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Differences in root biomass among wheat varieties shown by a qPCR assay wheat root DNA in soil samples

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Research Article

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Abstract

Aims. Root research on field-grown crops is hindered by the difficulty of estimating root biomass in soil. Root washing, the current standard method is laborious and expensive. Biochemical methods to quantify root biomass in soil, targeting species-specific DNA, have potential as a more efficient assay. We combined an efficient DNA extraction method, designed specifically to extract DNA from soil, with well-established quantitative PCR methods to estimate the root biomass of 22 wheat varieties grown in field trials over two seasons. We also developed an assay for estimating root biomass for black-grass, a common weed of wheat cultivation.
Methods. Two robust qPCR assays were developed to estimate the quantity of plant root DNA in soil samples, one specific to wheat and barley, and a second specific to black-grass.
Results. The DNA qPCR method was comparable, with high correlations, with the results of root washing from soil cores taken from winter wheat field trials. The DNA qPCR assay showed both variety and depth as significant factors in the distribution of root biomass in replicated field trials.
Conclusions. The results suggest that these DNA qPCR assays are a useful, high-throughput tool for investigating the genetic basis of wheat root biomass distribution in field-grown crops, and the impact of black-grass root systems on crop production.

Introduction

In the UK wheat is the single largest cereal crop, nationally accounting for 65% of total cereal production. While, historically, wheat breeding has focussed on the impact of above ground plant characteristics on yield, there is an increasing need to understand how root growth and root interactions with the soil environment; biological, chemical and physical, work together to influence yield (den Herder *et al.*, 2010). By exploring root biomass diversity within a set of historic varieties and breeders' lines, we seek to demonstrate the value of a phenotyping tool to plant breeders and researchers seeking to exploit the diversity available in germplasm collections. In addition to varietal differences, many agronomic practices are known to influence root establishment and biomass development, e.g. position within a crop rotation, nitrogen application and timing, cultivation method, seed rate, sowing date and plant growth regulator applications (Hoad *et al.*, 2001; Bayles *et al.*, 2002).

Root phenotyping of field crops is a developing science (George *et al.*, 2014). The current standard method of quantifying root biomass is to wash roots free from the soil and quantify as root length per unit volume of soil. Image analysis methods aid data capture (Bauhus and Messier, 1999; Zhu *et al.*, 2011), but the washing process is laborious and time consuming. The results obtained by these methods are informative with regards the proportions of fine to coarse roots, but results may not be transferable between different soil types (Kücke *et al.*, 1995). Field root phenotyping of wheat, using a 'core break – root count' method, showed considerable variation for deep root traits (Wasson *et al.*, 2014). 'Shovelomics' have been used to describe the root architecture of diverse wheat varieties, including modern and historic UK varieties and non-UK landraces (Fradgley *et al.*, 2020). Non-invasive geophysical methods, such as ground penetrating radar and electrical resistivity tomography, have been successful in measuring large tree roots (Butnor *et al.*, 2001; Paglis, 2013). However, these procedures are currently less informative for plants with fine root structures, where the root dimensions are similar to those of soil aggregates and pores (Amato *et al.*, 2009), although root electrical capacitance has been shown to correlate with root mass for barley in glasshouse experiments (Dietrich *et al.*, 2013).

The use of rhizotron-based systems for root characterisation is well established (James *et al.*, 1985), and being amenable to automation allow for repeated measurements during plant development (Lobet and Draye, 2013). However, rhizotrons, being artificial environments,

are somewhat removed from the field environment. Root biomass correlations between rhizotron and field were found to be high during the vegetative growth phases, but low during the reproductive growth phases (Watt *et al.*, 2013). Allied to rhizotrons are X-ray computed tomography (CT) systems capable of visualising detailed root structures in soil. Industrial micro-CT systems with resolutions of 500 nm or less (Mooney *et al.*, 2012), coupled with automated systems for sample presentation and data processing (Mairhofer *et al.*, 2012), are also a valuable tool for root phenotyping in rhizotrons.

Quantitative, species-specific DNA detection methods, coupled with robust soil extraction techniques, have been deployed to identify and quantify roots in soil. Real-time PCR has been used to differentiate between grassland species in mixtures of roots washed from soil (Mommer *et al.*, 2008), to quantify root ratios (Zhang *et al.*, 2014) and to measure roots from a mixed population of meadow grasses (Riley *et al.*, 2010; Haling *et al.*, 2011; Haling *et al.*, 2012). Detecting roots by DNA-based methods is however not straightforward (Mommer *et al.*, 2011): soil contains humic acids that are known to inhibit PCR by binding $MgCl_2$, so appropriate modification of DNA extraction methods is required. The concentration of plant DNA in soil has been shown to decline rapidly after plant death (Riley *et al.*, 2010; Bithell *et al.*, 2015), therefore the plant DNA in soil samples is largely derived from live roots. As roots comprise a small part of the total soil volume the most suitable PCR targets are those present at high copy number in the plant genome, e.g. ribosomal DNA internal transcribed spacer (rDNA ITS) regions. DNA-based assays targeting rDNA ITS were successfully used to assess root development under drought conditions in Australian wheat varieties (Huang *et al.*, 2013) and to assess responses to phosphorus by surface roots in wheat and barley (McDonald *et al.*, 2017). While the root biology community is aware of DNA-based methods, recent reviews suggest they have not gained wide acceptance (Tracy *et al.*, 2019; Gregory *et al.*, 2022).

