THE RESPONSE OF ROOT SYSTEM ARCHITECTURE TO SOIL COMPACTION

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Abstract

Soil compaction has been described as the most serious environmental problem caused by conventional agriculture, as it results in several stresses which may interact simultaneously, including increased soil strength, decreased aeration and reduced hydraulic conductivity. Root system architecture (RSA) is the arrangement of roots within the soil matrix and is important because the specific deployment of roots within the soil can determine soil exploration and resource uptake. As roots deliver water and nutrients to growing plants, whilst also providing anchorage, their importance cannot be overstated. Yet, our understanding of how roots interact with the surrounding soil, especially at the micro-scale level, remains limited because soil is an opaque medium, so preventing roots from being visualised without disturbing them. Destructive techniques are commonly employed for the analysis of RSA, however this can result in the loss of key information concerning root architecture, such as elongation rates and root angles and important soil characteristics such as soil structure and pore connectivity. However, X-ray Computed Tomography (CT) has been shown to be a promising technique for visualising RSA in an undisturbed manner. The species considered in this thesis were wheat (*Triticum aestivum* L.) and tomato (*Solanum lycopersicum* L.). Further information regarding the response of roots to soil compaction has been achieved through the use of X-ray CT, automatic root tracing software and novel image analysis procedures. Soil compaction significantly affected root length, volume, surface area, angle, diameter, elongation rates and root path tortuosity, however the influence of soil texture on root responses to soil compaction was significant. Moderate compaction benefits root growth in clay

soil, possibly due to the greater nutrient and water holding capacity, but adversely affected root growth in loamy sand. The results suggest that there is an optimum level of soil compaction for the different soil types. Roots elongated rapidly between 2-3 days after germination (DAG), it is hypothesised that is related to the mobilization of seed storage substances to the growing roots. The use of transgenic mutants of tomato with altered levels of abscisic acid (ABA) has provided a greater insight into the role of ABA in mediating root responses to soil compaction. This work will enable better phenotyping of plant varieties with enhanced root system traits for resource foraging and uptake. Knowledge of the responses of root systems in heterogeneous soil is vital to validate root phenotypes and overcome future food security challenges.

Publications arising from this work

Refereed journal articles included in the thesis

Tracy, S.R., Black, C.R., Roberts, J.A., Mooney, S.J. (2012) Exploring the interacting effect of soil texture and bulk density on root system development in tomato (*Solanum lycopersicum* L.). *Under review.*

Tracy, S.R., Black, C.R., Roberts, J.A., Sturrock, C., Mairhofer, S., Craigon, J., Mooney, S.J. (2012) Quantifying the Impact of soil compaction on root system architecture in tomato (*Solanum lycopersicum* L.) by X-ray micro-Computed Tomography (CT). *Annals of Botany,* 110, 511 – 519.

Tracy, S.R., Black, C.R., Roberts, J.A., McNeill, A., Davidson, R., Tester, M., Samec, M., Korošak, D., Sturrock, C., Mooney, S.J. (2011) Quantifying the effect of soil compaction on three varieties of wheat (*Triticum aestivum* L.) using X-ray micro-Computed Tomography (CT). *Plant and Soil*, 353, 195-208.

Tracy, S.R., Black, C.R., Roberts, J.A., Mooney, S.J. (2011) Soil compaction: a review of past and present techniques for investigating effects on root growth. *Journal of the Science of Food and Agriculture,* 91, 1528-1537.

Tracy, S.R., Roberts, J.A., Black, C.R., McNeill, A., Davidson, R., Mooney, S.J. (2010) The X-factor: Visualizing undisturbed root architecture in soils using X-ray computed tomography. *Journal of Experimental Botany*, 61, 311-313**.**

Other related refereed journal articles not included as part of this thesis

Zappala, S., Mairhofer, S., **Tracy S.R.,** Sturrock, C.J., Bennett M., Pridmore T., Mooney, S.J. (2012) The effect of soil moisture content on quantifying root system architecture in X-ray Computed Tomography images. *Plant and Soil*, Under review.

Helliwell, J., Sturrock, C., Grayling, K., **Tracy, S.R.,** Whalley, W.R., Flavel, R., Young, I.M., Mooney, S.J. (2012) Applications of X-ray Computed Tomography into studies of the soil physical environment. *European Journal of Soil Science*, Under review.

Mairhofer, S., Zappala, S., **Tracy, S.R.,** Sturrock, C., Bennett, M., Mooney, S.J. and Pridmore, T. (2012) RooTrak: Automated Recovery of Three-Dimensional Plant Root Architecture in Soil from X-Ray Micro-computed Tomography Images Using Visual Tracking. *Plant Physiology,* 158, 561-569.

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Lots of Love

Saoirse

X

CONTENTS

Abbreviations and acronyms

µCT	Micro Computed Tomography
2-D	Two dimensions
3-D	Three dimensions
4-D	Four dimensions
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AMF	Arbuscular mycorrhizal fungi
AMR	Acid molybdate reagent
ANOVA	Analysis of Variance
BC	Before Christ
c.	*circa*
cv.	*cultivar*
cvs.	*cultivars*
CT	Computed Tomography
CTN	Computed Tomography Number
DAG	Days after germination
DAT	Days after transplanting
DEFRA	Department for Environment, Food and Rural Affairs
DI	Deionised water
DW	Dry weight
Ed.	Editor
Eds.	Editors
FAO	Food and Agriculture Organization (of the United Nations)
Fig.	Figure

Figs.	Figures
GE	General Electric
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IDTC	Interdisciplinary Doctoral Training Centre
LLWR	Least limiting water range
MRI	Magnetic resonance imaging
NCED	9-*cis*-epoxycarotenoid dioxygenase
NERC	Natural Environment Research Council
NMR	Nuclear magnetic resonance
Nr	Never ripe
PGR	Plant Growth Regulator
PPFD	Photosynthetic photon flux density
psd_{cu}	Pore size distribution (coefficient of uniformity)
Roi	Region of interest
RSA	Root system architecture
SD	Standard deviation
SED	Standard error of the difference
SEM	Standard error of the mean
sp	superpromoter
Tel	Telephone
vs.	*versus*
UK	United Kingdom
USA	United States of America

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1. Chapter 1

Paper as published in *Journal of the Science of Food and Agriculture*

Chapter 1 is the main introductory chapter and is a review paper published in the *Journal of the Science of Food and Agriculture* (Tracy *et al*. 2011. 91, 1528–1537), so is presented in 'paper format'. The paper reviews past and present techniques used to investigate soil compaction and the effect on root growth.

Author contribution:

Project supervision performed by CR Black, JA Roberts and SJ Mooney

Literature review and construction of the paper performed by SR Tracy

2. Chapter 2

2.1 General Introduction

2.1.1 Summary of literature

The use of X-ray CT to examine undisturbed roots systems growing in soil was first implemented over two decades ago. Watanabe *et al. (*1992) first employed this approach to visualise the storage roots of Chinese Yam (*Dioscorea oppositifolia* L.) at a resolution of 2 - 5 mm. The earliest studies were limited to visualising plant species with coarse roots as the spatial resolution was not adequate to observe finer roots. Grose *et al.* (1996) used a medical scanner but were unable to resolve roots of wheat, cotton and radish <0.4 mm in diameter. Heeraman *et al*. (1997) undertook consecutive scans and obtained 3-D images of intact root systems of bush bean (*Phaseolus vulgaris* L.) using X-ray CT, although only to a depth of 0.8 cm. One of the first experiments that used a micro-tomography system was reported by Jenneson *et al*. (1999), who generated 3-D time-lapse images of growing roots of wheat (*Triticum aestivum* L.) seedlings to determine root length and volume, while Gregory *et al*. (2003) observed root growth for pre-germinated wheat and rape (*Brassica napus* L.) seedlings transplanted into a sandy loam soil in 25 mm diameter plastic containers with an image resolution of 100 µm. Kaestner *et al*. (2006) reconstructed the fine roots of two alder (*Alnus incana* L.*)* plants using X-ray micro-tomography. Similarly, Perret *et al*. (2007) used X-ray CT to visualise and quantify the roots of chick pea (*Cicer arietinum* L.). This approach, also used by Hargreaves *et al.* (2009) was able to measure root traits directly in barley (*Hordeum vulgare* ssp. *vulgare* and *spontaneum* L.). X-ray CT can be used successfully to examine the root responses to soil failure i.e. lodging (Mooney *et al.*, 2006). McNeill *et al*. (2007) visualised the

effect of interfaces between high and low bulk density soil horizons on root architecture. Therefore, previously unanswerable questions regarding effects of the 3-D soil environment on root architecture over time can now be addressed following the advent of X-ray CT scanning. The experiments reported in this thesis are some of the first to focus directly on the response of root architecture to soil compaction using X-ray CT.

2.1.2 Research aims and objectives

The overall aim was to investigate the response of root system architecture (RSA) to soil compaction. The project utilised X-ray CT technology to obtain non-destructive visualisations of roots growing in different soil textures at differing bulk densities and used state-of-the-art image analysis software to quantify the response at the micro-scale.

The overarching hypothesis is:

'The response of root system architecture to increasing soil compaction is determined by several soil physical characteristics, which can be visualised and quantified using non-destructive imaging technology'

The initial objectives were to:

1. Produce a scanning protocol suitable for scanning columns of uncompacted and compacted soil held at field capacity containing growing roots.
2. Develop image analysis procedures to quantify soil physical characteristics and segmentation of root systems.

Once these objectives had been achieved, the following questions were addressed:

- What is the response of roots to increasing soil bulk density and is this consistent for different soil types?
- What are the effects of soil compaction on root elongation rate and does this change over time?
- Is abscisic acid (ABA) able to mediate the response of the root systems to soil compaction?
- Can automatic root tracking algorithms be successfully applied to the research carried out?

2.1.3 Thesis structure

This thesis is primarily composed of published papers in 'paper format' and submitted papers. Each paper that is included as an experimental chapter has all the associated information relevant for that experiment. **Chapter 1** is the main introductory chapter and is a review paper published in the *Journal of the Science of Food and Agriculture* (Tracy *et al*. 2011. 91, 1528–1537), so is presented in 'paper format'. The paper reviews past and present techniques used to investigate soil compaction and the effect on root growth. **Chapter 2** provides a short summary of the key literature related to the research conducted. The research aims and objectives are also presented. **Chapter 3** offers a viewpoint of the use of X-ray CT to visualise undisturbed root architecture in soil. This has been published in the *Journal of Experimental Botany* (Tracy *et al*. 2010. 61, 311-313), so is presented in 'paper format'. **Chapter 4** is a general materials and method chapter and includes

experimental information and further analyses not presented elsewhere. **Chapter 5** used X-ray CT to quantify the effect of soil compaction on three Australian wheat varieties. Results from the automatic root tracking algorithm RootViz3D® are also presented. This was published in *Plant and Soil* (Tracy *et al.* 2012. 353, 195–208), so is presented in 'paper format'. **Chapter 6** visualised root elongation and RSA development in tomato for a 10 day period following germination in response to soil compaction. Daily quantification of root growth was possible and novel image analysis measurements were made. This was published in *Annals of Botany* (Tracy *et al*. 2012. 110, 511 - 519), so is presented in 'paper format'. **Chapter 7** investigated the effect of soil texture and bulk density in greater detail with the aim of determining typical responses of root architecture to incremental steps of increasing bulk density and through greater treatment replication. This paper is currently under review for publication. **Chapter 8** investigated the role of ABA in mediating root responses to increasing soil compaction using ABA mutants with differing internally generated ABA concentrations. **Chapter 9** provides a general discussion of the key results and findings. **Chapter 10** draws together the key conclusions from each experimental chapter; a section on possible further work is included.

3. Chapter 3

Paper as published in *Journal of Experimental Botany*

Chapter 3 offers a viewpoint of the use of X-ray CT to visualise undisturbed root architecture in soil. This has been published in the *Journal of Experimental Botany* (Tracy *et al.* 2010. 61, 311-313), so is presented in 'paper format'.

Author contribution:

Project supervision performed by CR Black, JA Roberts and SJ Mooney

General advice and draft editing performed by A McNeill and R Davidson, (University of Adelaide)

Literature review, practical work and paper construction performed by SR Tracy

4. Chapter 4

4.1 General Materials and Methods

As stated in Chapter 2, this thesis is primarily made up of published papers in 'paper format' or submitted papers. Each paper that is included as an experimental chapter contains detailed information relevant for each experiment. Information presented here comprises the part of the overall research that has not been presented elsewhere in the thesis.

4.1.1 Soil types

In Chapter 5, a sandy loam soil (Prospect Hill, Adelaide, Australia, 34^{o} 52' S, 138^{o} 30' E, A1 horizon, FAO Class: Brown Chromosol) was air-dried and sieved to <2 mm. For the rest of the experimental work undertaken a Newport series loamy sand (brown soil) and a Worcester series clay loam soil (argillic pelosol) from the University of Nottingham farm at Bunny, Nottinghamshire, UK (52.52 ° N, 1.07 ° W) were air-dried and sieved to <2 mm for packing into the columns.

4.1.2 Soil column packing

After sieving the soil to < 2 mm and weighing out the correct mass of soil per column to achieve the desired bulk density, the soil was swirled and poured in small amounts into the column. The swirling action was to distribute the different sized soil fractions evenly. After a small amount was poured in and compacted (if required) the surface of the layer was scarified to rough the surface, which proved essential at binding the layers together to prevent a layering effect. To ensure this

method did not created any distinct layers columns were scanned in the X-ray CT scanner to check for this. Figure 4.1 is an example image of a packed soil column; no layering present and the different micro-scale soil fraction sizes are evenly distributed throughout the column.

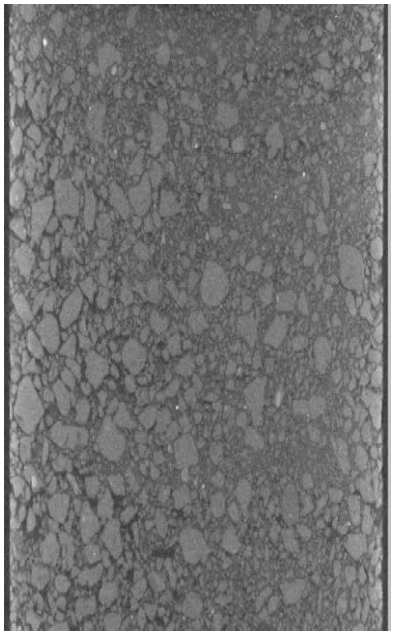

Figure. 4.1. Example image of a scanned loamy sand soil column at 33 µm.

4.2 Soil Chemical analysis

4.2.1 pH

A combined electrode was used to measure soil pH, which includes 'reference' and pH-sensitive glass electrodes incorporated into a single electrode stick. The pH meter was calibrated with pH 4.01 and pH 7.00 buffers using the 'buffer' and 'slope' controls. Approximately 5 g of air dried < 2 mm soil was measured into a centrifuge tube and 12.5 ml of de-ionised water was added. The tube was shaken for 30 min on an end-over-end shaker to attain equilibrium. After rinsing with de-ionised water the tip of the pH electrode was immersed into the soil suspension. After waiting 5 min for the

pH reading to stabilise, the result was recorded. The average pH value of the clay loam soil was 7.07 and the average pH value of the loamy sand was 7.13.

4.2.2 Nitrate

The amount of available nitrate in the soil was determined by mixing 10 g of air-dry <2 mm soil with 50 ml 1 M KCl and shaking for one hour. Standards of known nitrate concentration were prepared by appropriate dilutions of a standard stock solution. After filtration, 20 ml of the extractant solution was placed in a universal tube. Then, 3 ml ammonium chloride, 1 ml borax solution and 0.6 g of spongy cadmium were added to the tube. The tube was then shaken for 20 min. Afterwards 7 ml of this solution was transferred to a 50 ml flask, to which 1 ml of sulphanilamide solution was added and then gently swirled and allowed to stand for 5 min. Then 1 ml of N-1-napthylethylenediamine dihydrochloride was added and the flask was made up to 50 ml using de-ionised water. The flask was left for 10 min and the nitrate concentration measured from the absorbance of the solution on a spectrophotometer, with the wavelength set to 543 nm. The average nitrate concentration was 4.09 mg l^{-1} for the clay loam and 5.48 mg l^{-1} for the loamy sand.

4.2.3 Phosphorus

Extractable phosphorus concentrations of the soils were determined using the Olsen-P method. In triplicate, approximately 2.0 g of each soil was weighed into a 50 ml screw cap centrifuge tube and half teaspoonful of low phosphate charcoal was added. 30 ml of 0.5 M sodium bicarbonate was added and shaken end-over-end for 30 min before centrifuging at 2500 g for 15 min.

For calibration standards, 0, 1, 2, 3, 4 and 5 ml of the working phosphate standard (10 mg P l^{-1}) was added to 50 ml flasks, to give a calibration range expressed as 'amount' of P: 200, 400, 600, 800 and 1000 µg\l^{-1} P. 4 ml of the acid molybdate reagent (AMR) and 4 ml of the ascorbic acid solution were added before making the solution up to 50 ml with deionised water. The colour was allowed to develop for 20 min before reading the standards at 880 nm in a 1 cm cell.

After centrifuging, a 5 ml aliquot of the supernatant was added to a 50 ml flask, 2 ml 3 M H_2SO_4 was added to neutralise $NaHCO_3$. 4 ml of the AMR and 4 ml of the ascorbic acid solution was added before making up with deionised water. The colour was allowed to develop for 20 min before reading at 880 nm in a 1 cm cell after zeroing the spectrophotometer on the zero P standard. Using Appendix 4 from the DEFRA RB204 book of fertiliser recommendations, the phosphorus concentrations could be given DEFRA indices to determine whether the soil was deficient or surplus in those nutrients. For phosphorus concentrations the clay loam samples had an average DEFRA index of 2 (15.75 mg kg^{-1}), whereas the loamy sand samples had an average DEFRA index of 3 (29.65 mg kg^{-1}). The values obtained are typical of British agricultural soils.

4.2.4 Potassium

The potassium concentration of the soils was determined by mixing 10 g of air-dry <2 mm soil with 50 ml of 1 M ammonium nitrate and shaking for 30 min on a mechanical shaker. The solution was then filtered and the samples prepared for measurement by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

equipment. For potassium concentration, the clay loam samples had an average DEFRA index of 1, whereas the loamy sand samples had a value of 0.

All of the soil chemical analysis data correlated well with the average values stated on the NERC soil portal (http://www.bgs.ac.uk/nercsoilportal/).

4.3 Soil Hydraulic Properties

4.3.1 Water release curves

Using a combination of sand tables and pressure membrane apparatus, water release curves were obtained for both soil types. All data were subsequently fitted to the Van Genuchten-Mualem model using RETC software, (http://www.scisoftware.com/).

4.3.2 Sand Table

The sand table was prepared by filling the glass reservoir and rising above the base height of the table to ensure full saturation of the sand table with no air bubbles. Soil samples were prepared in cores packed to a bulk density of 1.2 Mg m^{-3} and placed flat on the sand table. Once saturation of the samples was achieved (0 kPa), the reservoir was lowered to the required pressure levels of -10 kPa, -30 kPa and -60 kPa. After equilibrating at each stage, the samples were weighed and then placed back on the sand table surface, ensuring good contact. After the final weight measurement was made, soil samples were oven-dried at 105 $^{\circ}$C for 24 hr then weighed.

4.3.3 Pressure Membrane Apparatus

Using a blade, the pressure membranes were cut into the correct sizes and placed on the plates, with the rubber 'O' rings placed at the top and bottom of the container. The mass of soil was carefully placed into the container and saturated with air-free water. The container was then sealed and bolted down to ensure a tight fit. Plastic collection tubes were filled with approximately 1 ml of oil to prevent evaporation of collected water. Individual tubes were then weighed and placed under the pressure plate outlets. The nitrogen gas was turned on and the pressure set to the isolator. Gas leakages were tested for using washing up liquid and observing bubbles; if any leaks were detected the individual container was cleaned and re-packed with soil. Once no gas leakages were present, the gas pressure was set to the lowest pressure of -200 kPa, collection tubes were weighed frequently and once equilibrated a higher pressure was set. This was repeated for pressures of -600, -1000 and -1400 kPa. After the final measurement, soil samples were oven dried at 105 $^{\circ}$C for 24 hr then weighed.

The water release characteristic curves for both soil types are displayed in Figures 4.2 and 4.3.

