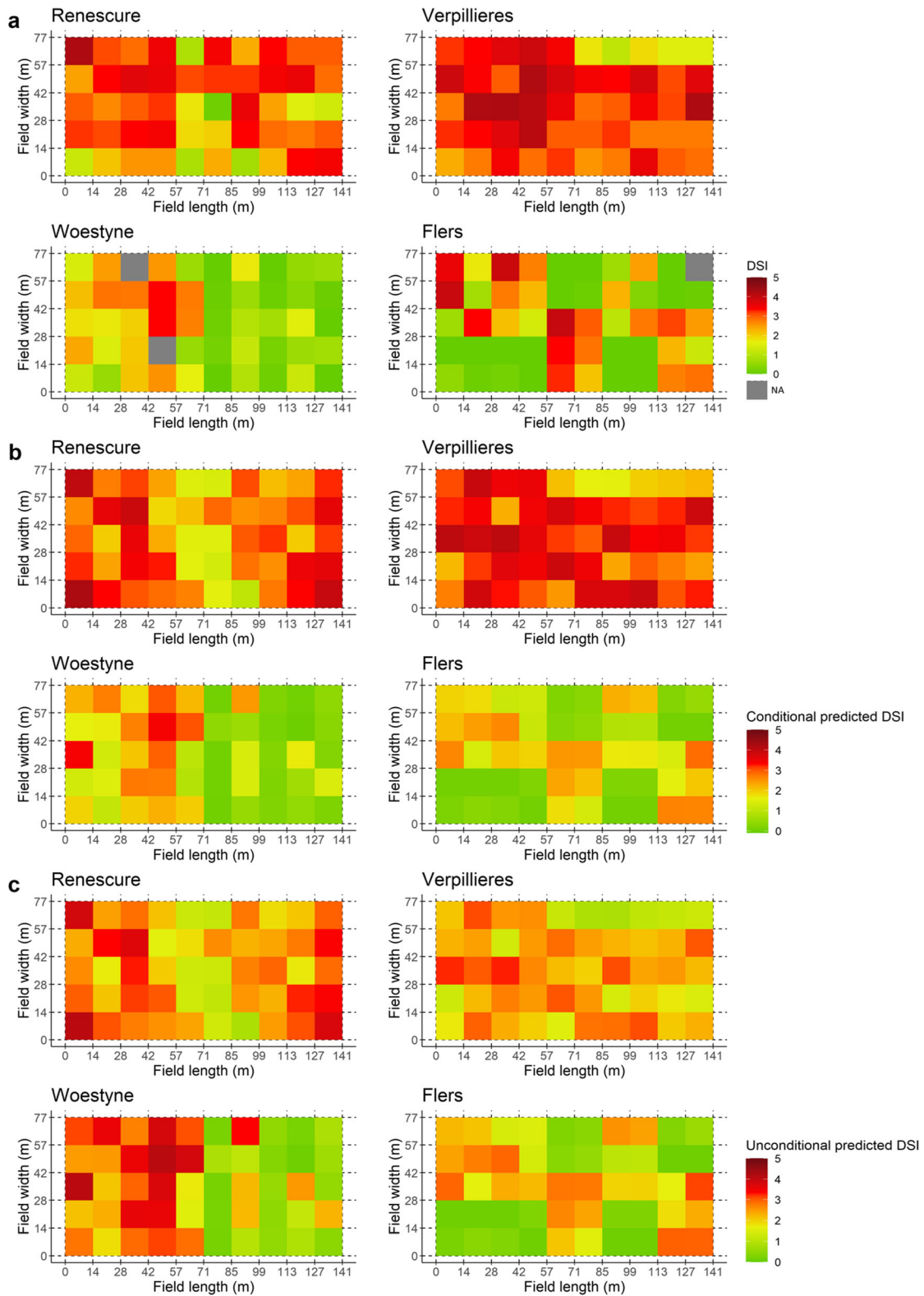


Fig. 6 Mapping of root rot disease severity indexes (DSIs) measured and predicted with the linear mixed model of soils sampled in the 1-ha plots of Renescur, Verpillieres, Woestyne and Flers fields. Each coloured square represents (a) a measured value or (b

and c) predicted values for one soil sample. **a** - Mapping of the mean DSIs measured by bioassays. **b** - Mapping of the predicted DSIs conditional on the mean random effect of each field. **c** - Mapping of the unconditional predicted DSIs