Black-grass (*Alopecurus myosuroides. Huds*) is an annual weed which presents a major problem to European cereal growers. Black grass is distributed all over the British Isles; but is most abundant in cultivated land in South-East England and has gradually developed resistance to many selective herbicides. Relatively low populations of 8–12 plants m^{-2} have been shown to have a significant impact on wheat grain yields (Naylor, 2008). An efficient method by which to measure root development of the crop and the weed is required to understand competition for water and nutrients in the field. While partitioning of total root biomass between weed and crop species in washed roots can be carried out using a variety of techniques (Mommer *et al.*, 2011), including infra-red spectroscopy (Meinen and Rauber, 2015) and biochemical analysis of plant waxes (Dawson *et al.*, 2000), species can only be reliably distinguished by sequencing the rDNA ITS region (Linder *et al.*, 2000). Species-specific quantitative PCR has been used to quantify root biomass of a single species in perennial grass swards (Haling *et al.*, 2012) and to determine the ratio of different species within mixed sward samples (Haling *et al.*, 2011).

In this study, we have developed semi-quantitative DNA-based assays able to estimate root biomass of field-grown wheat varieties and black-grass using root DNA extracted from soil core samples. We compared this qPCR assay to the results obtained with standard root washing procedures for estimating root biomass from soil cores. The qPCR assay was then used to compare differences in root biomass between wheat varieties, at different depths in field trials grown over two seasons. We discuss the power and limitations of this method, and outline the potential of this technology as a tool for plant breeders and root biologists seeking to exploit diverse germplasm including historic landraces and wild relatives.

Materials and methods

Wheat trial root-soil core sampling

Soil samples were collected from field trials over three growing seasons, soil cores being taken from within each plot (online Supplementary Table S1). In 2012, three wheat varieties were grown at Terrington St Clement, Norfolk with one plot per variety. In 2014 and 2015, trials were grown at Walpole St Andrew, Norfolk and Terrington St Clement, Norfolk respectively, with three replicate plots per genotype. Eighteen wheat varieties, two Reduced Height (*Rht*) near isogenic lines (NILs) of cv Mercia (Genetic Resources Unit, John Innes Centre, Norwich) and two BC1 (Xi19 × SHW218 where SHW218 is a synthetic hexaploid wheat *Ceta* × *Ae squarrosa*) lines were grown (online Supplementary Table S1). The wheat lines chosen for these trials were selected as they represented diverse root phenotypes based on information from rhizotube experiments (Karley *pers. comm*; Karley *et al.*, 2012) and were broadly representative of the diversity of UK wheat in the era 1946–2009. The wheat lines were planted in a randomised complete block field trial design (online Supplementary Materials Part S2). In 2012, soil cores were also taken from adjacent, uncultivated areas of the site. Soil data for each site were taken from the LANDIS Land information system (Landis, 2014; online Supplementary Materials Part S3).

Ten soil cores, measuring 1 m depth × 30 mm diameter, were sampled from each 10 × 2 m plot in accordance with standardised methods (White *et al.*, 2015). The soil cores were sampled when the wheat crop had reached growth stage (GS) 51–65 (Zadoks *et al.*, 1974). Five cores were sampled within the rows and five were taken between the rows, in accordance with the spatial sampling as proposed by Bengough *et al.* (2000). The cores were divided into four portions, representing 250 mm depth intervals in the soil profile. The four sections from the 10 plot cores were bulked into a single sample representing a depth interval, giving one sample at each of four depths per plot.

Soil cores were taken in the 2012 pilot trial and a subset of the 2014 trial for both root washing estimates of root length density (RLD) and for root biomass DNA (RBD) estimations using the PCR assay developed in this study. Soil cores were taken in the 2015 trial for RBD analysis. In the 2012 trial, cores were taken for RBD and RLD analysis from the one plot of each of three varieties; Alchemy, Oakley and Viscount, while in the 2014 trial cores were taken from three replicate plots of two varieties; Glasgow and Oakley. To assess black-grass root biomass additional cores were taken in the 2015 trial from three areas in the 'discard' planted surrounding the trial (variety Crusoe). These areas were judged by visual inspection as having high (300 black-grass heads m^{-2}), moderate (50 black-grass heads m^{-2}) and low (no discernible black-grass foliage) density black-grass populations. The black-grass population was estimated by counting the number of individuals within four quarter m^2 quadrats.