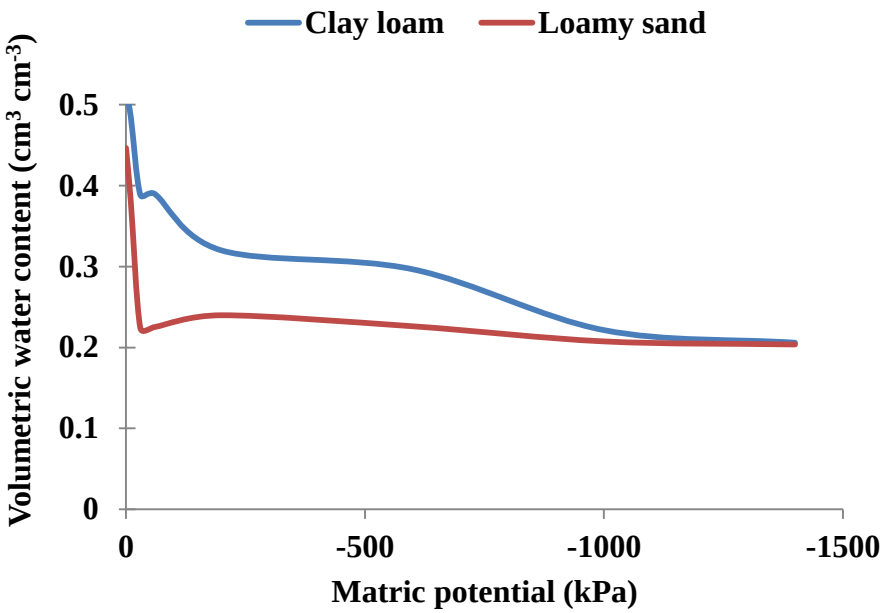

Figure.4.2. Water release curve of the clay loam and loamy sand soils of experimental values obtained

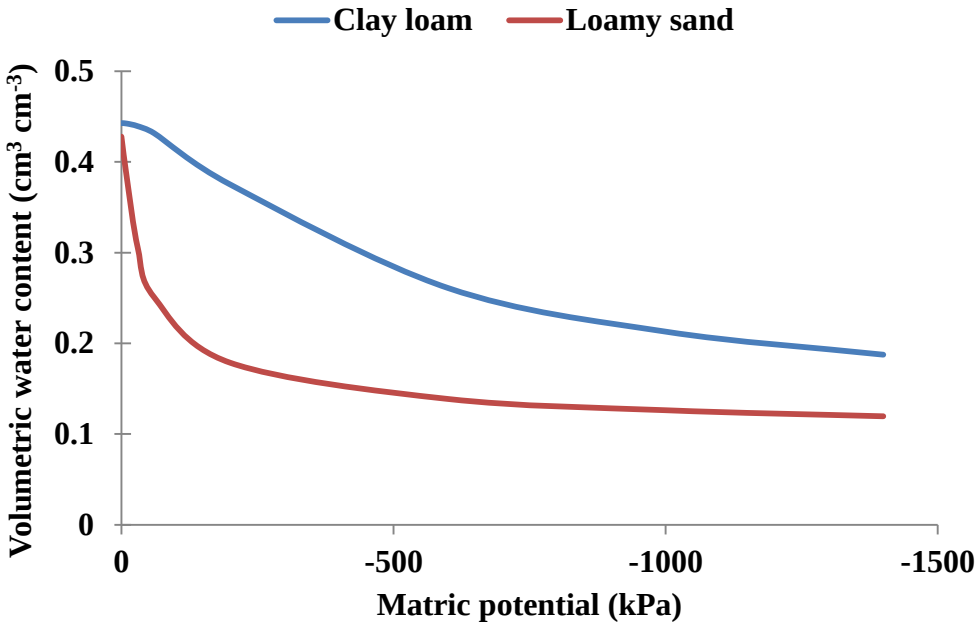

Figure.4.3. Water release curve of the clay loam and loamy sand soils of values fitted using the Van Genuchten-Mualem model

other voxels. The algorithm firstly creates a 3-D array of computed tomography number (CTN) values. Entries within this array are assigned values of 1 or 0 depending on whether they fall within a certain range of density values. If the CTN of a voxel falls within the predetermined range, the criterion that the connected voxel contains a root is satisfied (Lontoc-Roy et al. 2006). Another example of an image created by 3-D root tracking was produced by Perret et al. (2007), who admitted that some root laterals were missing as their visualisation algorithm volume-averaged CT matrices to generate a series of vertices and polygons, which formed the 3-D volume dataset. The resolution of the CT scanner they used also meant that some fine root segments could not be detected. Ideally, an automated process which is accurate and can be applied to various types of root and soil systems is required.

One approach which may resolve some of these difficulties is the application of the root tracking software, RootViz3D® (developed by Davidson, as reported by Tracy et al. (2010)), which utilises ideas from Oh and Lindquist (1999) and Pierret et al. (2002). This approach assigns a probability function (based on a Gaussian distribution) to determine whether specific pixels within images represent root material and can be used to provide 3-D visualisation of root distribution in undisturbed soil columns, enabling images to be captured as roots grow or encounter soil-borne stresses such as compacted soil layers. The main principle that was utilised is that root, soil and pore space can be described by probability distributions, which change in a predictable manner based on root diameter, soil type and moisture content. It has been shown that this automated and rapid methodology for root detection can remove the subjectivity of identifying 'root' voxels in noisy images and enable detailed visualisation of root architecture in situ (Tracy et al. 2010). The advantage of this approach is that, as it is based on a Gaussian distribution, it removes operator bias in thresholding, which can be significant (Baveye et al. 2010). One potential disadvantage is that it is computationally intensive, so it can take several hours to segment one core depending on the specification of the computer involved.

The objective of the present study was to examine the effect of contrasting soil microstructures associated with differing levels of compaction on root growth in three wheat (*Triticum aestivum* L.) cultivars which, in previous studies, had been shown to have different agronomic traits (Izanloo et al. 2008). The root tracking software, RootViz3D® was tested and appraised for its accuracy in segmenting root pixels from X-ray CT images of soil by comparison with semi-automated imaging methods and root washing techniques.

Materials and methods

Sample preparation and scanning procedures

A sandy loam soil (Prospect Hill, Adelaide, Australia, 34° 52' S, 138° 30' E, A1 horizon, FAO Class: Brown Chromosol) was air-dried and sieved to <2 mm. Soil composition was 66% sand, 21% silt and 13% clay. Columns (91 mm height×29 mm diameter) were packed to provide two levels of compaction with bulk densities of 1.1 and 1.5 g cm^{-3}, each with three replicates. The columns were packed with air dry soil in *c.* 1 cm deep layers. After compacting each layer, the surface was lightly scarified to ensure homogeneous packing and hydraulic continuity within the column (Lewis and Sjostrom 2010). Three South Australian bread wheat (*Triticum aestivum* L.) varieties, cvs. Excalibur, RAC875 and Kukri with differing agronomic traits, were chosen for study (Izanloo et al. 2008). Seeds were imbibed for 48 h before being planted 8 mm below the soil surface. The columns were weighed daily and sufficient water was added by pipette (*c.* 1–2 g) to ensure soil moisture content remained close to field capacity throughout the experiment.

The columns were scanned 2, 5 and 12 DAG using a SkyScan-1076 X-ray micro-CT scanner (Adelaide Microscopy, University of Adelaide) set at 100 kV and 100 μA, with a 1.0 mm aluminium filter and an image averaging of 4. Voxel resolution was 17.4 μm and each scan took 60 min to complete. As our pilot work demonstrated these scanner settings were the minimum required to achieve a workable image quality, scan times <60 min were not possible. The total number of image projections collected for individual columns at each sampling date was 898 with a total file size for each sample of *c.* 5 GB. Although the total height of each column was 91 mm, a limitation of this scanner meant that only a *c.* 20 mm deep section could be scanned in one scan

period. The columns were positioned so that scanning took place just below the seed to ensure the primary roots were sampled. After the final scan, the roots were washed from the soil and analysed using WinRHIZO® 2005c scanning equipment and software with root volume, with root surface area, with root length and with root diameter being measured. The images obtained were compared with the output from the X-ray micro-CT work.

Image processing and analysis

As the study was concerned with the impact and interaction between roots and soil, the soil pore characteristics were also measured in addition to root measurements. The original grey-level X-ray CT images were processed using ImageJ 1.42 software (http://rsbweb.nih.gov/ij/) after cropping to exclude the area outside the soil column. The resulting image size was 25.11×25.11 mm (1,116×1,116 pixels). A uniform contrast enhancement was applied to normalise all slices and reduce the effect of large differences in pixel grey-level of the same particle in neighbouring slices. This unwanted effect, known as beam hardening, occurs because the low energy (strongly attenuated) photons of a polychromatic beam are emitted with a higher velocity than the high energy (weakly attenuated) photons (Wildenschild et al. 2002). The proportion of low energy photons absorbed is therefore directly related to the X-ray path, giving an apparently higher attenuation at the boundaries of objects, as the beam passes from dense material to less dense material. To separate pores from the solid matrix, ImageJ automatic threshold algorithms were used. Different threshold algorithms were required for high and low density soil samples because a global threshold could not successfully be applied to all images due to significant differences in the greyscale values created by the different soil bulk densities. For high density soils, the *Maximum Entropy* Threshold (Sahoo et al. 1988) was used, while the *Otsu* threshold (Otsu 1979) was used for low density soil samples. It is preferable when examining large image datasets to use the same thresholding techniques to facilitate comparison between treatments. However, in this instance, the compaction treatments meant that there were significant differences in greyscale values between high and low density soil, rendering a global threshold unsuitable. To confirm this approach, a subset of images from both high and low density soils were compared with manual thresholding techniques, which confirmed the improved accuracy of the two automatic threshold algorithms when images are very different. The resulting binary images were analysed, using the *Analyze Particles* tool to provide information for total porosity, mean pore size and the coefficient of uniformity (psd_{cu}) i.e. the ratio of large to small pores (d_{60th} percentile/d_{10th} percentile) (Kézdi 1974), for each individual image (i.e. 898 per soil column). Pore spaces <5 pixels were excluded from the analysis and care was taken to ensure that no root material was classified as pore space during soil porosity analysis.

Root systems were non-destructively extracted from the greyscale CT images using the root tracking software RootViz3D® (http://www.rootviz3d.org/). This software assigns a probability function (based on a Gaussian distribution) to determine whether specific pixels within images represent root material. Grey values >100 for three phases namely, air/water filled pores, root material and the soil matrix were collected as separate input files. This was undertaken using ImageJ 1.42 software before processing with the RootViz3D® algorithm on the greyscale CT images. Probability density functions are automatically constructed by RootViz3D® for each of the three phases based on a Gaussian distribution. The root is traced from a starting point selected at the top of the root system. The software compares each pixel below this point with the greyscale values for the three phases and records the location of all those that are most similar to a root. Known points on the root architecture, where there are obvious branches in the root, are then compared. At this stage, if there are differences between the diameters, additional data points can be added around the branch, usually to add either more material on the root or soil. The program is re-run and the process repeated until the match is at a minimum. The final RootViz3D® outputs of the root architecture were analysed using Avizo® Fire 6.1 software. A semi-automated segmentation of the X-ray CT images based on erosion and dilation processes was also carried out in VG StudioMAX® 2.0 software using the *Region Growing* selection tool with no filtering, for comparison with the RootViz3D® outputs.

The results were analysed using a three-way general analysis of variance (ANOVA) conducted

using Genstat 12.1 and each column was analysed individually. The effect of compaction on the three cultivars was also evaluated using a three-way ANOVA containing cultivar, compaction and time and all possible interactions as explanatory variables. Normality was tested for by interpreting the plots of residuals and homogeneity of variance (homoscedasticity) by assessing the plot of residuals against fitted values. In each case the data were distributed normally, satisfying the assumptions underlying the general analysis of variance.

Results

Method validation

The ability of RootViz3D® to segment root voxels is shown by a selection of example plants (Fig. 1). From the RootViz3D® generated images, root growth in the three cultivars grown in the different soil bulk densities can be visualised and quantified over the experimental time period. The RootViz3D® images were compared with the semi-automated root segmentation and with the measurements collected from WinRHIZO®. We acknowledge that comparison of RootViz3D® images with 2D images obtained by WinRHIZO® software means that a 3D representation of an object is compared to a 2D object i.e. not the same. The semi-automated segmented images (Fig. 2a, c, e) confirmed the accuracy of the RootViz3D® software, as similar images of root architecture were provided by both approaches (Fig. 2a–f). The root volumes obtained using the semi-automated segmentation approach and the RootViz3D® software had a good correlation (r=0.82; Fig. 3), although the latter were consistently greater than those provided by the semi-automated segmentation technique performed using VGStudioMAX® 2.0.

The 2-D images provided by the WinRHIZO® software (Fig. 4) were useful for verification of the 3-D root outputs from RootViz3D® but, as the former approach is destructive i.e. it involves washing soil from roots, the roots could only be analysed after the final X-ray CT scan had been completed. It should also be noted that the WinRHIZO® images were of the entire

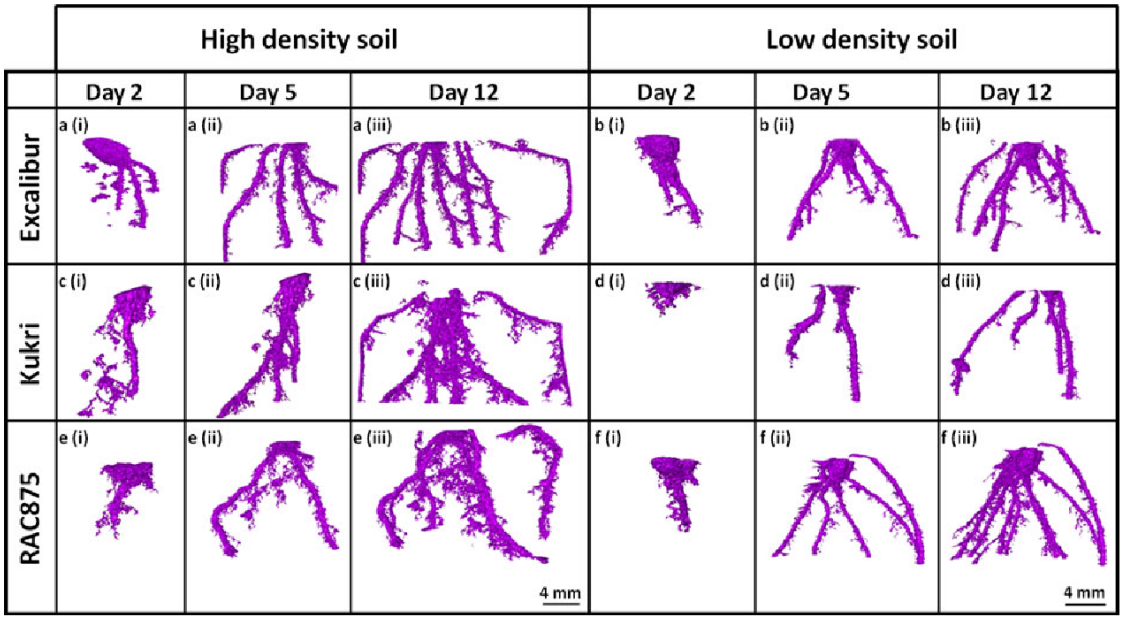

Fig. 1 RootViz3D® images of example root systems at 2, 5 and 12 DAG grown in high density soil; Excalibur **a** (i–iii), Kukri **c** (i–iii) and RAC875 **e** (i–iii) and low density soil; Excalibur **b** (i–iii), Kukri **d** (i–iii) and RAC875 **f** (i–iii). Scale bar represents 4 mm

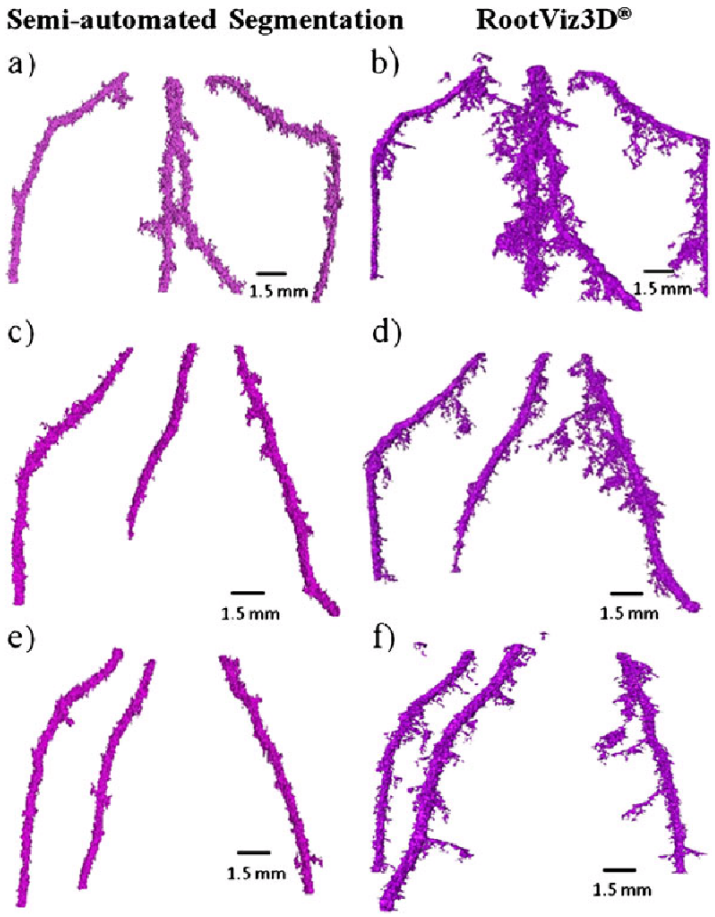

Fig. 2 Semi-automated segmented and RootViz3D® images of the root systems of 12 day old plants of (**a**, **b**) cv. Kukri grown in high bulk density soil (1.5 g cm^{-3}), (**c**, **d**) cv. Kukri grown in low bulk density soil (1.1 g cm^{-3}) and (**e**, **f**) cv. Excalibur grown in low bulk density soil (1.1 g cm^{-3}). Scale bar represents 1.5 mm

root system, whereas the X-ray CT images were limited to a smaller section, and hence do not represent the whole root system. To enable a detailed comparison of RootViz3D® outputs with the WinRHIZO® images, the latter were cropped to approximately represent the same size. The correlation between the root volumes obtained using WinRHIZO® (mean = 98.4 mm^3; standard error of mean, SEM = 11.9) and RootViz3D® (mean = 46.0 mm^3; SEM = 3.9) shown in Fig. 5 was not significant (r =0.53; Fig. 5); possible explanations are discussed below.

Root architecture analysis

The RootViz3D® images give further insights into 3-D root growth within undisturbed soil. For example, Fig. 4 shows RootViz3D® and WinRHIZO® images for cv. RAC875 at both low (Fig. 4a–b) and high (Fig. 4c–d) soil bulk density (1.1 and 1.5 g cm^{-3}). The roots produced at low bulk density were smaller in diameter (*c.* 0.65 mm) (P=0.006), and it was observed that they grew in relatively straight lines and had fewer root hairs than those produced at high bulk density. The latter were thicker (*c.* 0.73 mm) and followed a more convoluted growth path. The WinRHIZO® analysis also revealed a tendency towards lower mean total root length in the compacted treatments, although this not significant (P= 0.20). Root length was 98.4 mm in uncompacted soil (Fig. 6), compared to 85.2 mm at high bulk density (13% decrease). The difference in root length between cultivars was not significant (P=0.20) even though cv.

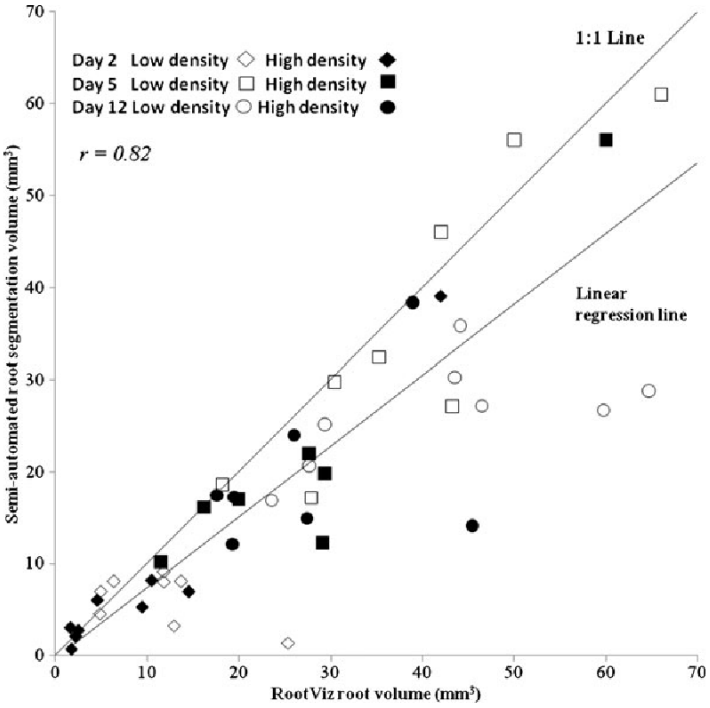

Fig. 3 Correlation between total root volumes estimated using the semi-automated segmentation and RootViz3D® approaches 2, 5 and 12 DAG in low and high bulk density soil (1.1 and 1.5 g cm^{-3} respectively). The linear regression and 1:1 *lines* are shown

Excalibur had an average length of 105 mm across both soil densities, whereas the average length for cvs. Kukri and RAC 875 was identical at 84 mm. However, the interaction between cultivar and soil density was significant (P=0.015), since cvs. Excalibur and RAC875 exhibited the largest difference in

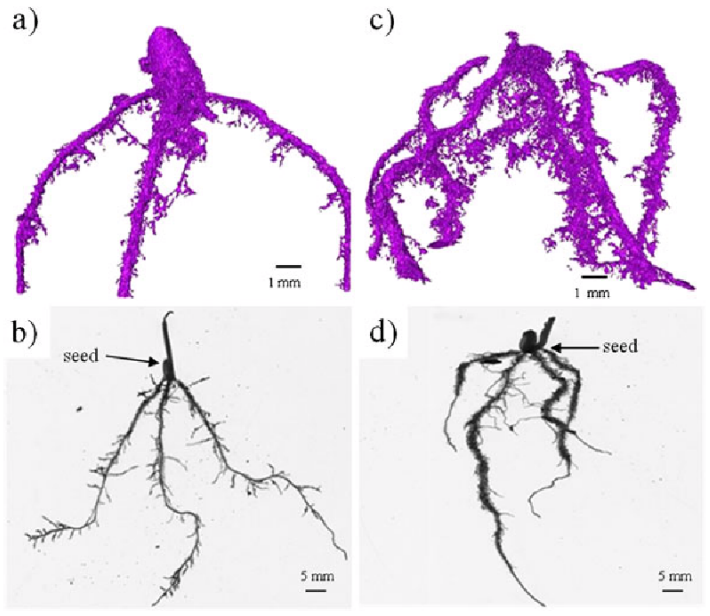

Fig. 4 RootViz3D® images of the roots of 12 day old cv. RAC875 plants grown on (**a**) low bulk density soil (1.1 g cm^{-3}) and (**c**) high bulk density soil (1.5 g cm^{-3}); (**b**, **d**) root systems of the same plants after extraction and analysis using the WinRHIZO® equipment. Scale bars represent 1 mm and 5 mm

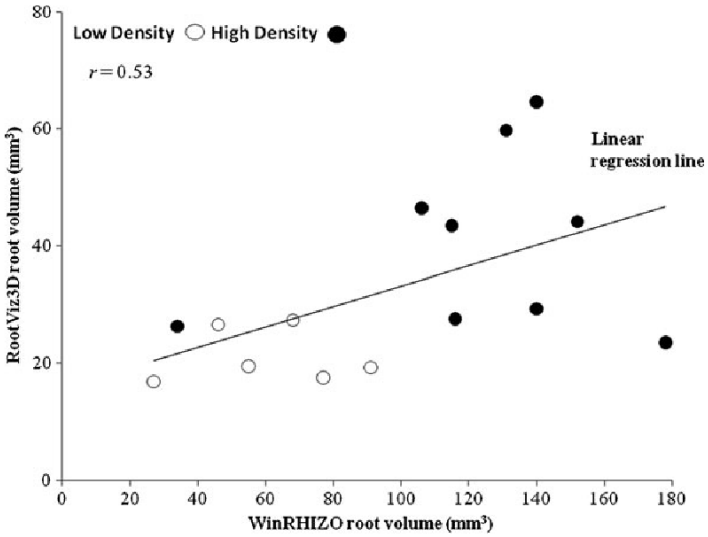

Fig. 5 Correlation between total root volumes at day 12 measured destructively using the WinRHIZO® technique and estimated using the RootViz3D® software for plants grown on low or high bulk density soil (1.1 and 1.5 g cm^{-3} respectively). The 1:1 *line* is shown

root length between bulk density treatments, with increases in total root length in the low density treatment of 51% and 12%, respectively.

