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Short communication

Quantifying potato pathogen DNA in soil

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ABSTRACT

An improved method for the direct extraction of DNA from soil involving processing of a relatively large sample (60 g) was developed. The accurate and reliable detection and quantification of the soil-borne potato pathogens *Colletotrichum coccodes* (black dot), *Rhizoctonia solani* (black scurf) and *Spongospora subterranea* (powdery scab) following inoculation of soils was demonstrated. With this method, low levels of target DNA (30–40 pg DNA/g soil) could be detected in field soils. DNA recovery was proportionate across a wide range of inoculum ($R^2 > 0.86$) and there was no effect of soil type on the recovery of *C. coccodes*. The method was used to assess levels of naturally occurring pathogen DNA in 122 soil samples obtained from commercial potato fields.

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1. Introduction

Soil-borne potato tuber blemish diseases including black dot (*Colletotrichum coccodes*), *Rhizoctonia solani* and powdery scab (*Spongospora subterranea*) affect the quality of seed and ware crops in the UK and world wide and have become more important in recent years due to the demand for washed potatoes with a high-quality skin finish (Lees and Hilton, 2003). In order to understand the relative importance of different sources of inoculum and the environmental factors influencing infection and disease development with the aim of developing effective control strategies, accurate and reliable methods for detecting and quantifying the pathogens in soil are required. Traditional methods of detecting soil-borne pathogens using plating and/or bait plant tests are time consuming and often not quantitative (Merz, 1989; Vincelli and Beaupré, 1989; Carnegie et al., 2003). The development of real-time PCR assays has made the rapid and accurate quantification of potato pathogen DNA possible (Cullen et al., 2001, 2002, 2005, 2007; Lees et al., 2002; van de Graaf et al., 2003; Cullen and Lees, 2007). However, the accurate and consistent quantification of target DNA in soil has previously proved problematic due to a number of factors including the binding properties of the soil structure, inhibitory compounds in different soil types and the small sample sizes usually processed. These issues are discussed in more detail by Ophel-Keller et al. (2008). The present investigation aimed to provide a reliable method for the extraction and quantification of DNA from soil samples that took these points into account and to investigate naturally occurring levels of the pathogens in commercial potato fields.

2. Materials and methods

2.1. Soil samples

Inoculum suspensions of three UK isolates each of *C. coccodes*, *R. solani* Anastomosis Group 3 and the unculturable plasmodiophorid pathogen *S. subterranea* were made according to the

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method previously described by Cullen et al. (2002). Inoculum consisted of a combination of mycelium and microsclerotia (*C. coccodes*), mycelium only (*R. solani*) and sporeballs (*S. subterranea*). Inoculum concentration (cfu/ml) was calculated by dilution plating for *C. coccodes* and *R. solani* and by direct microscopic counting using an haemocytometer for *S. subterranea* and inoculum was then used to infest field soils obtained from sites on which potatoes had never previously been grown.

Inoculum was added to loamy sand soil (400 g), resulting in final concentrations of 5.3, 10.6, 53, 106, 530, 2650 and 5300 colony forming units (cfu)/g soil of *C. coccodes*, 1.9, 3.8, 19, 38, 192, 958 and 1917 cfu/g soil of *R. solani* and 1000, 500, 100, 50, 10, 5 and 1 spore ball/g soil for *S. subterranea*. Sterile distilled water was added, in proportion, to those soils receiving less inoculum and control treatments had an equivalent volume of water only added. In addition, three soil types (loamy sand, sandy loam and silty clay) were inoculated with *C. coccodes* at a concentration of 3400 cfu/g soil.

Soil samples were collected from 122 commercial potato fields throughout the UK using a method adapted from the current UK potato cyst nematode (PCN) sampling strategy: a 1 kg sample of soil was obtained from an area of up to 4 ha by taking 100, 10 g sub-samples using a mini-auger at regular intervals along a W-shaped configuration across the field.