Wheat lines assessed for root biomass

The wheat lines grown in the 2014 and 2015 trials were selected based on genotypic diversity and phenotypic information from

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rhizotube experiments undertaken on a collection of 100 wheat varieties and breeder lines (Greenland *et al*., 2017). In addition, the two breeder lines SHW Xi19/(Xi19//SHW-218) > 18 and SHW Xi19/(Xi19//SHW-218) > 19 were included. These backcross-derived lines from the cross (Xi19/(Xi19//SHW-218)) were each descended from different BC_1 plants (plants XS-218 > 18 and XS218 > 19, respectively). SHW-218 is a synthetic hexaploid wheat supplied by CIMMYT, with the published pedigree Ceta/*Ae squarrosa* (895) (Gosman *et al*., 2014). Two near-isogenic lines (NIL) that harboured variation at the *Rht* (reduced height) locus in the background of variety Mercia were supplied by the Genetic Resources Unit, Norwich, UK. Additional data (including seasonality, *Rht*, presence or absence of the rye translocation 1B/1R and the predicted photoperiod response) on these varieties are provided by Alison Bentley (*pers. Comm*; online Supplementary Table S1).

Extraction of roots from soil samples by root washing

RLD were carried out at ADAS, Gleadthorpe on the cores sampled in the 2012 field trial, and at Rothamsted Research (RRes) on cores sampled in the 2014 field trial. RLD was not measured on the 2015 soil cores. The roots were extracted from the soil cores using a standard root washing system (Delta-T Devices Ltd, Burwell, Cambridge) and collected on a 550 µm wire mesh filter (ADAS) or 500 µm sieve (RRes). Root length was assessed using WinRHIZO software (Regent Instruments Inc. Sainte Foy, Qc, Canada) (White *et al*., 2015). Root biomass determined by soil washing was expressed as root length density (RLD), expressed as the length of roots recovered per volume of soil (cm/cm^3).

Extraction of DNA from soil samples

Soil samples were frozen within 3 h of collection and stored at −18°C. Samples were dried at 30°C in a re-circulating oven for a minimum of 72 h. The dried soil was milled using a Humboldt H4199.5F soil mill fitted with a 2 mm screen. The milled soil was sub-sampled by quartering to yield a laboratory sample. DNA was extracted from two 0.25 g portions of soil using a PowerSoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, USA) in accordance with the manufacturer's protocols; thus technical, DNA duplicates were obtained for each milled soil sample. The PowerSoil DNA extraction kit has been reliably reported to achieve DNA yields from soil equivalent to methods used in a commercial testing laboratory (Haling *et al*., 2011). While weighing the 0.25 g portions of soil we noted the presence of a small number of visible, but not necessarily evenly distributed, root fibres of up to 5 mm within the milled soil.

Preparation of root DNA calibration materials

We calibrated our RBD assay using DNA taken from lypholised roots of wheat variety Xi19 grown in horticultural sand and harvested at growth stage 20–23 (Zadoks *et al*., 1974). Root material was washed free of sand, rapidly frozen on 'dry ice', freeze dried, milled to a powder in a domestic coffee mill and stored at −18°C. DNA was extracted from 100 mg of dried root using the modified Tanksely method (Fulton *et al*., 1995) and re-suspended in 100 µl Tris – EDTA, pH8.0 at 1 mg/µl. DNA standards were prepared from this reference DNA as a series of 10-fold dilutions, allowing calibration in a five decade range of 1000–0.1 µg/µl. Black-grass calibration standards were prepared in the same way.

PCR quantification of root DNA in soil samples

Primers and fluorescent reporter probes were designed that targeted the wheat internal transcribed spacer region within the 5.8S ribosomal RNA gene (online Supplementary Table S2). The target sequence was acquired from NCBI Genbank AF438186.1 *Triticum aestivum* (Sharma *et al*., 2002), and the primers and fluorescent reporter probes were designed using Primer3 (Untergrasser *et al*., 2012). The primers were tested for specificity by PCR using DNA extracted from wheat, barley, faba bean, maize, oilseed rape and black-grass. The PCR products were visualised on a 1% agarose gel containing ethidium bromide (0.1 µg ethidium bromide/ml of gel solution). A black-grass target sequence was acquired from NCBI Genbank KM523760.1 (Soreng *et al*., 2015), and primers and fluorescent reporter probes designed using Primer3 (online Supplementary Table S2). The black-grass primers and fluorescent reporter probes were tested for specificity using DNA extracted from black-grass, wheat and barley.

Wheat root DNA from soil extracts was quantified by real-time PCR using an ABI 7900, running triplicate 6 µl reactions comprising 1.0 µl template DNA, 0.5 µl primers-probe solution, with primers and fluorescent reporter probes at 5 mM, 2.5 µl Thermo Fisher Scientific ABsolute Blue qPCR ROX Mix and 2.0 µl water (Thermo Fisher Scientific, 2014). Amplification was carried out using 10 min activation at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C, monitoring fluorescence at each cycle. The soil DNA extracts were quantified in a series of 15 PCR batches (384 well). The quantity of wheat root in each extract was calculated using SDS software (version 2.2, Applied Biosystems) with reference to serial dilutions of the reference DNA standard included with every batch. Soil DNA extracts were allocated to plates in plot number order, such that all technical replications of all soil depth samples from a plot were allocated before including extracts from the next plot. The quantity of root DNA (Root Biomass DNA – RBD) in each sample was expressed as wheat root dry weight (µg) per weight of air dried soil (g), rather than describing roots by reference to a quantity of DNA per unit mass of soil.