Mean total root volume for all cultivars calculated from the RootViz3D® images was substantially greater at high bulk density than at low bulk density (27.9 (SEM = 3.8) vs. 17.3 (SEM = 3.0) mm^3; P= 0.04). Mean root volume was greater at high bulk density in all cultivars at each sampling date (Fig. 7). No significant difference in treatment effects was found between cultivars or for the interactions between cultivar, treatment and time. Roots in the low density treatment of cv. Excalibur appeared to stop growing after Day 5, as root volume was 22.2 mm^3 at Day 5 and 18.8 mm^3 at Day 12 (Fig. 7b). The WinRHIZO® analysis also showed that the mean total root volume for all cultivars measured destructively at day 12 was significantly (P =0.032) greater at high bulk density (335 mm^3; SEM = 4.0) than at low bulk density (303 mm^3; SEM = 24.5). These values are much greater than the scanned RootViz3D® images, as the scanned section was *c.* 20 mm of the total soil column. The cropped 2-D images coinciding with the scanned area were also analysed using WinRHIZO® and showed a significant (P=0.007) difference in root volume. The high bulk density treatment images had a greater mean volume than the low bulk density images (124 mm^3 (SEM = 13.3) vs. 74 mm^3 (SEM = 12.6)). The difference between cultivars was also significant (P=0.006), as cvs. Excalibur and Kukri had overall greater root volume in high density soil, whereas cv. RAC875 had similar root volumes in high and low density soil.

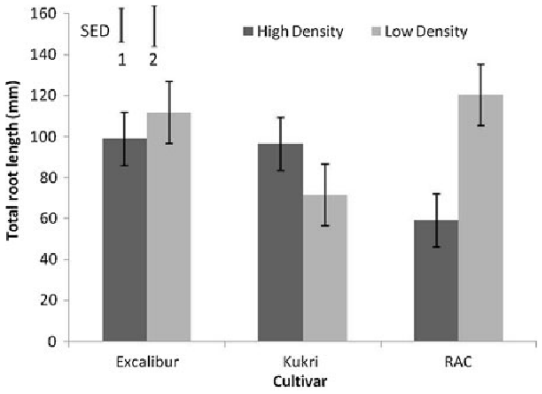

Fig. 6 Mean total root lengths in the low and high bulk density soil columns (1.1 and 1.5 g cm^{-3}). *Error bars* show double standard errors of the mean. *Vertical bars* show standard errors of the difference (SED) between treatment means for comparing differences between (1) bulk density treatments and (2) cultivars. ANOVA: P=0.20 (bulk density) P=0.15 (cultivar)

Soil porosity characteristics

Mean soil porosity (i.e. mean of classified pore space of 898 individual image slices per column for the given resolution) for all cultivars was greater at low bulk density (38%; SEM = 1.1) than at high bulk density (27%; SEM = 0.48) for all sampling dates (P<0.001;

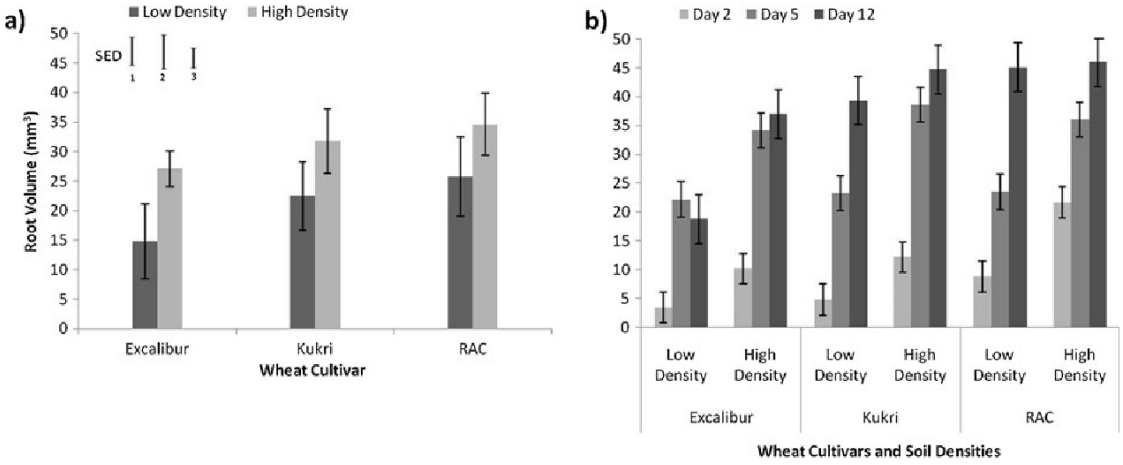

Fig. 7 Mean total root volumes derived from X-ray CT images for (**a**) plants of three wheat cultivars grown at low and high soil bulk densities (1.1 and 1.5 g cm^{-3}) and (**b**) 2, 5 and 12 DAG for plants of cvs. Excalibur, Kukri and RAC875 grown at low (LD) and high (HD) bulk density. Double standard errors of the mean are shown. *Vertical bars* show standard errors of the difference (SED) between treatment means for comparing differences between (1) bulk density treatments, (2) cultivars and (3) time. ANOVA: P=0.04 (bulk density) P=0.258 (cultivar) P=<0.001 (time)

Fig. 8a), and increased with time (P<0.001). This increase was much greater at low bulk density than at high bulk density (Fig. 8a). Thus, mean soil porosity in the low density treatment increased from 34% at Day 2 to 38% at Day 5 and 44% at Day 12; the corresponding increase in the high density treatment was from 26% at Day 2 to 28% at Day 12. Soil porosity showed no significant variation with depth in either of the compaction treatments (Fig. 8b & c). There was a significant difference in the interaction

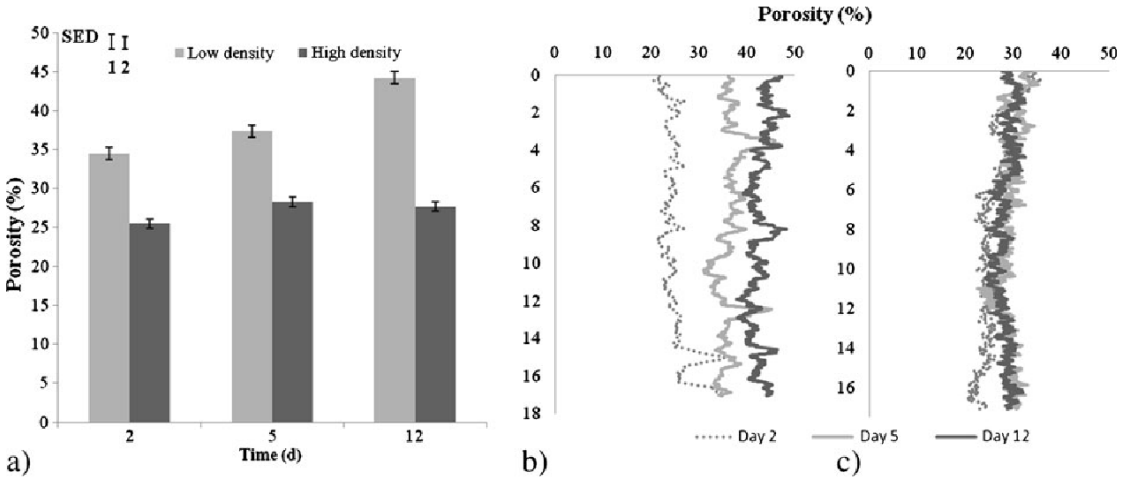

Fig. 8 Changes in mean soil porosity in the low and high bulk density treatments (1.1 and 1.5 g cm^{-3}) during the scanning period: (**a**) mean values for all cultivars 2, 5 and 12 DAG, and depth profiles in (**b**) low and (**c**) high bulk density columns containing an Excalibur plant. Double standard errors of the mean are shown in (**a**); *vertical bars* show standard errors of the difference (SED) between treatment means for comparing differences between (1) bulk density treatments and (2) time. ANOVA: P=<0.001 (bulk density) P=<0.001 (time)

between soil porosity and the different soil bulk densities at the different time intervals (P=0.003). No significant difference was found between cultivars.

Mean pore size was also greater in low bulk density soil (0.26 mm^2; SEM = 0.028) than in high bulk density (0.10 mm^2; SEM = 0.003) soil for all sampling dates (P<0.001), and increased significantly with time in low bulk density soil (P<0.001) but not in high bulk density soil, for which there was no significant change. The bulk density*time interaction was therefore significant (P<0.001). The increase in pore size in low bulk density soil was greatest between Days 5 and 12 as the mean pore diameter increased from 0.16 mm at Day 2 to 0.22 mm at Day 5 and 0.39 mm at Day 12. There was no significant difference between cultivars in terms of their influence on pore size.

The pore size distribution differed between the treatments as no large pores e.g. > −0.5 $\log^{10}mm^2$ were present in high bulk density soil, whereas, numerous large pores and few small pores were apparent in low bulk density (Fig. 9). The range of pore sizes was greater in low bulk density soil, as indicated by the greater number of pore size categories. However, the coefficient of uniformity (PSD_{cu}) does not support this conclusion as the mean value was much greater at high bulk density at than at low bulk density (2.20 vs. 1.55; P=0.016), suggesting that the range of pore sizes was greater at high bulk density. There was a significant difference between cultivars (P<0.001), as the mean coefficient of uniformity (PSD_{cu}) for columns containing cv. RAC875 was 2.69, compared to 1.47 and 1.46 respectively for cvs. Excalibur and Kukri. The bulk density*cultivar interaction was therefore significant (P=0.004).

Discussion

RootViz3D® systematically extracted greater root volumes from the X-ray CT images than the semi-automated segmentation technique (Fig. 3); we contend that this is because RootViz3D® was more successful at identifying pixels containing root material. The discontinuities apparent in the semi-automated segmented image shown in Fig. 2a provide evidence that this approach was unable to detect the entire root length and also failed to segment some lateral branching roots. By contrast, the RootViz3D® image (Fig. 2b) more fully reveals root architecture, clearly demonstrating the effectiveness of the probability algorithm at identifying root pixels from their grey scale (attenuation) value than the semi-automated segmentation tools routinely available in most image analysis software packages.

The X-ray CT images showed that root volume was greater in high bulk density soil than in low bulk density soil (P=0.04), which was confirmed by the destructive WinRHIZO® analysis performed at the end of the experimental period (P=0.05). This observation supports our conclusion that RootViz3D® does not preferentially segment greater quantities of root mass from images of high bulk density soil columns. The relatively poor correlation between the 2D WinRHIZO® and the RootViz3D® images could be explained by the difference in dimensions between the two imaging approaches (3D vs. 2D), because root morphology will be different in the 3D and 2D visualisations. Cropping a 2D section for comparison with the 3D images will introduce error, whereas the 2D images lack the architectural information that can be gained in 3D. The root volumes obtained by WinRHIZO® analysis were much greater than the volumes obtained from the X-ray CT images (Fig. 4; *c.* 100%). This difference could be attributed to the WinRHIZO® technique as this visualises root systems that have been physically removed from the soil matrix, so the contrast between air and root material was much greater, allowing the finest classes of roots to be more easily distinguished. Due to image resolution limitations and the poorer contrast and density differences between root material and soil material in the X-ray CT images, a reduced amount of root material was extracted from these X-ray CT images, using both RootViz3D® and semi-automated root segmentation. The amount of water held in the soil also influences the contrast between root and soil material, which could have introduced further difficulties in extracting the full root system from the greyscale X-ray CT images, compared to the WinRHIZO® technique.

Destructive root washing usually leads to some roots not being accounted for, as it would be near impossible to remove the entire root system and its fine lateral roots from the soil. It is likely that the roots were more difficult to extract from compacted soil by root washing, possibly because of increased

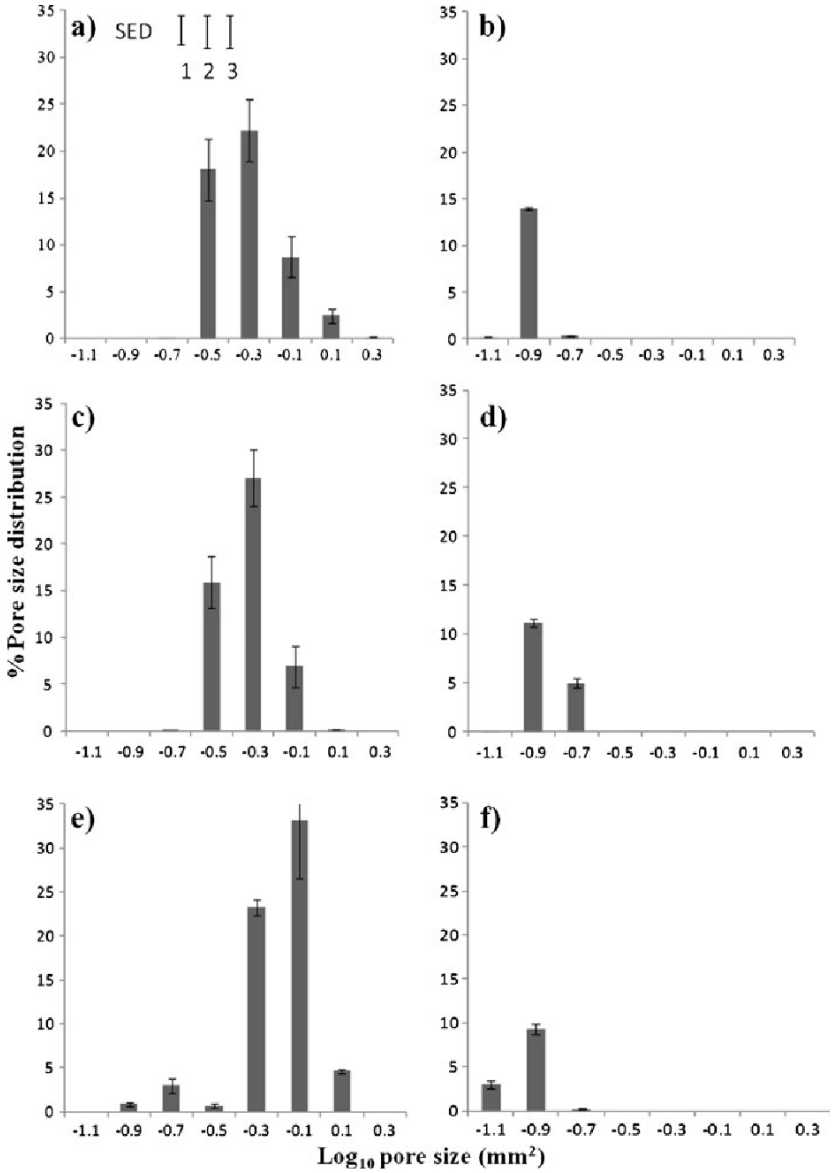

Fig. 9 Soil pore size distribution expressed as a percentage of the image area on Day 12 after germination for plants of cvs. Excalibur (**a**, **b**), Kukri (**c**, **d**) and RAC875 (**e**, **f**) grown on low (**a**, **c**, **e**) or high bulk density soil (**b**, **d**, **f**; 1.1 and 1.5 g cm^{-3} respectively). Double standard errors of the mean are shown. *Vertical bars* show SED values for comparing differences between (1) soil bulk densities, (2) cultivars and (3) time. ANOVA: P=0.016 (bulk density) P=<0.001 (cultivar) P=0.989 (time)

production of root exudates (Boeuf-Tremblay et al. 1995) or increased numbers of root hairs induced by accumulation of ethylene (Crossett and Campbell 1975). Destructive techniques also destroy the soil structure and do not give any information about how the roots are arranged architecturally in the 3D soil matrix, which is heterogeneous temporally and spatially. Yet, the WinRHIZO® technique remains a useful tool for the verification of root systems with the corresponding X-ray CT images.

The CT images enabled the root system architecture of the three cultivars to be recorded non-destructively and repeatedly. The mean root volume for plants of cv. RAC875 grown at high bulk density was much greater than at low density at Day 2, but this treatment effect diminished by Day 12, suggesting that the roots of this cultivar grew more rapidly in the low density treatment after Day 2 to produce a root volume similar to the high density treatment by Day 12. Varietal differences in agronomic traits may

explain why root volume increased progressively during the observation period in cv. RAC875 but showed little change in cv. Excalibur after Day 5.

Our findings substantiate previous studies which show that moderate compaction of some soil types may be advantageous for roots, suggesting the existence of an optimum degree of compaction for root growth (Bouwman and Arts 2000; Hamza and Anderson 2005). Mulholland et al. (1996) and Atkinson et al. (2009) showed that root and shoot growth were greatest in moderately compacted soil, possibly due to greater water retention and improved contact between roots and soil. Excessively loose soil may reduce crop yields, perhaps because roots preferentially grow through large pores, with the result that sufficient contact between roots and soil for effective extraction of water and nutrients is not achieved (Passioura 2002); the dominance of larger pore size classes at a low soil bulk density is clearly demonstrated by the pore size distribution data shown in Fig. 9. An intermediate level of soil compaction may therefore be beneficial as root-soil contact is limited when the soil is loose (bulk density of 1.1 g cm^{-3}), with the result that root length and surface area are reduced, preventing plants from extracting sufficient resources to sustain optimal growth.

An alternative explanation may be that the initial response of roots encountering compacted soil is an increase in radial expansion (Bengough et al. 2006), resulting in shorter, thicker roots. Croser et al. (2000) reported that the cortical cells of roots grown in compacted soil were shorter and wider than in roots grown in loose soil, while Materechera et al. (1991) concluded the root diameter of seedlings grown in compacted soil increased two-fold due to the increased diameter of cortical cells and, occasionally, the presence of additional cell layers (De Kroon and Visser 2003). The increased radial expansion of roots in high bulk density soil may also explain the observed increase in root volume. This conclusion is supported by the observed reduction in total root length and increase in root diameter at high bulk density (P=0.006).

The increase in total soil porosity (i.e. between image slices) with time in the low bulk density treatment (P<0.001; Fig. 8), suggests that the production of roots physically altered soil structure and caused the soil particles to aggregate; this effect became increasingly pronounced over time and as the quantity of root material present increased. The presence of roots increases aggregation of soil particles and porosity following exudation of chemical compounds. Repeated cycles of wetting and drying and the presence of root exudates alter soil structural properties and increase porosity over time (Czarnes et al. 2000). It has also been suggested that exudates may stabilise soil structure in the rhizosphere by increasing the strength of bonds between soil particles, thereby aiding aggregation, whilst also decreasing the wetting rate (Czarnes et al. 2000). However, it has also been proposed that the influence of mucilage on soil hydraulic properties is related to soil organic matter content and not root-derived mucilage (Whalley et al. 2004). However, an increase in soil porosity with time was not found in compacted soil, which contained a greater quantity of root material. Root exudation is also thought to increase when wheat experiences compaction stress (Brady and Weil 1999).

Although total root volume was greater in high bulk density soil, exploration of the available soil volume was reduced relative to the low bulk density treatment, as shown by a reduced total root surface area (18 mm^2 at high density vs. 20 mm^2 at low density). The decrease in total root length and associated increase in root diameter resulted from the increased radial expansion induced by the greater soil resistance to root growth. These findings are supported by the validation checks of the RootViz3D® output shown in Figs. 3 and 5. We conclude that the greater root volume at high bulk density is a direct consequence of a combination of the thicker, but shorter, roots resulting from the increased radial expansion at the root tip (Bengough et al. 2006), increased water retention associated with the greater number of smaller pore size classes (Fig. 9) and improved root-soil contact which would have ensured adequate supplies of water and nutrients to support plant growth.