2.2. DNA extraction from soil and real-time PCR assay

The soil DNA extraction method of Cullen et al. (2001) was adapted by substituting the initial processing procedures, sonification and bead beating, with the milling protocol described below. Air-dried soil samples (1 kg) were mixed thoroughly. Four replicate sub-samples (60 g each) were taken from each inoculated soil for processing. For field soils, four replicate sub-samples (60 g) were taken from four of the soils and for all others (118) a single 60 g sub-sample was taken. Soil sub-samples were placed in a Retsch milling bowl (Planetary Ball Mill PM 400) with 120 ml extraction buffer (SPCB: 120 mM sodium phosphate, 2% CTAB (hexadecyltrimethyl-ammonium bromide), 1.5 M NaCl; pH 8.0) and 12 stainless steel ball bearings (20 mm diameter) and milled at 300 rpm for 5 min until a fine soil suspension was created. Triplicate 1.5 ml aliquots of the soil suspension (equivalent to 0.75 g soil) were transferred to 2 ml tubes and kept on ice until further processing. Milling bowls were cleaned with 96% ethanol and 0.2 M NaOH between samples. The soil suspension was centrifuged (1820 g for 5 min) and the supernatant removed. Subsequent DNA extractions and purification through a Micro-Bio-Spin column followed the method of Cullen et al. (2001). The detection and quantification of *R. solani*, *C. coccodes* and *S. subterranea* using real-time PCR (TaqMan) assays were carried out according to the methods of Cullen et al. (2002), Lees et al. (2002) and van de Graaf et al. (2003), respectively. All samples were tested in duplicate. Results were expressed as pg DNA/g soil (*C. coccodes* and *R. solani*) or spore balls/g soil (*S. subterranea*).

2.3. PCR inhibition

Twelve field soils (seven sandy loam, three sandy clay, two loamy sand mineral, hereafter referred to as sand, clay and mineral, respectively) were used to investigate PCR inhibition according to soil type. Pure DNA of *C. coccodes* was diluted to 10 pg DNA/μl and used to spike total DNA (previously extracted from each of the soils). Target DNA (*C. coccodes*) was quantified in spiked and un-spiked soil samples, the difference in values being attributed to the contribution of the *C. coccodes* DNA spike. Pure *C. coccodes* DNA samples were also assayed to determine the quantity of detectable DNA within the aliquot used to spike the soil DNA.

3. Results

3.1. Quantification of pathogen DNA in soil

When inoculum (ranging from 5.3 to 5300 cfu/g soil of *C. coccodes*) was added to soil, a consistent and proportionate amount of DNA was extracted and quantified across the range of inoculum levels ($R^2 = 0.86$) (Fig. 1A). At the lowest concentra-

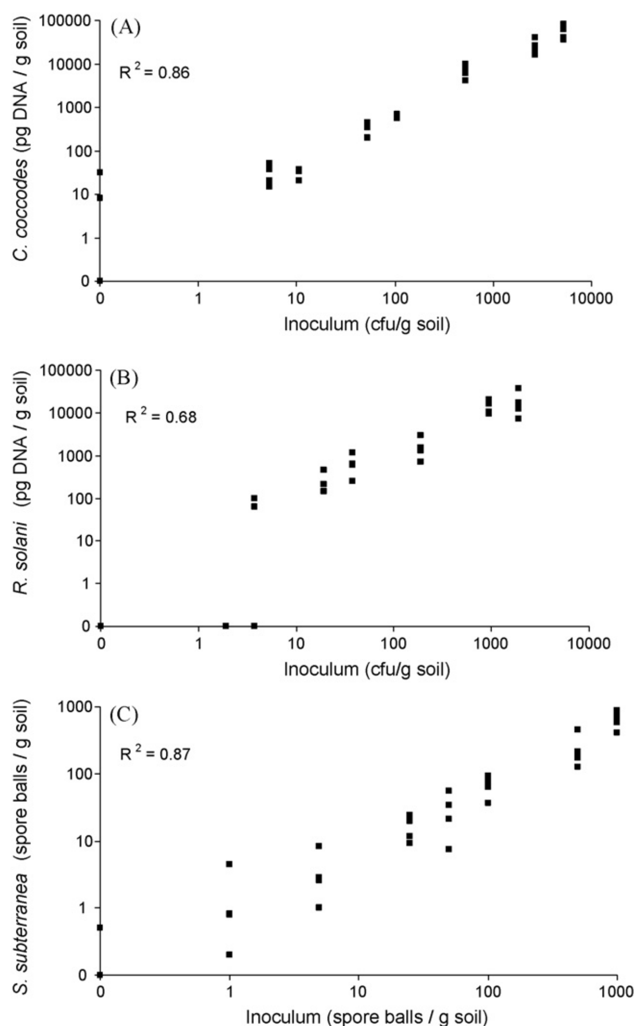

Fig. 1 – Quantification of DNA by real-time PCR following the addition of (A) *Colletotrichum coccodes* (cfu/g soil), (B) *Rhizoctonia solani* (cfu/g soil) and (C) *Spongospora subterranea* (spore balls/g soil) to soil. Data points represent four replicates for each inoculum level.

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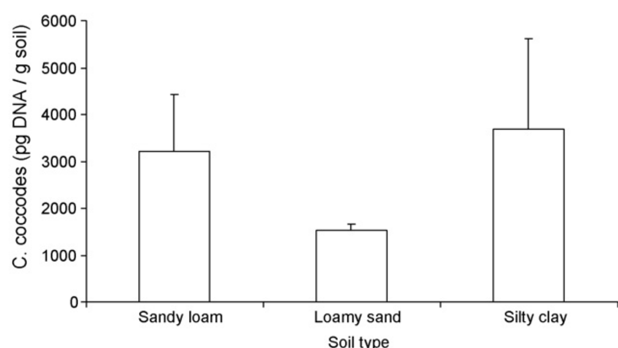

Fig. 2 – Quantification of *C. coccodes* DNA (pg/g soil), following the addition of 3400 cfu/g soil of inoculum to three different soil types. Values are the mean of three replicates (±standard error).