Data analysis

All qPCR data were processed using Applied Biosystems SDS 2.2, and the results collated and analysed in Microsoft Excel. Analysis of variance (ANOVA) was carried out using Genstat 12.1.0.3338, and correlations and regressions using R-stat (version 3.0.1). All statistical analyses were carried out on original data, without prior averaging of technical duplicates. As part of the data quality control process, we inspected the technical DNA duplicates for gross errors likely to have arisen from sampling large root fibres in one of the two technical duplicates. Three measurements (out of 1056) were removed that had RBD values greater than 500 µg/g, being at least 10-fold higher than their paired DNA technical replicate sample.

Where comparisons were made between estimates of RLD and RBD, correlations were calculated in R-stat. Data from the 2014 and 2015 wheat trials were subject to analysis by REML linear mixed model implemented in Genstat using the model (equation 1):

$$\mathrm{RBD}_{ijkl} = \mu + v_i + d_j + y_k + vd_{ij} + vy_{ik} + dy_{jk} + vdy_{ijk} + r_{jk} + t_{jkl} + p_m + e_{ijklm} \quad (1)$$

where, B_{ijkl} is the RBD of the ith wheat line in the jth year in the kth field replication in the lth technical replication; wheat line, depth

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and year were treated as fixed effects while field and technical replication and plate allocation were treated as random effects.

When the model was amended to include additional data (a) (e.g. seasonality, *Rht*, etc.) the wheat line term was nested within additional data (equation 2).

$$\begin{aligned} \mathrm{RBD}_{ijkl} = \mu + a_h + a_h v_i + d_j + y_k + ad_{hj} + ay_{hk} + dy_{jk} \\ + ady_{hjk} + avy_{hik} + avd_{hij} + avdy_{hijk} + r_{jk} + t_{jkl} \\ + p_m + e_{hijklm} \end{aligned} \quad (2)$$

where, B_{ijkl} is the RBD of the *i*th wheat line in the *j*th year in the *k*th field replication in the *l*th technical replication; additional data, wheat line, depth and year were treated as fixed effects while field and technical replication and PCR batch were treated as random effects.

The RBD data were regressed against the root depth for each wheat line profile and modelled for the best fit using the 'poly' function in R, applying linear, quadratic or cubic models, and selecting the model yielding the lowest residual as the best fit. The coefficients calculated from the results of these regressions were used to generate equations to predict RBD at depth. Integration of these equations allowed calculation of the proportion of RBD within a defined range of soil depths, which in turn allowed prediction of the soil depth containing 50 and 95% of all roots (D_{50} and D_{95}) (Schenk and Jackson, 2005) using 'solver' in Microsoft Excel.

Estimates of variance were obtained by fitting a linear mixed model in R using the lme4 package (Bates *et al.*, 2015) and the model given in equation 3:

$$\mathrm{RBD}_{ijkl} = \mu + v_i + y_j + vy_{ij} + r_{jk} + t_{jkl} + e_{ijklm} \quad (3)$$

where, RBD_{ijkl} is the RBD of the *i*th wheat line in the *j*th year in the *k*th field replication in the *l*th technical replication.

All effects, apart from the mean (μ) were treated as random effects. Variance components associated with the random effects (variety, *v*; year, *y*; field replicate, *r*; technical replicate, *t* and the error term, *e*) were estimated using REML as implemented in the lmer function. Broad sense heritabilities were calculated using the method of Piepho and Möhring (2007) as shown in equation 4:

$$H^2 = \frac{v_v}{\left(v_v + \frac{v_{vy}}{2} + \frac{v_{vyr}}{6} + \frac{v_{vyrt}(\text{base error})}{12}\right)} \quad (4)$$

Results

Development of wheat and black-grass specific qPCR assays for soil extracted DNA

Given the impact of black-grass on wheat production, and the levels of black-grass contamination that can be found on farm, it was considered of value to develop qPCR assays that could distinguish between wheat and black-grass roots. This enabled us to ensure that the root biomass we were assessing in this study of wheat phenotype variability was wheat root DNA, and not contamination from black-grass.

The soils for which DNA extraction methods were developed had textures described as sandy loam, sandy silt loam, silt loam, silty-clay loam, clay loam and fine loam over clay. We found the PowerSoil DNA extraction kit yielded DNA of sufficient quantity and quality to carry out qPCR, however, the DNA yield was not sufficient to assess DNA concentration or quality on an agarose gel. Single copy gene targets did not give reliable PCR results using genomic DNA (data not shown), however when PCR was carried out using primers targeting the ribosomal internally transcribed spacer (ITS) region amplification products were obtained for the majority of soil samples tested. The calibration of the qPCR system showed the expected log – linear response between concentration and Ct (cycle threshold). Amplification efficiencies were between 0.982 and 1.135 across all plates, with correlation coefficients in the range 0.981–0.995 over a five decade range of 1000–0.1 μg/μl. An ANOVA of RBD values obtained from the technical, DNA replications showed no significant difference between RBD values ($F = 0.13$, $P = 0.722$).