The combination of X-ray CT scanning and RootViz3D® root tracking approach provides a greater insight into how roots interact with the surrounding soil than previous methods. With X-ray CT becoming more widely available and associated advances in scanning speeds, it is now possible to visualise roots non-invasively, repeatedly within the 3-D soil matrix and establish how root architecture is affected by soil conditions, both spatially and

temporally. As current CT approaches are sufficiently refined to identify varietal differences in root distribution in space and time, this technology will aid the development of models of root functional architecture which will be critical to the formation of sustainable cropping systems (Pierret et al. 2007). This study has demonstrated that imaging approaches based on root tracking are better at segmenting root pixels in CT images than techniques based on global thresholding, which are subjective and potentially open to operator bias. RootViz3D® can be used to provide a dynamic characterisation of the responses of different cultivars of individual species to variation in soil physical conditions such as compaction.

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soil physical properties individually and at specific time points, as their importance changes as the root system becomes established.

Keywords: *Solanum lycopersicum*; root system architecture; soil compaction; soil texture; X-ray micro-Computed Tomography (µCT); root washing

1. Introduction

Soil compaction is an increase in the number of soil particles per unit volume, leading to increasing bulk density and penetration resistance to growing roots and reducing soil porosity (Batey and McKenzie, 2006). Pore size distribution is significantly affected by compaction as the larger macropores in particular are compressed, increasing the proportion of smaller pore sizes (Marshall and Holmes, 1988), thereby reducing the movement of water and air through the soil profile. These major effects on soil physical properties can also inhibit root growth, reducing the growth and yield of both annual and perennial species (Unger and Kaspar, 1994). Yet, our understanding of root system development in heterogeneous soil remains fragmented.

The relative proportions of sand, silt and clay particles, also known as soil texture, has a major role in determining the extent to which specific soils become compacted and the possible effects on root growth (Alameda and Villar, 2012). Soils with different textures have provided contrasting results in previous studies; for example, when the coarser grain particles in sandy soils are compacted they are often positioned in rigid point-to-point contact, with the result that they cannot easily move to accommodate growing roots (Batey and McKenzie, 2006). Soils composed predominantly of finer particles such as clay and silt can pack more closely within a defined volume, however a large overall volumes of pore space remains to allow growing roots to extend more easily (White, 2006). This contrast in soil mechanics means that comparison of the results of studies using different soil textures may not provide a clear understanding of the response of roots to soil compaction. Few

recent studies of the effects of compaction have examined soils of differing texture within the same experiment (Gregorich *et al.,* 2011; Alameda and Villar, 2012; Chen and Weil, 2012; Gao *et al.,* 2012). Studies that consider a continuous range of bulk densities may provide new information as most previous research has typically considered only a discrete and widely spaced range of bulk densities. Some researchers have noted a bell-shaped response of root growth to compaction, with growth being reduced at the lowest and highest bulk densities (Arvidesson, 1999; Alameda and Villar, 2009; Atkinson *et al.,* 2009a). The observation that moderate bulk densities (1.3 – 1.5 Mg m^{-3}) enhance root growth is supported by other workers (Bouwman and Arts 2000; Hamza and Anderson 2005; Tracy *et al.,* 2012a) and this effect is more pronounced in nutrient-rich loamy soils. Arvidsson (1999) attributed this positive effect to a greater nutrient concentration per unit volume of soil and suggested that improved root-soil contact enhances nutrient uptake, a view supported by an increase in leaf N concentration in work by Alameda and Villar, (2009). However, in sandy soils, increases in bulk density decrease root growth and alter root morphology, possibly due to the absence of any increase in nutrient availability following compaction due to nitrate leaching from the coarser-grained soils in high precipitation areas (Lynch and Brown, 2012).

Bulk density varies within individual soil profiles due to the pressure exerted by farm machinery, soil management practices, overburden pressure and biological activity (Whalley *et al.,* 1995); this heterogeneity and other environmental constraints induce markedly different variations in root system architecture (RSA) within individual species (Hodge *et al.,* 2009). However, few studies have examined effects on elongation rate when roots encounter areas of differing soil strength (Goss

and Russell 1980; Bengough and Young, 1993; Bengough *et al.*, 1994). Roots grown in compacted soil exhibit a more tortuous root path (Konôpka *et al.,* 2009; Tracy *et al*., 2012b) and branching may differ from the usual herringbone pattern (Becel *et al.*, 2012). The increased forces imposed on root systems growing in compacted soil reduces the size and increases the irregularity of root distribution, resulting in a smaller volume of soil being exploited (Grzesiak *et al.,* 2012), requiring water and nutrients to travel greater distances to reach the nearest root (Tardieu, 1994).

Phenotypying protocols are required to understand the 'root phenes' controlling RSA, which are related to resource uptake (Lynch and Brown, 2012). Several techniques can be employed when measuring roots (Mancuso, 2012); the two main techniques employed in the present study were root washing followed by WinRHIZO® scanning, which has the advantage of being rapid so numerous samples can be analysed, and X-ray micro-Computed Tomography (µCT), which provides repeated, non-destructive three-dimensional (3-D) images of RSA *in situ* (Mooney *et al.,* 2012), albeit with a reduced sample throughput. The aim of this study was to explore the response of root systems to differences in soil type and bulk density and the effect of their interaction. The objectives were to: (1) test the hypothesis that intermediate bulk densities are optimum for root growth; (2) dissect the influence of bulk density at different soil textures on root growth and function; and (3) visualise and quantify root elongation in soil containing layered bulk density treatments to gain a greater understanding of root behaviour at bulk density boundaries mimicking contrasting soil horizons.

2. Materials and methods

2.1 Sample preparation

Soil was obtained from the University of Nottingham experimental farm at Bunny, Nottinghamshire, UK (52.52 ° N, 1.07 ° W). A Newport series loamy sand (brown soil) and a Worcester series clay loam (argillic pelosol) were air-dried and sieved to <2 mm. Soil was packed to various bulk densities (listed below) into plastic columns (70 mm height x 30 mm diameter).

Destructive root architecture experiment: Columns were uniformly packed to provide bulk densities of 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} with 12 replicates per treatment for both soil types, giving a total of 120 columns. The columns were packed with air-dry soil in *c*. 1 cm deep layers. After compacting each layer, the surface was lightly scarified to ensure homogeneous packing and hydraulic continuity within the column (Lewis and Sjöstrom, 2010).

X-ray Computed Tomography (CT) boundary experiment: Columns were packed to provide a compacted layer (1.6 Mg m^{-3}) over an uncompacted layer (1.2 Mg m^{-3}) (C/UC) or an uncompacted layer (1.2 Mg m^{-3}) over a compacted layer (1.6 Mg m^{-3}) (UC/C), with three replicates of each treatment, giving a total of 12 columns. To achieve the treatment that had a compacted layer over an uncompacted layer (C/UC), columns were prepared with the compacted layer at the base of the column (due to the greater force required to compact the mass of soil) and then gently turned over,

so that the compacted layer was at the top of the column. Two glass beads were placed either side of the column as reference objects for use during image analysis.

After preparation, all columns in both experiments were wetted thoroughly and placed in a growth room with day/night temperatures of 28/22 °C and a 12 h photoperiod; PPFD was 226 µm m^{-2} s^{-1}. Seeds of tomato (*Solanum lycopersicum.* L) *cv*. Ailsa Craig were imbibed for 48 h before being planted 5 mm below the soil surface. The columns were initially kept inside a transparent propagator to maintain high humidity and were weighed daily and sufficient water added to ensure soil moisture content remained near field capacity throughout the experiment.

2.2 Root scanning and image analysis

Destructive root architecture experiment: Roots were washed from the soil and analysed three days after transplanting (DAT) and at 10 DAT; six replicates per treatment were analysed on both dates. The washed roots were analysed using WinRHIZO® 2002c scanning equipment and software to determine root volume, surface area, length and diameter. Plant dry weight was measured as described by Rowell (1996) after being dried at 100 °C for 40 h.

X-ray CT Boundary experiment: Columns were scanned daily for 10 DAT using a Phoenix Nanotom® (GE Measurement & Control Solutions) X-ray µ-CT scanner set at 110 kV and 180 µA, with a 0.1 mm copper filter and an image averaging of 1. Pixel/voxel resolution was 24 µm and each scan took 20 min to complete. The total number of image projections collected for individual columns at each sampling date

was 1200 with a file size of *c*. 15 GB. The columns were positioned so that scanning occurred just below the seed to ensure the primary roots were sampled. The columns were initially scanned in a randomised order during the photoperiod; scan order and the timing of each scan were subsequently kept constant so that the columns were scanned at 24 h intervals to ensure all treatment combinations were equally exposed to any diurnal variation in root growth and avoid systematic error.

Root systems were non-destructively extracted by segmentation from the greyscale µCT images using the *Region Growing* selection tool in VG StudioMAX® 2.1 software. The root system models segmented from the µCT image data were used for quantitative determination of root length, volume, surface area, mean diameter and maximum rooting depth. Root length was expressed in two ways, vertical length (i.e. maximum rooting depth) and primary root length determined using the *Polyline* tool in VGStudioMAX® 2.1. Tortuosity of the root path (the ratio of actual path length compared to the shortest possible path) was measured by comparing the length of the primary root to the vertical depth of the root system. The elongation rate of the primary root was recorded daily.

2.3 Statistical analysis

The results were analysed by three-way general analysis of variance (ANOVA) containing soil type, bulk density, time and all possible interactions as explanatory variables using Genstat 13.1. Each column was analysed individually, with measurement date set as a polynomial contrast. The effect of treatments on root parameters was tested by including polynomial contrasts fitted to the day factor in

the analysis; significant treatment*linear interactions indicate that the specific root characteristic being analysed differed among treatments. This approach facilitated the testing of whether the linear component of changes with time differed between treatments to a greater extent than expected from the residual variation, instead of simply considering the overall effect of time. Normality was tested by interpreting the plots of residuals and homogeneity of variance (homoscedasticity) by assessing the plot of residuals against fitted values. In each case, the data were distributed normally, satisfying the assumptions underlying the general analysis of variance.

3. Results

Destructive root architecture experiment: The effect of soil type and bulk density on root volume differed between treatments as values were greatest at high bulk density in the clay loam but decreased with increasing bulk density in the loamy sand, particularly at 10 DAT (Fig. 1A, B; $P<0.001$). The bulk density*soil type interaction was significant for both time points because root volume differed between soil types (Fig. 1A, B; $P<0.001$); mean root volume for both time points was substantially greater in the clay loam than in the loamy sand (101 *vs.* 44 mm^3). At 10 DAT, root volumes were greater in the clay loam in the 1.4, 1.5 and 1.6 Mg m^{-3} treatments ($P<0.001$). At 3 DAT, mean values for the loamy sand and clay loam soils were 16 and 21 mm^3 respectively (Fig. 1A) compared to 73 and 179 mm^3 at 10 DAT (Fig. 1B). Root volumes in the clay loam soil were greatest at the two highest bulk densities, 46 and 30 mm^3 respectively in the 1.5 and 1.6 Mg m^{-3} treatments at 3 DAT (Fig. 1A) and 227 and 276 mm^3 at 10 DAT (Fig. 1B). The effect of bulk density on root volume was significant at 3 DAT ($P<0.001$) but not at 10 DAT. This pattern was seen for several rooting characteristics, as described below. At 3 DAT, mean root volumes in clay loam were 12, 11, 8, 46 and 30 mm^3 at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively compared to 14, 24, 12, 19 and 11 mm^3 respectively in the loamy sand. The bulk density*soil type*time interaction was significant ($P<0.001$), highlighting the changes in the influence of bulk density and soil type between 3 and 10 DAT.

Unsurprisingly, the patterns for root surface area (Figs. 1C, D) closely matched those for root volume as the effect of bulk density was significant at 3 DAT ($P<0.001$) but not at 10 DAT. At 3 DAT, mean surface area in the clay loam was 97, 97, 77, 277 and 209 mm^2 at bulk densities of 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively compared to 104, 161, 150, 137 and 107 mm^2 in the loamy sand. Root surface area was greatest at the higher bulk densities in the clay loam but no trend was apparent for the loamy sand, although the values tended to be greatest at intermediate bulk densities (1.3 and 1.4 Mg m^{-3}). Surface area was greater in the clay loam than in the loamy sand at 10 DAT ($P<0.001$), but not at 3 DAT. Mean values were respectively 151 and 132 mm^2 for the clay loam and loamy sand at 3 DAT compared to 768 and 345 mm^2 respectively at 10 DAT. The bulk density*soil type interaction was significant at both time points ($P<0.001$) due to the different responses to bulk density shown by roots grown in the different soil types. The bulk density*soil type*time interaction was also significant ($P<0.01$), again emphasising the differing interactions between bulk density and soil type at 3 and 10 DAT.

The impact of bulk density on root diameter was significant at 3 DAT (Figs. 1E, F; $P<0.001$) but not at 10 DAT. Root diameter increased slightly with increasing compaction, although this response was more pronounced in the clay loam, although the mean values were reduced at 1.4 Mg m^{-3}. At 3 DAT, mean root diameter in the clay loam was 0.48, 0.43, 0.42, 0.67 and 0.59 mm at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively, compared to 0.52, 0.57, 0.32, 0.54 and 0.42 mm^2 respectively in the loamy sand. Mean root diameters were 0.52 and 0.48 mm in the clay loam and loamy sand at 3 DAT, compared to 0.87 and 0.82 mm at 10 DAT. The bulk density*soil type interaction was significant ($P<0.001$) as root diameter did not show

a uniform response across all treatment combinations. Figure 2 clearly illustrates the influence of bulk density on root diameter as the primary root became progressively thicker as bulk density increased, particularly in the loamy sand (Fig. 2B). Other deleterious effects of increasing bulk density included reductions in the number and length of lateral roots, inducing poorer root architectures. The detrimental response to increasing bulk density was more apparent in the loamy sand, in which mean particle size was greater.

The influence of bulk density on the total root system length was significant at 3 DAT (Fig. 3A; $P<0.001$) but not at 10 DAT. The mean values at 3 DAT were 6, 7, 6, 13 and 12 cm at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively in the clay loam, compared to 6, 9, 15, 8 and 8 cm respectively in the sandy loam. The difference in total root length between soil types at the higher bulk densities was much greater at 10 DAT than at 3 DAT (Fig. 3A). The influence of soil type on total root length was significant at day 10 DAT ($P<0.001$) but not at 3 DAT. Total root length was greater in the clay loam than in the loamy sand for all bulk densities at 10 DAT, when the mean values for the former were 29, 21, 27, 31 and 31 cm at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively, whereas the corresponding values for the loamy sand were 16, 19, 15, 12 and 8 cm respectively. The bulk density*soil type interaction was significant ($P<0.001$) as total root length did not show a uniform response to bulk density in both soil types.

The response of bulk density on root dry weight was significant at 3 DAT (Fig. 3C; $P<0.001$), but not at 10 DAT. At 3 DAT, mean root dry weights in clay loam were 0.0010, 0.0008, 0.0004, 0.0108 and 0.0075 g at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3}

respectively compared to 0.0011, 0.0038, 0.0012, 0.0032 and 0.0010 g respectively in the loamy sand. At both time points, root dry weight in the clay loam was greatest at 1.5 and 1.6 Mg m^{-3} (Figs. 3C, D). At 10 DAT, root dry weight increased with bulk density in the clay loam, but decreased slightly with increasing bulk density in the loamy sand (Fig. 3C). The effect of soil type was significant (Fig. 3D; $P<0.001$) at 10 DAT but not at 3 DAT. The mean value for all bulk densities was slightly greater in the clay loam than in the loamy sand at both time points (0.0041 *vs*. 0.0021 g at 3 DAT and 0.0103 *vs*. 0.0031 g at 10 DAT ($P<0.001$). The soil type*bulk density interaction was significant (Figs. 3C, 3D; $P<0.001$) as root dry weight responded differently to increasing bulk density in the two soil types.

The effect of bulk density on shoot height was significant at 3 DAT (Fig. 4A; $P<0.001$) but not at 10 DAT. At 3 DAT, mean shoot heights in the clay loam were 25, 23, 18, 34 and 31 mm at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively; the corresponding values for loamy sand soil were 30, 35, 17, 28 and 24 cm respectively. At 3 DAT, shoot height was greatest at the highest bulk densities (1.5 and 1.6 Mg m^{-3}) in the clay loam, but was greatest in the lowest bulk densities (1.2 and 1.3 Mg m^{-3}) in the loamy sand (Fig. 4A). However, by 10 DAT, shoot height in the loamy sand was greatest at intermediate bulk densities (1.3 and 1.4 Mg m^{-3}). The influence of soil type on shoot height was significant at 10 DAT (Fig. 4B; $P<0.001$), but not at 3 DAT, the opposite of the effect found for bulk density. Mean values for all bulk densities were slightly higher for the loamy sand i.e. 27 *vs*. 26 mm for the clay loam at 3 DAT and 46 *vs*. 39 mm at 10 DAT ($P<0.01$). The soil type*bulk density interaction was significant (Figs. 4A, B; $P<0.001$), as the effect of both factors was significant at different time points.

The effect of bulk density on shoot dry weight was significant at both time points (Figs. 4C, 4D; P<0.001). At 3 DAT, mean shoot dry weight in the clay loam was 0.0019, 0.0011, 0.0016, 0.0029 and 0.0032 g at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively compared to 0.0083, 0.0071, 0.0079, 0.0099 and 0.0118 g at 10 DAT. For the loamy sand, mean shoot dry weights were 0.0024, 0.0027, 0.0012, 0.0020 and 0.0016 g at 1.2, 1.3, 1.4, 1.5 and 1.6 Mg m^{-3} respectively at 3 DAT, compared to 0.0113, 0.0077, 0.0053, 0.0050 and 0.0038 g respectively at 10 DAT. The response of shoot dry weight to soil type was not significant at 3 DAT but was at 10 DAT (Fig. 4D; P<0.001); mean values for all bulk densities were slightly greater in the clay loam at both time points. Mean shoot dry weight for all bulk densities did not differ between the clay loam and the loamy sand at 3 DAT, but differed significantly at 10 DAT (0.0090 *vs.* 0.0066 g respectively; P<0.001). The soil type*bulk density interaction was again significant (Figs. 4C, D; P<0.001) as the effects of both variables differed between time points.

X-ray CT Boundary experiment: Surprisingly, root volume was not significantly affected by compaction treatment but was affected by soil type, being greater in the loamy sand after day 8 (Fig. 5A; P<0.05). In the C/UC treatment, mean root volume for all time periods was 21 mm^3 in the clay loam compared to 23 mm^3 in loamy sand (Fig. 5A; P<0.05); the corresponding values for the UC/C treatment were 18 *vs.* 20 mm^3 (Fig. 5A; P<0.05). Although the effects of compaction treatment on root volume were not significant, in 80% (16/ 20) instances root volume was greater in the C/UC treatment of both soil types (Fig. 5A). The lack of significance may be explained by the large error bars from 8 DAT onwards, when variability in the

values for root volume increased. As expected, the influence of time on all root characteristics examined was significant ($P<0.001$).

No significant effects or interactions were detected for root surface area (Fig. 5B), apart from the influence of time ($P<0.001$). Again, this may be explained by the large error bars, as 75% (15/ 20) of the root surface area values were greater in the C/UC treatment (Fig. 5B). Maximum root depth showed no detectable treatment effects, although the interactions between soil type*bulk density and soil type*bulk density*time were significant (Fig. 5C; $P<0.05$ and $P<0.01$ respectively). Plants grown in the C/UC treatment of clay loam achieved the deepest rooting depths from 4 DAT (Fig. 5C), and 80% of the values were greater in C/UC treatment of the clay loam. Plants grown in the UC/C treatment of the loamy sand subsequently achieved greater root depths, with 90% of the values being greater than in the C/UC treatment (Fig. 5C). In the C/UC treatment, mean root depth for all time intervals was 25 mm in clay loam *vs.* 19 mm^3 in the loamy sand (Fig. 5C); the corresponding values for the UC/C treatment were 20 *vs.* 23 mm^3. No detectable treatment effects were found for primary root length but the soil type*bulk density and soil type*bulk density*time interactions were significant (Fig. 6A; $P<0.01$ and $P<0.001$). Primary root length was greatest in the C/UC treatment of the clay loam (Fig. 6A) and in the UC/C treatment of the loamy sand. Mean primary root length for all time periods was 37 mm in the C/UC treatment of the clay loam, compared to 26 mm^3 in loamy sand (Fig. 6A); the corresponding values for the UC/C treatment were 26 *vs.* 28 mm^3.

The tortuosity of the root system was increased by compaction (Fig. 6B; $P<0.05$) and was greater in the C/UC than in the UC/C treatment. In the former, the mean tortuosity value for clay loam was 1.60, compared to 1.42 for loamy sand; the corresponding values for the UC/C treatment were 1.34 *vs.* 1.23. Tortuosity values were greatest in the C/UC treatment of both soil types. Mean tortuosity values were greater in clay loam than loamy sand for all sampling dates (Fig. 6B $P<0.01$), and were also greatest during the first few days of the sampling period in all treatments (Fig. 6B; $P<0.001$). Measurements of root elongation rate during the 10 day sampling period (data not presented) showed that the soil type*bulk density interaction was significant ($P<0.05$) and elongation rate was typically greater in the C/UC treatment of the clay loam and the UC/C treatment of the loamy sand. Mean values in the C/UC treatment were 4.62 *vs.* 2.64 mm d^{-1} for clay loam and loamy sand respectively; the corresponding values for the UC/C treatment were 2.80 *vs.* 3.39 mm d^{-1}. Overall, elongation rate was greatest in the C/UC treatment and between 1 - 4 DAT.

Measurements of root volume, diameter and length above and below the bulk density boundary (Fig. 7) revealed no significant treatment effects (numerical data not shown). Lateral root number was greatest in the C/UC compaction treatment and in the clay loam soil ($P<0.05$; Fig. 8). Mean values in the UC/C treatment of the clay loam and loamy sand were respectively 7.0 and 5.3 roots $plant^{-1}$; the corresponding values for the C/UC treatment were respectively 10.7 and 7.0 roots $plant^{-1}$.