A second biological barrier is the presence of *A. euteiches* at very low inoculum levels in field soils and its capacity to cause severe symptoms on pea crops from IDs less than 10 oospores *per* gram of soil (Heyman, 2008). Therefore, it is necessary to accurately quantify inoculum levels below the qPCR limit of detection obtained by Gangneux et al. (2014). Unfortunately, qPCR is subject to soil inhibitors that impede the improvement of its sensitivity threshold on soil DNA (Albers et al., 2013; Matheson et al., 2010). The presence of PCR-inhibiting substances after soil DNA extraction and purification commonly requires a sufficient DNA sample dilution before proceeding to a qPCR assay, and this results in a limited sensitivity for rare target detection (Albers et al., 2013). The richness of several soils in PCR inhibitors coupled with the low inoculum levels of *A. euteiches* in field soils have been described as a cause of unsuccessful qPCR quantification (Heyman, 2008). ddPCR, which is based on the PCR mix partition in up to 20,000 droplets and on the endpoint reading of the PCR, is less affected than qPCR by soil PCR inhibitors (Racki et al., 2014). It allows for high amounts of soil matrix DNA to be used, hence a major gain in sensitivity compared to qPCR. In our study, up to 200 ng of soil matrix DNA *per* PCR mixture were required for an accurate and reproducible quantification of the multicopy Ae_ITS1 target. As discussed, soils rich in organic matter like Renescure or Woestyne were the most limiting ones for determining the sensitivity threshold of the method. The large amounts of clay, humic acids and phenolic compounds they contain can inhibit the PCR. In addition, their large content in soil DNA, resulting from an important biological activity, leads to a greater dilution of the DNA samples and consequently of the targets. Thus, for the same ID level and for an equivalent number of ITS copies *per* *A. euteiches* genome, these soils required a greater DNA quantity in the ddPCR mixture for target detection than the siltier Verpillieres and Flers soils did. If necessary, ddPCR offers the flexibility to increase the matrix DNA quantity up to 1 µg *per* reaction mixture, which could theoretically enhance the method sensitivity by a factor of 5 (Droplet Digital PCR Applications Guide, Bio-Rad). However, improving the method sensitivity is required for robust quantification of the single copy Ae_76pb target in naturally infested soil samples. More numerous soil DNA extracts and the merging of the ddPCR results before the Poisson calculation is a potential option but requires far more DNA extractions

and induces a higher analysis cost than the multicopy target Ae_ITS1 does. For example, we had to analyse DNA from 50 soil samples from each field to obtain a representative value of ID from the quantification of the single copy marker. Recovery of and enrichment in soil oospores before DNA extraction using density flotation or wet sieving could be alternative strategies to enhance sensitivity but they are time consuming (Debode et al., 2011; Kraft, 1990; Wang et al., 2006). Therefore, the gain in sensitivity provided by the use of multicopy ITS1 sequences is essential for the quantification method.

A third barrier to be overcome to obtain representative and reproducible measurements is the heterogeneous distribution of *A. euteiches* inoculum in naturally infested soils. Its propagules are mainly dispersed in plant debris of heterogeneous sizes from previous host legume crops (Schren, 1960). The soil is altogether a complex matrix, and special attention to soil homogenisation, particle size and quantity is needed to minimise sampling errors (Petersen et al., 2005). We made an important effort by carefully determining the appropriate amount of dry soil and the best milling procedure to optimise these parameters before DNA extraction. These improvements are in accordance with: i) a decrease of the fundamental sampling errors, related to the variability of particle size and compositional distribution within each sample, and ii) a decrease of the grouping and segregation error, which arises from heterogeneity within a given sample (Gerlach & Nocerino, 2003; Petersen et al., 2005). Our study shows that DNA extraction from 2 g of milled dry soil followed by a ddPCR quantification of the targeted sequence in the presence of *EcoR*I provides an efficient and reproducible procedure to quantify *A. euteiches* ID in naturally infested soil. It is also a better compromise in terms of cost and efficiency compared to DNA extractions with higher soil quantities requiring greater lysing matrix and buffer quantities.