Wheat primers were tested for specificity against a range of field crops grown in the UK. Amplicons were obtained for wheat and barley DNA, but there was no reaction with maize, oilseed rape or faba bean DNA. The wheat primers were also tested against black-grass and found to produce no amplification. With the black-grass primers amplicons were obtained only with black-grass DNA, there was no amplification with wheat and barley DNA. Soil extracts for cores taken from an area of bare soil within the 2012 trial site gave no PCR amplification with wheat ITS primers.

Comparison between the DNA-based and root washing assays

Root biomass, as measured by the DNA-based PCR assay (RBD; μg dry roots/g air dried soil) was compared to root length density (RLD: cm/cm^3) at the four depths taken through the soil profile in the 2012 and 2014 trials (Fig. 1; Table 1). High Pearson correlations were found in both the 2012 ($r = 0.7947$; df = 10; $P = 0.002$) and the 2014 ($r = 0.674$; df = 22; $P < 0.001$) trials, while combining the data from the two seasons gave a value of $r = 0.702$ (df = 34, $P < 0.001$). Examining the wheat varieties independently also showed good correlations between RBD and RLD measurements; Alchemy $r = 0.918$ (df = 2, $P = 0.082$), Glasgow $r = 0.762$ (df = 10, $P = 0.004$), Oakley $r = 0.735$ (df = 14, $P < 0.001$) and Viscount $r = 0.992$ (df = 2, $P = 0.007$). The DNA qPCR method therefore provided a good estimate of root biomass, even at the lower depths where lower RLDs were found.

Comparison of root biomass between wheat lines and soil depth in the 2012 trial

A one-way ANOVA of the 2012 RLD data indicated that differences in root content by depth were highly significant ($F = 182.9$; $P < 0.001$), with RLD values decreasing with soil depth, but that differences between varieties were not significant ($F = 0.17$; $P = 0.846$). A one-way ANOVA of the 2012 RBD data also highlighted significant differences in root biomass by depth ($F = 6.83$; $P < 0.003$), but not between varieties ($F = 1.03$; $P = 0.375$).

Comparison of root biomass between wheat lines and soil depth in the 2014 and 2015 trials

For soil cores sampled from the 2014 and 2015 trials, a linear mixed-model analysis of RBD showed highly significant differences between varieties ($P < 0.001$), depths ($P < 0.001$) and the interactions between varieties × depth ($P < 0.001$). However, while no significant difference between years, a lines × year ($P < 0.001$) effect was seen, indicating that the root biomass produced by each

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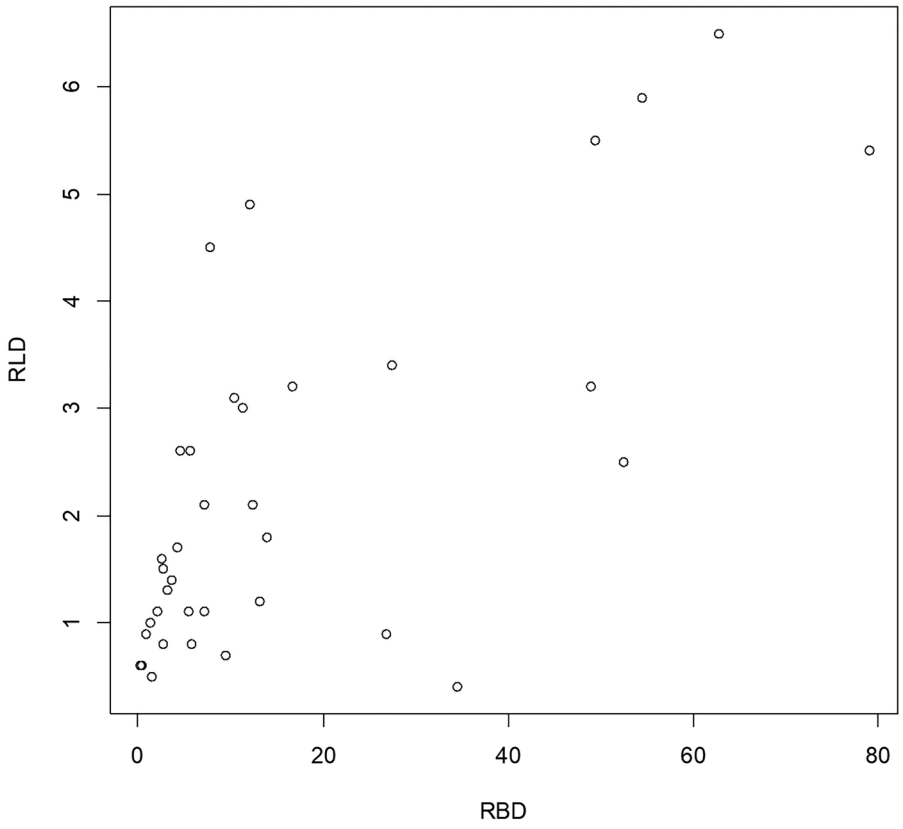

Figure 1. Scatter plot for DNA-based (RBD; µg dry roots/g air dried soil) and root washing assays (RLD: cm/cm^3) for wheat varieties in 2012 and 2014 field trials. Pearson's correlation between RBD and RLD for all varieties is 0.702 (df = 34, P value ≤ 0.001).

wheat line differed between the 2014 and 2015 field trials (online Supplementary Table S7).