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Tracy et al. Fig. 1

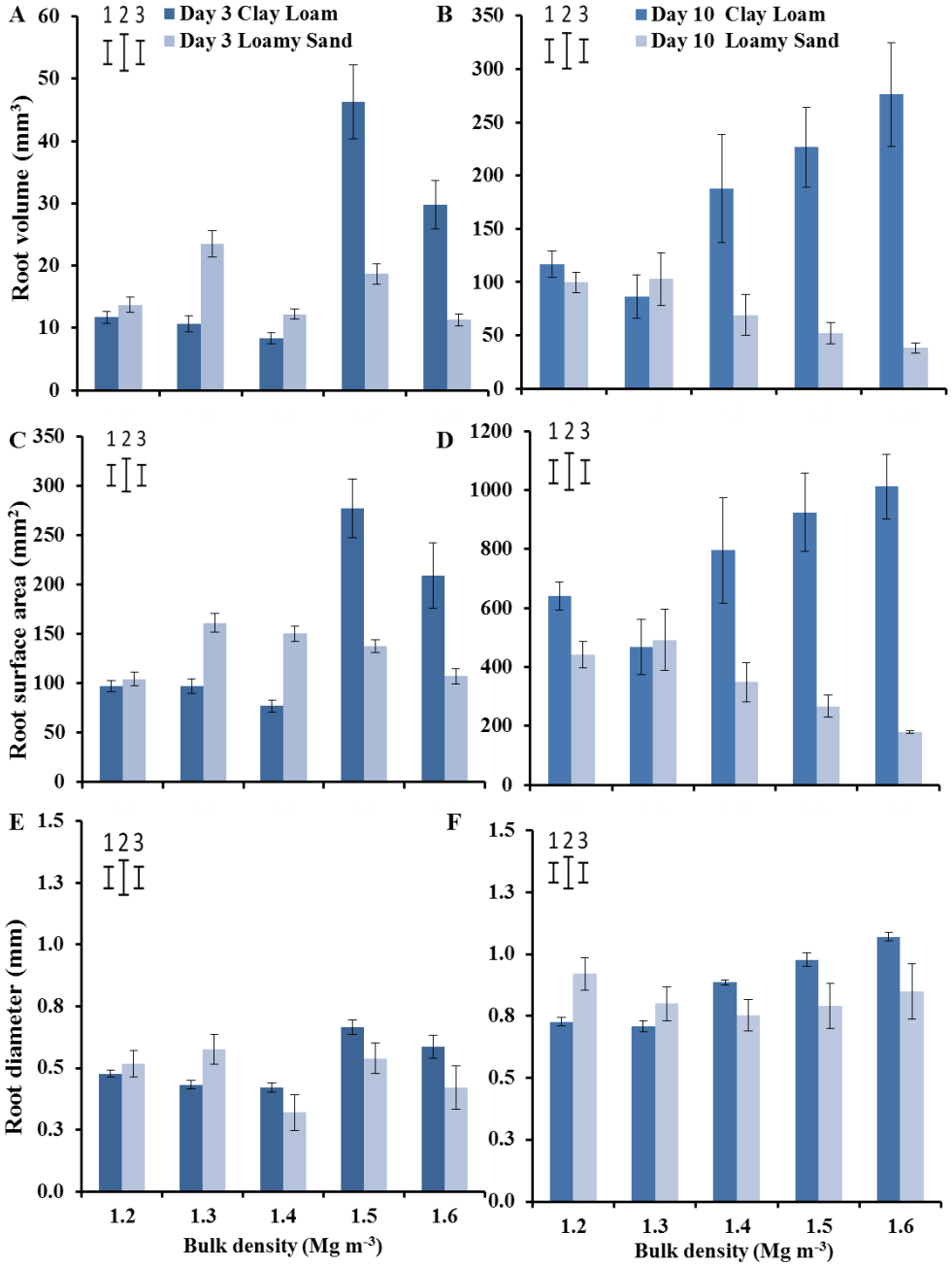

Figure. 1. WinRHIZO® analysis values at 3 (A, C, E) and 10 (B, D, F) days after transplanting (DAT) at bulk densities ranging from 1.2 to 1.6 Mg m^{-3} for mean root volume (A, B), root surface area (C, D) and root diameter (E, F). Error bars associated with the histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) soil type, (2) compaction treatment and (3) time.

Tracy et al. Fig. 2

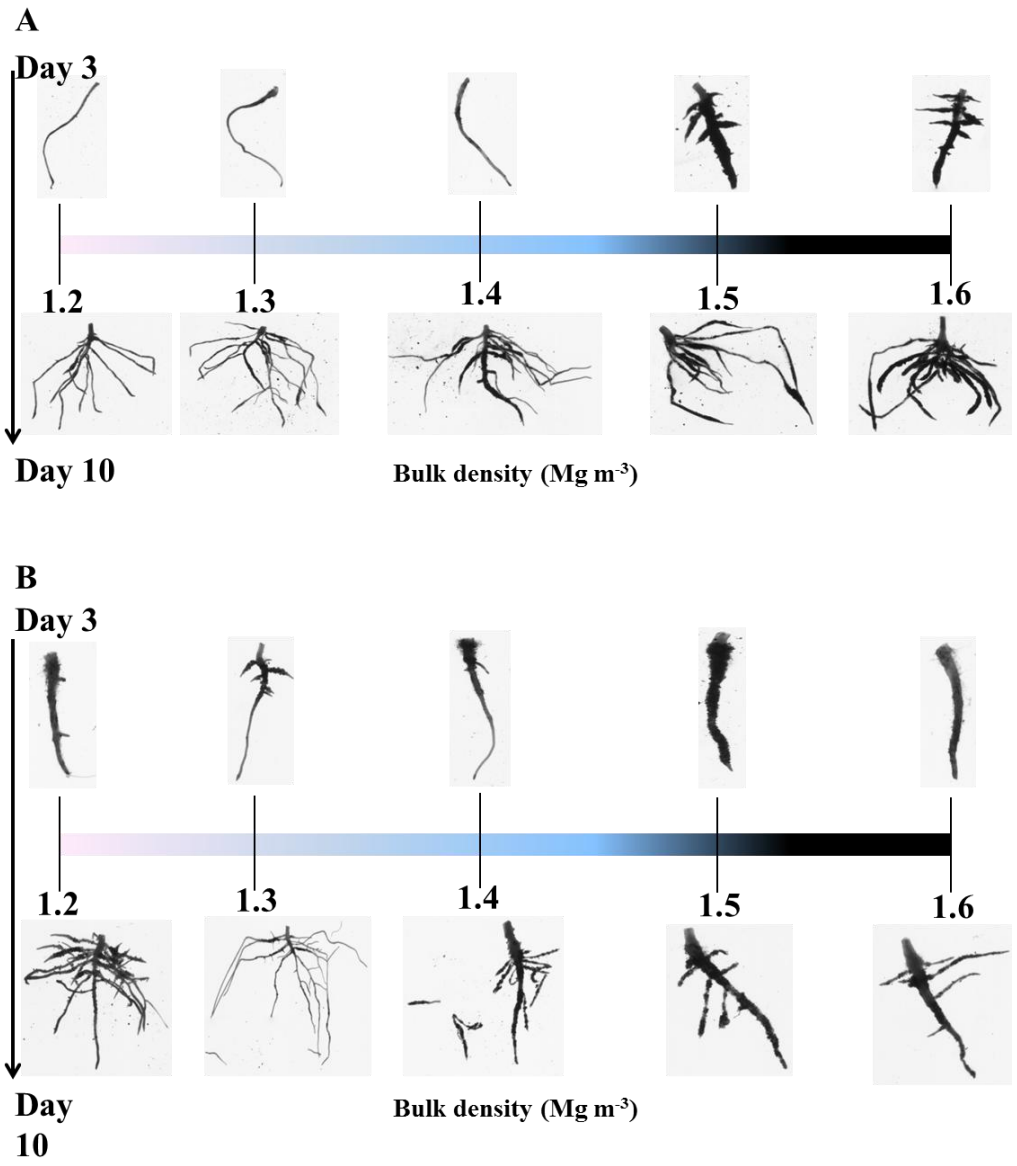

Figure. 2. Diagram showing typical root systems grown in clay loam (A) and loamy sand (B) soil at all bulk densities and destructively harvested at 3 (upper row) and 10 days after transplanting (DAT; lower row). Gradient bar represents increasing bulk density from left to right.

Tracy et al. Fig. 3

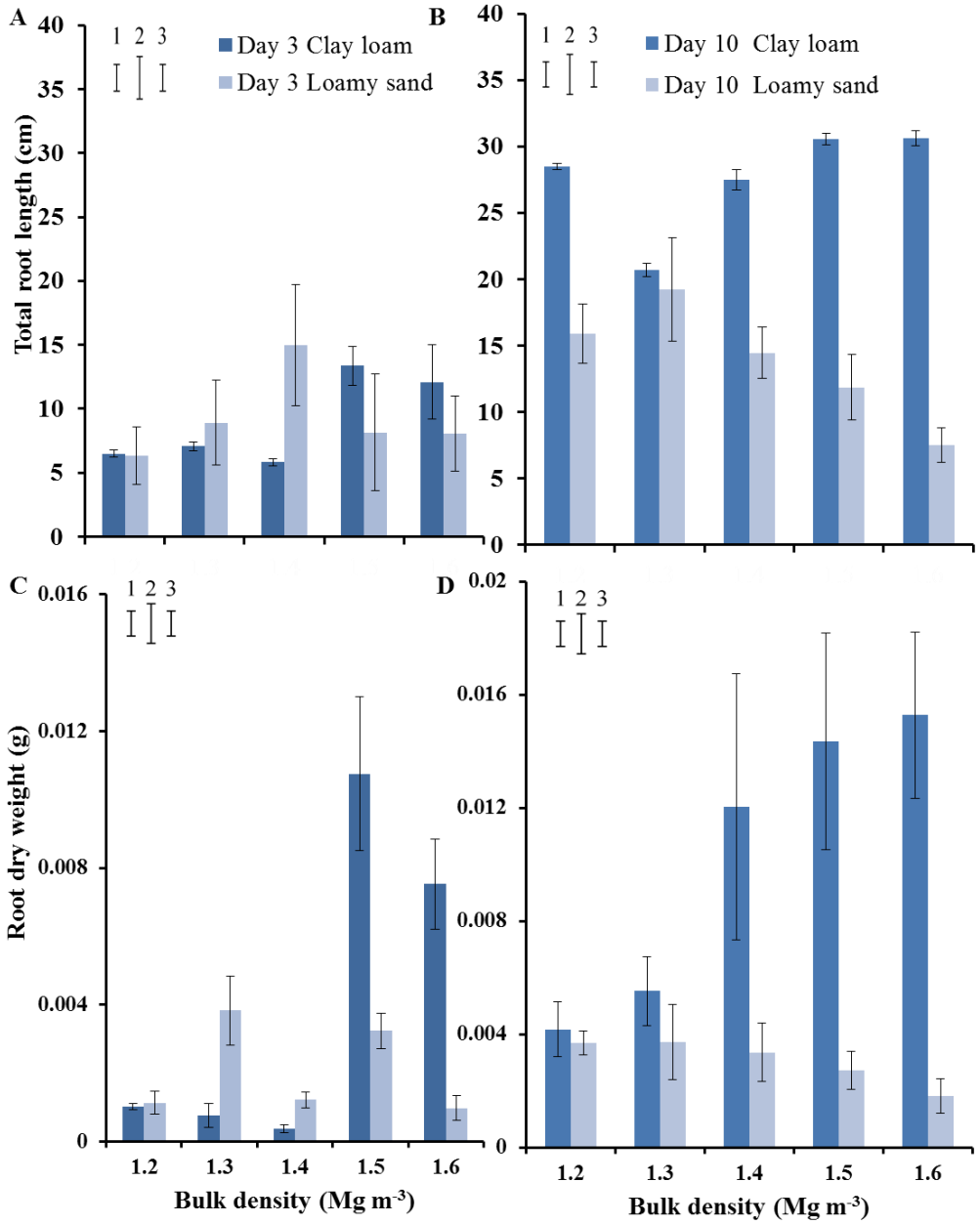

Figure. 3. WinRHIZO® analysis values at 3 (A, C) and 10 (B, D) days after transplanting (DAT) at soil bulk densities ranging from 1.2 to 1.6 Mg m^{-3} for total root length (A, B) and root dry weight (C, D). Error bars associated with the histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) soil type, (2) compaction treatment and (3) time.

Tracy et al. Fig. 4

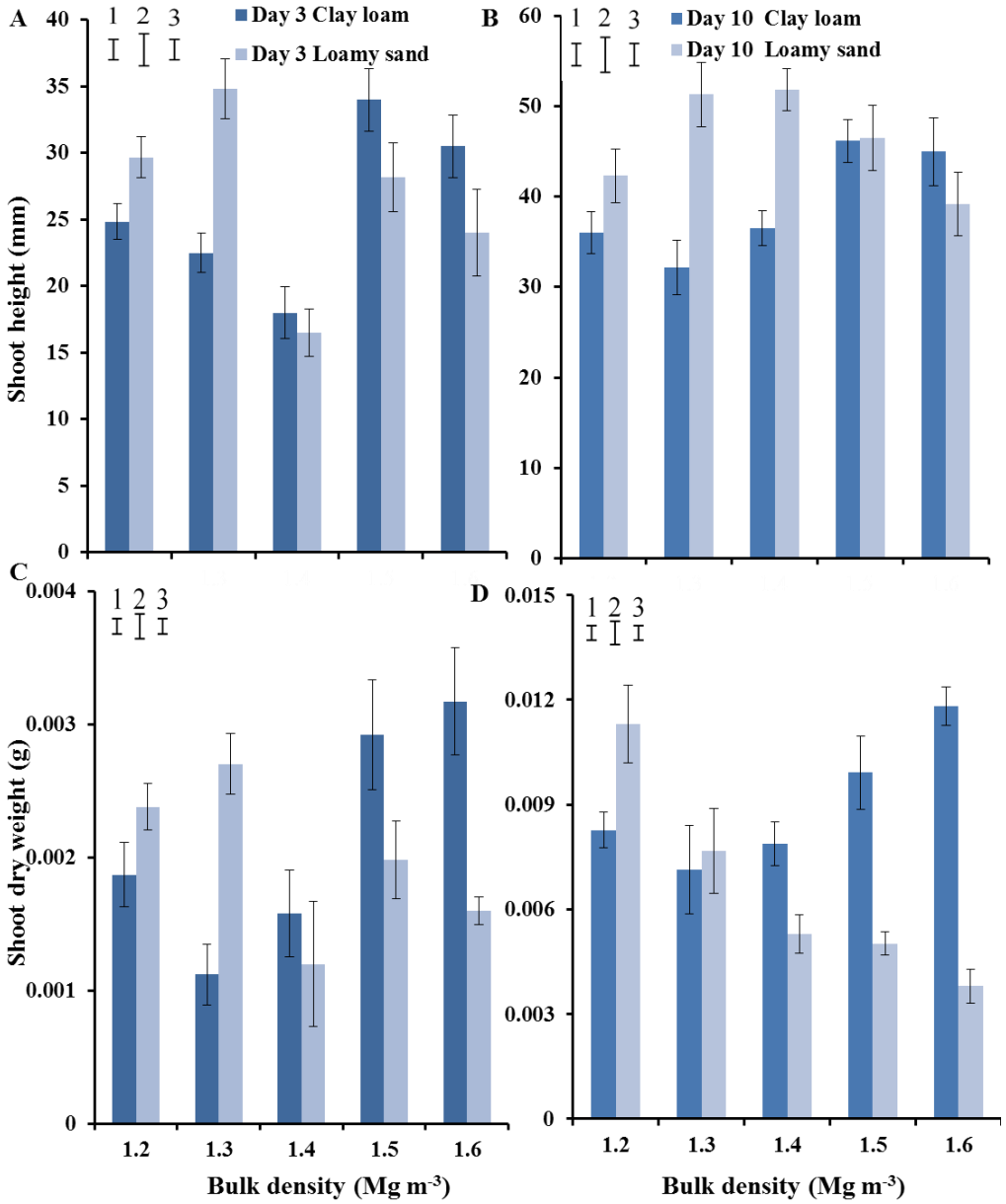

Figure. 4. WinRHIZO® analysis values at 3 (A, C) and 10 (B, D) days after transplanting (DAT) at soil bulk densities ranging from 1.2 to 1.6 Mg m^{-3} for shoot height (A, B) and shoot dry weight (C, D). Error bars associated with the histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) soil type, (2) compaction treatment and (3) time.

Tracy et al. Fig. 5

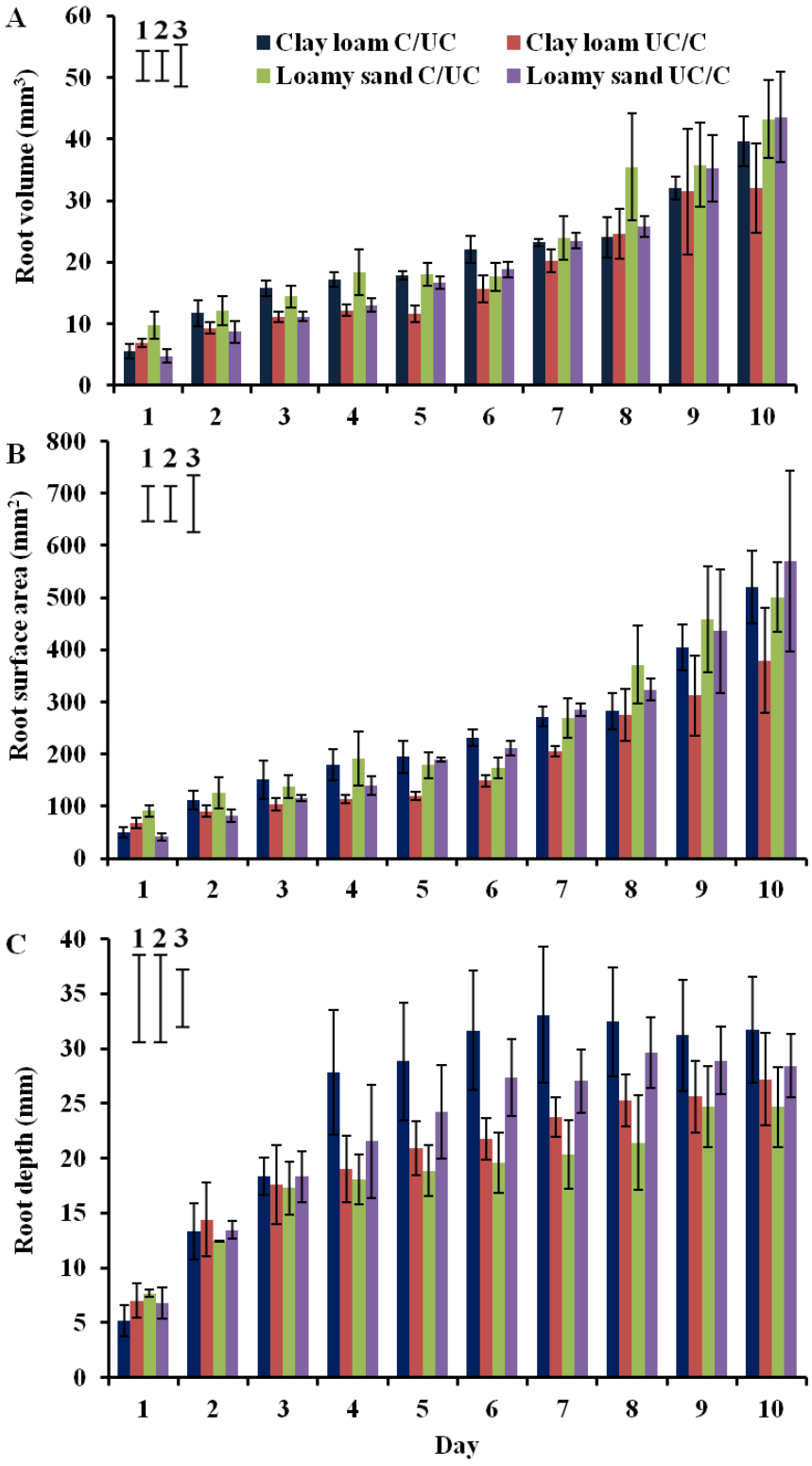

Figure. 5. Semi-automated segmented mean values for (A) root volume, (B) root surface area and (C) root depth during the 10 day observation period for both compaction treatments and soil types. Error bars associated with the histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) soil type, (2) compaction treatment and (3) time.