Table 1 – Number and percentage field soils tested (n = 122) which were found to have detectable levels of pathogen contamination. Maximum level of detection is given.

Pathogen	Number of soils contaminated (%)	Max. contamination level
Colletotrichum coccodes	92 (75)	7,100 pg DNA/g soil
Rhizoctonia solani	17 (14)	17,000 pg DNA/g soil
Spongospora subterranea	92 (75)	148 spore balls/g soil

tion (5.3 cfu/g soil) *C. coccodes* DNA was detected in all four replicates (mean 30.4 ± 17.0 pg DNA/g soil). There was no significant effect of soil type (loamy sand, sandy loam and silty clay) on the detection of *C. coccodes* DNA (pg/g soil) following the addition of 3400 cfu/g soil of inoculum (Fig. 2).

The extraction and quantification of *R. solani* following the addition of inoculum to soil (ranging from 1.9 to 1913 cfu/g soil) was slightly less consistent than for *C. coccodes*, but the quantification of target DNA across the range of concentrations was proportionate to the level of inoculum added (R^2 = 0.68) (Fig. 1B). At 3.8 cfu/g soil *R. solani* DNA was detected in two of the four replicates (mean 40.2 ± 48.7 pg DNA/g soil).

Following the addition of *S. subterranea* inoculum to soil (1–1000 spore balls/g soil), target DNA was consistently and proportionately extracted and quantified across the range (R^2 = 0.87) (Fig. 1C). At the lowest inoculum concentration (1 spore ball/g soil), *S. subterranea* DNA was detected in all four replicates (mean 1.6 ± 1.9 spore balls/g soil).

Of the 122 field soils sampled, 75% were found to be contaminated with *C. coccodes* (maximum level 7100pg DNA/g soil), 14% with *R. solani* (maximum level 17,000 pg DNA/g soil) and 75% with *S. subterranea* (maximum level 148 sporeballs/g soil) (Table 1). These soils, which were predominantly sandy loam, but included clay, organic, mineral, loam and sand soil types were specifically selected and may not be representative of UK fields in commercial potato production. At low levels of natural pathogen infestation, for example when *C. coccodes* was detected at levels <10 pg DNA/g soil, real-time PCR detection of target DNA was possible in two of the four sub-samples. Similarly, *R. solani* was detected in two or fewer sub-samples when the mean DNA levels were <150 pg DNA/g soil and DNA equivalent to fewer than two spore balls/g soil *S. subterranea* was not consistently detected in all sub-samples.

3.2. *Effect of soil type on PCR inhibition*

The concentration of *C. coccodes* DNA in the aliquot used to spike the soil samples was 9.4 pg DNA/assay (±1.02 standard error). The *C. coccodes* DNA attributable to the addition of this aliquot of *C. coccodes* used to spike each of the 12 soil samples tested was not significantly different from the amount detected in the control sample (ANOVA; p = ns) (Fig. 3).

4. Discussion

A prerequisite for accurately determining pathogen populations in field soils is an appropriate soil sampling methodology: one that addresses the need to collect a sample that is both representative of the whole field and also realistic with regards to time and cost. Limited information on pathogen

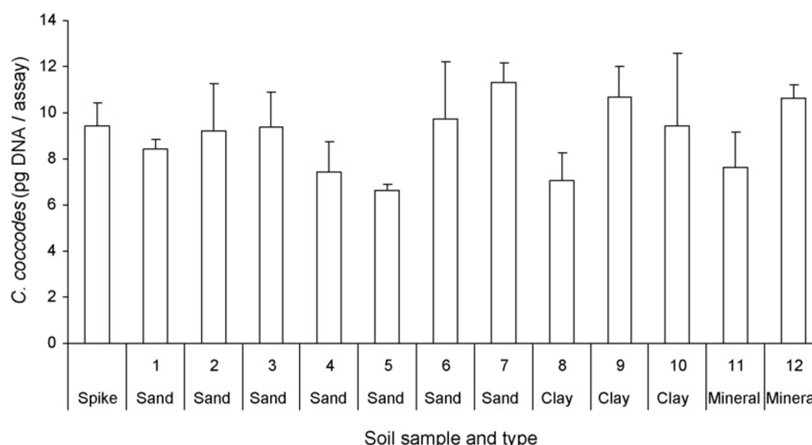

Fig. 3 – Quantification of a known concentration of *C. coccodes* added as a DNA spike to soil DNA samples prior to real-time PCR reaction, mean of three replicates (±standard error).