In general, the highest RBD values were found in the upper soil profiles and the lowest values at depth, with all 22 wheat varieties tested (Table 2 and online Supplementary Table S4). At each depth RBD varied between 0.7–721 µg/g (0–250 mm), 0.9–394 µg/g (250–500 mm), 0.0–119 µg/g (500–750 mm) and 0.0–42.3 µg/g (750–1000 mm). More than 50% of the measured RBD was in the upper 500 mm of the soil profile in all, but two of the plots sampled in each field trial (data not shown). The proportion of RBD in the upper 500 mm of the soil profile averaged 79% in 2014 and 88% in 2015. Regression analysis showed that a quadratic fit best described the variation in RBD with depth, for all wheat lines. The regression equations were integrated and used to calculate D_{50} and D_{95} by the method of Schenk and Jackson (2005). The values for D_{50} had a range of 274–620 mm below the soil surface, with a mean of 459 mm. The values for D_{95} had a range of 695–976 mm below the soil surface, with a mean of 876 mm. The mean results over 2 years are shown in Table 3 and the full results are given in online Supplementary Table S3. The values for D_{50} and D_{95} allow rapid identification of shallow rooting and deep rooting wheat lines, and indicate that wheat lines Norman and SHW Xi19/(Xi19//SHW-218) > 18 are shallow rooting, while varieties Cadenza and Xi 19 are deep rooting.

In the 2012 and 2014 trials RLD data were only obtained for four wheat varieties, with only one variety in common between the 2 years. This was insufficient to conduct an analysis of variation between wheat lines.

Influence of key genetic traits on RDB values

The wheat lines included in these analyses of root biomass varied in their seasonal growth habit, their photoperiod response alleles (*Ppd*), in *Rht*, and in the presence/absence of the rye translocation (1B/1R) (online Supplementary Table S1). Highly significant differences ($F = 18.67$, $P < 0.001$) were found in RBD values between wheat lines with different seasonal growth habits, with spring types having the greater average RBD values within the soil profile, followed by alternative and winter types. Variation at the *Rht* loci was also associated with variation in the RBD phenotype ($F = 2.71$, $P < 0.050$), with *Rht* showing a significant interaction with trial year ($F = 3.61$, $P = 0.013$). No significant variation in the RBD values was accounted for by the presence or absence of the rye translocation ($F = 0.47$, $P = 0.506$), or variation at the *Ppd* loci ($F = 1.73$, $P = 0.096$).

The variation in RBD values associated with *Rht* loci was significant in 2014 ($P < 0.001$), but not in 2015 ($P = 0.128$). In 2014, wheat lines harbouring wild-type alleles and *Rht2* had greater average RBD throughout the soil profile than those harbouring *Rht1* and *Rht8*. This trend was not observed in the 2015 data. These observations may be linked to differences in the weather conditions at the 2014 and 2015 test sites. In 2014, the winter and spring temperatures were uncharacteristically high (anomaly 1.8 and 1.6°C) relative to the 30-year average (1981–2010), while conditions in 2015 were closer to the 30-year average (anomaly 0.3 and 0.2°C) (http://www.metoffice.gov.uk/climate/uk/summaries/) (online Supplementary Table S4).

Heritability of the RBD phenotype

Broad sense heritability for total RBD in the soil profile was calculated as 0.16, while the heritability of RBD was 0.11 in the upper 250 mm of the soil profile, 0.21 in the profile at 250–500 mm depth, 0.00 in the profile at 500–750 mm depth and 0.43 in the profile at 750–1000 mm depth. These results suggest that

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Table 1. DNA-based (RBD; µg dry roots/g air dried soil) and root washing assays (RLD: cm/cm3) for wheat varieties in 2012 and 2014 field trials

Trial	Rep	Depth	Variety	RBD	RLD
Terrington 2012 Pilot experiment	A	0–250	Alchemy	41.2	3.2
	A	250–500	Alchemy	8.4	2.1
	A	500–750	Alchemy	5.3	1.4
	A	750–1000	Alchemy	0.2	0.6
	A	0–250	Oakley	9.1	3.0
	A	250–500	Oakley	6.1	1.7
	A	500–750	Oakley	1.2	0.9
	A	750–1000	Oakley	9.1	0.6
	A	0–250	Viscount	40.8	3.2
	A	250–500	Viscount	13.2	1.8
	A	500–750	Viscount	6.1	1.1
	A	750–1000	Viscount	0.9	1.0
Terrington 2014	A	0–250	Glasgow	62.8	6.5
	A	250–500	Glasgow	27.4	3.4
	A	500–750	Glasgow	7.1	1.1
	A	750–1000	Glasgow	2.1	1.1
	B	0–250	Glasgow	7.7	4.5
	B	250–500	Glasgow	4.6	2.6
	B	500–750	Glasgow	3.1	1.3
	B	750–1000	Glasgow	5.8	0.8
	C	0–250	Glasgow	12.0	4.9
	C	250–500	Glasgow	10.4	3.1
	C	500–750	Glasgow	2.6	1.6
	C	750–1000	Glasgow	2.7	0.8
	A	0–250	Oakley	49.4	5.5
	A	250–500	Oakley	12.4	2.1
	A	500–750	Oakley	26.8	0.9
	A	750–1000	Oakley	34.5	0.4
	B	0–250	Oakley	54.5	5.9
	B	250–500	Oakley	5.6	2.6
	B	500–750	Oakley	2.7	1.5
	B	750–1000	Oakley	9.4	0.7
	C	0–250	Oakley	79.0	5.4
	C	250–500	Oakley	52.5	2.5
	C	500–750	Oakley	13.2	1.2
	C	750–1000	Oakley	1.4	0.5

Pearson's correlation between RBD and RLD for all varieties is 0.702 (df = 34, P value ≤ 0.001).