Tracy et al. Fig. 6

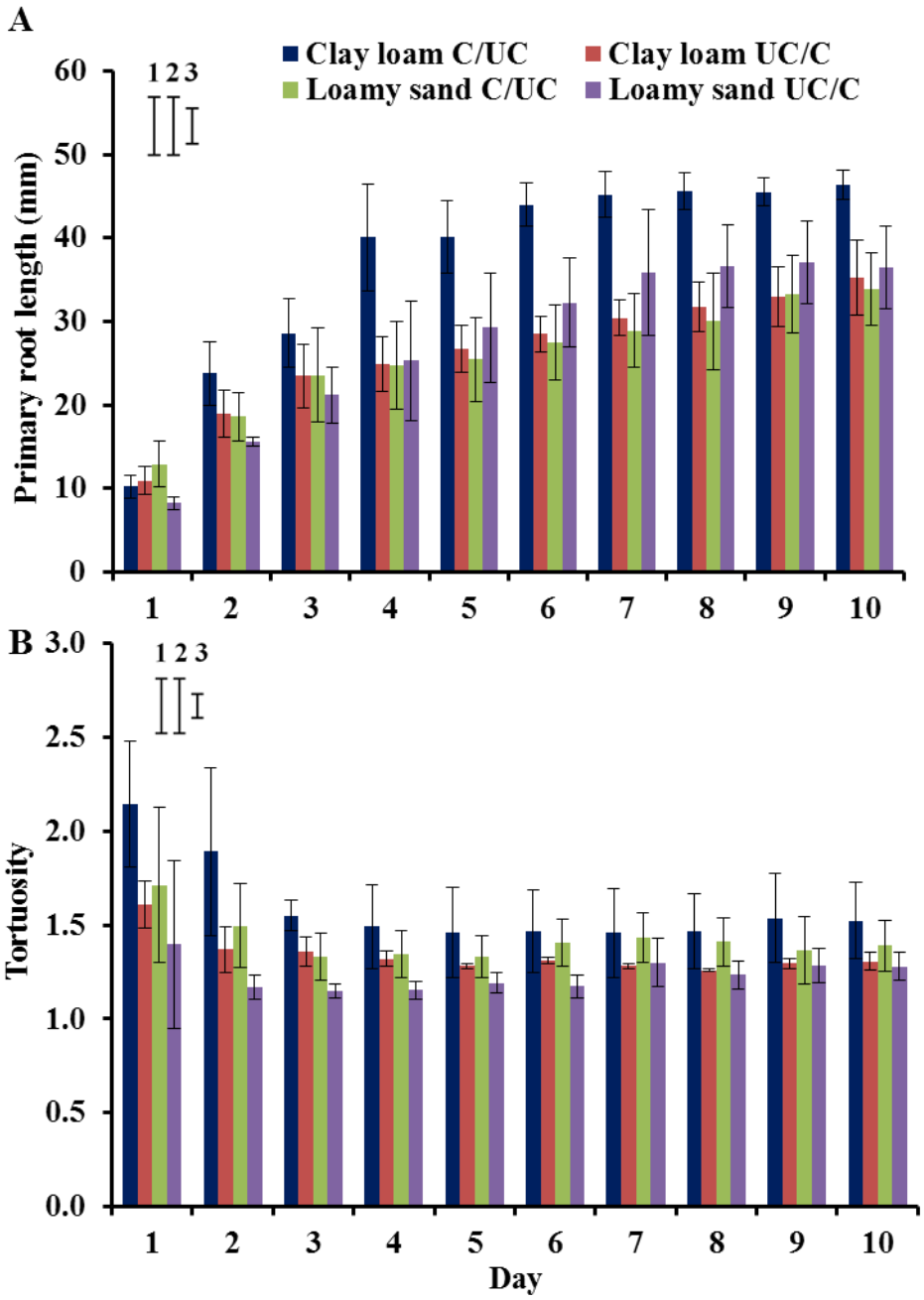

Figure. 6. Mean primary root length (A) and tortuosity (b) values for both compaction treatments and soil types. Error bars associated with the histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) soil type and (2) compaction treatment.

Tracy et al. Fig. 7

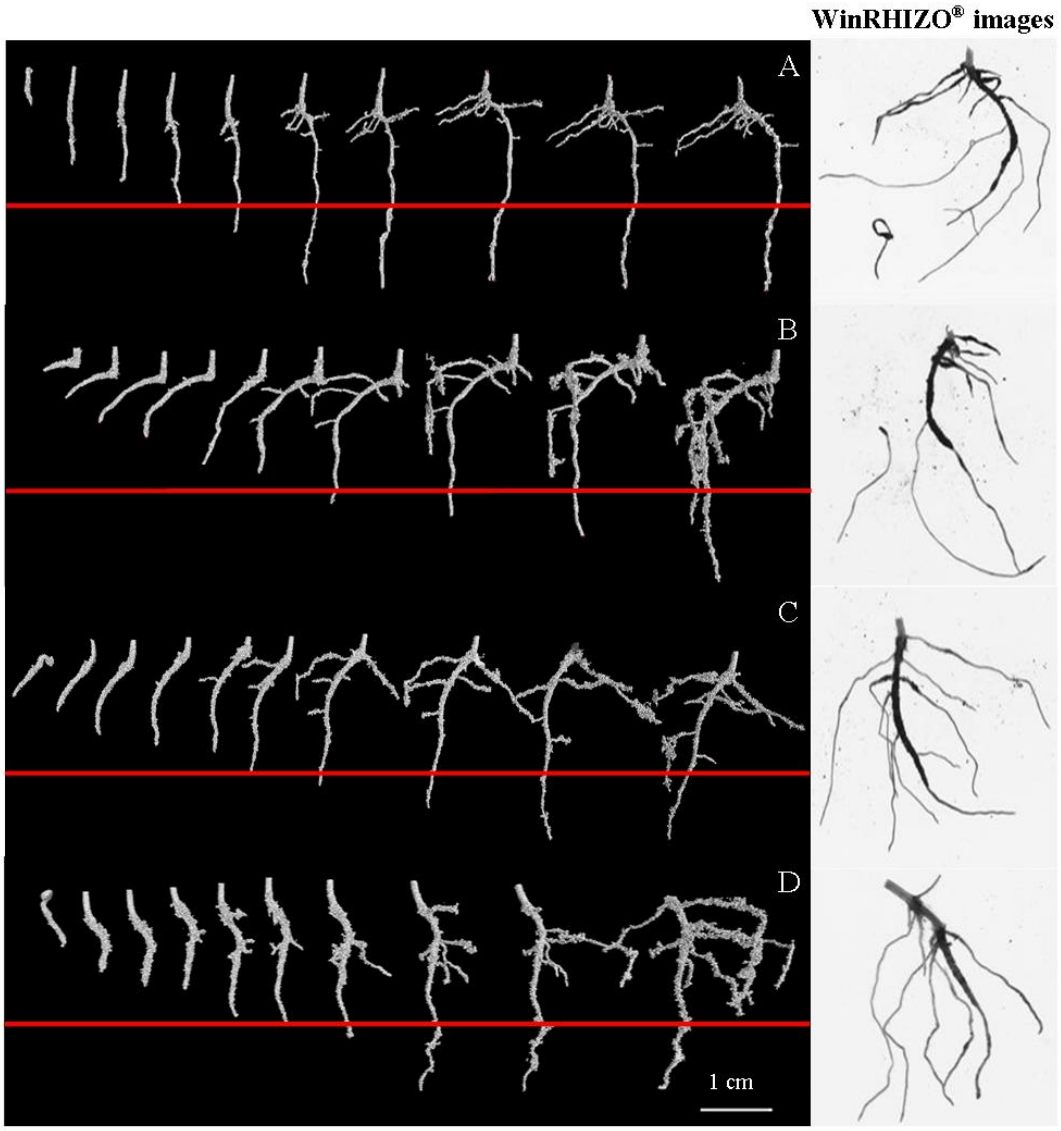

Figure. 7. Root growth over 10 consecutive days and final destructive WinRHIZO® root images (white background) for plants grown in the C/UC and UC/C treatments of the clay loam (A and B) and loamy sand (C and D). Horizontal line represents the location of the bulk density transition. Scale bar indicates 1 cm.

Tracy et al. Fig. 8

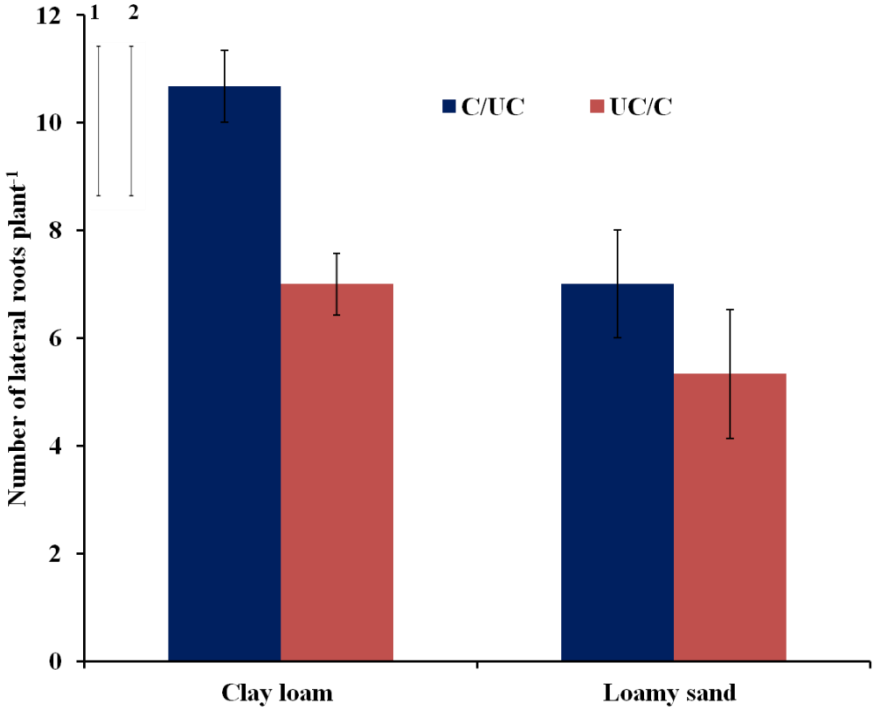

Figure. 8. Mean lateral root numbers for both compaction treatments and soil types. Error bars associated with the histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) soil type and (2) compaction treatment.

8. Chapter 8

Chapter 8 investigated the role of ABA in mediating root responses to increasing soil compaction, through the use of ABA mutants with previously demonstrated differing internally generated ABA concentrations. This paper will be submitted to a scientific journal, so is presented in 'paper format'.

Author contribution:

Project supervision performed by CR Black, JA Roberts and SJ Mooney

ABA measurement undertaken by I Dodd (Lancaster University) and SR Tracy

Practical work and construction of paper performed by SR Tracy

Dissecting the role of abscisic acid in the response of roots to soil compaction

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Number of Figures - 10; Number of Tables – 0

Abbreviations:

ABA – Abscisic acid

RSA – Root system architecture

DAT – Days after transplantation

DI – Deionised water

DW – Dry weight

3-D – Three dimensions

4-D – Four dimensions

Tracy et al. Fig. 3

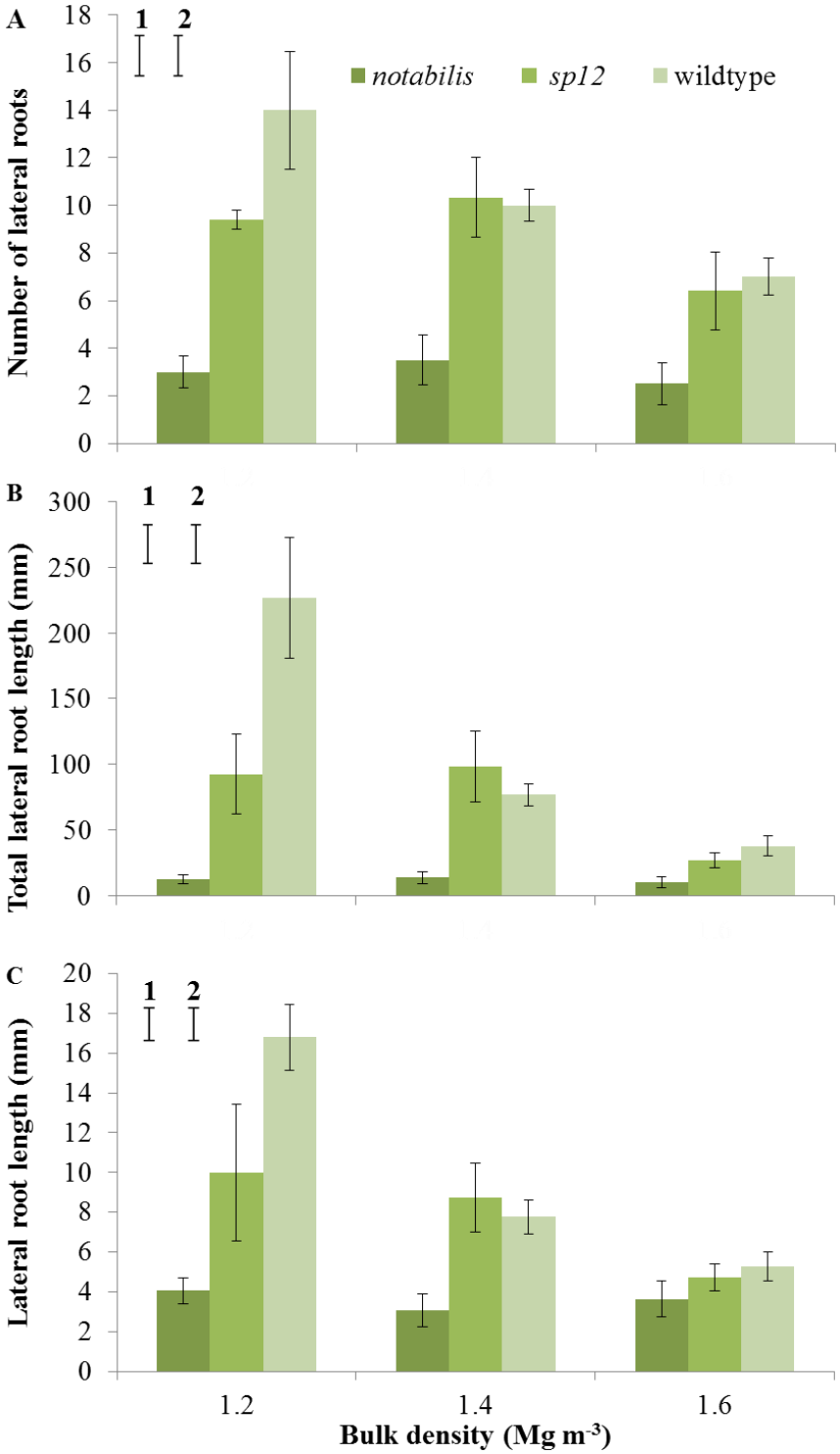

Figure. 3. X-ray µCT-derived mean values for (a) number of lateral roots, (b) total lateral root length and (c) lateral root length for all compaction treatments and genotypes. Error bars associated with histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) bulk density and (2) genotype.

Tracy et al. Fig. 4

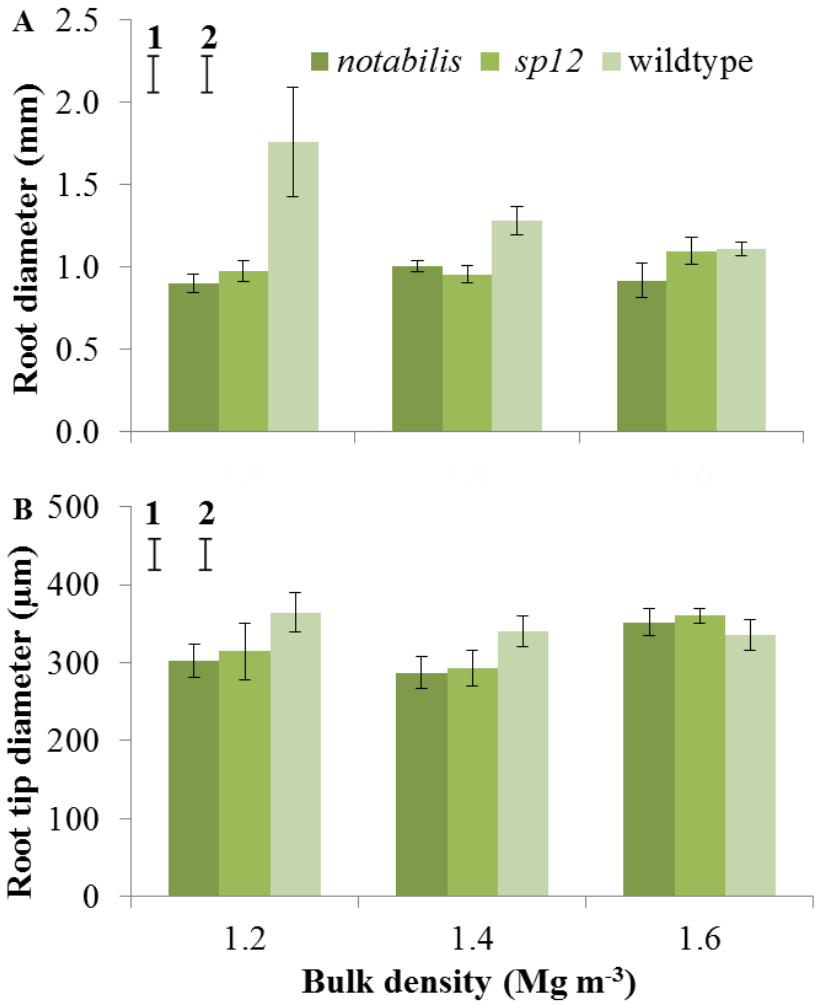

Figure. 4. WinRHIZO® analysis values for mean (a) root diameter and (b) root tip diameter measurements determined by confocal microscopy for all compaction treatments and genotypes. Error bars associated with histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) bulk density and (2) genotype.

Tracy et al. Fig. 5

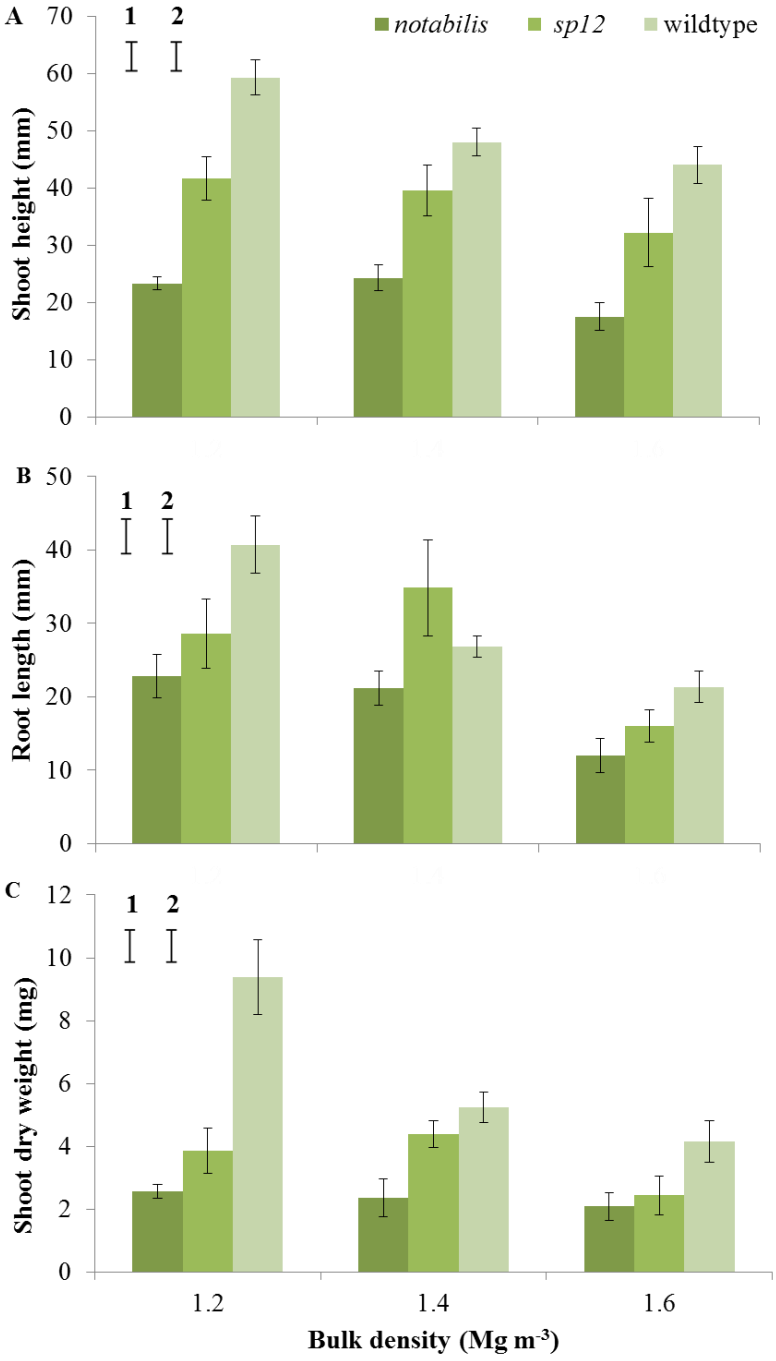

Figure. 5. Destructive analysis values for (a) shoot height, (b) root length and (c) shoot dry weight for all compaction treatments and genotypes. Error bars associated with histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) bulk density and (2) genotype.