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distribution patterns and/or appropriate sampling procedures for potato pests and pathogens other than potato cyst nematode (PCN) in soils exists (e.g. Basu et al., 1977; Gilligan et al., 1996) although relevant information resulting from studies of soil-borne cereal pathogens is available (Heap and McKay, 2004). The sampling strategy used in the current study was based on the current statutory PCN sampling procedure in Scotland in which a 1 kg sample of soil is collected from 100 points. This sampling method is in broad agreement with the protocol of Ophel-Keller et al. (2008) recommended for the sampling of cereal pathogens in soil and proved to be successful for the detection of *C. coccodes* and *S. subterranea* at low levels in naturally infested fields. Detection of *R. solani* was variable despite the high sensitivity of the assay and this might be attributable to the patchy distribution of pathogen and/or its presence at levels close to the limits of detection. Further work may be required to ensure low levels of *R. solani* present in field soils are being detected adequately.

Previously reported direct soil DNA extraction methods for plant pathogens have used very small amounts of soil, up to a maximum of 10 g (Bell et al., 1999; Cullen et al., 2001; Kernaghan et al., 2007) and many of the commercially available kits use <1 g of soil. The direct soil DNA extraction method described in the current study begins with a 1 kg sample, from which a 60 g sub-sample is processed—at least a sixfold increase in sample size from previously reported methods. Thorough mixing of the soil prior to the sub-sample being processed, to ensure homogeneity, in combination with the increased sample volume used in this study resulted in the good correlation between the quantification of DNA from inoculated soils and the amount of inoculum initially added. When only very small quantities of target pathogen are present (i.e. close to the assays limit of detection) the pathogen may not be detected in a single sub-sample. If these very low levels are considered to be important in terms of disease risk then further consideration of the number of sub-samples taken may be required; only through the validation of soil diagnostics for each disease can these judgments be made.

Inconsistent detection of pathogens from soil extracts have been widely attributed to inhibitors of the PCR by co-extracted soil chemicals (Cullen and Hirsch, 1998; Yeates et al., 1998; Menking et al., 1999; Miller et al., 1999; Schneegurt et al., 2003). However, no significant inhibition of the *C. coccodes* PCR reaction was observed in the 12 spiked soils investigated in this study which included sand, clay and mineral soils, all of which were used in commercial potato production. This indicates that the PVPP purification step is removing inhibitors from the total DNA extract adequately. Martin-Laurent et al. (2001), attributed differences in the amount of DNA extracted from three different soils to differences in the soil properties. However, we found no significant difference in the amount of DNA extracted from three different soil types. It may be that the milling procedure which reduces the soil slurry into a very fine emulsion, breaks down soil particle structure thus reducing the DNA binding capacity of soil particles.

Due to the problems in extracting and quantifying target DNA in soil outlined above, very little data exists on the estimation of potato pathogen populations in natural systems. Merz (1993) surveyed 78 soils from potato producing areas of Switzerland and reported that soils highly contaminated with *S. subterranea* had inoculum densities greater than 500 spore - balls/g of soil as determined using a baiting technique described by Merz (1989). A study in America by Qu et al. (2006) tested 17 field soils and found them to be contaminated with *S. subterranea* levels between 0 and 14,400 sporeballs/g of soil, using a competitive PCR assay. Nakayama et al. (2007) using a competitive PCR technique tested 29 potato fields and found the highest spore ball density to be 105 spore balls/g soil. In this work the maximum level of *S. subterranea* infestation we found in 122 soils sampled was 148 spore - balls/g of soil. van de Graaf et al. (2005) infested soils with *S. subterranea* spore balls and found inoculum levels as low as 5 spore balls/g soil resulted in 52% of plants with powdery scab, indicating that only very low levels of infestation are needed to cause disease.

The development of this technique to extract and quantify potato pathogen DNA in soil has allowed research into the factors affecting black dot development on progeny tubers to be carried out for the first time (Brierley et al., unpublished data). Few studies that attempt to link pathogen levels in soil to disease risk have previously been described. Kernaghan et al. (2007) were able to relate real-time PCR calculated DNA concentrations of *Cylindrocarpon destructans* in soil samples (5 g) to disease index on American ginseng and Ophel-Keller et al. (2008) recently described a routine DNA-based testing service for soil-borne cereal diseases and identified disease risk categories, but did not describe the method used for soil DNA extraction. With the advances set out in this paper the links between naturally occurring inoculum levels and resulting disease in potatoes can be investigated, and the validation of real-time PCR diagnostics for predicting disease risk is currently being investigated. This method is also likely to be appropriate for the extraction and quantification of other soil-borne organisms.

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