RBD, particularly RBD at depth should be amenable to selection by plant breeders.

Black-grass observations

In 2015, soil cores were taken within the wheat trial from areas with 'low', 'moderate' and 'high' black-grass. As expected, black-grass RBD values in 'low' black-grass areas were 0.0 µg/g dry soil. In 'moderate' black-grass areas between 0.0 and 2.5 µg/g dry soil and in 'high' black-grass areas ranged from 1.9 to 18.2 µg/g dry soil (Table 4). In the soil cores taken from the 'high' density black-grass area, over 70% of the black-grass root RBD was in the top 250 mm of the soil profile, while in the 'moderate' density black-grass area, over 90% of the root biomass was in this upper profile suggesting that in denser black-grass patches roots tend to grow deeper. Our black-grass sampling design did not allow any conclusions to be drawn on whether 'high' black-grass densities inhibit wheat root development, but our results show that the qPCR tools developed in this study would be of value in future, crop–weed interaction studies.

Discussion

Traditional root washing methods used to assess root development in field experiments are time consuming. In this study, we have developed a robust, qPCR method to reliably measure root biomass of wheat and the major weed of cereal crops, black-grass, down to soil depths of 1 m. We show that the qPCR assay can distinguish wheat from among most other major agricultural crops, and from black-grass. The ability to exclude weed roots from the total root density represents an advance over conventional root washing methods, while the ability to quantify black-grass root biomass relative to wheat root biomass will be useful in competition experiments to determine the impact of weeds on wheat production.

Despite the inherent variation present within the PCR technology (Karlen *et al.*, 2007), the estimate of root biomass as determined by RBD correlated extremely well with classical root washing RLD measurements in both the 2012 and 2014 field trials. While RLD and RBD quantify roots in soil by length per volume and weight per weight respectively, both are measures of root biomass within the soil. Both methods have a degree of uncertainty: RLD underestimates the biomass of fine roots and includes the roots of both the crop and weeds while RBD underestimates the biomass of larger roots. However, the correlations obtained suggest that the results by either method would discriminate between accessions or agronomic treatments in the same way, allowing researcher to arrive at similar conclusions.

In general root density decreased with soil depth. However, the RBD assay did identify distinct differences between wheat varieties in root distribution through the soil profile, some varieties from the 22 tested being better at producing roots at depth, with a significant interaction between varieties and depth being observed in both the 2014 and 2015 field trials. A variety × year effect was also observed, indicating that root production was significantly influenced by the different climatic and environmental growing conditions prevalent in the 2014 (Burkees Field, silty clay loam) and 2015 (Willow Tree Field, silt loam/sandy silt loam) trials.

Spring wheat varieties were found to produce more root biomass, having higher RBD values, than alternative and winter wheat varieties. The *Rht* alleles were also found to have a potential influence on root formation, a significant interaction being found between *Rht* allele and trial year, showing a significant interaction in 2014, but not 2015.

The RBD assay was capable of discriminating between wheat and black-grass in the same soil DNA extraction. The black-grass RBD values reflected the above-ground black-grass population density. Our black-grass sampling design did not allow any

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Table 2. Root biomass density from soil cores collected from the 2014 and 2015 trials showing the mean for each variety at each depth

Tables of means Variate: μg/g dry soil	Depth			
Variety	0–250	250–500	500–750	750–1000
Alchemy	93.65	27.04	22.00	6.58
Avalon	116.83	69.76	35.05	10.44
Beaver	40.47	15.86	7.18	6.17
SHW Xi19/(Xi19//SHW-218) > 18	77.07	28.01	6.26	−3.25
SHW Xi19/(Xi19//SHW-218) > 19	147.75	67.24	33.02	19.10
Buster	44.32	17.58	16.60	2.28
Cadenza	50.81	28.54	27.69	16.62
Cappelle Deprez	68.64	22.93	11.79	1.82
Glasgow	32.39	17.38	5.41	4.74
Hereward	33.19	16.27	7.22	5.18
Mercia	32.10	11.95	5.78	2.16
Mercia Rht8	63.28	22.51	6.99	3.83
Mercia Rht8 D1B	44.11	21.51	0.56	−2.71
Norman	110.30	46.19	19.69	10.54
Oakley	44.39	18.17	4.52	4.47
Paragon	106.52	48.62	20.39	1.95
Rialto	35.06	25.10	6.07	0.50
Robigus	36.44	9.04	2.33	−1.37
Savannah	85.36	44.05	15.52	3.67
Soissons	44.89	40.00	28.18	8.03
Spark	91.68	42.47	7.77	3.87
Xi19	216.17	90.83	9.90	10.58
Mean	73.43	33.23	13.63	5.24

Additional information is shown in online Supplementary Table S4.

conclusions to be drawn on whether 'high' black-grass densities inhibit wheat root development, but our results show that the qPCR tools developed in this study would be of value in future, crop–weed interaction studies.