Tracy et al. Fig. 6

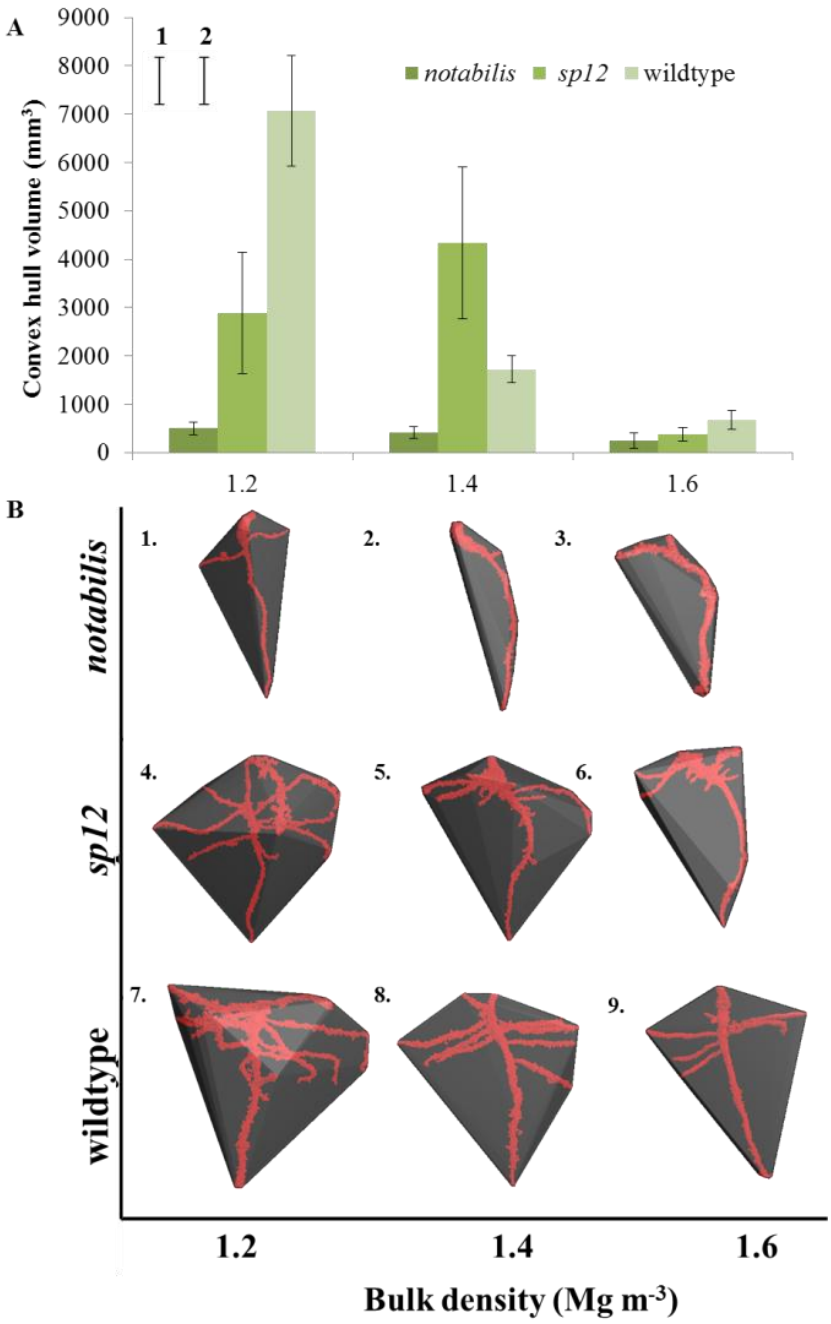

Figure. 6. X-ray µCT-derived mean values for (a) convex hull volume for all compaction treatments and genotypes. Error bars associated with histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) compaction and (2) genotype. (b) Typical root systems grown in uncompacted, intermediate and compacted soil are shown for *notabilis* (1, 2 and 3), *sp12* (4, 5 and 6) and wildtype plants (7, 8 and 9) plants.

Tracy et al. Fig. 7

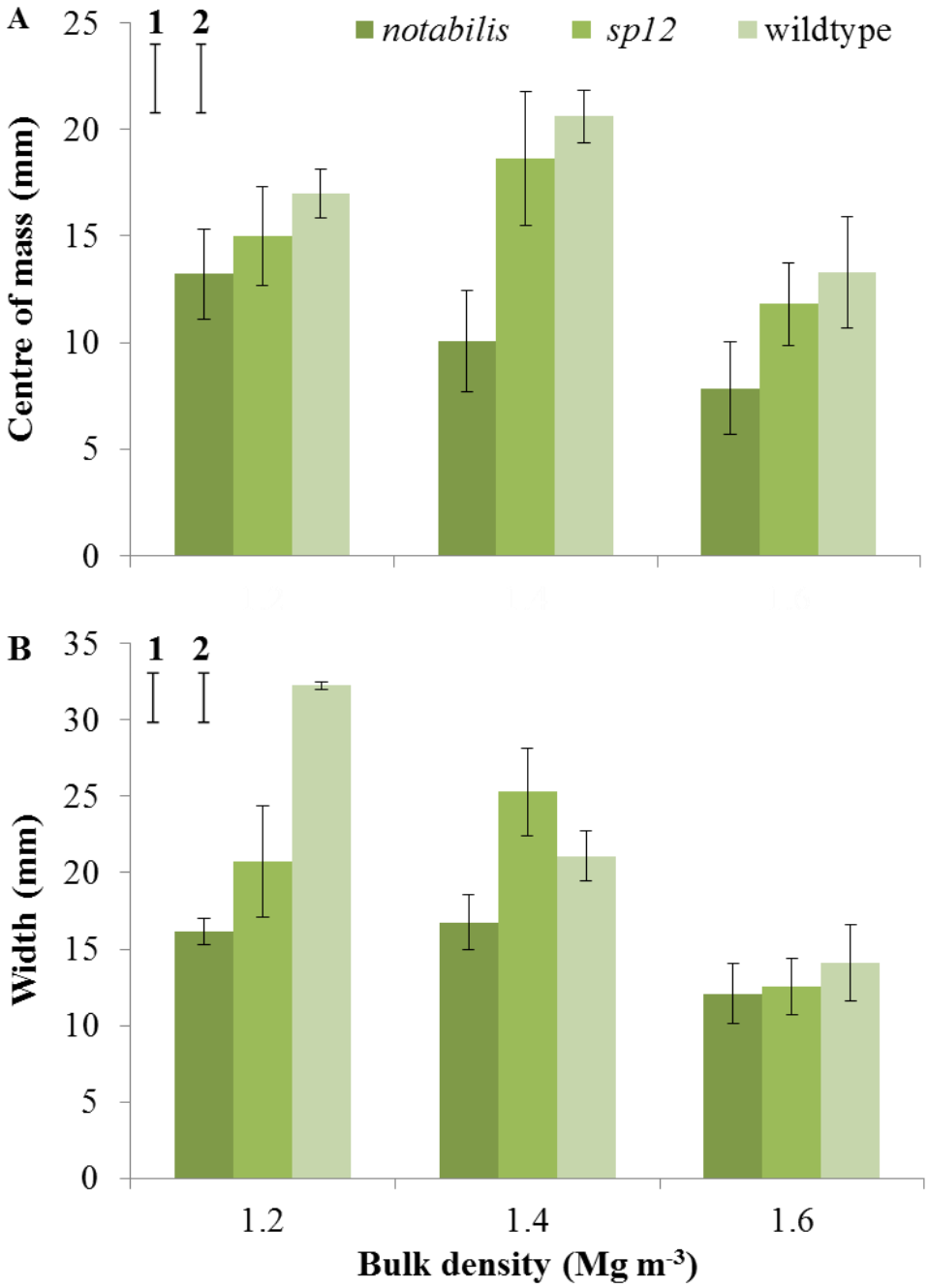

Figure. 7. X-ray µCT-derived mean values for (a) centre of mass and (b) maximum horizontal rooting width for all compaction treatments and genotypes. Error bars associated with histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) bulk density and (2) genotype.

Tracy et al. Fig. 8

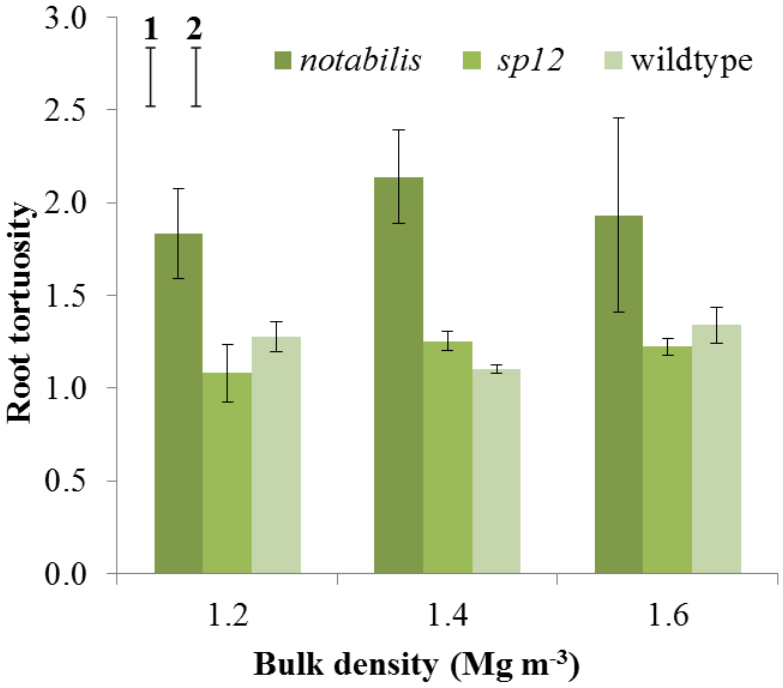

Figure. 8. X-ray µCT derived mean values for tortuosity of the root path for all compaction treatments and genotypes. Error bars associated with histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) bulk density and (2) genotype.

Tracy et al. Fig. 9

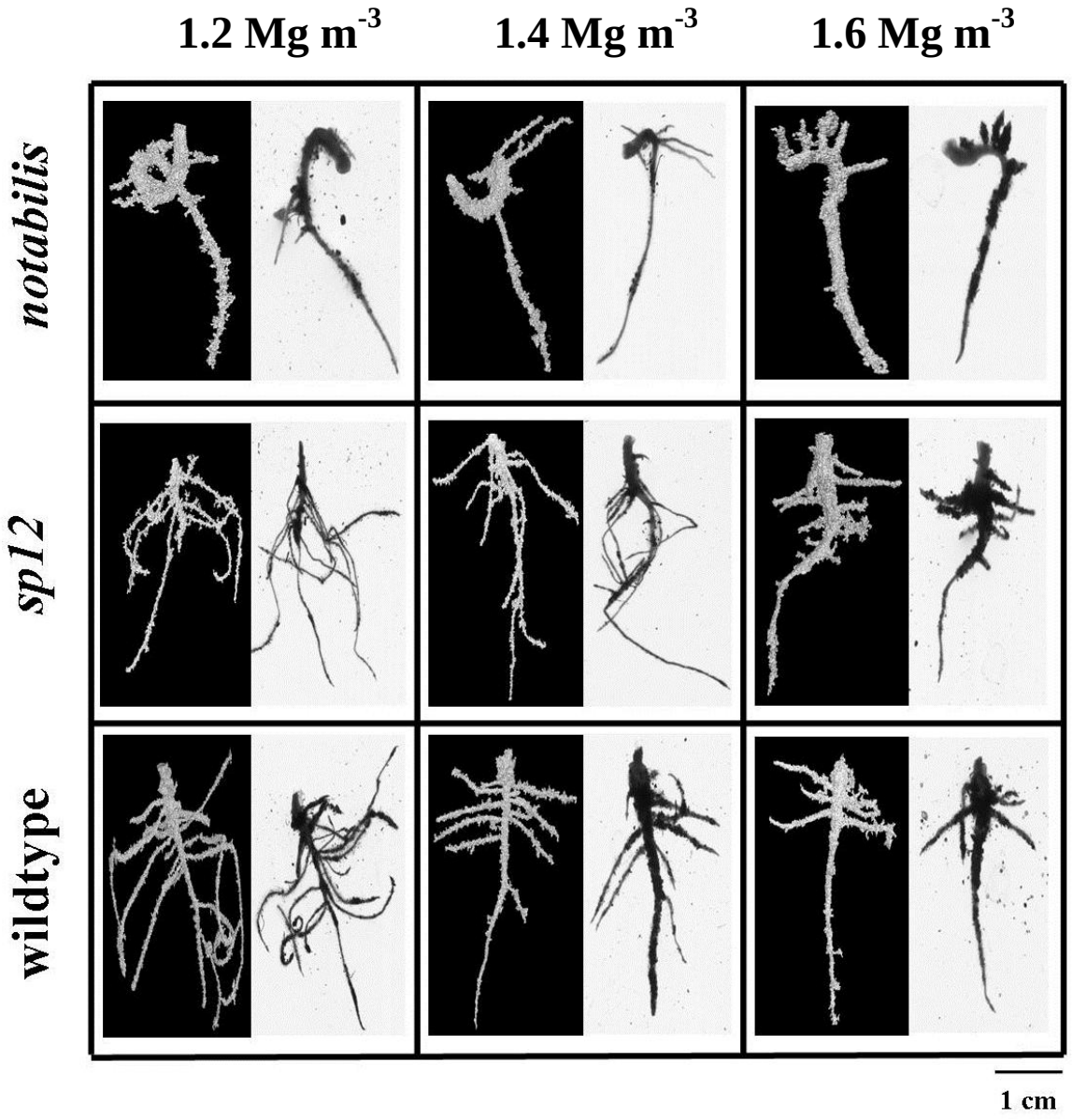

Figure. 9. Examples of segmented individual root systems (black background) and their associated final destructive WinRHIZO® root image (white background) for all compaction treatments and genotypes. Scale bar indicates 1 cm.

Tracy et al. Fig. 10

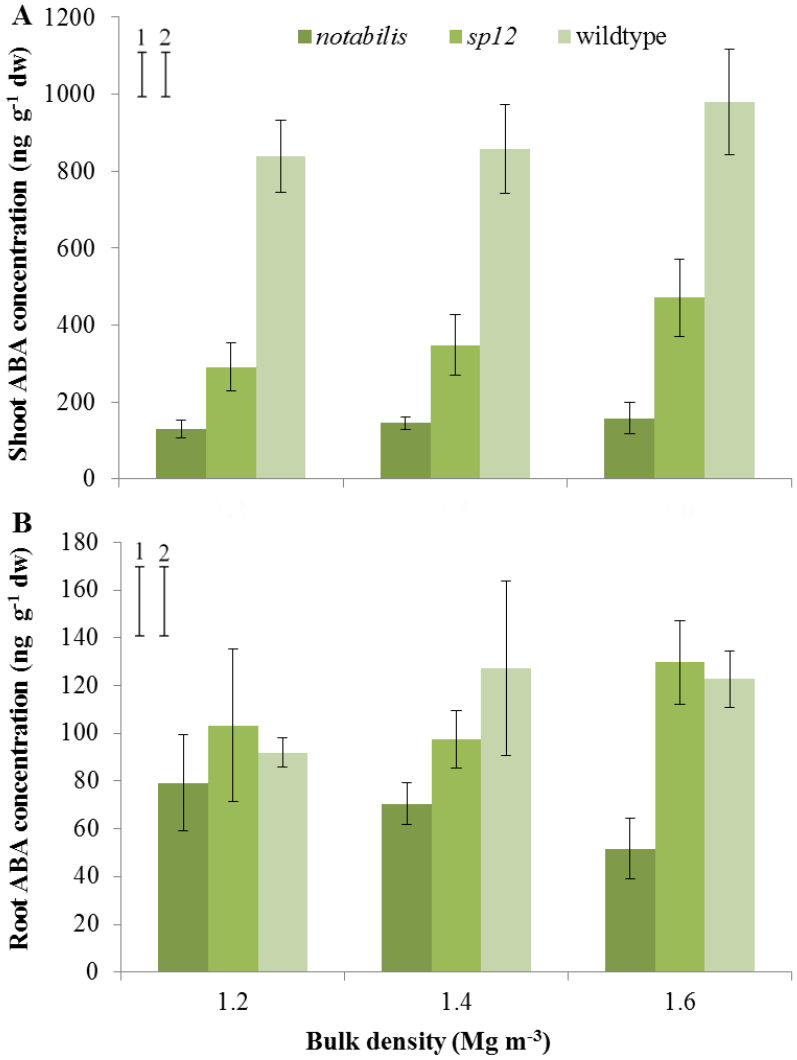

Figure. 10. ABA concentrations measured in (a) shoots and (b) roots. Error bars associated with histograms show double standard errors of the mean. Standard errors of the difference (SED) are shown for (1) bulk density and (2) genotype.

9. Chapter 9

9.1 General Discussion

9.1.2 Response of root system architecture to soil bulk density

This thesis has shown that several aspects of root architecture are affected by increases in soil bulk density (Table. 1). For example, root diameter (Chapters 5 and 6) and root tip diameter (Chapter 6) were significantly greater in plants grown in compacted soil. Chapter 7 clearly demonstrated the response of root diameter to incremental increases in bulk density (Figs. 1 & 2 in Chapter 7), an effect which was greater in the coarser grained loamy sand soil. The effect of compaction was already significant at 3 DAG and the bulk density*soil type interaction was significant ($P<0.001$) for root diameter. However, the experiment involving layers of contrasting bulk density described in Chapter 7 revealed no difference in root diameter between the different horizons, in agreement with the results reported by Bengough and Young (1993).

Table. 9.1: An overview of root characteristic responses observed in the different soil types in this thesis

Chapter	Soil	Root volume	Root surface area	Root diameter	Primary root length	Total length	Convex Hull volume	Vertical root depth	Tortuosity
5	Sandy loam	Increased*	N/A	Increased**	Decreased	Decreased	N/A	N/A	N/A
6	Loamy sand	Decreased**	Decreased**	Increased*	Decreased	Decreased*	Decreased*	Decreased	Increased**
6	Clay loam	Decreased	Decreased	Increased*	Decreased	Decreased*	Decreased*	Decreased	Increased
7	Loamy sand	Decreased	Decreased	Increased	Decreased	Decreased	N/A	Decreased	Increased
7	Clay Loam	Increased	Increased	Increased	Increased	Increased	N/A	Increased	Increased
8	Loamy sand	Decreased**	Decreased**	No effect	Decreased**	Decreased**	Decreased**	Decreased**	No effect

** = significance @ P<0.01

* = significance @ P<0.05

These increases in root diameter were accompanied by increases in root volume. Although this rooting characteristic is often used as a measure of root growth (Dupuy *et al.,* 2010), direct association of root volume with the successful establishment of root systems may be misleading as it is possible that, although plants often produce shorter, thicker roots in response to compaction, they may nevertheless have total root volumes similar to plants with longer, thinner roots. However, the rooting depth and soil volume exploited by plants for the uptake of essential resources can be very different. This problem in determining root system responses is discussed further below. Nevertheless, root volume remains a useful and insightful characteristic when considered together with other architectural characteristics. For example, in Chapter 5, root volumes for all three wheat varieties examined were greater at 1.5 Mg m^{-3} than at 1.1 Mg m^{-3}. This finding substantiates previous work showing that moderate compaction of some soil types may be advantageous, suggesting the existence of an optimum degree of compaction for root growth (Bouwman and Arts 2000; Hamza and Anderson 2005; Atkinson *et al.,* 2009a). Mulholland *et al.* (1996) and Alameda *et al.* (2012) showed that root and shoot growth were greatest in moderately compacted soil (1.4 - 1.5 Mg m^{-3}), possibly due to greater water retention and improved contact between roots and soil. Excessively loose soil may reduce crop yield, perhaps because roots preferentially grow through large pores, with the result that sufficient contact between roots and soil for effective extraction of water and nutrients is not achieved (Passioura, 2002); the dominance of larger pore size classes at a low soil bulk density (1.2 Mg m^{-3}) is clearly demonstrated by the pore size distribution data shown in Fig. 9 in Chapter 5. Root systems are also required

to provide adequate physical support and anchorage, if the soil is too loose, the likelihood of lodging may increase, especially in cereals such as barley (*Hordeum vulgare* L.; Berry *et al.*, 2006).

Root surface area is undoubtedly an important root trait as it determines the area in contact with the surrounding soil particles. Root surface area decreased in response to increases in soil compaction in Chapter 5 (although not significantly), Chapter 6 ($P<0.001$) and Chapter 8 ($P<0.001$) across all genotypes examined. In Chapter 7, the influence of bulk density was significant during early seedling growth and differences between soil types were apparent, leading to a significant bulk density*soil type interaction due to the different responses to bulk density shown by roots growing in the different soil types. The images presented throughout the thesis show that roots grown in uncompacted soil were typically longer and thinner, with a greater surface area:volume ratio, representing a more efficient use of photoassimilates for root production (Paula and Pausas, 2011).

Although moderate soil compaction is known to enhance root attributes such as root volume, effects on root architectural traits are generally somewhat negative. Alameda and Villar (2009) found that, although moderate soil compaction may increase total biomass (Fig. 3 in Chapter 7), rooting architecture was negatively influenced (Fig. 2 in Chapter 7). Commonly studied variables such as biomass and root length density do not provide critical insight into how the root system branches and explores the surrounding soil matrix over time and in response to local variation in soil compaction

(Becel *et al.*, 2012). Distortion of root morphology arises from an increased penetration resistance and a consequent increase in root diameter (Figs. 1 & 2 in Chapter 7), which has been shown to reduce total root length and fine root number (Chassot and Richner, 2002). In Chapter 6, it was shown that lateral roots emerged *c*. 2 d sooner in plants grown in uncompacted soil. Although lateral root number was not affected, the maximum diameter and total length of lateral roots were lower, although not significantly. In Chapter 8, lateral root number was greatest in the Compacted/Uncompacted treatment (Fig. 8 in Chapter 7), while in Chapter 8 lateral root number was greater at the lowest bulk density of 1.2 Mg m^{-3} ($P<0.01$). Total lateral root length also decreased as bulk density increased ($P<0.001$). Differences between genotypes were apparent as the ABA-deficient genotype (*notabilis*) showed no differences in lateral root characteristics across the three bulk density treatments examined, whereas the wildtype showed a clear decrease in lateral root number and length as bulk density increased. This may provide further evidence that ABA is involved in lateral root emergence (De Smet *et al.*, 2006). Overall, increased bulk density resulted in poorer root architecture, with thicker roots and fewer wide-spreading lateral roots, especially in the loamy sand soil; the consequently reduced provision of root branches within the soil matrix would have negative consequences for soil exploration and resource acquisition.