Validation of the optimised ddPCR assay with naturally infested soils

Despite the efforts to reduce the sampling and measurement errors by overcoming the biological and technical barriers of the molecular quantification of *A. euteiches* inoculum in naturally infested soil, CV values were high when we validated the optimised ddPCR assay on naturally infested soil samples. An

overall median CV of 44% was observed in the measurements carried out in triplicate on 200 soil samples, i.e. 600 soil homogenisations, DNA extractions/purifications, and ddPCR analyses. Considering the previously discussed technical barriers, CV values reflect the accumulation of errors at each step of the process, from soil sampling to target quantification. Thus, they encompass the representativeness of the sample replicates after homogenisation, the technical errors linked to the analysis steps such as dilution and pipetting, DNA extraction, purification, and quantification steps, and the potential errors of the ddPCR assay. Robotising the process could reduce the technical errors related to the analysis steps. Finally, a large part of these CVs is due to the scarcity of the *Ae_ITS1* target in soil DNA, in the order of a few molecules *per* ng of soil DNA. For example, the CV was particularly high, sometimes greater than 150%, in low infested soil samples from the Flers and Woestyne fields with IDs less than 5 *A. euteiches* diploid genomes *per* gram of dry field soil. Apart from these extreme values, these CVs were quite consistent with those observed in soils artificially inoculated with *A. euteiches* oospores (Gangneux et al., 2014): they ranged from 0.5% to 78.6% with median values of 20, 26, and 46% for inocula of 10, 100, and 1000 oospores *per* gram of soil, respectively. Other assays for plant pathogen quantification in artificially inoculated soil samples showed inter-sample repeatability only slightly lower than that of the ddPCR applied on naturally infested soil samples presented in this study (Almquist et al., 2016; Wallenhammar et al., 2012).

Relationship between the inoculum density and the disease severity index in naturally infested field soils

A logarithmic relationship between potential disease severity and *A. euteiches* ID was previously established in disinfected soil artificially inoculated with oospores (Gangneux et al., 2014; Malvick et al., 1994; Persson et al., 1999; Sauvage et al., 2007). We established a significant relationship between pea root rot disease severity and *A. euteiches* ID in naturally infested soils. Thus, we demonstrated the predictive value of *A. euteiches* inoculum quantification in naturally infested soils via our method.

In addition, the *A. euteiches* DNA we detected in naturally infested soils probably originated from viable and active resting oospores – the primary inoculum and the pathogenic disease units. Extracellular DNA (eDNA) released from *A. euteiches* dead cells, DNA from non-pathogenic mycelial propagules or DNA from non-viable cells were probably present but in limited quantities. The mycelial form of the pathogen is only active on living plants and does not grow as a saprophyte on dead plant material (Kjoller & Rosendahl, 1998). Moreover, the degradation rates of fungal or oomycete eDNA is quite high in moist soils favourable to biotic and DNase activity (Herdina et al., 2004; Kunadiya et al., 2020; Pietramellara et al., 2008). For example, *Phytophthora cinnamoni* eDNA has a half-life of only 2 to 7 days and rarely persists up to 90 days in wet soils, but it could persist for up to 378 days in dry soil conditions (Kunadiya et al., 2020). The soils of Renescure, Verpillieres, Woestyne, and Flers were sampled in autumn, four to five months after the last pea crop, and they were subjected to daily and seasonal fluctuations in humidity and temperatures. Thus, *A. euteiches* eDNA should have been mainly degraded since the last mycelial development within its host. Soil sampling during a dry summer right after the cultivation of a sensitive legume may lead to an overestimation of the viable pathogenic inoculum and should be avoided.

The significant relationship between \log_{10} -transformed *A. euteiches* IDs and logit-transformed DSIs of the linear mixed regression made it possible to predict pea root rot disease severity from very low inoculum densities. This relationship highlighted the very low inoculum levels required to induce severe root rot symptoms. In France, a DSI measured on composite soil samples must be below 1 to grow peas without risking yield losses. We reached this threshold at a mean ID of only 5 *A. euteiches* diploid genomes *per* gram of dry soil. Models established on artificially inoculated soils predicted similar inoculum levels to reach this threshold (Gangneux et al., 2014; Persson et al., 1999; Sauvage et al., 2007). Knowing the biology of the pathogen, this prediction is quite realistic, as one oospore produces about 300 to 400 motile zoospores via the development of the zoosporangium. Zoospores swim to the host roots by chemotactic attraction (Sekizaki et al., 1993), what leads to a high probability of pea root infestation. Besides, an equivalent low inoculum level required for

infection has been reported for *A. cochlioides* on sugarbeet, a phylogenetically close pathogen with similar physiological characteristics (Almquist et al., 2016). Our results confirm the usefulness of a sensitive tool such as ddPCR to accurately quantify *A. euteiches* IDs below 10 oospores *per* gram of dry soil in naturally infested pea fields, make a relevant predictive diagnosis and assess the risk of pea root rot disease.