Compared with current methods we can see that the RBD assay has both strengths and weaknesses. Cores can be taken at any point in the growing season, allowing root biomass accumulation in the field to be assessed throughout the growing season. The soils assayed in this study had textures described as sandy loam, sandy silt loam, silt loam, silty clay loam, clay loam and fine loam over clay, with RBD working equally as well in all these soil types. Basically the method can be implemented in any soil that can pass through a mill. Removal of roots by washing from heavy soils requires prolonged sample pre-treatment with sodium hexametaphosphate solution and use of a hydropneumatic elutriation system (Thivierge *et al.*, 2015).

Processing time for a batch of samples is likely to be less than that required for soil washing assays. The time required for soil milling is approximately 20 min per plot (four depth horizons), for extractions of a batch of 12 plots (four depth horizons, extracted in duplicate) is approximately 1 day and for the DNA assay (qPCR set-up, running and data collation; four depth horizons, extracted in duplicate, PCR in triplicate) approximately half a day. Apart from a soil mill, the equipment needed is available in many research facilities.

The RBD assay makes the assumption that the ratio of ribosomal DNA to genomic DNA does not differ between wheat varieties or the developmental stage of the plant (Huang *et al.*, 2013). For example, in older roots the cortex dies leaving only the stele, thus older, larger roots may be under-represented by the RBD assay. Conversely, very fine roots, which are difficult to wash from soil samples, may be under-represented in the RLD assay (Sierra *et al.*, 2003). Clearly, the RBD method does not allow a detailed dissection of root architecture; for example, rooting angles or the ratio of fine to coarse roots. However, the DNA-based method does allow root development to be economically studied in field situations throughout the growing season.

Despite the limitations, the RBD assay allows cost-effective estimation of root biomass within the soil profile, supporting studies of rooting behaviour between different wheat genotypes and an exploration of the effects of differing agricultural practices on root development. In developing this RBD assay as a standard method to be adopted by the research community, we would seek to develop standardised calibration materials and agreement on

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Table 3. Estimates of the soil depths (mm) containing 50 and 95% of all roots (D50 and D95) for each variety

Depth (mm)	D_{50}	D_{95}
Wheat variety		
Alchemy	−492	−936
Avalon	−597	−952
Beaver	−482	−872
SHW Xi19/(Xi19//SHW-218) > 18	−274	−738
SHW Xi19/(Xi19//SHW-218) > 19	−613	−976
Buster	−398	−961
Cadenza	−573	−953
Cappelle_Desprez	−451	−827
Glasgow	−305	−907
Hereward	−392	−904
Mercia	−458	−759
Mercia_Rht8	−359	−887
Mercia_Rht8_D1	−567	−915
Norman	−380	−782
Oakley	−442	−844
Paragon	−514	−812
Rialto	−312	−903
Robigus	−448	−841
Savannah	−453	−695
Soissons	−486	−893
Spark	−488	−966
Xi19	−620	−948
Overall mean	−459	−876

A table of D50 and D95 for each year is given in online Supplementary Table S5.

Table 4. The biomass of wheat and black-grass roots measured at four different depths in the soil profile using the DNA-based assay (RDB), sampled from three black-grass population densities

Black-grass population	Depth (mm)	Black-grass µg/g soil	Wheat µg/g soil
Low black-grass infestation 0 heads per m^2	0–250	0.0	98.9
	250–500	0.0	77.9
	500–750	0.0	69.3
	750–1000	0.0	21.8
Medium black-grass infestation 50 heads per m^2	0–250	2.5	32.5
	250–500	0.1	16.5
	500–750	0.0	49.9
	750–1000	0.0	21.5
High black-grass infestation 300 heads per m^2	0–250	18.2	42.7
	250–500	1.9	8.9
	500–750	2.1	3.8
	750–1000	2.4	4.8

the basis by which results are declared, that would allow comparable results to be shared by the root research community.

The effect of *Rht* on root development has been demonstrated in experiments on seedlings grown on germination paper (Schmidt *et al.*, 2022) or paper rolls (Khadka *et al.*, 2021) and on mature plants in growth tubes (Subira *et al.*, 2016), ours is the first study to demonstrate the effect in a field-grown crop. The effect of vernalisation genes on root development has been demonstrated in hydroponics (Smirnova and Pshenichnikova, 2021) and pot experiments (Arifuzzaman *et al.*, 2016), here we show an effect in a field-grown crop. While our results are not unexpected, to demonstrate these effects in a relatively small field experiment in a mature crop show the potential of the qPCR system to reveal subtle differences in root biomass phenotypes in experimental lines or to reveal the potential of diverse germplasm held in genebanks.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1479262124000492

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