The greater bulk density and less well connected and continuous pore space in compacted soil are likely to have been responsible for the greater tortuosity of roots, as they may have buckled as a result of physical impedance imposed by the soil and been forced to follow more convoluted pathways. In Chapter 6,

tortuosity of the root path was greater in plants grown in compacted soil ($P<0.01$), while in Chapter 7 roots in the Compacted/Uncompacted treatment, which initially experienced the higher bulk density had the greatest tortuosity values ($P<0.05$). In Chapter 8, the ABA-deficient *notabilis* exhibited the most tortuous pathway, implying that, in the absence of wildtype ABA concentrations, its roots were poor navigators of the soil matrix. Soil compaction affected total root length ($P<0.05$; Chapter 6 and $P<0.001$ at 3 DAG; Chapter 7). In the bulk density transition experiment (Chapter 7), plants in the Compacted/Uncompacted treatment of the clay loam achieved the deepest rooting depths from 4 DAG (Fig. 5 in Chapter 7), but plants in the Uncompacted/Compacted treatment of the loamy sand subsequently achieved greater rooting depths. In Chapter 8, rooting depth was greatest at the intermediate bulk density of 1.4 g cm^{-3} ($P<0.01$). Surprisingly, few significant differences were found in all experiments for root depth and primary root length, possibly due to the young age of the seedlings. A limitation of the X-ray CT scanner used in this study is that plants must be grown in small (<5 cm diameter) containers to fit into the scanner chamber and also to achieve a high resolution, because there is a trade-off between object diameter and spatial resolution. Therefore the plants could only be grown for a maximum of two weeks to avoid the root system becoming pot bound. Although it was vital that root confinement did not reduce plant performance and influence the results, as stated by Poorter *et al.* (2012), pot size is not usually a limiting factor for the relatively young plants examined.

9.1.3 Response of root system architecture to soil type

Clear differences were found in root morphometrics between the clay loam and loamy sand. In Chapter 7, differences were observed for all root and shoot attributes, highlighted by the significant interactions between bulk density and soil type for root volume, surface area, diameter, total root length and dry weight, shoot height and shoot dry weight. Plants grown in soils of differing texture displayed contrasting responses to increased bulk density, as plant growth was typically greater at the higher bulk densities of 1. 5 and 1.6 Mg m^{-3} in the clay loam, but was greatest at 1.3 Mg m^{-3} in the loamy sand. When sandy soils are compressed, the grains create a closely compacted medium which cannot easily expand to allow roots to penetrate (Batey and McKenzie, 2006), whereas the pores within the smaller particle sizes of the clay loam can be compacted but still retain a large overall volume of micropores, allowing roots to elongate as the particles move apart more readily (Hamblin, 1985). This may explain why in Chapter 6 effects on root morphology were more pronounced in the loamy sand than in the finer textured clay soil and elongation rates were greatest in the compacted treatment of the clay loam but in the uncompacted treatment of the loamy sand (Fig. 7 in Chapter 6).

The interaction between bulk density and soil type was significant for maximum rooting depth ($P<0.05$) and primary root length ($P<0.01$) in the bulk density transition experiment (Chapter 7). The greatest rooting depths in the clay loam soil were in the Compacted/ Uncompacted treatment, in which initial root establishment occurred at the highest bulk density (1.6 Mg m^{-3}), whereas the opposite trend occurred in the loamy sand as rooting depth was greatest in

the Uncompacted/Compacted treatment in which germination occurred at the lower bulk density of 1.2 Mg m^{-3}. These findings obtained using X-ray CT confirmed the results from the destructive root architecture experiment (Chapter 7) as root growth was greatest at high bulk densities in the clay loam and at low bulk densities in the loamy sand. Chapter 7 also demonstrated that the influence of soil texture was rarely significant at 3 DAG but was significant at 10 DAG for root surface area, total length and dry weight, shoot height and shoot dry weight. It has been suggested that the influence of soil texture on root growth in compacted soil originates partly from differences in nutrient availability (White, 2006), suggesting that at 3 DAG, seedlings were still reliant on seed storage reserves and increased bulk density was the greatest barrier to growth, whereas the influence of soil texture and nutrient supplies was more important for plant growth at 10 DAG. The size of the individual seeds was not determined, although larger seeds contain greater quantities of reserve substances and outperformed plants produced by smaller seeds in terms of root length and coping with stresses (Wulff, 1986).

In Chapter 5, a sandy loam soil (Prospect Hill, Adelaide, Australia, FAO Class: Brown Chromosol) was used comprising 66 % sand, while a Newport series loamy sand (brown soil) containing 79 % sand was used in Chapter 7. In Chapter 5, the growth of wheat roots as determined by X-ray CT was greater at 1.5 Mg m^{-3} than at 1.1 Mg m^{-3}, whereas in Chapter 7 root growth in wildtype tomato seedlings declined as bulk density increased from 1.2 Mg m^{-3} to 1.6 Mg m^{-3}, perhaps because the greater proportion of sand in the Newport soil exacerbated the adverse effect of soil compaction, or possibly because tomato

is more sensitive to soil compaction than other species (Mulholland *et al.*, 1999b). There is substantial variation between plant species, and even varieties, in their ability to cope with increased bulk density and soil strength. The response of roots of several cereal plant species to soil compaction was shown to be related to root shape (circular or flattened), which determined the cellular deformations in the cortex and vascular cylinder (Lipiec *et al.*, 2012). Although pea plants are particularly sensitive to compaction, and have therefore been used in several studies (Taylor and Ratliff, 1969b; Wilkins *et al.*, 1976; Bengough and Young, 1993; Bengough and Mackenzie, 1994; Croser *et al.*, 2000), differences in sensitivity nevertheless occur between cultivars (Vocanson *et al.*, 2006). The effects of bulk density and soil type on root growth are therefore tightly interconnected and taking account of only one of these variables within specific experiments does not inform researchers of the complete effect of soil physical properties on root growth (Alameda and Villar, 2012). Chapters 6 and 7 clearly demonstrated the importance of using contrasting soil types when investigating root responses to differing bulk density treatments.

9.1.4 Response of root elongation rate to soil physical properties

The lack of experimental information on the 4-D responses of roots to soil compaction *in situ* has resulted primarily from the difficult and time-consuming nature of procedures for extracting roots from soil to characterise root system architecture over extended growth periods (Imhoff *et al*., 2010). Indeed, as recently as 2010, Pagés *et al*. stated that measurements of the

elongation rate of individual roots remained a challenge. The use of X-ray μCT has enabled the root systems of individual plants to be visualised over periods of days to measure root traits non-destructively. In Chapters 6 and 7, the elongation rates of the primary roots of tomato plants grown at different bulk densities were quantified daily for 10 days. Primary root elongation was most rapid between 2-3 DAG in all treatments before declining and plateauing, showing no significant variation between treatments. This finding suggests that forming a significant early root presence for anchorage and resource capture is extremely important and the main task of the seed storage substances is to provide the newly emerged radicle with sufficient resources to anchor the root system securely in the soil and enable the shoot to develop its initial photosynthetic capacity (MacIssac *et al.,* 1989). Research into α-amylase activity and starch breakdown has also shown that the onset of polysaccharide breakdown occurs four days after seed imbibition (2 DAG in the present study), leading to rapid mobilisation of sucrose to the growing roots (Murata *et al.,* 1968; Daussant *et al.*, 1983). The observation that root elongation rate was greatest between 1-4 DAG and subsequently levelled off is consistent with the hypothesis that primary root elongation is primarily driven by seed reserves until *c*. 4 DAG. In Chapter 7, root elongation rate was greatest in the C/UC treatment, suggesting that roots are programmed during the early growth stages to cope with the soil environment that germinating seedlings initially encounter. Work by Schmidt (2011) also found that elongation rates were greatest when the seedlings were surrounded by soil providing adequate root-soil contact. However, in dry soil, good root-soil contact might be disadvantageous as maintaining this might result in the loss of water to the dry

soil (Schmidt, 2011). Other workers have shown that roots decrease in diameter in dry soils to prevent such an occurrence (Carminati *et al.,* 2009).

As Chapter 6 showed that most lateral roots were produced following rapid primary root elongation during the first few days after germination, it is likely that initial primary root growth coincided with the period when seed reserves were still abundant and prior to the development of significant photosynthetic capacity. Previous studies have also shown that root elongation is most rapid when plants are still reliant on seed storage reserves and before the newly produced leaves became the predominant assimilate source (Renau-Morata *et al.,* 2012). The sharp decline in primary root elongation to a relatively stable rate after 3 DAG may reflect a transition between the initial establishment phase, supported primarily by seed reserves, and subsequent preferential allocation of remaining seed reserves and newly acquired water, mineral nutrients and photosynthate to support lateral root formation and shoot growth (Copeland and McDonald, 2001). Interestingly, this decrease in primary root elongation after 3 DAG occurred in all compaction treatments and soil types, suggesting that soil compaction may be a secondary rather than primary stress factor in terms of its impact on root growth during the early stages of seedling establishment. However, due to the lack of non-destructive quantitative experimental evidence concerning root elongation rates in soil, this is an area of research that requires much further investigation.

9.1.5 The use of X-ray CT in root:soil interaction studies

The work presented here has built upon the previous pioneering research using X-ray CT in environmental and agricultural sciences (Watanabe *et al.,* 1992; Heeraman *et al.,* 1997; Jenneson *et al.,* 1999; Gregory *et al.,* 2003; Mooney *et al.,* 2006; Perret *et al.,* 2007). Technological advances in the hardware and software have allowed further questions on root:soil interactions to be investigated. The shorter scan times now available have enabled repeated measurements of important rooting characteristics simultaneously and non-destructively over extended periods. The improvements in detector capabilities and reconstruction software have meant that fine roots, such as those of *Arabidopsis thaliana*, can be visualised and quantified (Lucas *et al.,* 2011). This thesis has contributed further evidence that X-ray CT is a powerful tool for studies of root:soil interactions and, with further advancements, the throughput of samples will increase. However, the real strength of this technique lies in its combination with plant genetic and mechanistic physiological approaches to obtain a more precise understanding of the consequences of soil physical properties for root growth.

9.1.6 Application of automatic root tracking algorithms and novel image analysis techniques

Chapter 5 showed that the automatic root tracking algorithm, RootViz3D® (developed by R. Davidson), systematically extracted greater root volumes

from X-ray CT images in all treatments than the semi-automated segmentation technique and did not show any bias towards specific treatments. Although the outcome of the root segmentation by image analysis is inevitably related to the scan quality and contrast between root material and non-root objects, this study has shown that the tracking algorithm can be used successfully in field soils at field capacity. As highlighted in Chapter 3, the use of automatic root tracking algorithms is vital to remove the subjectivity of segmenting root voxels from the CT images and would provide an objective tool for research groups worldwide to standardise root segmentation techniques so that different studies can be more easily compared. As advances in computer science are made, the algorithms will improve still further. RooTrak (Mairhofer *et al.*, 2012), developed towards the end of the research described here, is one such tool for extracting accurate root volumes of different plant species in a variety of soil types from X-ray CT images.

A significant portion of current root research aims to optimise root system architecture by understanding how efficient root systems are and how the specific deployment of root branches within the heterogeneous soil environment corresponds to resource uptake (Smith and De Smet, 2012). To maximise the information gained from each scanned sample, image analysis techniques were employed to gather an improved insight into root responses to soil compaction. In Chapter 6, measurements of convex hull volume, centroid and maximum width of the root systems were derived using a new extended version of RooTrak created by Stefan Mairhofer. Plants grown in compacted soil consistently had smaller convex hull volumes and a reduced maximum

spread of lateral roots compared to those growing in uncompacted soil. The centre of mass of the root system, calculated by the centroid value, was deeper for plants grown on uncompacted soil than for those grown on compacted soil. As stated previously, using only root volume as a measure of root growth can be misleading and considering other root structural descriptors, such as convex hull volume, in unison with traditional morphological measurements would provide a more powerful characterisation of root responses to soil compaction. In Chapter 8, convex hull volumes were used to characterise the ability of the root systems of ABA mutant genotypes to explore the available soil volume effectively. The ABA-deficient mutant, *notabilis*, displayed a poor rooting phenotype in all bulk density treatments and explored smaller soil volumes. Chapter 8 also aimed to elucidate the role of ABA in determining root diameter and cortical cell expansion. Although no significant trends were apparent, mean root diameter was smallest in the ABA-deficient mutant *notabilis*, in agreement with Mulholland *et al.* (1996b).

In Chapter 6, connectivity of the pore space was analysed using the *Defect Analysis* module in VG StudioMAX® 2.1 software. This allowed 3-D visualisation and quantification of the pore space, highlighting how the arrangement of soil particles and pore space may vary greatly over very short (*c*. 5 µm) distances. The images used to analyse soil pore architecture showed that roots growing in compacted soil potentially have thousands of discrete pore spaces to explore, but very little connected pore space to extend through (at 24 µm resolution). It was also possible to overlay and align the segmented

root systems to their corresponding surrounding soil environment (Fig. 9.1). Although not statistically significant, compaction appeared to influence lateral root angle, as this was closer to 90 ° than in plants grown in uncompacted soil, possibly due to the presence of large areas of horizontally connected pore space in the compacted soil columns, which the lateral roots were able to exploit, similarly to how roots in the field exploit cracks and biopores (Passioura, 1991; Bengough *et al.*, 2011).

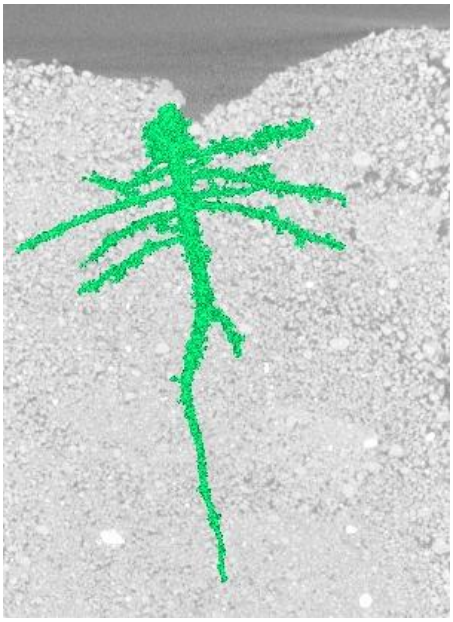

Fig. 9.1. Segmented root system of a tomato plant growing in uncompacted (1.2 Mg m^{-3}) loamy sand soil. Roots have been falsely coloured green for clarity.

10. Chapter 10

10.1 Conclusions

The following key conclusions can be drawn from the research presented here:

- Soil compaction significantly affects root system architecture and root growth is restricted at the higher bulk densities by the consequent increase in soil compaction, which roots must overcome to extend. The rooting architecture of plants grown on high bulk density soil (1.6 Mg m^{-3}) typically exhibited increased root diameters, shorter root lengths and rooting depths, fewer lateral roots, smaller convex hull volumes and surface areas and a reduction in root and shoot biomass.

- The effects of bulk density differed between soil types as root system architecture was negatively affected by increasing bulk density in the loamy sand, whereas moderate compaction (1.4 – 1.5 Mg m^{-3}) had some positive consequences in the clay loam soil. The results challenge the assumption that root growth is more severely impeded in compacted clay soils as the present study showed that moderate compaction of this type enhanced root growth, possibly due to the greater nutrient and water holding capacity associated with the increased bulk density, or because the greater cohesive properties of clay soils increased resilience to physical stresses (Horn *et al.,* 1995).

- The results suggest there is an optimum level of soil compaction, but this depends on soil type. If the soil is too loose, the roots cannot explore the soil matrix adequately, reducing growth rate. However, if the soil is too compact, the increased mechanical resistance experienced by roots also restricts their growth.

- There are several methods for segmenting root systems from X-ray CT images using a variety of image analysis software packages. However, due to the subjective nature of such techniques and operator bias, automated root tracking algorithms have shown great promise and offer an objective method that all research groups would be well advised to consider. In Chapter 5, the automated root tracking algorithm RootViz3D® showed a greater sensitivity in discriminating fine roots from grayscale values than user eyesight.

- The influence of plant growth regulators or chemical messengers in mediating root responses to increased soil bulk density requires further investigation. Results from this project suggest that abscisic acid has a role in maintaining optimal root architecture, especially of lateral roots, in an adequate volume of soil. The effect was most clearly observed at the intermediate bulk density of 1.4 Mg m^{-3}. Abscisic acid may also be involved in determining root diameter.

- Roots elongated rapidly between 2-3 DAG and it is hypothesised that this is related to the mobilisation of seed storage substances to the

growing roots. After this period, the plant becomes reliant on edaphic resources and carbon supply from the shoots, so root elongation rate declined and became constant for the remainder of the experimental period. However, due to the lack of experimental information on the 4-D responses of roots to soil compaction *in situ*, further work to determine the source of the plant's nutrient and carbon supplies whilst quantifying root elongation rates over short intervals is required.

10.2 Further work

- Although the work reported here used field soils rather than sand or agar, the columns were packed using soil sieved to < 2 mm. The use of structured field soils or cores taken directly from the field would be preferable in future studies to represent the natural environment more realistically. Scanning undisturbed field cores would enable the rhizosphere of field crops to be visualised and quantified non-destructively at a microscopic resolution and would provide a more accurate representation of rhizosphere processes under field conditions; however, substantially greater replication would be required to account for the greater variation in soil moisture content and physical characteristics encountered in heterogeneous soil cores. An example image of a scanned field soil core containing a wheat plant is shown in Figure. 10.1. Although it may prove harder to draw firm conclusions regarding the effect of soil structure on rooting architecture due to the

greater variability in soil structure, this is the direction that this type of research must follow, albeit with greater replication.

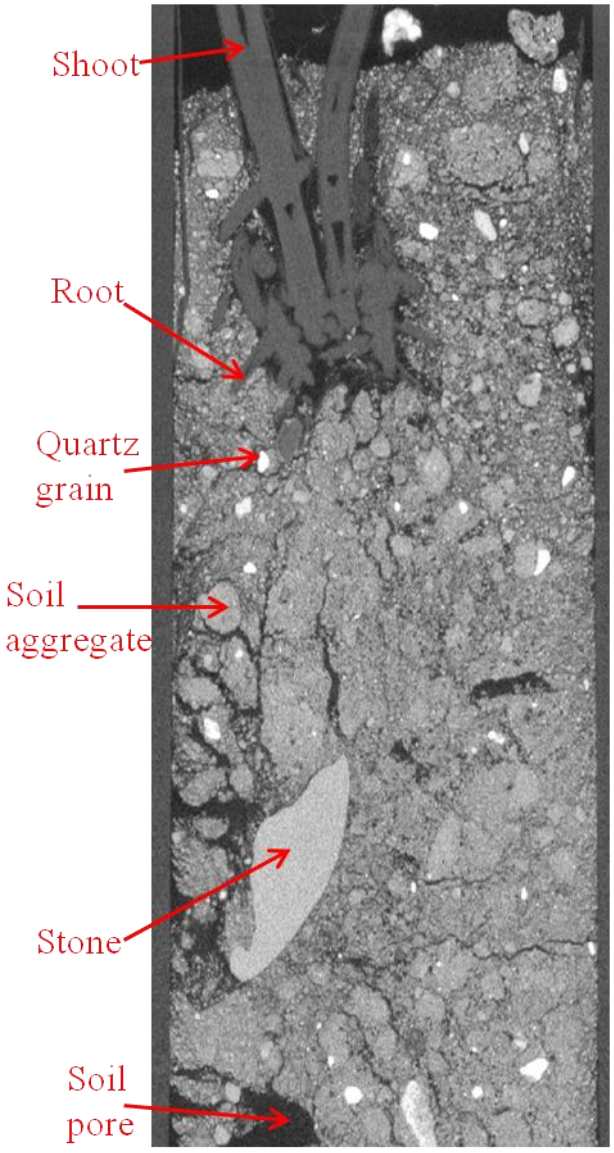

Figure. 10.1. Example image of a scanned field core containing a wheat plant (20 cm height, 5 cm diameter).

- For this thesis, only soil physical properties and their influence on root growth were investigated. However, chemical (e.g. nutrient provision) and biological factors (e.g. microbial community) or the interactions between adjacent plants would give further insight into how roots

operate below ground and overall rhizosphere processes. Being able to visualise root hairs and fungal hyphae easily would also be a major advance, as the entire rhizosphere could be viewed at high resolution. Studies of further stresses (e.g. drought, salinity) and soils held at different moisture contents to elucidate effects on root system architecture would again provide further information on root deployment in soil.

- The age of the plants investigated was limited due to the column size they could be grown in. Further research using older and larger root systems would provide greater reliability in predicting root responses throughout the life cycle of individual plants. Schmidt (2011) suggested that older plants may be more sensitive to physiochemical soil stresses, irrespective of water or nutrient supplies, and so could provide further insight into the effects of stress factors on root growth. However, Souch *et al.* (2004) found that the seedling phase was most sensitive to soil compaction as the roots are younger and thinner, and so experience a greater resistance. Some X-ray CT scanners are able to accommodate larger soil columns (up to 2 m depth), which could contribute to research efforts here. With recent advances in scanner detector technology and robotics, to aid in transporting these inevitably larger samples, it is probably only a matter of time before such systems are more widely available in laboratories undertaking rhizosphere research. However, the same trade-offs between container diameter and spatial resolution would remain.

- Throughout this thesis it has been hypothesised that, during the early days of seedling establishment, plants are reliant on seed-derived nutrients, after which there is a switch to edaphic resources, which may influence root elongation during these early days. However, this is an area that is lacking in supporting experimental data, possibly due to the difficulties of quantifying root elongation *in situ*. Further work to determine the sources of assimilate supplies or by limiting photosynthetic activity would provide key information.

- This project has also highlighted the possibility of using X-ray CT within plant phenotyping protocols, as it can visualise and quantify differences in root architecture between individual cultivars within species. Knowledge of which root characteristics are most important to quantify, and when during the growth cycle, is still urgently required and will be particularly valuable in enabling large high-tech plant phenotyping laboratories to accumulate substantial databases concerning the influence of environmental factors on root growth and function, which is vital for food security by maximising crop efficiency.

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