The confidence interval of the predicted mean DSI was acceptable. However, the mean ID values of the three technical replicates and the mean DSIs values of three technical replicates of the soil samples were used to set up the model. Thus, part of the technical variance was not taken into account in the model to better represent biological variance. The presence of several other pea pathogens such as *Pythium* spp. in symptomatic pea roots could be partly at the origin of the unexplained variance of the model, as the bioassay scoring encompassed similar symptoms from other pea pathogens potentially present in the soil samples. The presence of other soil-borne fungi in symptomatic roots, such as non-pathogenic or pathogenic *Fusarium* spp., is also known to create synergistic effects on symptom severity (Peters & Grau, 2002; Willsey et al., 2018). As different primers and probes targeting *Fusarium* spp. involved in the pea root rot complex are already available (Willsey et al., 2018), a multiplexed ddPCR approach simultaneously targeting different microorganisms associated with the *A. euteiches* pea root rot complex would probably improve the prediction. This involves that future methodological development in ddPCR equipment proposes more channels than the two currently provided.

Significant field random effects increased the unconditional prediction interval. These effects could be due to the addition of several field-specific parameters such as *A. euteiches* aggressiveness, and differences in the soil receptivity to pea root rot disease. However, we found no significant difference in the aggressiveness levels of the different *A. euteiches* isolates collected and tested in the pathogenicity bioassays. More globally, French *A. euteiches* isolates mostly belong to pathotypes I and II and exhibit a low diversity of aggressiveness on pea genotypes (Quillevère-Hamard et al., 2018; Wicker et al., 2001). Differences in field soil receptivity to pea root rot have already been assessed (Persson et al., 1999), and soil suppressiveness to *A. euteiches* disease due to Ca content has also been studied (Heyman et al., 2007; Persson, 1998).

Moreover, the probability for the presence of soil-borne pathogens associated with *A. euteiches* on plant root tissues depends on edaphic parameters such as soil pH, Fe²⁺ and K⁺ (Zitnick-Anderson et al., 2020).

Overall, as a result of the significant relationship between pea root symptom severity and *A. euteiches* inoculum density, the predicted DSIs conditional on the mean field random effects and the unconditional predicted DSIs showed the same spatial patterns as DSIs actually did in the four soils. In the low infested fields of Flers and Woestyne, *A. euteiches* was distributed in foci, as already described for *A. euteiches* and for other soil-borne pathogens (Anees et al., 2010; Moussart et al., 2009). Foci were larger, more or less confluent and more difficult to distinguish from each other in the highly infested fields of Renescure and Verpillières. This scattered-cluster pattern of the inoculum requires adapted sampling strategies to reliably assess the disease risk at the field scale. Otherwise, soil sampling may miss inoculum foci, leading to under- or over-estimations of the inoculum level. Accurate and rapid molecular assays are thus effective tools for the processing of large amounts of soil samples needed to model soil-borne pathogen distribution at the field scale and to assess the risk of infested areas within a field. Nevertheless, molecular assay reagents are expensive, and analysing samples taken on a large scale for disease risk mapping may have a significant economic cost. In this context, a major challenge remains the defining of sampling strategies representative of the scattered soil-borne pathogen inoculum to optimise the sampling size.

We only analysed four fields, ranging from low to highly infested soils. They encompassed a wide variety of pathological situations illustrated by the heterogeneous distribution of DSI foci in the pea production basin of the north of France. The model we propose will have to be confirmed with pathological and pedoclimatic situations existing in other pea-producing regions, in France or elsewhere, for general validation. Yet, it is already usable as such and should be implemented by diagnostic laboratories to be tested by pea producers.

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Code availability R scripts developed in this study are available in a supplementary information file.

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Data availability All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Conflicts of interest/competing interests The authors have no conflicts of interest to declare that are relevant to the content of this article.

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